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Transcriptomics of learning

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Transcriptomics of Learning

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Dedication

I'd like to dedicate this work to my mother, whom through adversity raised me and taught me to persevere.

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Abstract

Learning is a basic and important component of behavior yet we have very little empirical information about the interaction between mechanisms of learning and evolution. In our work, we are testing hypotheses about the neurogenetic mechanisms through which animal learning abilities evolve. We are able to test this directly by using experimentally evolved populations of flies, which differ in learning ability. These populations were previously evolved within the lab by creating worlds with different patterns of change following theoretically predicted effects on which enhanced learning will evolve. How has evolution acted to modulate genes and gene expression in the brain to accomplish the behavioral differences observed in these populations? We report results from work characterizing the differences in gene expression in the brains of populations of Drosophila that evolved in environments favoring learning from paired populations evolving under control conditions. Using olfactory conditioning in the t-maze, we first show that flies which evolved enhanced learning in an oviposition context also have a generalized enhanced learning ability. We dissected brains from flies following experience learning in the t-maze and analyzed pooled samples using RNAseq. We completed a factorial design of comparing the brains of flies from high learning populations with control populations and in each of two conditions: after conditioning and without conditioning. Following differential gene expression analysis, we found differences within known suites of genes as well as novel transcripts. We have also found evidence of predicted trade-offs between immune response and

cognitive capacity. We present these data, as well as results from gene ontology analyses.

Combining predictions from behavioral ecology with experimental evolution is a powerful approach to assessing the suites of genetic and neurological changes associated with the evolution of complex behavioral traits, like learning. By analyzing the genomic mechanisms of what has evolved under experimental conditions, we can make a great step forward in understanding the evolution of learning and of plasticity in general.

Chapter 1

Historical background

1.1 Introduction

In this chapter, I give a brief overview on the history of studies of learning and memory, particularly with regards to *Drosophila* and mechanisms. I then discuss theoretical and experimental aspects of the evolution of learning and approaches from experimental evolution. Finally, I give an overview of some proposed trade-offs and interactions with learning, primarily with the immune system.

1.2 Early days in memory research

The concept of learning as we know it is a process that provides an organism with the capacity to store information from experiences and later on retrieve that information as needed. Although the study of learning and memory have historically formed separate fields, learning can be studied from the perspective of memory since both terms require the capacity for information storage and retrieval. The field of memory has been thoroughly dissected since the early 19th century and a great deal of understanding regarding its subtleties has been achieved.

Thanks to the advent of technology and multidisciplinary collaborations, the way memory operates and the molecular mechanisms involved have been dissected and are better understood. Although, in order to achieve our current molecular understanding, it took over a century of debate starting with debates for instance, on whether memory was stored in the brain or in the heart ventricles,

and more modernly, if the brain was a system of independent brain regions responsible for different behaviors or not (1).

Perhaps the most known theories about learning and memory are those originally proposed by Pavlov in which he proposes the concept of classical conditioning describing that an organism can learn to associate a cue formerly neutral with either a positive or negative reward (2). The Pavlovian paradigm opened the field to studying the conditioning phenomena under different contexts and sensory modalities. Contextually speaking, conditioning can either be aversive or appetitive. Aversive conditioning involves something like a shocking experience such as electric or mechanical shock or a poor-tasting food, while appetitive involves some sort of reward such as access to sucrose, a safe location, or a potential mate ((90). Conditioning has been studied from all sensory perspectives: olfactory, auditory, visual and spatial orientation. Due to challenges with the techniques required to study each modality, aversion learning has ended up being the preferred context in which to work, leaving appetitive learning virtually untouched for half a century and revisited in the late 1980's and mid 1990's (3,4). Olfactory and spatial orientation received more attention originally, yielding great breakthroughs for long asked questions in the field. Over time a much deeper understanding of aversive olfactory memory formation and consolidation was achieved when compared to the other modalities (5). In 1949 "The organization of behavior" by Hebb was published and it proposed a mechanism in which brain cells function and cooperate with each other in order to provide a basis for learning (6,7).

1.2.1 The Hippocampus and the Mushroom Bodies (MB)

Thanks to the spatial orientation memory research taking place in the mid 1900's, it was proven that certain brain regions are responsible for behavioral capacities. More specifically, it was shown that the hippocampus plays an active role in spatial memory and memory associations in mammals (8). This finding suggested that there must be an ancestral brain region playing the role of the mammalian hippocampus in simpler organisms, such as invertebrates. Memory had already been studied and observed in invertebrates but not yet in such depth nor from such anatomical perspective. In 1974, Quinn and Benzer showed that *D. melanogaster* learns to avoid electric shock (9). This opened the doors to forward genetics in memory on fruit flies and led to the identification of the first known mutant named "dunce" which possessed a learning deficiency (10). Simultaneously in the 1970's, more invertebrate animal models were used to study memory and continued the search for the hippocampus ancestral organ. It was concluded through the honeybee that the mushroom bodies (MBs), particular structures present within the brain of different species of invertebrates, are responsible for the memory and spatial orientation capacities (11,12). By 1985, Heisenberg had shown that the MBs play a role in olfactory memory in *D. melanogaster* (13).

1.2.2 Learning Phases

Thanks to the technical foundation laid at this point in time, the different memory phases were already dissected and better understood. For instance, it was

determined that there exist different forms of memories, for instance consolidated memories and memories that do not require consolidation. Consolidation is the process in which information learned through an experience is transferred, in mammals, from the hippocampus into the cerebral cortex (5,14,15). The consolidation independent memory is also known as short-term memory (STM) and there is debate whether a consolidation independent mid-term memory (MTM) exists (5,14,15). The consolidation dependent learning events have been dissected into two different kinds, protein synthesis dependent and protein synthesis independent. These were discovered thanks to the use of anesthetics, which are known to have an amnesia-inducing effect if exposed at the time of consolidation. Organisms that exhibit resistance to the deleterious effects that anesthetics have on memory are believed to have what is known as anesthesia resistant memory (ARM). Organisms possessing the normal consolidation dependent memory that becomes ablated when exposed to anesthetics is called long-term memory (LTM) (5,14,15). This form is protein synthesis dependent, and this synthesis becomes disturbed when exposure to anesthetics occurs during consolidation (5,14,15,16).

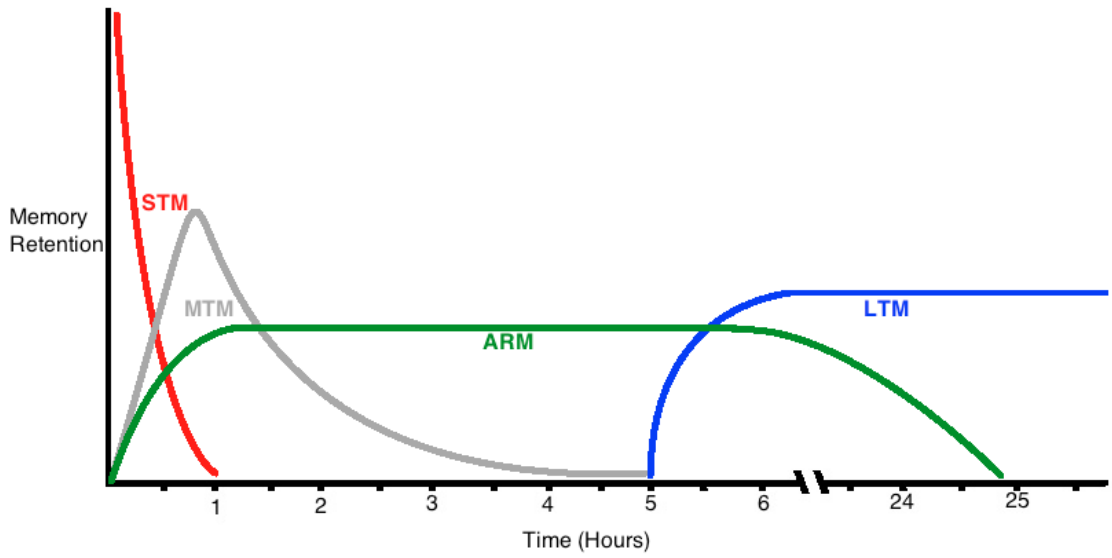


Figure 1.1 Learning Phases. This figure depicts the time course of the different proposed forms of memory. The x-axis gives the time from the experience that is learned. The y-axis is memory retention, from perfect retention of the learned information to no retention and forgetting. Short-Term Memory (STM) forms immediately and also degrades quickly. Anesthesia resistant memory (ARM) is observed as quick as STM but decays after the 24 hour range and is a protein independent process. It is believed to be an alternate information retrieval mechanism independent from LTM, which is protein dependent. LTM requires a longer consolidation period of about 5 hours, but has a much longer retention duration. (20)

The understanding of the subtleties of each memory phase has allowed for the thorough design of protocols that better elicit certain kinds of memory based on the length, intensity and repetition of conditioning protocols. It is known that for STM, conditioning happens through a relatively short exposure and a few repetitions suffice.

It has been established that ARM is induced with a massed conditioning protocol, which entails six or more consecutive conditioning cycles. A spaced conditioning protocol is required for LTM, which entails six or more conditioning cycles with 10 minute rest periods between each conditioning cycle (5,15,16,21).

1.2.3 Molecular and technological breakthroughs

Due to the advent of technology in the genetics, molecular biology and biochemistry fields, molecular work took place with the aims of unraveling the mechanisms involved in the learning deficient drosophila mutant found by Quinn. It was determined that the first learning deficient mutant “Dunce” (dnc) lacked a cyclic adenosine monophosphate (cAMP) diesterase (17,18) and the other learning deficient mutant “Rutabaga” (rut) lacked Ca_2/calmodulin-sensitive adenylyl cyclase activity (19). At the time, these were huge accomplishments given the techniques and amounts of labor to screen the genome and actually pinpoint the mutations. These findings shed light over a pathway that involves a nuclear protein known as the cAMP recognition element binding protein (CREB). This pathway has been known to be highly involved with the capacity to learn,

but it is now known that it is only involved on LTM. [Figure1.2]

During the 1990's, the technological momentum that started a decade earlier had only gotten stronger and facilitated an even greater advent in molecular, genetic and cellular understanding in the field, allowing for the development of transgenic tools and novel gene expression techniques that changed the nature and scope of research in the field. In 1998, Dubnau and Tully released a thorough review of the state of the field in regards to gene discovery. In this review, they thoroughly explain how the pathways involved in learning had been tested and proven by generating mutants unable to learn and later on partially rescuing their capacity to learn by injecting cDNA encoding the healthy gene being studied. It is this same paper in which the term of vertical integration, the translational potential of the discoveries made in *D. melanogaster,* is proposed (20).

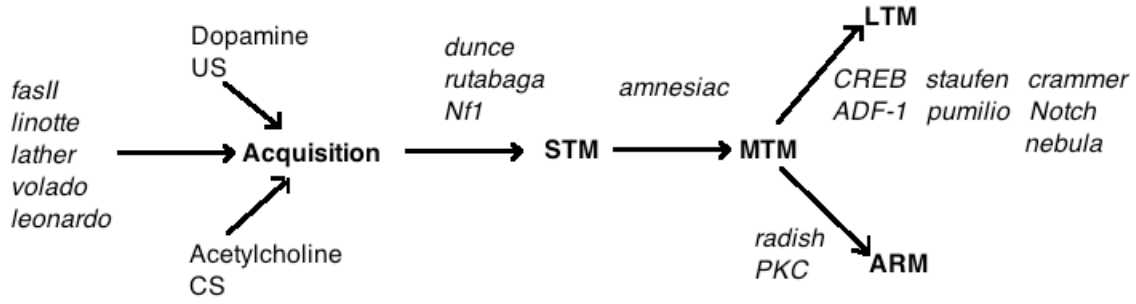


Figure 1.2 Memory Pathways

The above diagram depicts the pathway for memory formation and known genes related to each stage. *fasII, linote, latheo, volado* and *leonardo* are genes known to be involved in the capacity to acquire new information. Dopamine is related to triggering unconditioned stimuli (US) acquisition of information, whereas acetylcholine is related to conditioned stimuli (CS) acquisition of information. The genes *Rutabaga, Dunce* and *Nf1* are related to the capacity to consolidate the acquired information into STM. *Amnesiac* is necessary to further consolidate the STM information into MTM. It is believed that an organism either has ARM or LTM, which are the longer spanning memory forms. ARM is contingent on the *radish* and *PKC* genes, whereas LTM is contingent on *CREB, NOTCH, Stauffen/Pumillio, crammer* and *nebula* (20).

1.2.4 The evolution of learning

Brains are the products of evolution, and there are many theories that propose when learning should evolve (and they all revolve around patterns of change). While there are many comparative studies of learning, direct studies of evolution are much more rare. Conditioning ability was artificially selected in the late 1980's, with high learning flies and poor learning flies selected in each of those directions(90). About two decades later, Moore released "The evolution of learning", an extensive compendium that identifies the multiple possible kinds of learning viewed from multiple perspectives and on multiple organisms. It also explores the distinctions between each kind of learning and the possible evolutionary relationship between them (22). Shortly after, at the beginning of the 2000's, Mery and Kawecki released "Experimental evolution of learning ability in fruit flies" in which they show that *D. melanogaster* populations kept under certain conditions more favorable for learning showed a greater capacity to make the right choice under the oviposition paradigm. They showed that after only 14 generations, there is a significant difference in the experimental populations (23).

While incredibly important, this work considered only one form of environmental change, and the richness of earlier theoretical work still requires addressing. Considering only one form of change actually presents a paradox, especially since change can both promotes and inhibit the evolution of learning. Learning is still accurate in an unchanging environment for instance, yet too much change makes learning of little use. (24). Dunlap & Stephens (2009) solve this

paradoxical problem of change by splitting change into two components: the certainty of using a particular behavior and the reliability of the cues being used for learning when to employ a given behavior. In other words, it doesn't only matter if the environment changes, but it also matters if the correct signals are being delivered and properly processed in order to lead to the right decision making. Through an NSF grant, their hypothesis was more fully tested by evolving nine treatments of 12 replicates each of populations of flies under a gradient of the two aforementioned variables, the environmental certainty and the reliability of the best action being taken. The environmental certainty was manipulated by changing probability that eggs placed on either pineapple or orange flavored agar would result in fitness: being reared for the next generation. The second type of change, reliability, was manipulated through the quinine pairing in the first phase that could then inform the females' later placement of eggs. A reliable quinine pairing would indicate accurately where not to place eggs in the second phase. On that same 2009 paper, Dunlap proposed what is known as the flag model (24) (See figure 1.3).

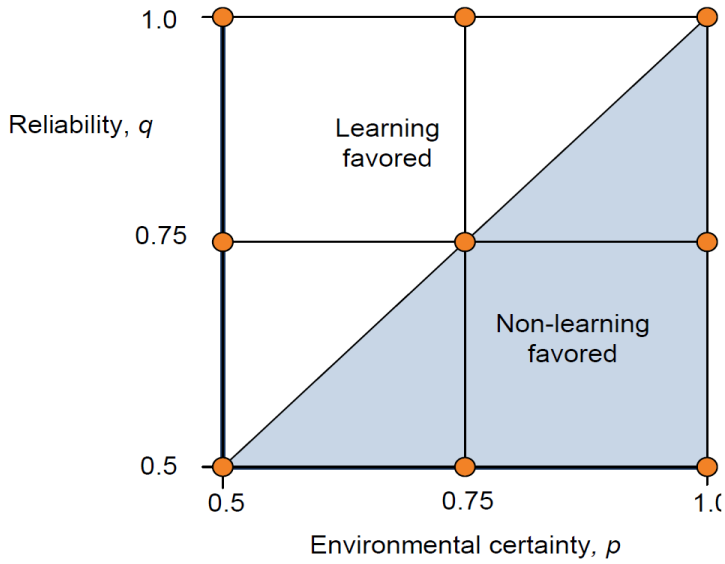


Figure 1.3 Flag Model (24)

This model is a visual organization of the two variables used in the experimental evolution design, the environmental certainty and the reliability of the best action taken. Based on the ratios of each, the theory would predict either greater learning or unlearned preference to evolve.

Around the same time, Keene and Wadell delivered another review in which over 37 memory related genes had been identified in *D. melanogaster*. This paper reviews useful genetic tools that had been developed by designing transgenic lines that provide the capacity to the researcher to control turning genes on or off based on temperature or light exposure and also to visualize different cellular events through the expression of a reporter gene in a particular cell type (21).

1.2.5 Evolve and Re-Sequence (E&R)

Once again thanks to technological improvements, high-throughput sequencing, also known as next generation sequencing (NGS), was developed. The field of bioinformatics experienced a great leap forward due to the greater computational power achieved at the time. NGS opened the possibility to sequence entire genomes in short periods of time and at accessible prices to the average researcher. That changed the scope of analysis and dissection of behavior from the genetic and genomic perspective (25). The conversation towards further dissecting the evolution of cognitive traits started to happen, which unraveled another discussion on whether the technology was available for this analysis at that point (26, 27, 28, 29). One important application that combines the experimental evolution approach with NGS is known as “evolve and re-sequence” (E&R). E&R basically proposes sequencing a population before and then again after experimentally evolving it. Using this approach, one

can gain understanding of the gene dynamics taking place in the evolutionary process and also better understand how the different selective pressures affects the organism at a molecular level (30,31).

1.2.6 Energetic and Life History Trade-offs in the Evolution of Learning

Learning and cognitive capacities are complex mechanisms that require a great deal of energy and investment from an evolutionary perspective. In order to evolve such mechanisms, an organism must benefit from such investment with the ultimate goal of surviving and reproducing.

To understand the evolution of cognition, the field has looked into brain size for correlation with cognitive capacity. From that perspective, the expensive tissue hypothesis (ETH) was developed when observing the gut vs. relative brain size ratio in primates and humans (40). The ETH refers to the economics regarding the allocation of resources based on the metabolic costs each tissue type has. There has been quite a great deal of controversy in regards to the ETH, and alternative hypothesis have been formulated due to conflicting results under different contexts and model organisms (41,42,43). Ectothermic animals are believed to be the most applicable group for studying such purposes due to the greater cost for them to maintain brain tissue (44).

One alternative hypothesis is known as the “energetic trade-off” (ETOH) hypothesis, which states that in order to allocate more resources to the brain, other systems become compromised such as reproductive success, fecundity or even development (41). Therefore, ETOH assumes that two expensive

systems do not co-evolve due to the energetic conflicts. Tissues such as gut and brains have been negatively correlated and confirmed in cichlids and anurans, supporting the ETOH (44,45,46). Relative brain size has also been compared and correlated with other traits, such as sexual traits (47), mate search (48), gender (49), fecundity (50) and innate immunity (51). In some cases the correlation has been negative, and in other cases the correlation has been positive, such as in the case of sexual traits.

In the case of innate immunity, it has been observed through the tissue graft rejection paradigm in guppies that innate immunity is negatively correlated with brain size, but not adaptive immunity (51).

1.2.7 Innate Immunity in Drosophila

Innate immunity is a complex system that a host has evolved to protect itself against infection from foreign organisms (32). The broadness of immunity achieved by an organism may provide the fitness an organism requires to survive given the conditions in the environment. Insects are much simpler than mammals in many ways, yet insect innate immunity is very complex and highly conserved. This is the reason why *D. melanogaster* has been a great tool for its dissection (33). A great deal of understanding has been achieved in mammal immunity thanks to *D. melanogaster* (34, 35, 36).

It has been determined that at the time of infection, the innate immune system detects the molecular signatures on each organism through the pathogen-associated molecular patterns (PAMPS) through the pattern recognition

receptors (PRR). This then leads to the expression of antimicrobial peptides (AMPs) in fat cells to then be released into the haemolymph for pathogen recognition and clearance (35, 37, 38). We now know of seven families of AMPs: Attacins, Cecropins, Defensin, Diptericin, Drosocin, Drosomycin and Metchnikowin (39).

There is recent findings from a different research group with pre-print evidence of AMP's being involved in LTM modulation (86).

It has been hypothesized that an evolutionary trade-off exists between memory and immunity. Because of that hypothesis, it has been tested and observed in both *D. melanogaster* and *B.terrestris,* that there is no trade-off but actually a positive relationship between learning and immune response (91,92).

Chapter 2

2.1 Introduction

Having the capacity to recall information from past experiences is an adaptation that allows organisms to make decisions in order to ensure survival. It is also a process that has captivated fascination in humans since ancient times (1). Many breakthroughs have been achieved in regards to dissecting learning and its basic theoretical intricacies. Pavlov's associative and classical conditioning paved the way for scientists of multiple backgrounds to tackle the subjects of learning and memory during the first half of the 20th century (2). During the second half of the 20th century, a technological advent led to a great leap in discoveries. Many interdisciplinary and translational efforts dissected the function of different parts of the brain and were able to pinpoint that the hippocampus is responsible for memory association and spatial orientation in mammals. These efforts also determined that insects have the mushroom bodies (MB) which serve the same function as the hippocampus. The MB occupy a great percentage of the actual brain in many insects such as *D.melanogaster* (8,11,12,13,21). Through ambitious and labor intensive techniques, science has created a breach that each decade has exponentially deepened along side the greater computational power achieved in the fields of electronics and computer science (9,17,18,19,20,21,25). A great deal of molecular work has been successfully accomplished, dissecting the learning pathways and its

mechanisms (20, 21). Thanks to this work, the kinds of learning and various gene pathways that are involved in each kind of learning are now understood (20). Once the field was set for a genomic conversation of learning to take place, the subject of how learning evolved came to be. The theory suggests that this learning adaptation only takes place when it is economically favorable for an organism to invest in the machinery involved with the capacity to learn. In other words, there has to be a reward for being able to recall former experiences, and in nature this reward comes in the form of vertical gene transfer or procreation. Mery and Kawecki showed that *D. melanogaster* can evolve learning in only 14 generations based on the rate of change of the environment (23). Cognitive tissue is believed to be an expensive investment though, therefore evolving such machinery happens only under conditions that require proper decision making, such as a highly changing environment (24,44,45,46). Additionally, the right decision-making is contingent on the execution of the action, which can be contingent on the proper signal processing capacities. This awareness allowed for the theory of the evolution of cognition (24). This theory states that both the rate of change in the environment and the certainty of the best action taken determine whether learning or non-learning (innate-bias) will be favored. The theory was tested under the oviposition paradigm by experimentally evolving populations of wild *D. melanogaster* under gradients of the aforementioned variables. The rate of change in the environment was controlled and lines of flies were evolved based on different parameters (see figure 1.3 Flag model).

The model was first tested in 2009, in which populations with different learning capacities was allowed to be evolved (24). As the techniques of Next-Gen Sequencing (NGS) have matured, Evolve and Resequence can be applied to more experimental systems. (25,26,27,28,29). E&R refers to sequencing an organism before and after submitting it to experimental evolution treatment (30,31). The recently evolved high-learning lines from the factorial experimental evolution test of the flag model provide an excellent opportunity to apply these genomic techniques to experimental evolution of a complex phenotype.

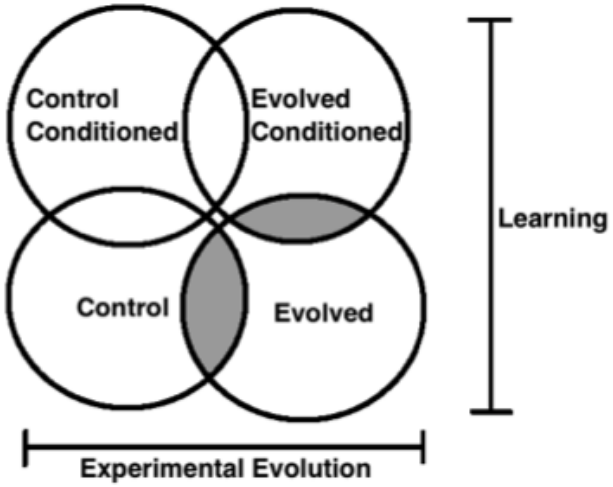


Figure 2.1.2 Flower Chart: The flower diagram breaks down the experimental design in order to dissect the gene dynamics with regard to both experimental evolution treatment and actual learning. The differentially expressed genes between control and experimentally evolved populations are the ones expected to be related to the experimental evolution treatment. The differentially expressed genes when comparing the experimentally evolved population before and after learning are expected to be related to the actual process of learning. Only two overlapping petals are shaded because in this work we only focus on the results of the evolved vs. controls and evolved vs. evolved conditioned.

The lines of flies evolved under the constantly changing environment with the highest reliability of best action were found to have evolved higher cognitive capacities. In order to test whether these lines of flies had evolved a general capacity for higher learning across contexts, the experimental populations were behaviorally tested for short-term memory (STM) using a different paradigm and two novel stimuli to see if the higher cognition transferred to a different context. The experimental populations showed greater learning than their control pairs (see Figure 2.3.1.1).

Because mushroom bodies are associated with memory in *D. melanogaster*, we collected whole brains for the analysis. In order to accommodate RNA Pool-Seq, we collected a minimum of 40 brains per sample. Samples were collected immediately after learning was shown. RNA was purified and mRNA libraries were prepared in-house. Libraries were sent for High-output sequencing to the DNA core at the University of Missouri in Columbia. Samples were sequenced using a NextSeq platform aiming for >35 million reads per sample. The resulting data was ~35GB that were first aligned to the *D. melanogaster* genome (UCSC dm3) and then analyzed through Cufflinks & DE basespace workflow to unveil the statistically significant differentially expressed (DE) genes between treatments. Once the lists of DE genes were ready, they were submitted for gene ontological analysis using GOrila (52, 53) (see Figures 2.3.3.1.1.- *2.3.3.2.5)*.

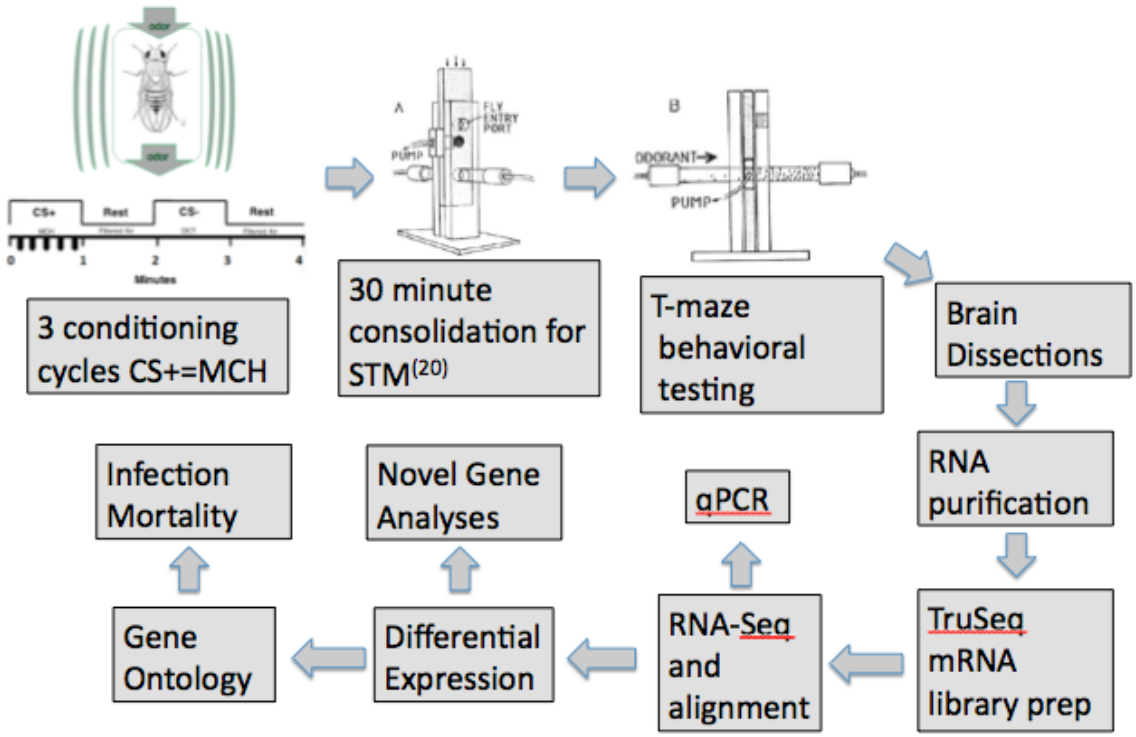


Figure 2.1.3 Experimental Design: Flies were conditioned against 4-Methycyclohexane (MCH) and allowed to consolidate for 30 minutes. Flies were immediately tested and individuals that showed learning were separated and immediately put in ice for their brains to be dissected. Within 30 minutes, samples were kept in -80 ˚C until 40 brains were reached per sample. RNA purification and validation were performed, followed by mRNA library prep for Illumina sequencing. Libraries were sequenced and aligned, followed by differential expression (DE) analysis that unveiled novel and notable genes. The DE data allowed for the ontological analysis, which unveiled immune related processes further tested through an oral bacterial infection mortality assay and qPCR.

These analyses yielded a great deal of information, including novel genes, annotated genes of uncertain function, and known genes highly involved in different processes and functions. In this Chapter, I report on the most outstanding results, which mainly relate to an observed learning-innate immunity co-evolution and potential immune trade-offs observed after learning took place.

These findings require addressing the work that has taken place to assess the evolution of cognition from a relative brain-size perspective. We know that a bigger brain is capable of greater cognition, but cognitive tissue is expensive and brain-size has shown patterns of fluctuations based on different evolutionary conditions. What is known as the energetic trade-off hypothesis (ETOH), which is an alternative for the expensive tissue hypothesis (ETH), proposes that energetic investment in biological systems follow economic dynamics and constraints based on resource availability and allocation (40, 41).

It has been observed through the tissue graft rejection paradigm in guppies evolved for greater brain size that innate immunity is negatively correlated with brain size, but not adaptive immunity (51). Our results conflict with those findings, as we have observed an actual co-evolution of innate immunity alongside cognition. As mentioned, , *D.melanogaster* immunity is very complex, making it a suitable model organism to study immunity in mammals, and a great deal of understanding about human immunity has been achieved thanks to *D.melanogaster* (33, 34, 35, 36).

2.2 Materials and Methods

2.2.1 Experimental evolution

Populations were evolved following the 'flag model' framework (See figure 1.3). This theory uses components of change in the environment to predict when learning is favored evolutionary: the certainty with which the best action in a given environment changes and the reliability of cues available for learning (78,79). Wild-caught populations of flies were evolved using an oviposition aversion learning paradigm, as maternal choice of where to lay eggs has a large effect on fitness. Our lab is maintaining populations of flies with evolved enhanced learning, currently at over 150 generations of selective pressure.

2.2.2 Behavioral testing

3-5 day old females were sexed into populations of 30 individuals in new food agar vials using ice treatment to immobilize flies. Flies were then allowed to recover at 24˚C and >60% humidity for 4 days before testing to diminish any physiological/cognitive effects caused by the ice treatment. All testing occurred in an environmental chamber under red LED light conditions. 12 populations were tested on each possible treatment: non-conditioned, conditioned against 4-Methylcyclohexane (MCH), and conditioned against 3-Octanol (OCT). Flies were conditioned using 3 consecutive STM conditioning cycles. (See figure 2.2.2.1)

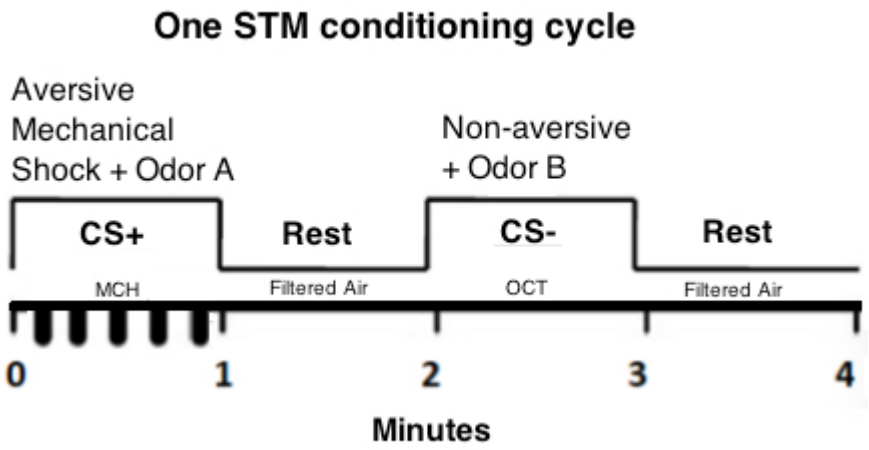


Figure 2.2.2.1 One STM conditioning cycle: Depicted above is the sequence of events in one conditioning cycle. Starting with coupled aversive mechanical shock with odor A. Mechanical shock was delivered by vortexing the flies at 2000 RPM for one second every five seconds for the entire coupled stimuli (CS+). Starting second 61, odor A and mechanical shock are replaced by activated charcoal filtered air for a whole rest minute. Starting second 120, flies are exposed to odor B without any aversive stimulus and is known as non-coupled stimuli (CS-). Starting second 180, filtered air is delivered for a whole rest minute. One STM conditioning protocol requires three consecutive STM conditioning cycles taking a total of 12 minutes.

This 4 minute protocol is repeated 3 consecutive rounds for a total of 12 minutes with 3 paired CS+ conditioning events and 3 unpaired CS- events. Populations of conditioned flies were then allowed to rest for 30 minutes. Following this resting period, populations were loaded one at a time into the t-maze and simultaneously exposed to both olfactory cues, one from each direction. Flies making each choice, MCH and OCT, were removed from the maze within each choice vial and frozen. These flies were then counted, allowing us to quantify the learning capacities of the experimentally evolved populations in the t-maze context.

2.2.3 Sample Collection

A subset of flies from the behavioral testing was then used in sample collection. These flies were first behaviorally tested for their capacity to make the right choice upon conditioning using the t-maze. As soon as the choice consistent with learning was observed, flies were placed on ice and their brains were dissected within 30 minutes of testing. The tissue was homogenized using RNase-free pestles (Fisher Scientific, Waltham, Massachusetts) and stored in ~10µl of Trizol (Life Technologies, Carlsbad, California) per brain and left to incubate at room temperature for 5 minutes. Then, the Trizol volume was doubled and samples were stored at -80˚C.
A total of 16 samples was collected. First, 8 samples were collected during the Fall and Spring of 2016, each containing 40 brains. The lines used were

the experimentally evolved populations for higher learning (J2 & J10) with their respective controls (C2 & C10) both before learning (samples J2-O, C2-O, J10-O, C10-O) and after learning (samples J2+O, C2+O, J10+O, C10+O). These 40 brain-containing samples were used for downstream RNA-Seq purposes. During the fall of 2017 the same 8 samples were collected again following the exact same protocol, but this time aiming for 20 brains each to perform qPCR and RNA-Seq validation.

2.2.4 RNA purification

Total RNA was purified with the RNeasy mini kit (Qiagen, Hilden, Germany) using the manufacturer's protocol. RNA quantity and quality were assessed using an Epoch Nanospec (BioTek, Winooski, Vermont), a Qubit 2.0 Fluorometer (Life Technologies, Carlsbad, California) and a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, California).

2.2.5 *mRNA library preparation*

mRNA libraries were prepared from 350ng of total RNA per sample using the TruSeq Stranded mRNA LT Sample Prep Kit (Illumina, San Diego, California) following the manufacturer's protocol using 13 PCR amplification cycles. mRNA libraries were quantified and qualified using Qubit 2.0 Fluorometer and Agilent 2100 Bioanalyzer.

2.2.6 RNA-Seq

High-throughput sequencing services were performed at the University of Missouri DNA Core Facility. A single 75 bp paired-end high-output Illumina Nextseq run was performed, yielding >35 million reads per sample with Q30 scores >96%.

2.2.7 Bioinformatics

2.2.7.1 Alignment and DE

Alignment was performed using STAR 2.5(73), and then validated using bowtie Differential expression was performed using Cufflinks assembly & DE (Basespace Workflow) 2.1.0 (74) with the UCSC dm3 reference genome. An RNA-Seq Heatmap was generated from the Cufflinks assembly and a DE list of significant filtered genes using DESeq2 (75).

2.2.7.2 Ontologic analysis

Gene ontology analysis was performed using Gorilla (52,53). Significantly differentially expressed genes with a p-value < ~5.21 x 10^{-2} were run against the background gene list of ~9300 genes. From this, ~5700 genes were associated with GO terms.

Visualization diagrams were produced with REViGO using the GOrilla generated GO terms and p-values with similarity=0.9. The Whole Uniprot database was selected for GO term reference and the simRel score for

semantic similarity measures (77).

2.2.7.3 Novel gene analysis

Novel gene analysis was performed using the integrative genomic viewer (IGV)(75), as explained in Thorvaldsdottir, Robinson and Mesirov 2012 (76).

2.2.7.4 Gene Network analysis

The network plot was generated using esyN as in (89) to assess public data looking for interactions and processes in biological systems.

2.2.8 qPCR

For RNA-Seq validation purposes, qPCR was performed on cDNA as explained in Taylor, S. 2010 (87).

Reverse transcription was performed using ProtoScript II First Strand cDNA Synthesis Kit (NEB, Ipswich, Massachusetts) following manufacturers protocols using 48.8ng of starting total RNA per sample. Using a CFX96 Bio-Rad (Hercules, California). The RNA had been collected from 20 brains per sample using the same protocol used for the RNA-Seq.

Luna Universal Probe One-Step RT-qPCR Kit (NEB, Ipswich, Massachusetts) was used following manufacturers protocol using a 1:32 dilution of the cDNA. Primers were designed using the NCBI Primer-Blast tool as explained in (68 and 87) and value oligos were ordered at 25nm and lyophilized from Invitrogen (Carlsbad, California).

Primers used:

AttC

AttC_F_2: TAAGCAAGGCCGTTGGAACT

AttC_R_2: GCCGTGTCCATGATTGTTGTAG

Dro

Dro_F_1: GCATACCGCGGAGAAGTCAT

Dro_R_1: CGATGGGAACCCCTCATTGT

GAPDH

GAPDH_F:TTATCAACGAGACGCACGAG

GAPDH_R:ACGGCCAAGATCAAGGTATG

2.2.9 Functional Tests of Observed Differences

Following the RNA-Seq results, we conducted an additional experiment testing the function of the observed immune effects.

2.2.9.1 Infection Mortality Assay

We performed an oral infection protocol as in Apidianakis and Rahme, 2009 (67) on both evolved and control populations by knocking 6 replicates of 10 females into a vial with 2% agar and 5% sucrose covered with a Whatman filter paper disc wetted with 170ul of 5% sucrose solution containing 6.4 x 10^9 PA01 bacterial cells previously pelleted from 4ml at OD_{600}=1.6. Flies were

kept at 24℃, >60% humidity, and surviving flies were counted two times daily for 3 weeks.

2.2.9.2 Bacterial Stocks

For each experiment, PA01 cultures were freshly inoculated in LB agar plates from glycerol stocks kept at -80℃ under a sterile hood. Single colonies were then picked after 24 hours of incubation at 37℃. 5ml of LB media was inoculated and left to incubate at 37℃ in a circular shaking incubator at 120rpm using a ridged Erlenmeyer flask for 24 hours. Subculturing would follow by first diluting (1:100-1:200) overnight cultures in 5ml of LB media until an OD_{600} of 0.05 would be reached. Then 95 ml of LB media were added and left to incubate at 37℃ in a circular shaking incubator until an OD_{600} of ~3 was reached. 1ml of media at OD_{600}=3 contains ~3-5x10^9 bacterial cells.(67)

2.2.10 Analysis and Data

2.2.10.1 Behavioral Confirmation of Experimentally Evolved Effects

For behavioral testing, we performed a repeated measures ANOVA, with repeated measures on each replicate line, nested within evolutionary treatment (high learning, or control), and a main effect of the pairing of the conditioned stimulus. Here we specifically predict a significant interaction between CS+ pairing and evolutionary history.

2.2.10.2 Infection Mortality Assay:

For the infection mortality assay, we performed a repeated measures ANOVA, on the mean numbers of flies alive within each line, with a repeated measure of with repeated measure of time. These measures were performed on data normalized from the individual time points of mortality across the vials. Here, the mean of each control line or evolved high learning line was normalized against its own non-infection control vials. This allowed each line to serve as its own control for infection.

2.2.10.3 qPCR: Relative gene expression was calculated using the double delta Ct method, which compares an internal housekeeping gene (GAPDH) with a target gene using CFX manager software from Bio-Rad (Hercules, California).

2.2.10.4 RNA-Seq: Alignment was performed using STAR 2.5.1, which uses a seed search followed by clustering, stitching and scoring (73). Gene Ontology analysis was performed using GOrilla as in 52, 53.

2.2.10.5 Data access: All sequencing data will be submitted to NCBI Gene Expression Omnibus (GEO) (http://www.ncbi.nlm.nih.gov/geo/) at the time of submission of this work to a journal for publishing.

2.3 Results

2.3.1 Behavioral testing

The ANOVA revealed a statistically significant effect of the CS+, indicating that the pairing of the odorant with mechanical shock affected behavior. We also found the predicted interaction between evolutionary history, or selection type, and CS+. As shown in Figure 2.3.1.1., high learning flies show a strong difference in choice of MCH following different conditioning pairings, whereas the control lines do not show this difference in behavior.

	Univariate Tests of Significance for PI MCH (Spreadsheet2 in Sasha Final Data.stw) Over-parameterized model Type III decomposition							
Effect	Effect (F/R)	SS	Degr. of Freedom	MS	Den.Syn. Error df	Den.Syn. Error MS	F	p
Intercept	Fixed	0.617803	1	0.617803	2.00000	0.001815	340.3477	0.002925
CS+	Fixed	1.636166	1	1.636166	66.00000	0.110394	14.8211	0.000270
Line(Selection Type)	Random	0.003630	2	0.001815	66.00000	0.110394	0.0164	0.983696
Selection Type	Fixed	0.017339	1	0.017339	2.00000	0.001815	9.5521	0.090676
CS+*Selection Type	Fixed	1.243896	1	1.243896	66.00000	0.110394	11.2677	0.001312
Error		7.286035	66	0.110394				

Table 2.3.1.1. Repeated measures ANOVA of the behavioral testing.

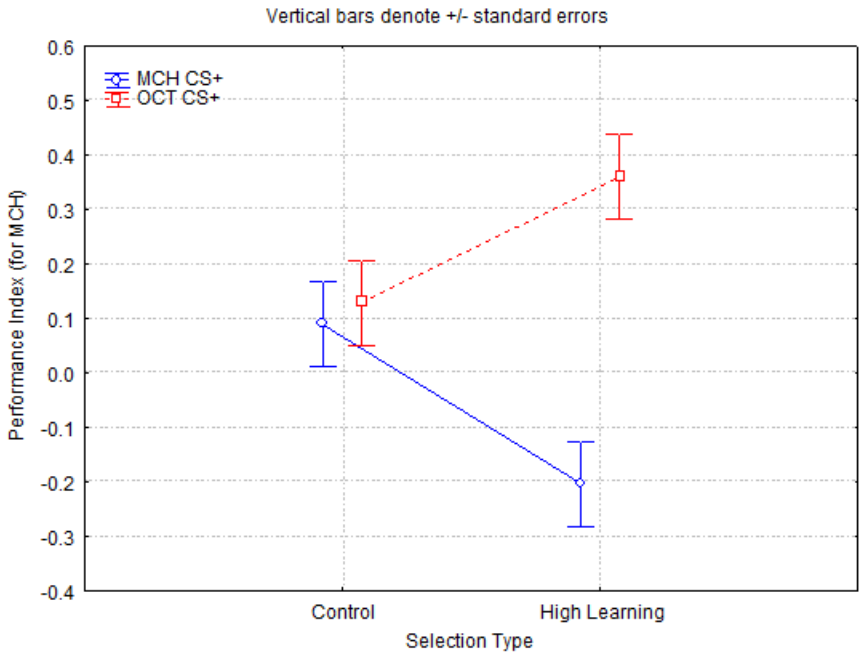


Figure 2.3.1.1. Behavioral Testing: Significantly greater learning was observed on experimental populations than on control populations. High learning is on the right vs. controls on the left. The greater the slope between the points, the greater the behavioral difference between lines.

2.3.2 Differential Expression

The differential expression analysis revealed a difference in 19 significantly differentially expressed genes related to the experimental evolution treatment and 91 significantly differentially expressed genes related to learning. The significance threshold used was $q<0.005$. From which, I focused on a subset of genes in figure 2.3.2.1

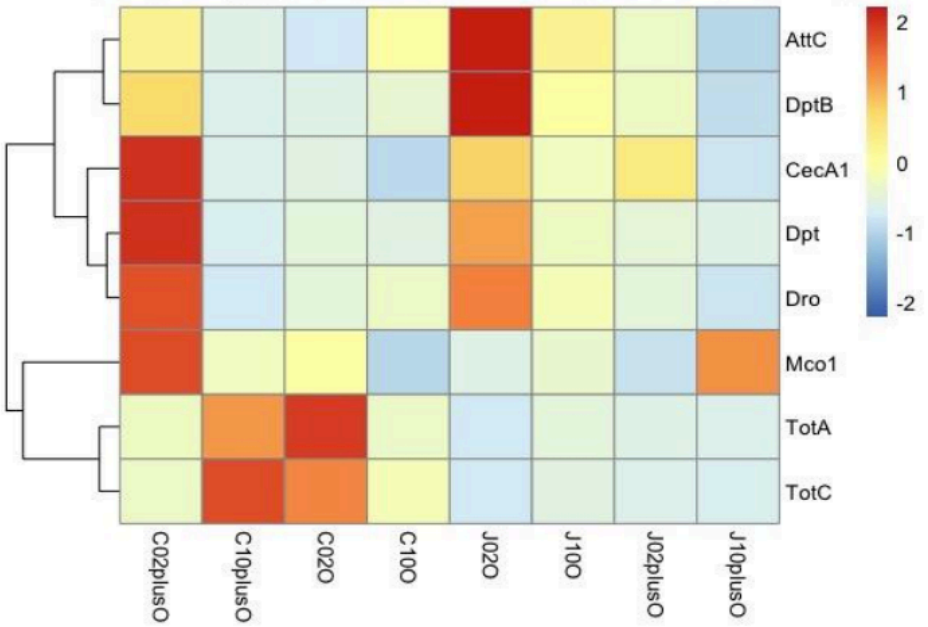


Figure 2.3.2.1 Innate Immunity DE: J02O & J10O are evolved populations that chose OCT without conditioning. C02O & C10O are the paired controls for each evolved population that chose OCT without conditioning. J02plusO & J10plusO are the evolved populations that chose OCT after conditioning. C02plusO & C10plusO are the paired controls for each evolved population that chose OCT after conditioning. The top cluster on the chart (AttC, DptB, CecA1, Dpt, Dro) includes all immune-related genes that show an increased expression in the experimental evolution treatment. Interestingly, the same cluster of genes shows significant repression upon conditioning on the evolved population, suggesting a potential energetic trade-off between learning and innate immunity.

The bottom cluster (TotA and TotC) contains stress response genes involved in energy metabolic processes (84). Mco1 is a plasma membrane-bound multicopper oxidase that oxidizes substrates in the heamolymph (85).

2.3.3 Gene Ontology

2.3.3.1 Experimental evolution for greater learning (Evolved vs. Controls)

The ontological processes unveiled related to the experimental evolution treatment are listed in 2.3.3.1 GO Experimental Evolution Process Table. Three main processes were unveiled through the ontological analysis. The main process was response to bacterium and immune response, mainly associated with genes Dro, AttC, DptB, TotA and TotC. The second one is chitin-metabolic process, which is related to glucosamine-containing compound metabolic process and amino sugar metabolic process based on the genes involved (CG34282, CG14645, CG34220). The third one is stress response related, including UV and heat response, with genes TotA and TotC associated with such GO processes.

2.3.3.1 GO Experimental Evolution Process Table

Description	FDR q-value	Enrichment	Genes
response to bacterium	4.73E-03	24.5	Dro, AttC, DptB, TotA, TotC
response to other organism	1.87E-02	16.14	Dro, AttC, DptB, TotA, TotC
response to external biotic stimulus	1.28E-02	16.05	Dro, AttC, DptB, TotA, TotC
response to biotic stimulus	9.64E-03	16.05	Dro, AttC, DptB, TotA, TotC
multi-organism process	2.84E-02	12.3	Dro, AttC, DptB, TotA, TotC
defense response to Gram-positive bacterium	2.84E-02	47.36	Dro, AttC, DptB
humoral immune response	3.35E-02	42.62	Dro, AttC, DptB
chitin metabolic process	7.27E-02	31.57	CG34282, CG14645, CG34220
glucosamine-containing compound metabolic process	8.43E-02	28.9	CG34282, CG14645, CG34220
cellular response to UV	7.80E-02	113.66	TotC, TotA
amino sugar metabolic process	7.26E-02	28.41	CG34282, CG14645, CG34220
antibacterial	9.52E-	94.72	Dro, AttC

humoral response	02		
response to external stimulus	9.87E-02	7.78	Dro, AttC, DptB, TotC, TotA
aminoglycan metabolic process	9.43E-02	24.01	CG34282, CG14645, CG34220
cellular response to heat	2.40E-01	54.12	TotA , TotC
response to UV	2.47E-01	51.66	TotA, TotC
defense response to bacterium	2.47E-01	16.24	Dro, DptB
cellular response to light stimulus	2.41E-01	49.42	TotA, TotC
immune response	3.10E-01	14.45	Dro, DptB

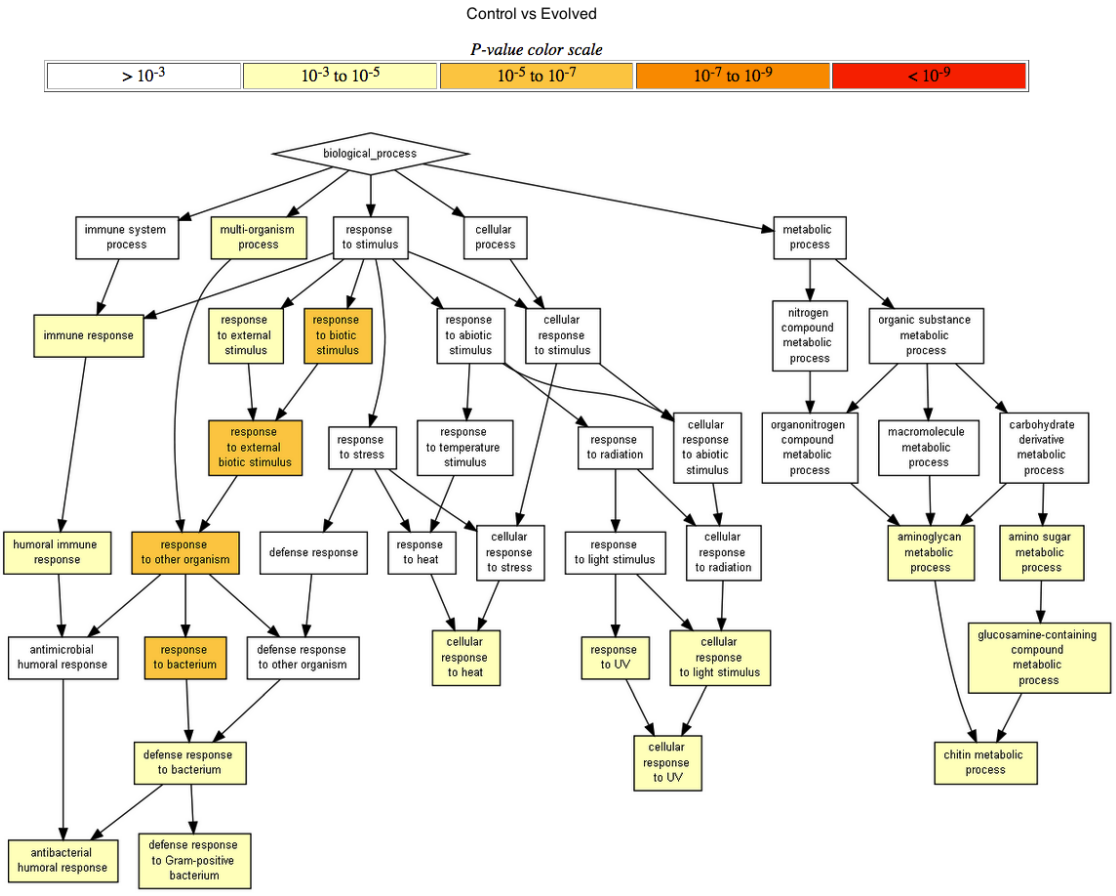


Figure 2.3.3.1.1. GO Experimental evolution Process GOrilla Flowchart. This flowchart shows the relationship between GO processes and depicts the p-value using the color scale bar on top. It was generated simultaneously with GO Experimental Evolution Process Table 2.3.3.1 and is a different visual representation of the same results.

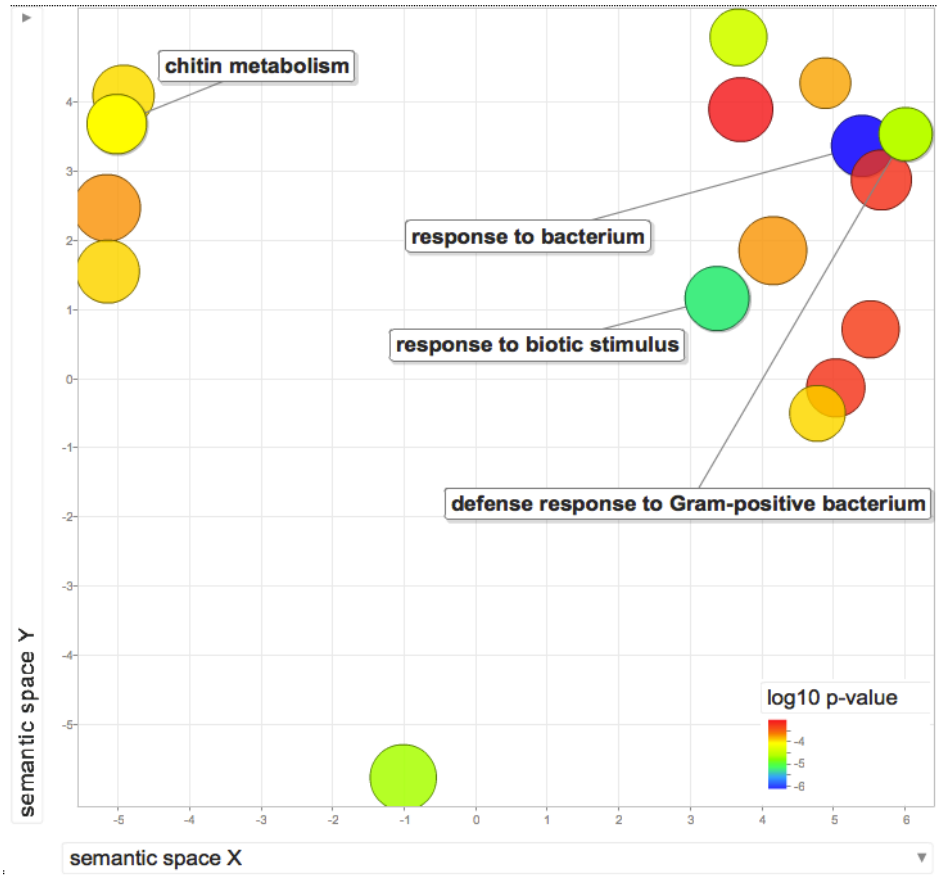


Figure 2.3.3.1.2 GO experimental evolution process ReViGO Plot. This figure was also generated using the data from GO Experimental Evolution Process Table 2.3.3.1 and it depicts the same results in a different visual representation. X and Y axes are irrelevant, and the color coding denotes p-value as explained by the color legend.

In contrast to the vast processes being evolved, the sole ontological function observed upon experimental evolution treatment was chitin binding with the associated genes CG34282, CG14645, and CG34220 (Table 2.3.3.2).

2.3.3.2 GO Experimental Evolution Function Table

Description	FDR q-value	Enrichment	Genes
chitin binding	1.88E-01	32.17	CG34282, CG14645, CG34220

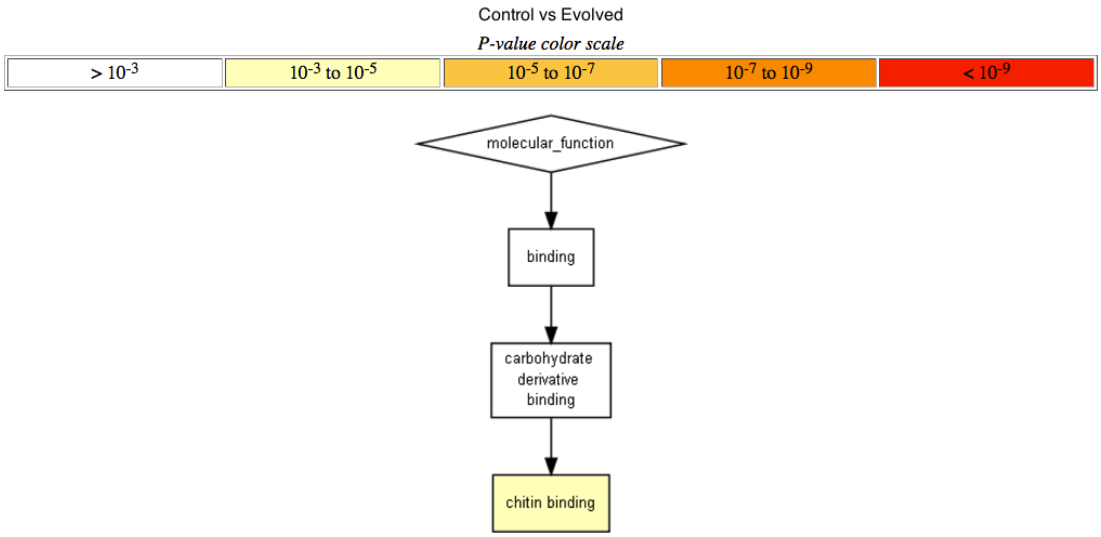


Figure 2.3.3.1.3 GO Experimental evolution Process Gorilla Flowchart. This flowchart shows the relationship between GO functions and depicts the p-value using the color scale on the top. It was generated simultaneously with GO Experimental Evolution Function Table 2.3.3.2 and is a different visual representation of the same results.

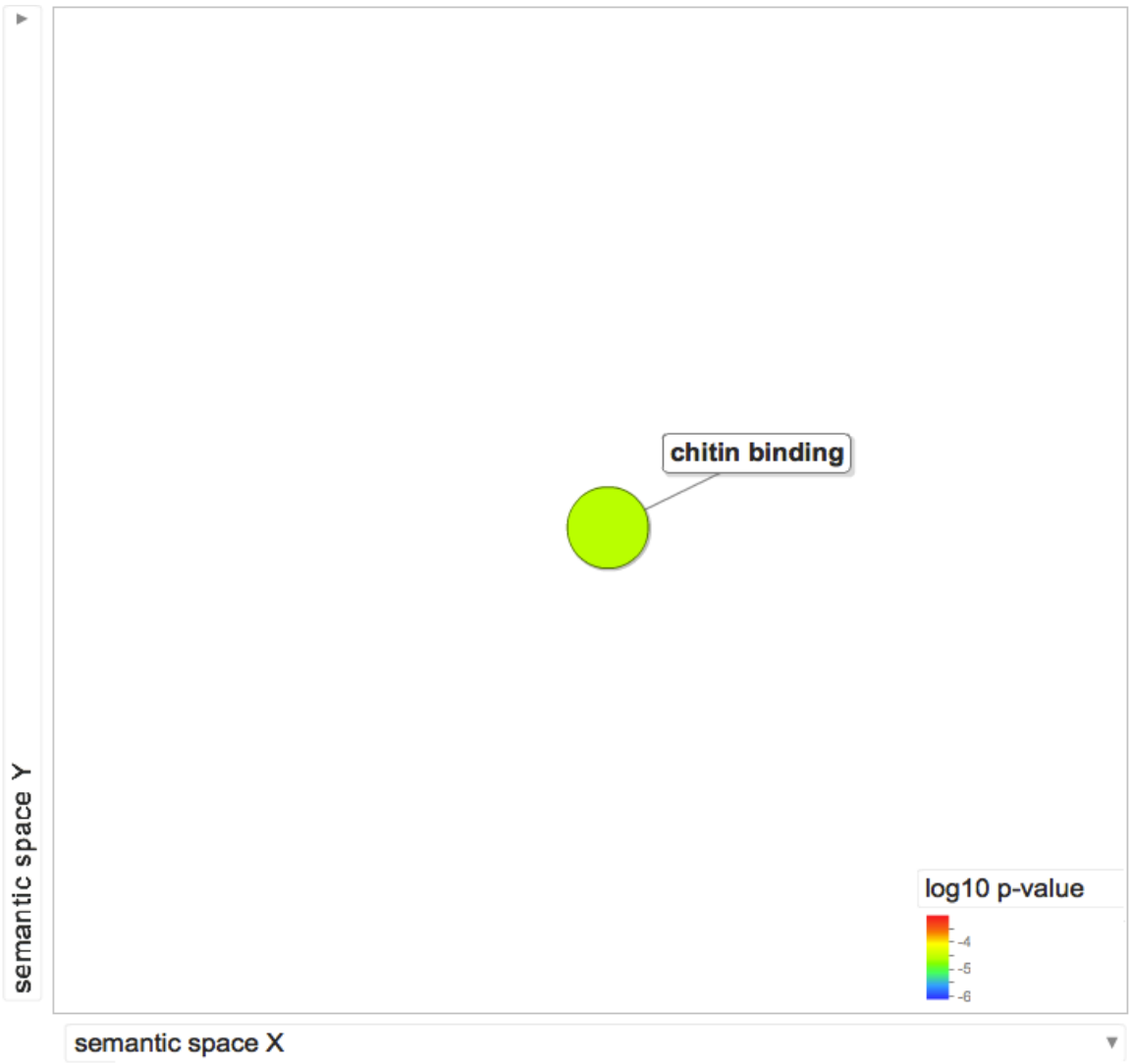


Figure 2.3.3.1.4 GO Experimental Evolution Function ReViGO Plot. This plot was generated using the data from GO Experimental Evolution Function Table 2.3.3.2 and it depicts the same results in a different visual representation. X and Y axes are irrelevant, and color coding denotes p-value as explained by the color legend.

2.3.3.2 Learning GO Process (Evolved vs Evolved Conditioned)

The main ontological processes observed after learning took place were translation related and sensory perception of smell related. It is important to point out the ribosomal protein genes involved with this translational difference upon learning, which are RpL38, RpS4, RpS30, RpL32, RpS2, RpL36A & RpS25 (Table *2.3.3.2.1)*.

2.3.3.2.1 GO Learning Process Table

Description	FDR q-value	Enrichment	Genes
cytoplasmic translation	6.52E-03	12.6	RpL38, RpS4, RpS30, RpL32, RpS2, RpL36A, RpS25
translation	6.19E-01	5.7	RpL38, RpS4, RpS30, RpL32, RpS2, RpL36A, RpS25
peptide biosynthetic process	4.13E-01	5.7	RpL38, RpS4, RpS30, RpL32, RpS2, RpL36A, RpS25
amide biosynthetic process	3.94E-01	5.48	RpL38, RpS4, RpS30, RpL32, RpS2, RpL36A, RpS25
peptide metabolic process	3.25E-01	4.69	RpL38, RpS4, RpS30, RpL32, RpS2, RpL36A, RpS25, CG40470
cellular amide metabolic process	4.90E-01	4.3	RpL38, RpS4, RpS30, RpL32, RpS2, RpL36A, RpS25, CG40470
sensory perception of smell	5.64E-01	10.01	Arr1, Obp56g, Obp56f, Obp56e

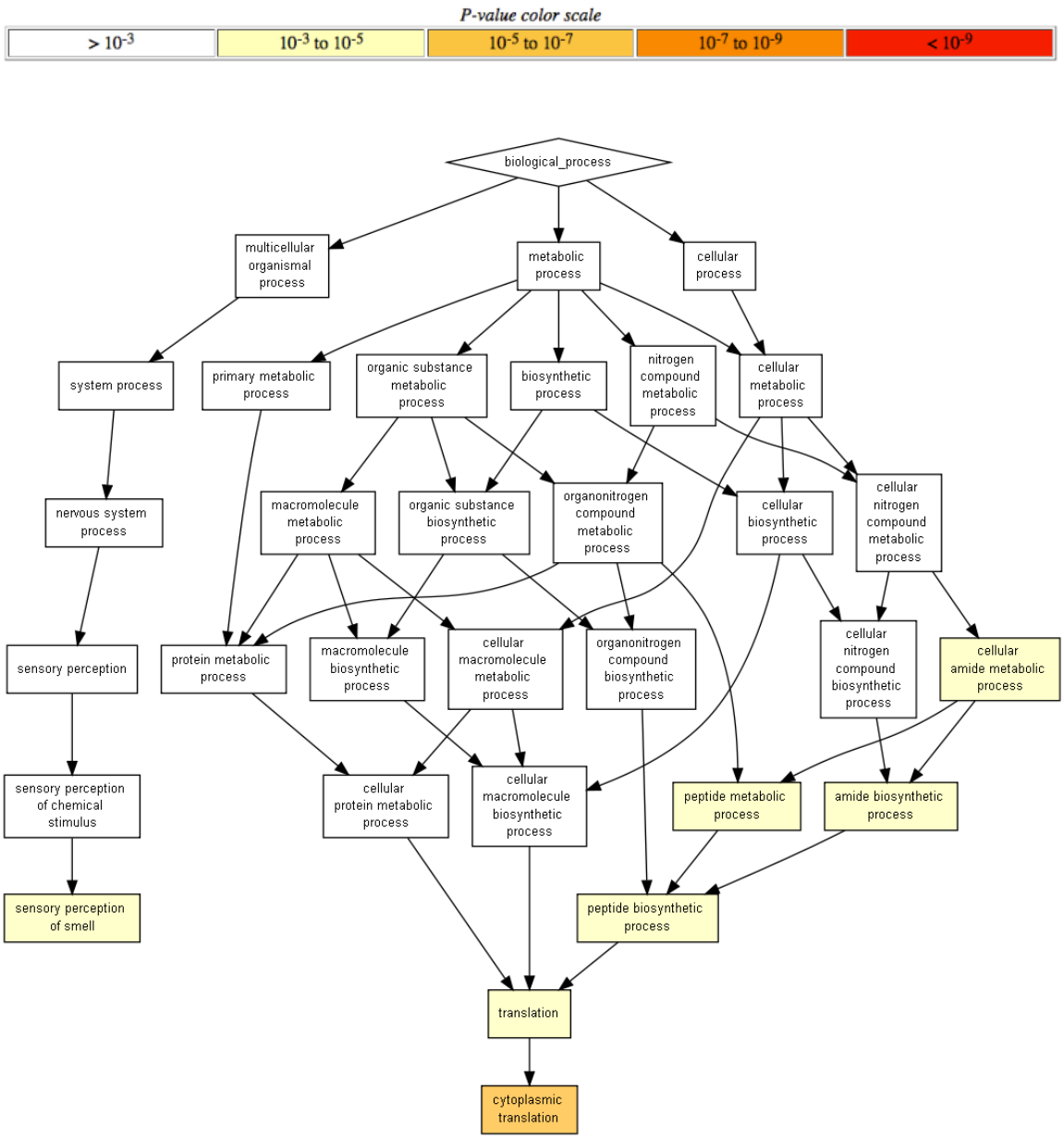


Figure *2.3.3.2.1* GO Learning-Process Gorilla Flow Chart. This chart shows the relationship between GO processes and depicts the p-value using the color scale on the top. It was generated simultaneously with GO Learning Process Table *2.3.3.2.1* and is a different visual representation of the same results.

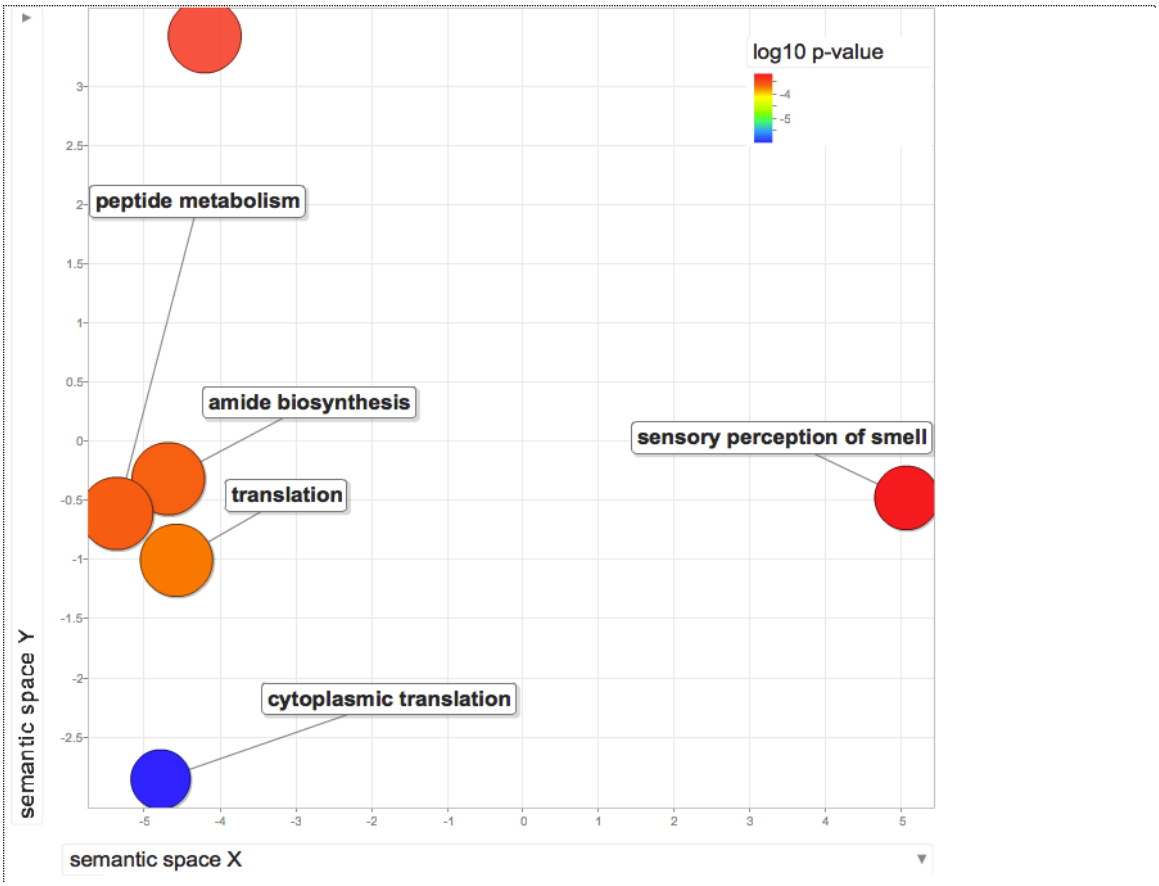


Figure *2.3.3.2.2* GO Learning Process ReViGO Plot. This plot was generated using the data from GO Learning Process Table *2.3.3.2.1* and it depicts the same results in a different visual representation. X and Y axes are irrelevant, and color coding denotes p-value as explained by the color legend.

Regarding the ontological functions observed after learning took place, several structural constituents of the ribosome were pointed out. Ribosomal proteins are extremely abundant and often come up as significant in RNA-Seq experiments reason why these could potentially be false positives. Regardless, the ribosomal protein genes RpS4, RpS30, RpL32, RpS2, RpL36A, RpS25 came up as significantly differentially expressed. Additionally, carboxylic-ester hydrolase activity was shown, with the genes CG10175, bmm, Glt, CG5966, being associated (Table 2.3.3.2).

2.3.3.2.2 GO Learning Function Table

Description	FDR q-value	Enrichment	Genes
structural constituent of ribosome	1.35E-01	6.84	RpS4, RpS30, RpL32, RpS2, RpL36A, RpS25
carboxylic ester hydrolase activity	9.96E-02	10.69	CG10175, bmm, Glt, CG5966,

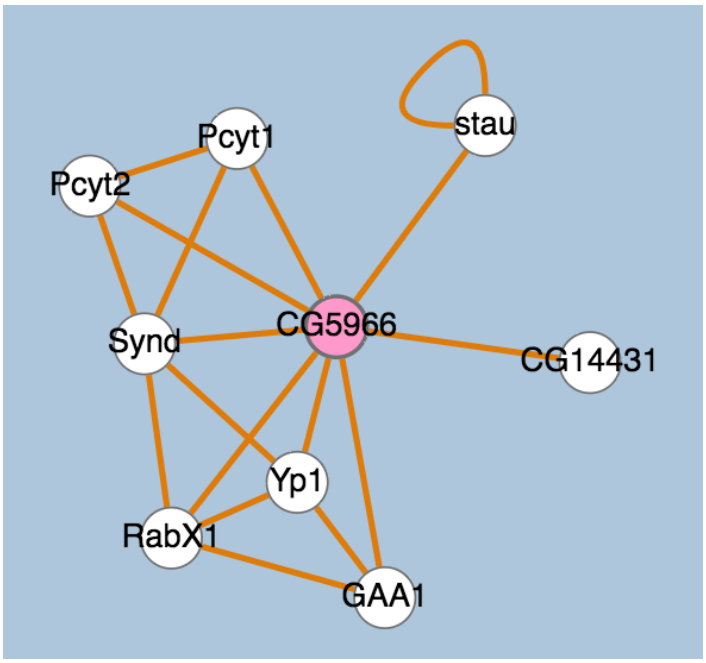


Figure 2.3.3.2.3: Easy Network Diagram. This diagram shows the relationship of the candidate gene involved in the act of learning, CG5966, with the Staufen protein pathway, which is a very well known pathway involved in LTM (20). CG5966 encodes for an mRNA that when folded has affinity to the Staufen protein coding sequence (88).

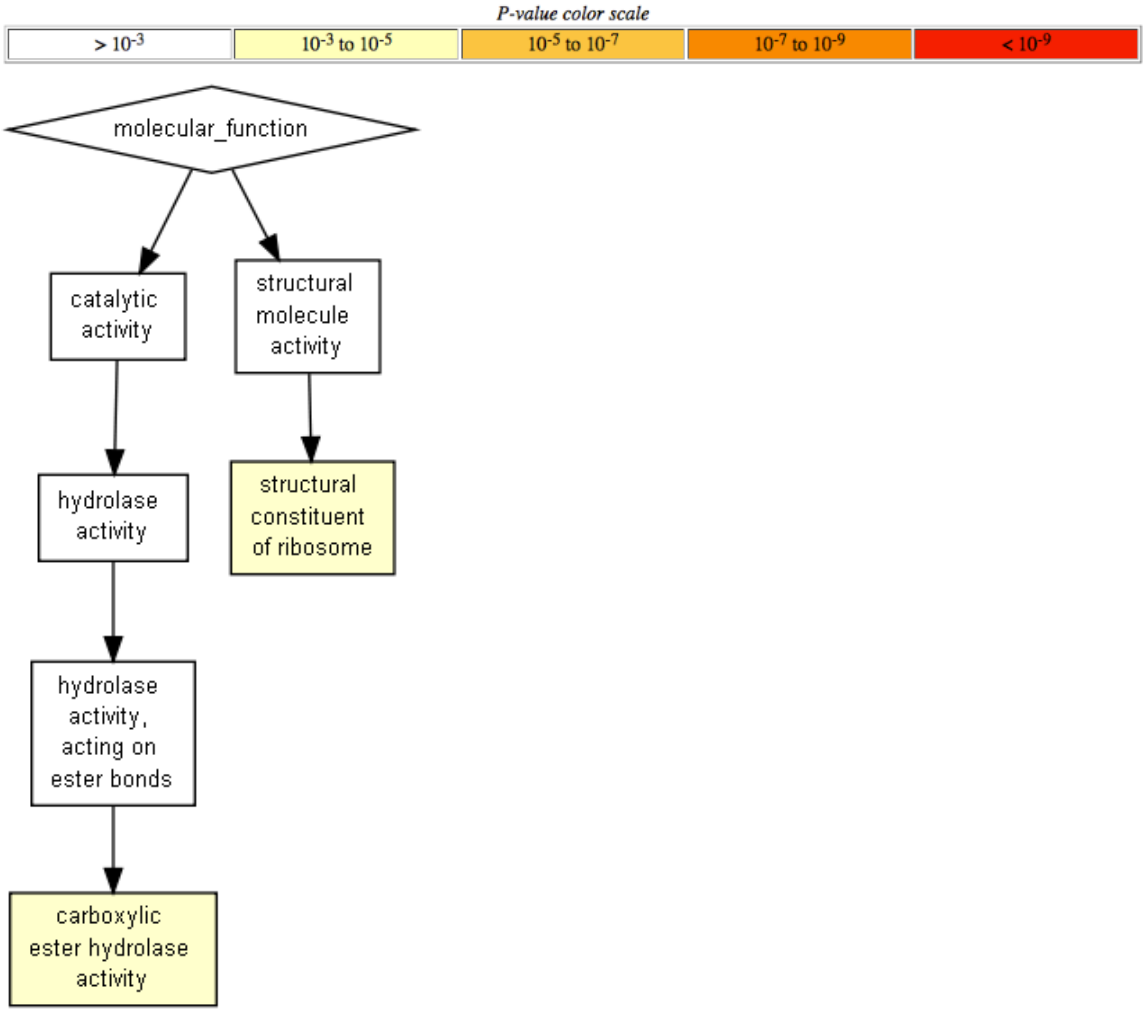


Figure 2.3.3.2.4 GO Learning Function Gorilla Flow Chart. This chart shows the relationship between GO functions and depicts the p-value using the color scale on the top. It was generated simultaneously with GO Learning Function Table 2.3.3.2 and is a different visual representation of the same results.

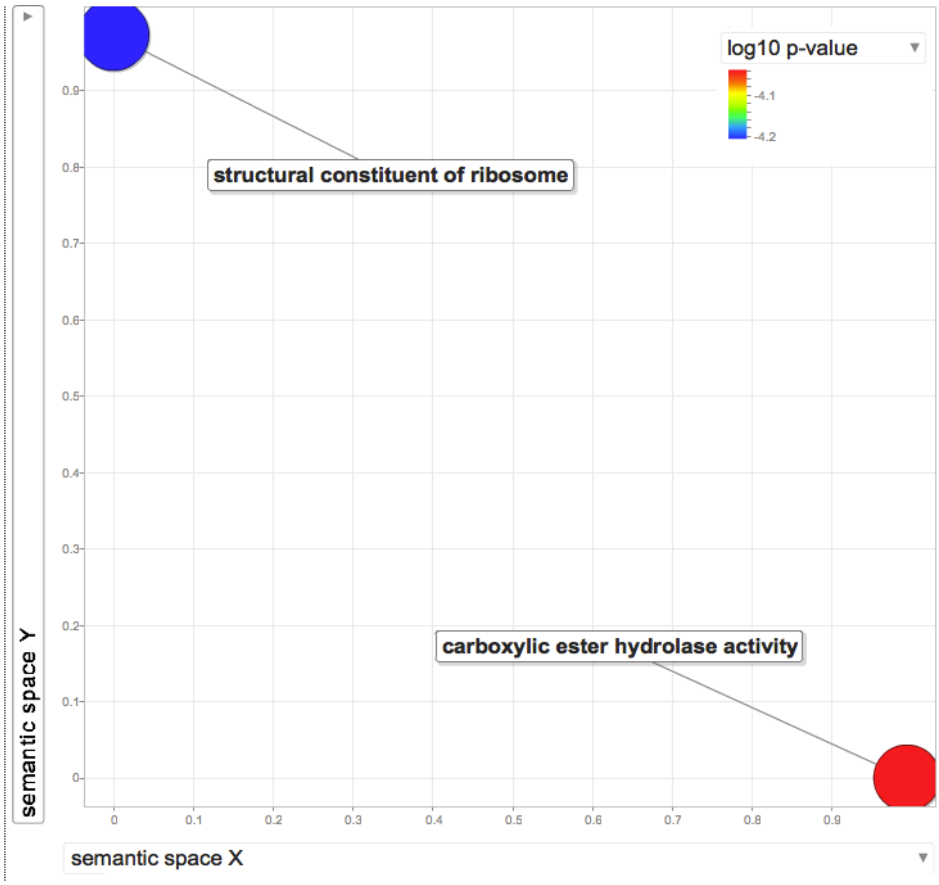


Figure *2.3.3.2.5* GO Learning Function ReViGO Plot. This plot was generated using the data from GO Learning Function Table 2.3.3.2 and it depicts the same results in a different visual representation. X and Y axes are irrelevant, and color coding denotes p-value as explained by the color legend.

2.3.4 qPCR

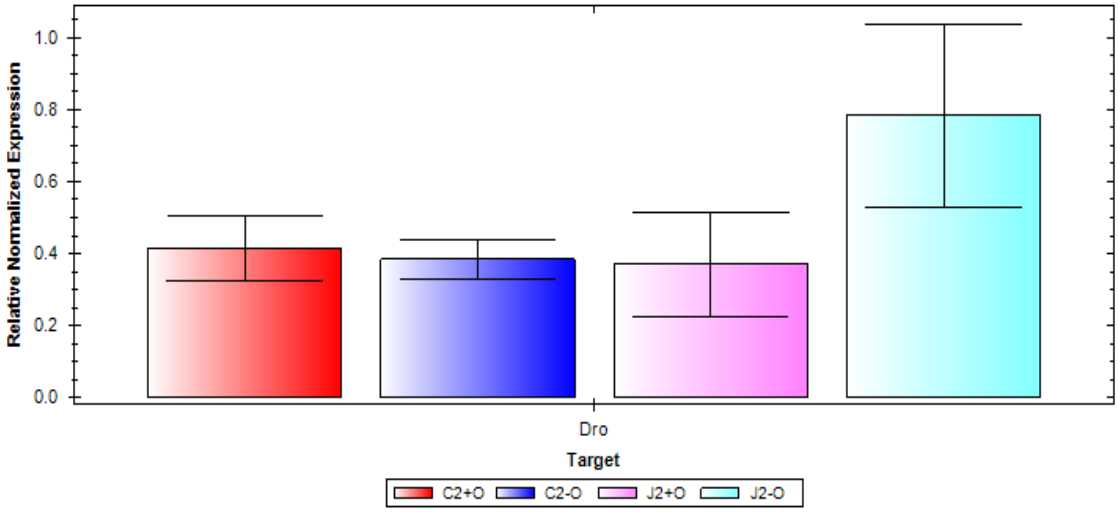


Figure 2.3.4.1 Dro qPCR. Shows the difference in Drosocin (Dro) gene expression on control populations C2 and evolved J2. Controls before learning (C2-O Blue) have a certain expression which increases upon experimental evolution treatment (J2-0 Teal). Although after learning its expression decreases (J2+O Pink).

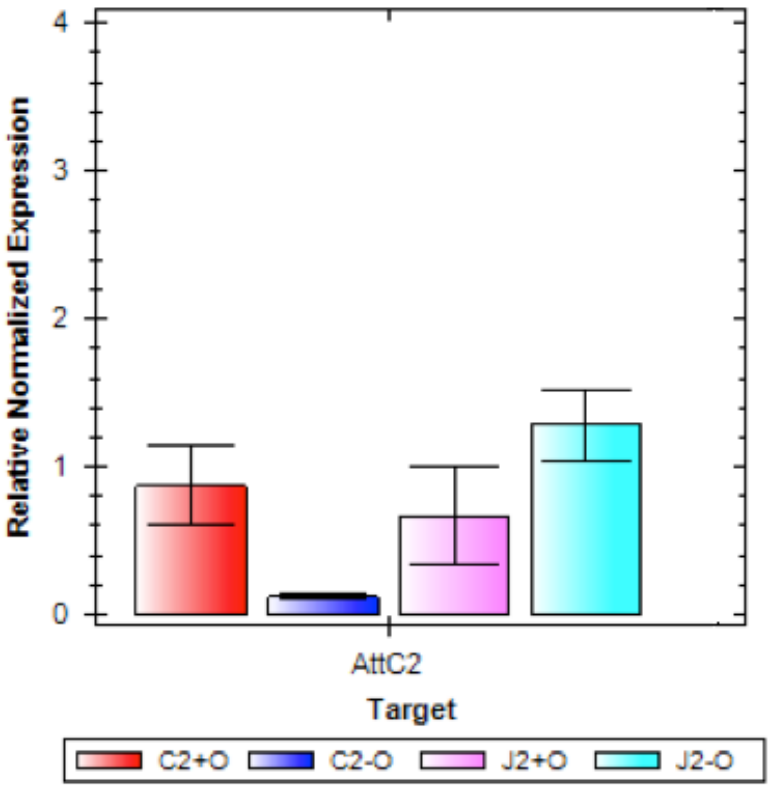


Figure 2.3.4.2 AttC qPCR. Depicts the changes in gene expression of the Attacin C (AttC) gene, on both Controls (C2) and evolved (J2) populations, Controls before learning (C2-O Blue) show a certain expression level whereas the evolved (J2-O Teal) show a much greater expression before learning, The evolved populations after learning (J2+O) show a decrease in the expression of this immune related gene.

2.3.5 Infection Mortality Assay

The results of the statistical analysis can be found in table 2.3.5.1. Here we find a significant effect of time: mortality changed with time. We also find a significant interaction between time and the evolutionary history: flies in control lines died more frequently with time than flies in evolved populations.

Table 2.3.5.1 Repeated Measures Analysis of Variance (ANOVA) of infection mortality.

Effect	Repeated Measures Analysis of Variance (Spreadsheet in Workbook1) Sigma-restricted parameterization Effective hypothesis decomposition; Std. Error of Estimate: 3.4947 **SS**	Degr. of Freedom	MS	F	p
Intercept	114.5280	1	114.5280	9.37751	0.092137
Var1	131.6909	1	131.6909	10.78280	0.081556
Error	24.4261	2	12.2130		
TIME	52.7981	31	1.7032	2.56050	0.000822
TIME*"Var1"	73.4018	31	2.3678	3.55969	0.000010
Error	41.2405	62	0.6652		

Effects of greater mortality due to oral bacterial infection were evident in multiple instances on the control populations as suggested by the RNA-Seq results. The experimental evolution treatment may have aided in the evolution of a stronger innate immune response observed on the experimental populations. (Figure 2.3.5.1).

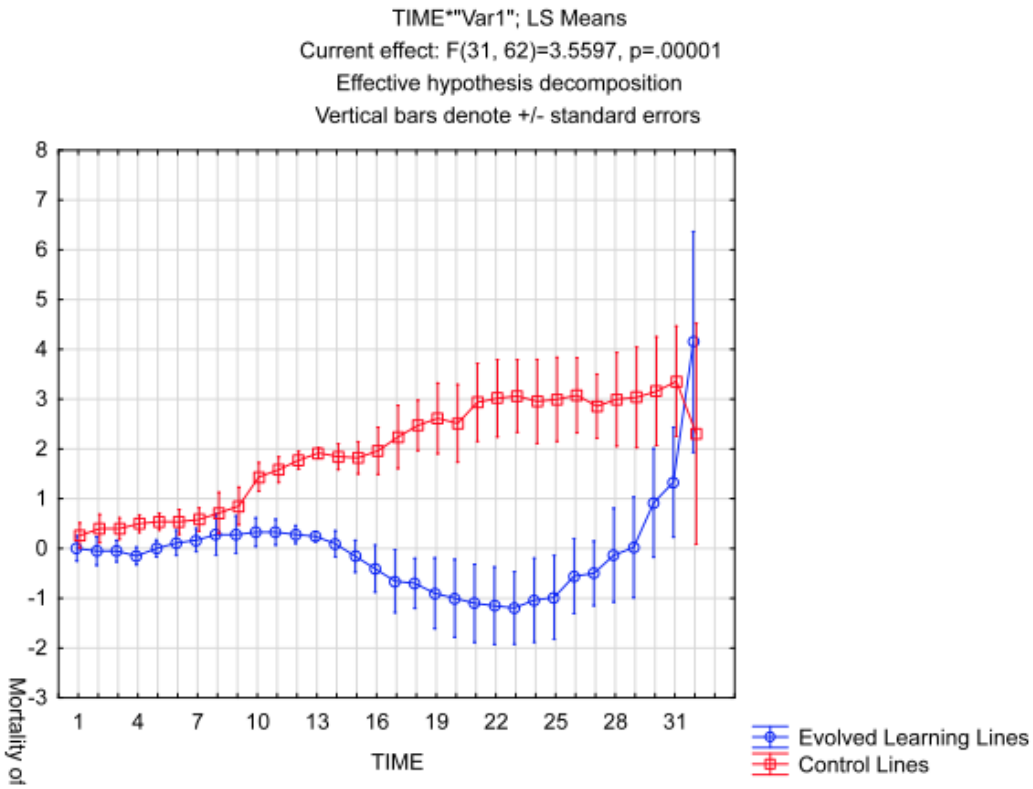


Figure 2.3.5.1 Infection mortality Assay. The red scatter plot depicts mortality of control populations, while the blue scatter plot depicts mortality of experimentally evolved populations. Control populations showed a significantly greater mortality due to oral bacterial infection with *P.aeruginosa* (PA01).

2.3.6 Experimental evolution for higher learning novel and notable DE gene table (Controls vs Evolved)

Novel genes marked with (-)

gene_id	gene	locus	p_value	q_value
XLOC_000963	CG16826	chr2L:13347772-13349020	5.00E-05	0.0276053
XLOC_003789	AttC	chr2R:9281209-9286873	5.00E-05	0.0276053
XLOC_003885	Dro	chr2R:10633465-10634219	5.00E-05	0.0276053
XLOC_004225	Dpt	chr2R:14753269-14753765	5.00E-05	0.0276053
XLOC_004226	DptB	chr2R:14754635-14755890	5.00E-05	0.0276053
XLOC_005009	CG34220-DCBP4	chr2R:5920792-5937571	5.00E-05	0.0276053
XLOC_005345	Cyp6a8	chr2R:10774675-10776515	5.00E-05	0.0276053
XLOC_005965	CG3906	chr2R:19398808-19399748	5.00E-05	0.0276053
XLOC_006438	- Col	chr2RHet:3264997-3265575	5.00E-05	0.0276053
XLOC_007184	CG14125-CDBP1, CG43896-CDBP2	chr3L:11967436-11976864	5.00E-05	0.0276053
XLOC_009600	- Vil	chr3LHet:482665-483521	5.00E-05	0.0276053
XLOC_009685	CG14645-*DCBP3*	chr3R:160600-165636	5.00E-05	0.0276053

XLOC_010547	CG34282	chr3R:14520973-14521504	5.00E-05	0.0276053
XLOC_010656	TotA	chr3R:16692570-16697735	5.00E-05	0.0276053
XLOC_010657	TotC	chr3R:16698709-16699302	5.00E-05	0.0276053
XLOC_012855	CG7567	chr3R:25412958-25413826	5.00E-05	0.0276053
XLOC_013370	- Mar	chrM:12733-14057	5.00E-05	0.0276053
XLOC_015950	- Isa	chrX:21539025-21539933	5.00E-05	0.0276053
XLOC_015980	CR41602	chrXHet:184013-196434	5.00E-05	0.0276053

2.3.6 Novel genes

2.3.6.1 Gene id: XLOC_006438

Gene Name: (-) Novel unnamed gene #1, now "Collana" (COL).

Locus: chr2RHet:3264997-3265575

CG41363 is a gene that has been withdrawn from flybase. We have transcripts of that gene on both controls and evolved populations. Although, we only have the read TCONS_00017446 on the evolved populations. This is because it is one of the most significantly differentially expressed novel constructs. Based on the pattern of constructs obtained, there is resemblance of TCONS_00017446 and TCONS_00017447 being two exons alternatively spliced out on the controls but conserved on the evolved lines (see Figure 2.4.1.2).

Further proteomic analysis will be performed on the sequence encoded in TCONS_00017446 and TCONS_00017447, plus further genomic analysis on the potential novel gene experimentally evolved for higher learning formerly annotated as CG41363 that we will now refer to as *Collana (CO*L).

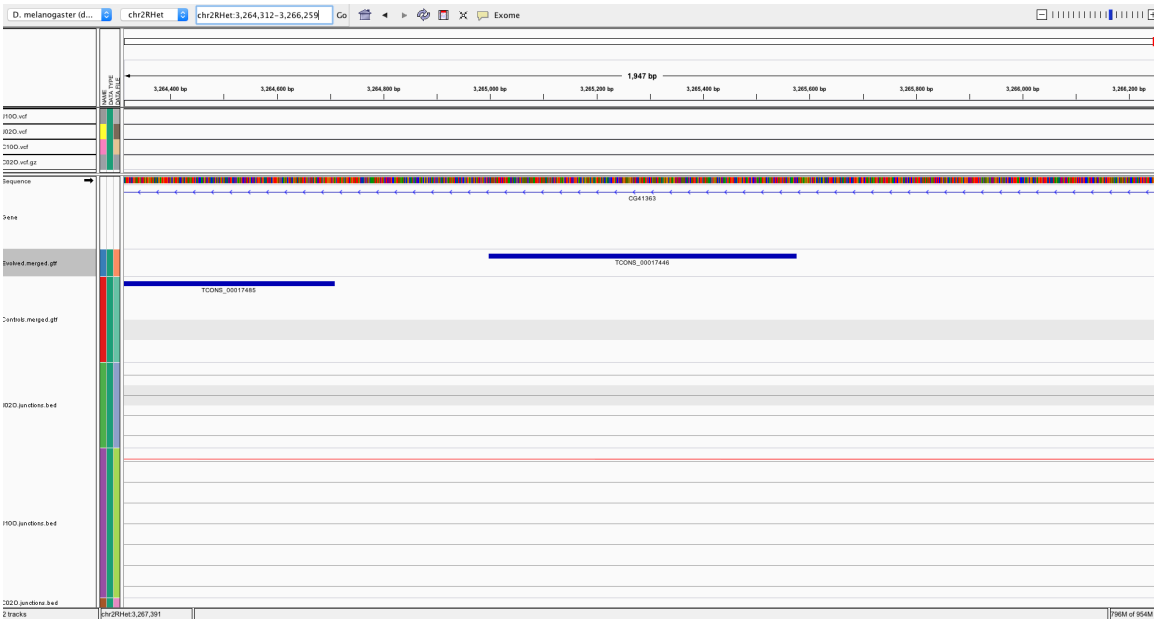


Figure 2.3.6.1. TCONS_00017446. Depicts the DE construct.

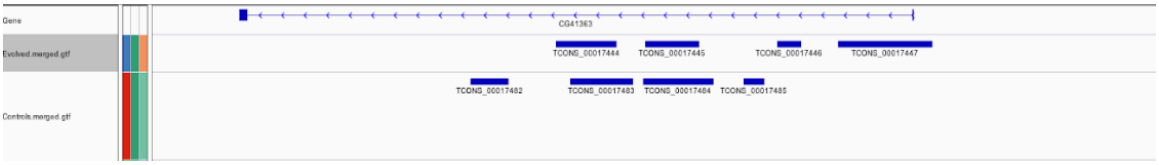


Figure 2.3.6.2. Collana.Shows a broader genomic area of the construct on figure 2.3.6.1, allowing for the visualization of adjacent reads captured when sequencing, exposing what resembles to be a gene potentially differentially spliced on the evolved populations than on the controls.

2.3.6.2 Gene id: XLOC_009600

Gene Name: (-) Novel unnamed gene #2, now Vilca (VIL)

Locus: chr3LHet:482665-483521

It appears that there are two novel genes in this region. One that encodes 5'→3' and one that encodes 3'→5'. The 3'→5' is the one most differentially expressed, with construct TCONS_00026107 being expressed on the evolved but not controls.

On the evolved, 3'→5' seems to be composed of TCONS_00026109,260108, 26107, 26064, 26063, 26062 and 260106. On the controls the 3'→5' is composed of TCONS_00026217,26216,26173,26172,26171.

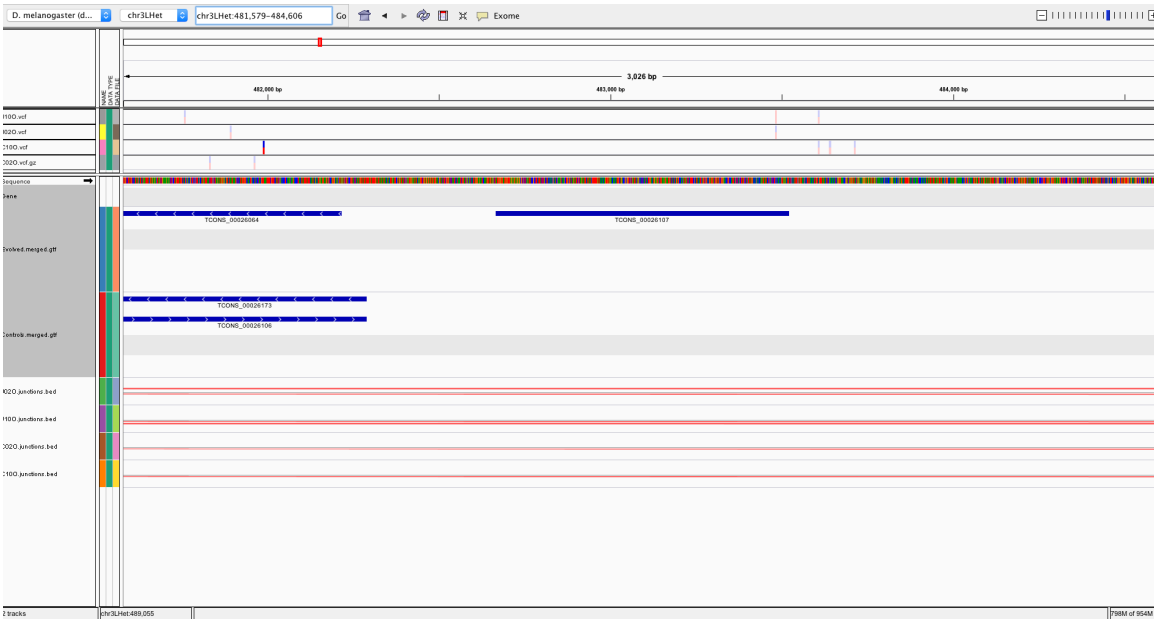


Figure 2.3.6.2.1 TCONS_00026107. Depicts in blue the DE construct TCONS_00026107 on evolved populations but not on controls.

Figure 2.3.6.2.2 Vilca. Shows the adjacent genomic region from construct TCONS_00026107. Unveiling other constructs resembling exons from the same gene but differentially spliced in evolved population than controls. Notice the genome reference does not have a gene annotated on such loci.

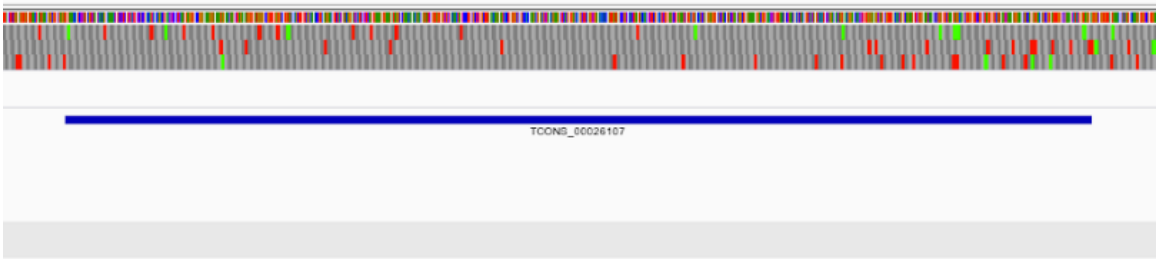


Figure 2.3.6.2.3 Vilca TSS. Depicts in blue the DE construct from a very close perspective allowing one to observe the codon possibilities based on the genomic sequence. There are initiation codons depicted in bright green coding 3’->5’ being a potential translationtion start sites (TSSs).

2.3.6.3 Gene ID: XLOC_013370

Gene Name: (-) Novel unnamed gene #3, now Marko (MAR)

Locus: chrM:12733-14057

In this case we see the differential splicing of construct TCONS_00037059 and 37058 on the controls, but constructs are conserved on the evolved (Figure 2.3.6.3.1). There is evidence of a TSS that matches the 5'→3' sense (Figure 2.3.6.3.2).

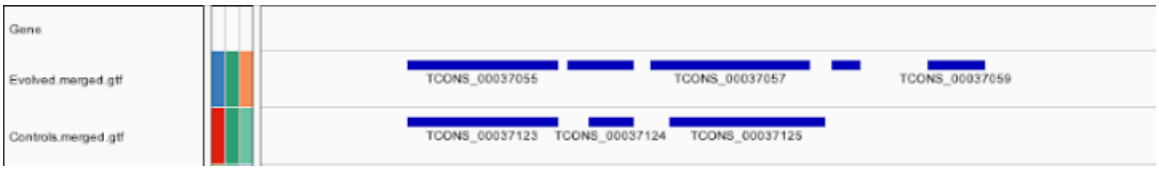


Figure 2.3.6.3.1. TCONS_00037059. Depicts in blue the DE constructs TCONS_00037059 and 37058 on evolved populations but not on the controls.

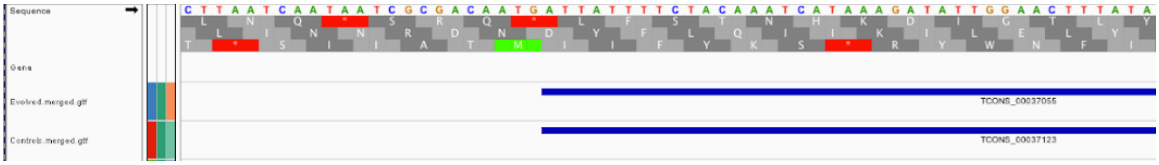


Figure 2.3.6.3.2. Marko TSS. Depicted in green a potential TSS on the first exon of the novel construct set on a 5'-->3' sense.

2.3.6.4Gene ID: XLOC_015950

Gene Name: (-) Novel unnamed gene #4, now Isa

Locus: chrX:21539025-21539933

This transcript seems to be a single coding gene not previously annotated, but that has been lost on the evolved lines.

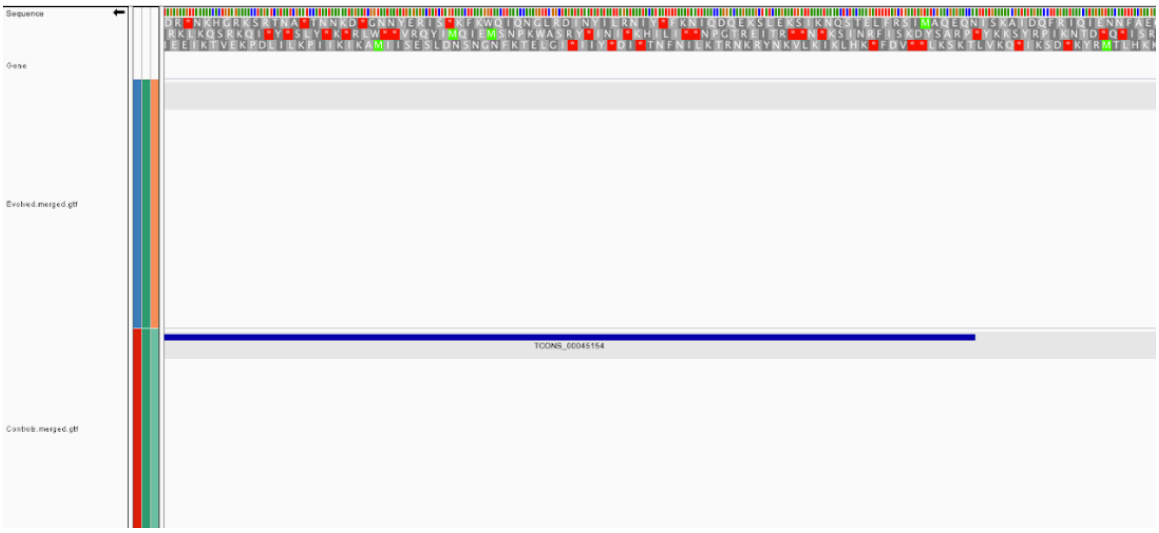


Figure 2.3.6.4.1 Isa. Depicts the 3’ end of the DE construct and allows for the visualization of a potential translation TSS when coded 3’→5’.

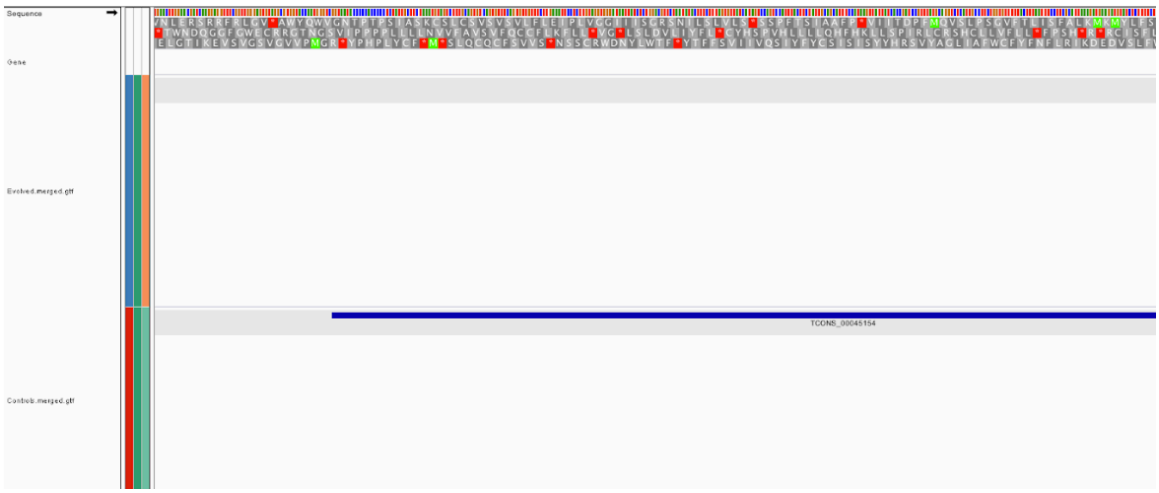


Figure 2.3.6.4.2. Isa TSS. Depicts the 5’ end of the DE construct and allows for the viualization of a potential translation start site when coding 5’→3’.

2.3.7 Chitin binding genes

2.3.7.1 Gene ID: XLOC_007184

Gene name: CG14125 & CG43896, now Dunlap Chitin Binding Protein #1 & #2

Locus: chr3L:11967436-11976864

Controls use a different TSS than the evolved populations. This is a Chitin binding protein and the longer transcript observed on controls, potentially leads to the chitin binding domain blocked or somehow disturbed when protein is folded.

Figure 2.3.7.1.1. DCBP1. Depicts the DE construct and shows the longer isoform on the controls.

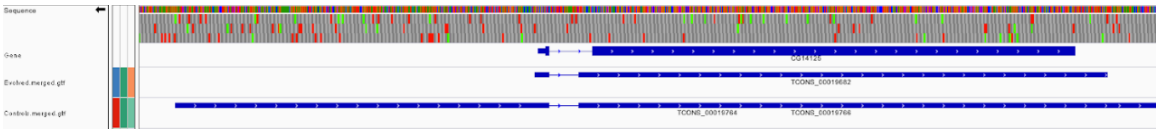


Figure 2.3.7.1.2. DCBP1 TSS. Depicts both isoforms observed on controls and evolved populations. It also allows for the visualization of the translation start site.

2.3.7.2 Gene ID: XLOC_009685

Gene Name: CG14645, now Dunlap Chitin Binding Protein #3 (DCBP3)

Locus: chr3R:160600-165636

This is another Chitin Binding Protein, and controls show a construct much longer than the actual encoded gene, which could have its chitin binding capacity truncated or compromised somehow.

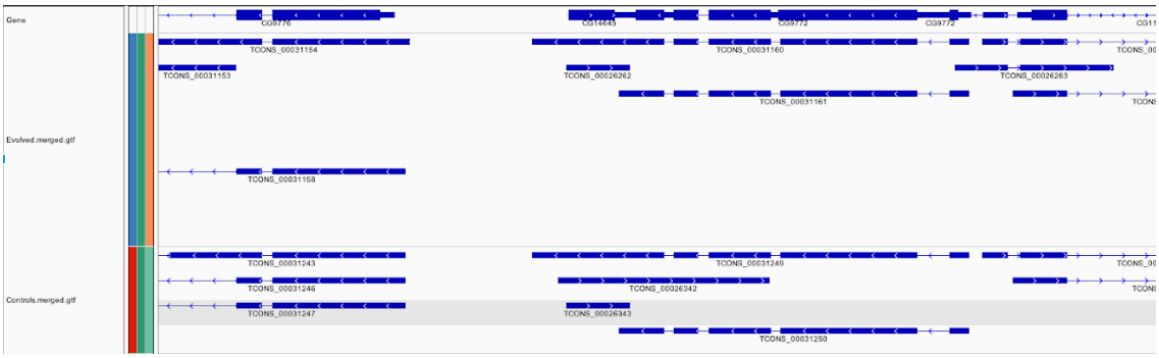


Figure 2.3.7.2.1 DCBP3. Depicts the DE construct TCONS_0026262 which is normaly expressed on the evolved population but on the controls seems to be a longer isoform which could again have the chitin binding domain compromised when protein folding.

2.3.7.3 Gene ID: XLOC_005009

Gene Name: CG34220, now Dunlap Chitin Binding Protein #4 (DCBP4)

Locus: chr2R:5920792-5937571

Apparently, the controls have a different isoform of this chitin binding protein, potentially truncating the chitin-binding domain in turn affecting its activity. The gene CG43220 is expressed three times more in the evolved lines.

Figure *2.3.7.3.1*. DCBP4. Depicts the DE constructs observed on both control and evolved populations, showing a different isoform present on the controls potentially affecting the chitin binding domain and affecting its activity.

2.3.7.4 Gene ID: XLOC_010547

Gene Name: CG34282, now Dunlap Chitin Binding Protein 5 (DCBP5)

Locus: chr3R:14520973-1452150

This chitin binding protein has 3 single nucleotide polymorphisms (SNPs) on J10. It would be interesting to check if the mutations fall in the chitin-binding domain or if they facilitate the chitin binding capacity in any way.

Figure *2.3.7.4.1*. DCBP5. Depicts the potential single nucleotide polymorphisms SNPs only on the evolved J10 but not controls.

2.3.7 Learning novel and notable DE gene table (Evolved vs Evolved Conditioned)

gene_id	gene	locus	p_value	q_value
XLOC_000017	Hop	chr2L:295121-297449	5.00E-05	0.00978727
XLOC_000065	Lsp1beta	chr2L:898499-901316	5.00E-05	0.00978727
XLOC_000620	Glt	chr2L:8672662-8679812	5.00E-05	0.00978727
XLOC_001047	CG34166	chr2L:14743192-14743879	5.00E-05	0.00978727
XLOC_001240	Arr1	chr2L:18078268-18081307	5.00E-05	0.00978727
XLOC_001411	CG2617	chr2L:20682367-20714599	5.00E-05	0.00978727
XLOC_001524	CG1416	chr2L:21757852-21795447	5.00E-05	0.00978727
XLOC_002087	Rack1	chr2L:7821033-7827405	5.00E-05	0.00978727
XLOC_002101	RpL36A	chr2L:8041105-8042909	5.00E-05	0.00978727
XLOC_002211	RpS2	chr2L:9896264-9897552	5.00E-05	0.00978727
XLOC_003282	CG8343	chr2R:2064522-2068224	5.00E-05	0.00978727

XLOC_003528	trpl	chr2R:5641201-5653023	5.00E-05	0.00978727
XLOC_003789	AttC	chr2R:9281018-9286873	5.00E-05	0.00978727
XLOC_004219	Dpt	chr2R:14753269-14753765	5.00E-05	0.00978727
XLOC_004220	DptB	chr2R:14754895-14755672	5.00E-05	0.00978727
XLOC_004279	Obp56e	chr2R:15599599-15600437	5.00E-05	0.00978727
XLOC_004692	RpL38	chr2R:402892-403963	5.00E-05	0.00978727
XLOC_005585	CG43202	chr2R:14267775-14269611	5.00E-05	0.00978727
XLOC_005616	CG15096	chr2R:14695860-14704000	5.00E-05	0.00978727
XLOC_005674	Obp56g	chr2R:15670971-15671548	5.00E-05	0.00978727
XLOC_005969	CG10332,IM18	chr2R:19488436-19489296	5.00E-05	0.00978727
XLOC_006242	-	chr2R:11589835-11590261	5.00E-05	0.00978727
XLOC_006659	Hsp83	chr3L:3192968-3197631	5.00E-05	0.00978727
XLOC_006988	Cp15	chr3L:8721580-8722166	5.00E-05	0.00978727
XLOC_007198	RpL10Ab,snoRNA:Psi18S-920	chr3L:11815950-11819002	5.00E-05	0.00978727

XLOC_007226	Lsp2	chr3L:12122492-12124969	5.00E-05	0.00978727
XLOC_007286	RpS4	chr3L:13034855-13037334	5.00E-05	0.00978727
XLOC_007935	-	chr3L:24527385-24527997	5.00E-05	0.00978727
XLOC_008514	Hsp26	chr3L:9368479-9370527	5.00E-05	0.00978727
XLOC_008671	Muc68D	chr3L:11760318-11765261	5.00E-05	0.00978727
XLOC_008990	Dbp73D,PGRP-SB1	chr3L:16717777-16723918	5.00E-05	0.00978727
XLOC_009107	CG3819	chr3L:18913529-18915208	5.00E-05	0.00978727
XLOC_009666	-	chr3LHet:493182-493910	5.00E-05	0.00978727
XLOC_010424	CG5399	chr3R:11520962-11522864	5.00E-05	0.00978727
XLOC_010686	CG4783	chr3R:15754998-15834234	5.00E-05	0.00978727
XLOC_010735	RpS30	chr3R:16676237-16677149	5.00E-05	0.00978727
XLOC_010985	Ela	chr3R:20690853-20693456	5.00E-05	0.00978727
XLOC_011033	CG5107,CR43457,CR45651	chr3R:21327305-21331769	5.00E-05	0.00978727
XLOC_011133	CG31077	chr3R:22822662-22889921	5.00E-05	0.00978727

XLOC_011220	CG33346,CG9989	chr3R:24494943-24497750	5.00E-05	0.00978727
XLOC_011370	CG18673	chr3R:27088319-27089600	5.00E-05	0.00978727
XLOC_011835	RpS25	chr3R:7040749-7045786	5.00E-05	0.00978727
XLOC_011919	Hsp70Ba	chr3R:8291174-8293498	5.00E-05	0.00978727
XLOC_012342	ninaE	chr3R:15711976-15713928	5.00E-05	0.00978727
XLOC_012457	CG31174,CG34148,CR45046,fit	chr3R:17690889-17710369	5.00E-05	0.00978727
XLOC_012596	Hsp68	chr3R:19880015-19883212	5.00E-05	0.00978727
XLOC_012945	CG7829	chr3R:25642459-25643486	5.00E-05	0.00978727
XLOC_012968	RpL32	chr3R:25869062-25872039	5.00E-05	0.00978727
XLOC_013166	-	chr3R:9865529-9866117	5.00E-05	0.00978727
XLOC_013213	-	chr3R:20970950-20971352	5.00E-05	0.00978727
XLOC_013925	CG5966	chrX:5882823-5886673	5.00E-05	0.00978727
XLOC_014057	GIIIspla2	chrX:8046980-8053646	5.00E-05	0.00978727
XLOC_014218	Pa1	chrX:11252871-11262142	5.00E-05	0.00978727

XLOC_014354	Rtc1,Yp3	chrX:13651194-13655989	5.00E-05	0.00978727
XLOC_014524	CG4928	chrX:16807212-16834906	5.00E-05	0.00978727
XLOC_000669	Mco1	chr2L:9416587-9426363	0.0001	0.0160687
XLOC_001806	CG16712	chr2L:3696217-3696658	0.0001	0.0160687
XLOC_001984	Tig	chr2L:6412314-6423310	0.0001	0.0160687
XLOC_002063	CG15818	chr2L:7410908-7412229	0.0001	0.0160687
XLOC_002627	CG5953,mir-4943,snoRNA:Me28S-C1237	chr2L:16508077-16532877	0.0001	0.0160687
XLOC_004838	CG30497,CG45093	chr2R:3624521-3670142	0.0001	0.0160687
XLOC_009141	CG9451	chr3L:19491296-19493898	0.0001	0.0160687
XLOC_010327	CG9297	chr3R:9597216-9605039	0.0001	0.0160687
XLOC_011459	CG43131	chr3R:934399-934900	0.0001	0.0160687
XLOC_012017	-	chr3R:9866305-9867449	0.0001	0.0160687
XLOC_012556	CG10175	chr3R:19347985-19356708	0.0001	0.0160687
XLOC_014797	CG13360,CR44469	chrX:678305-685119	0.0001	0.0160687

XLOC_000214	CG3523	chr2L:3056372-3144836	0.00015	0.0221219
XLOC_002487	NimB2	chr2L:13963506-13967299	0.00015	0.0221219
XLOC_004843	cathD	chr2R:3709615-3711074	0.00015	0.0221219
XLOC_006259	-	chr2R:14905725-14906154	0.00015	0.0221219
XLOC_013133	-	chr3R:5905596-5906102	0.00015	0.0221219
XLOC_014191	CG2145	chrX:10820299-10823788	0.00015	0.0221219
XLOC_005343	Cyp6a8	chr2R:10774675-10776515	0.0002	0.0279636
XLOC_008342	BG642312,CG42714	chr3L:6225561-6227182	0.0002	0.0279636
XLOC_008844	bmm	chr3L:14769595-14779512	0.0002	0.0279636
XLOC_013352	-	chr3RHet:2368982-2369528	0.0002	0.0279636
XLOC_007568	CG7589	chr3L:17463226-17467617	0.00025	0.0340696
XLOC_011268	Obp99a,ppk19,ppk30	chr3R:25490500-25501143	0.00025	0.0340696
XLOC_001867	hoe1	chr2L:4908580-4932384	0.0003	0.0393878
XLOC_004280	Obp56f	chr2R:15600899-15601481	0.0003	0.0393878

XLOC_009381	-	chr3L:798617-799293	0.0003	0.0393878
XLOC_003105	-	chr2L:14485046-14485525	0.00035	0.0428193
XLOC_003472	CR44274	chr2R:4830999-4831800	0.00035	0.0428193
XLOC_004193	CG45087	chr2R:14424271-14426921	0.00035	0.0428193
XLOC_011453	CG2016	chr3R:782720-787073	0.00035	0.0428193
XLOC_013302	CG40198	chr3RHet:2503742-2506152	0.00035	0.0428193
XLOC_013908	CG12239	chrX:5686172-5688576	0.00035	0.0428193
XLOC_009359	CG40470	chr3L:23826868-23983366	0.0004	0.0473231
XLOC_010632	CG14297,Rh2	chr3R:14723842-14726741	0.0004	0.0473231
XLOC_013041	chp	chr3R:27022635-27036452	0.0004	0.0473231

2.4 Discussion

We found a large number of differentially expressed genes both in comparison of evolved high learning flies and their control lines, and how expression of genes changed after conditioning. Gene ontology analyses revealed expected patterns from learning, but also unexpected strong effects of immune function and chitin binding function. An experimental comparison of high learning lines and control lines under infection by a bacterial pathogen revealed that this difference in expression of immune genes is also functional: high learning lines of flies survived longer than control flies.

A total of four potential novel genes have been identified and placeholder names have been assigned. Each of these potential novel genes show different isoform expression patterns on both evolved and control populations and also potential translation start sites (TSS). (See 2.3.6 Novel genes). Also, total of five chitin binding proteins have been identified and placeholder names have been assigned. Each of these chitin binding proteins show different isoform expression patterns (See 2.3.7 chitin binding genes).

Upon DE analysis, it became evident that innate immunity was being affected by the experimental evolution (see Figure 2.3.2.1).

Once GO analyses were complete, it became clear that the experimental

evolution treatment for greater learning also led to the co-evolution of greater innate immunity and humoral immune response, supporting the positive evolutionary relationship between learning and immunity formerly observed in experimentally evolved populations of *D.melanogaster* and *B.terrestris* (91, 92). Even more interestingly, once learning was observed, innate immunity became repressed on the experimental populations, which could be the first molecular evidence for a cognition-innate immunity energetic trade-off (see figure 2.3.2.1).

Additionally, through the GO analysis other functions and processes were unveiled. Several chitin-binding proteins have been pinpointed as the sole ontological function differentially expressed on the experimental populations. These findings at first were confusing, as chitin molecules were not expected to be found within *D.melanogaster* brains. However, it has been shown that higher eukaryotes do express chitinases and chitin synthetases for the biosynthesis and degradation of hyaluronic acid, which seems to require a chitin primer when synthesized (55, 56, 57) (see Figures 2.3.3.1.1, 2, 3 & 4). It has also been shown that chitin has a neurodegenerative effect. It tends to agglomerate in neurons and has been found in Alzheimer's disease (AD) brain samples (58, 59, 60). These data together suggest that the chitin-binding proteins expressed on the greater learning populations may provide some sort of neuro-protection from chitin agglomeration, perhaps ensuring the proper functioning of neurons and aiding in the greater cognitive capacities observed.

Furthermore, when observing the ontological results of the experimentally evolved population before and after learning, it was revealed that in regards to processes, there is greater translation, peptide metabolism and sensory perception of smell upon learning. This makes sense as the learning events will trigger gene expression, and the learning is taking place through olfaction. With regard to function, structural constituents of the ribosome and carboxylic ester hydrolase activity were revealed upon learning in the experimental populations (see Figures 2.3.3.2.1, 2 ,4 & 5).

Structural constituents of the ribosome are known to have an effect on the fidelity of translation (62,63). Five structural constituents of the ribosome have been determined to be expressed upon learning: ribosomal protein s4, L38, S30, L32, S2, L36A and S25. To our knowledge, this is the first time these specific structural components of the ribosome have been associated with learning. At the same time, its important to point out the fact that due to their ubiquitious expression, ribosomal proteins often come as significant although they may be false positive reuslts. Carboxylesterases (CarEs) are a family of enzymes known to be involved in the process of insecticide detoxification in insects (61). CarEs have also been associated with pheromone, cholesterol and fatty acid metabolism, as well as with heroin and cocaine (64). The main genes related are brummer (*bmm)* and Glutactin (*Gln)*. *Brummer* is a known lipase that regulates fat storage and availability in *D. melanogaster*. It is known to lead to obesity when repressed and to deplete fat reservoirs when over expressed (65). It is possible that proper

phospholipid biosynthesis and storage may confer a greater neuronal connectivity due to better insulation. Glutactin is a CarEs that resembles an Acetylcholine esterase but lacks the catalytic serine residue (66). It is possible that through this mechanism of arresting Acetylcholine, its availability is being fine-tuned. Additionally, the gene CG5966 was associated with the CarEs GO function and is a gene encoding for a lipase, but its RNA has been co-immunoprecipitated with the Staufen protein (88). The relationship was addressed through the easy Network (esyN) tool based on former publications (88,89). The Staufen/Pumillio pathway have been already determined to play a role in LTM, and the interactions of these RNAs may play a role in the fine-tuning of its availability or range of interactions (20).

Our discoveries regarding the cognition/innate immunity co-evolution contradict those regarding artificial selection for relative brain size vs. innate immunity discovered in guppies (51). Because of the theoretical controversy, we further tested innate immunity by orally infecting both control and experimental populations with a *P.Aeruginosa* strain, PA01. This species was selected since it is known to produce PAMPs that are recognized by the Diptericin (Dpt) gene, which encodes for an AMP for systemic recognition and clearance *in D. melanogaster*. The Dpt gene is one of the innate immunity significantly DE genes in the experimental populations (54). The experimental populations showed significant greater innate immunity than

controls as suggested by the RNA-Seq results (see Figure 2.3.5.1).

2.4.1 RNA-Seq validation

qPCR was performed on cDNA reverse transcribed from RNA previously purified from 20 brains per sample collected following the same protocols than for the RNA-Seq. Gene expression patterns support the greater immunity observed upon experimental evolution for greater learning. Although, regarding the energetic trade off observed after learning, some immune genes had a different expression pattern. This could be due to the smaller sample size used per population in the validation leading to potential bias or non-representative population results since our populations were evolved from a wild caught population, from Fenn Valley MI, and have a great deal of variance. Due to the labor-intensive nature of the research and the time constraints posed by the timeline a Masters Thesis has, sample size was reduced in half to 20 brains per sample. There are a few elements to consider also, such as the fact that sample collection for RNA Seq took place through the fall of 2016 and the spring of 2017 whereas sample collection of brains for RNA-Seq validation took place during the fall of 2017. There is evidence of rapid seasonal evolution of innate immunity in *D. melanogaster,* which means that innate immunity may oscillate in 10 month cycles and should be accounted for in future experimental design furthering these discoveries (69). There is also evidence of decreased learning and increased fecundity during the fall and increased learning during the spring in

wild *D. melanogaster* populations (70). The sample collection timeline does coincide with the learning and innate immunity discoveries on wild populations of *D. melanogaster* and the trend observed in the results of the RNA-Seq and the validation through qPCR. Additionally there are small subtleties in the rearing of *D. melanogaster* populations that can have drastic effects. Subtleties such as food quality and egg per vial ratio are believed to have trans-generational effects on up to 5 generations in the future, and effects on parental investment made by the female at the time of oviposition (71,72). Those two variables are unfortunately subject to human error and one single mishap can have serious repercussions.

2.5 Future Directions

With regard to the chitin-binding neuroprotective hypothesis as an experimentally evolved trait for greater learning, further liquid-chromatography mass-spectrometry (LCMS) work could be of great value. If the chitin binding proteins are actually preventing the chitin primer from agglomerating in the neurons and that is having a neuro-protective effect on the flies conferring them greater learning, then the haemolymph of the evolved population could have greater levels of chitin primers than the controls. PhD Candidate Michael Manino from Dr. Alexei Demchenko's lab at the chemistry department at the University of Missouri-St. Louis devised a synthesis protocol for a chitin primer to be used as a standard when observing the levels of chitin binding proteins in the haemolymph of both control and experimentally evolved populations. Once again due to time constraints I was not able to execute those experiments. I look forward to someone accomplishing this future direction.

In another direction, average relative brain size per population will be determined with the aid of immuno-histochemistry and confocal microscopy. This is currently in progress at the Dunlap lab under Cell and Molecular Biology Masters Student Mladen Senicar. Through confocal microscopy and immunohistochemistry, chitin binding domains can be localized in both experimental and control populations using an anti-Chitin Binding Domain

(anti-CBD) Monoclonal Antibody as the primary antibody. Also, technology has allowed for mRNA localization and visualization through tagged cDNA probes targeting the mRNAs of interest (80). Hopefully someone will observe where these mRNAs and chitin binding domains are localizing as well as their relative concentrations within the MB.

In regard to the novel and notable genes listed and discussed, each candidate in my opinion is worth further dissection through novel genome editing tools, such as CRISPR-Cas9, to further understand their actual function and involvement in the capacity to learn and the actual process of learning.

One cost-effective and potential way to investigate gene function could be through the oral delivery of custom designed polyamides for the genes of interest. It has been shown to be an effective treatment for gene expression control allowing for the gain or loss of gene function in *D.melanogaster* (81). Dr. James Bashkin from the chemistry department at the University of Missouri-St. Louis is an expert in the polyamide field and an advisor eager to support furthering these discoveries and able to design and synthesize these polyamides to target the genes of interest.

Further testing of the innate immunity-learning trade-offs observed in the RNA-Seq is already in progress under Biochemistry and Biotechnology

Masters student Jill Lee as her Masters Thesis.

Thanks to Dr. Lon Chubiz's guidance and generous support, the DNA libraries for Whole Genome Sequencing (WGS) of the experimentally evolved populations and their controls is also in progress. This will allow us to perform other kinds of genomic analyses and compare with the transcriptomic data unveiled in this work.

The furthest this study can be taken would be re-evolving all populations making certain changes in the experimental evolution protocol in order to account for noise sources that caused trouble in this study. Sources of noise such as the wild populations with high variability, exposure to unknown volatiles through the cocktails of organic volatiles present in the orange and pineapple odors used and exposure to potential different sugar sources as potential sources of bacterial exposure. The flies themselves carry an extensive microbiome and under different conditions such as rich caloric agar media bacterial growth conditions can be optimal for exponential growth and un-balances to take place, all potentially affecting the evolution of *D. melanogaster* innate immunity (82).

Designing artificial wild type populations with known allelic frequencies based on the percentage of females present and carrying specific alleles of interest in each population could be great for future work. Like this, we could

isolate different mechanisms through which learning may evolve and we will be able to further dissect the subtle effects based on the known genomic past. This can now be achieved thanks to the Drosophila Genome Reference Panel (DGRP). The DGRP lines have been fully sequenced and are available to researchers. Also, if high-throughput automation is available, selective pressure should rather be used under the associative aversive and appetitive olfactory paradigm using an automated T-maze instead of evolving them under the oviposition paradigm. As matter of fact, as an RA in the Dunlap lab, I built and furthered the automated T-maze as in Jiang, H. 2015 (83). More units can be replicated and experimental evolution under this alternate paradigm may be closer to reality.

2.6 References

1.- Spurzheim, J.G.The Physiognomical System of Dr. Gall and Spurzheim. Baldqan, Cradock and Joy, London, 1815.

2.Pavlov, I.P. Conditioned Reflexes . Oxford University Press, London, 1927.

3. Bouton,M.E.,and Peck,C.A.(1989).Context effects on conditioning ,extinction, and reinstatement in an appetitive conditioning preparation. Anim.Learn. Behav. 17,188–198. doi:10.3758/BF03207634

4. Koch,M.,Schmid,A.,and Schnitzler,H.-U.(1996).Pleasure-attenuation of startle

is disrupted by lesions of the nucleus accumbens. Neuroreport 7,1442–1446.

doi:10.1097/00001756-199605310-00024

5. Isabel, G. and Preat, T. Molecular and System Analysis of Olfactory Memory

in Drosophila. Learning and Memory a comprehensive reference. Chapter 4.0.7. Elsevier. 2008

6. Hebb, D.O. The Organization of Behavior. John Wiley & Sons, New York, 1949.

7.Mc Gaugh, J., Searching for Memory in the Brain: Confronting

the Collusion of Cells and Systems. Bertoni, F. Neural Plasticity and Memory: From Genes to Brain Imaging, Frontiers in Neuroscience, Taylor & Francis

Group LLC. 2007.

8. Scoville, W.B. and Milner, B. Loss of recent memory after bilateral hippocampal lesions. J. Neurol. Neurosurg. Psychiatr., 20, 11, 1957.

9. Quinn WG, Harris WA, and Benzer S (1974) Conditioned behavior in Drosophila melanogaster. Proc. Natl. Acad. Sci.USA 71: 708–712.

10. Dudai, Y.; Jan, Y. N.; Byers, D.; Quinn, W. G.; Benzer, S. (1976). "Dunce, a Mutant of Drosophila Deficient in Learning". Proceedings of the National Academy of Sciences. 73 (5): 1684–1688. Bibcode:1976PNAS...73.1684D. doi:10.1073/pnas.73.5.1684

11. Menzel R, Erber J, and Masuhr T (1974) Learning and memory in the honeybee. In: Barton-Brown L (ed.) Experimental Analysis of Insect Behaviour, pp. 195–217. Berlin: Springer.

12. Erber J, Masuhr T, and Menzel R (1980) Localization of short-term memory in the brain of the bee Apis mellifera. Physiol.Entomol. 5: 343–358.

13. Heisenberg, M., Borst, A., Wagner, S. & Byers, D. Drosophila mushroom body mutants are deficient in olfactory learning. J. Neurogenet. 2, 1–30 (1985).

14. Quinn, W.G., and Dudai, Y. (1976). Memory phases in Drosophila.

Nature 262, 576–577.

15. Tully, T., Preat, T., Boynton, S. C. & Del, V. M. Genetic dissection of consolidated memory in Drosophila. Cell 79, 35–47 (1994).

16. Tempel, B.L., Bonini, N., Dawson, D.R., and Quinn, W.G. (1983).

Reward learning in normal and mutant Drosophila. Proc. Natl. Acad. Sci. USA 80, 1482–1486.

17. Byers D, Davis RL, and Kiger JA Jr (1981) Defect in cyclic AMP phosphodiesterase due to the dunce mutation of learning in Drosophila melanogaster. Nature 289: 79–81.

18. Davis RL and Kiger JA Jr (1981) Dunce mutants of Drosophila melanogaster: Mutants defective in the cyclic AMP phosphodiesterase enzyme system. J. Cell Biol. 90:101–107.

19. Livingstone MS, Sziber PP, and Quinn WG (1984) Loss of calcium/calmodulin responsiveness in adenylate cyclase of rutabaga, a Drosophila learning mutant. Cell 37: 205–215.

20. Dubnau, J. and Tully, T. (1998) Gene Discovery in Drosophila: New Insights for Learning and Memory. Annu. Rev. Neurosci. 1998. 21:407–44

21. Keene, A. and Wadell, S. Drosophila olfactory memory: single

genes to complex neural circuits. Nature Rev. Neuroscience. Volume 8, May 2007.

22. Moore, B. (2004) The evolution of learning. Biol. Rev. (2004), 79, pp. 301–335. Cambridge Philosophical Society 301

DOI: 10.1017/S1464793103006225

23.Mery, F. and Kawecki, T. Experimental evolution of learning ability in fruit flies. PNAS, October 29, 2002. vol.99. no.22. p.14277

24.Dunlap, A.S. and Stephens, D. Components of change in the evolution of learning and unlearned preference. Proc. R. Soc. B (2009) 276, 3201–3208 doi:10.1098/rspb.2009.0602

25.Tucker, T., Marra, M., Friedman, J.M. (2009). "Massively Parallel Sequencing: The Next Big Thing in Genetic Medicine". The American Journal of Human Genetics. 85 (2): 142–54. doi:10.1016/j.ajhg.2009.06.022. PMC 2725244. PMID 19679224

26. Croston,R., Branch,C.L., Kozlovsky, D.Y., Dukas, R., Pravosudov, V.V. et al. 2015. Heritability and the evolution of cognitive traits. Behav Ecol. 26: 1447–1459.

27. SmuldersTV. 2015. Interpreting measurements of heritability: comment on Croston et al. Behav Ecol. 26: 1461– 1462.

28. ThorntonAWilsonAJ. 2015. In search of the Darwinian Holy Trinity in cognitive evolution: comment on Croston et al. Behav Ecol. 26: 1460– 1461.

29. R. Croston C.L. Branch D.Y. Kozlovsky R. Dukas V.V. Pravosudov The importance of heritability estimates for understanding the evolution of cognition: a response to comments on Croston et al. Behavioral Ecology, Volume 26, Issue 6, 1 November 2015, Pages 1463–1464, https://doi-org.ezproxy.umsl.edu/10.1093/beheco/arv192

30.Schlötterer, C.,Kofler, R.,Versace, E., Tobler, R., Franssen, S.U. (2015) Combining experimental evolution with next-generation sequencing: a

powerful tool to study adaptation from... Heredity, Nature publishing group. DOI: 10.1038/hdy.2014.86

---

31. Versace, E. and Reisenberger, J. (2015) Large-scale assessment of olfactory preferences and learning in Drosophila melanogaster: behavioral and genetic components. PeerJ, 3:e1214; DOI 10.7717/peerj.1214

32. Grasso, P.; Gangolli, S.; Gaunt, Ian (2002). Essentials of Pathology for Toxicologists. CRC Press. ISBN 978-0-415-25795-4. Retrieved 30 August 2011.

33.Watson, F.L., Puttmann-Holgado, R., Thomas, F., Lamar, D.L., Hughes, M., Kondo, M., Rebel, V.I., Schmucker, D., 2005. Extensive diversity of Ig-superfamily proteins in the immune system of insects. Science 309, 1874–1878.

33.Muller, U., Vogel, P., Alber, G., Schaub, G.A., 2008. The innate immune system of mammals and insects. Contrib. Microbiol. 15, 21–44

34. Anderson, K.V., 2000. Toll signaling pathways in the innate immune response. Curr. Opin. Immunol. 12, 13–19.

35. Hoffmann, J.A., 2003. The immune response of Drosophila. Nature 426, 33-38.

36. Kimbrell, D.A., Beutler, B., 2001. The evolution and genetics of innate immunity. Nat. Rev. Genet. 2, 256–267.

37. Li, J. et al (2017) Achilles is a circadian clock-controlled gene that regulates immune function in Drosophila. Brain, Behavior, and Immunity 61

(2017) 127–136

38. Imler, J.L., Hoffmann, J.A., 2000. Signaling mechanisms in the antimicrobial host

defense of Drosophila. Curr. Opin. Microbiol. 3, 16–22

39. Hetru, C., Troxler, L., Hoffmann, J.A., 2003. Drosophila melanogaster antimicrobial

defense. J. Infect. Dis. 187 (Suppl 2), S327–S334

40. Aiello, L. and Wheeler, P., (1995) The Expensive-Tissue Hypothesis. The Brain and the Digestive System in Human and Primate Evolution. Current Anthropology. Volume 36, Number 2.

41.Isler, K., and C. van Schaik. 2006a. Costs of encephalization: the energy trade-off hypothesis tested on birds. Journal of Human Evolution. 51:228–243.

42. Pitnick, S., K. E. Jones, and G. S. Wilkinson. 2006. Mating system and brain size in bats. Proceedings of the Royal Society B 273:719–724.

43. Navarrete, A., van Schaik, C.P., and Isler, K. (2011). Energetics and the evolution of human brain size. Nature 480, 91–93.

44. Liao, W.B., Lou, S.L., Zeng, Y., and Kotrschal, A. 2016. Large Brains, Small Guts: The Expensive Tissue Hypothesis Supported within Anurans. The American Naturalist, vol. 1 8 8 , no. 6.

45.Tsuboi,M., A. Husby, A. Kotrschal, A. Hayward, S. D. Buechel, J. Zidar, H. Løvlie, and N. Kolm. 2015. Comparative support for the expensive tissue hypothesis: big brains are correlated with smaller gut and

greater parental investment in Lake Tanganyika cichlids. Evolution 69:190–200.

46.Kotrschal, A., B. Rogell, A. Bundsen, B. Svensson, S. Zajitschek, I. Brannstrom, S. Immler, A. A. Maklakov, and N. Kolm. 2013. Artificial selection on relative brain size in the guppy reveals costs and benefits of evolving a larger brain. Current Biology 23:168–171.

47. Kotrschal, A., Corral-Lopez, A., Zajitschek, S., Immler, S., Maklakov, A. and Kolm, N. 2015.Positive genetic correlation between brain size and sexual traits

in male guppies artificially selected for brain size. J . Evol. Biol . 2 8 (2 0 1 5) 8 4 1 – 8 5 0

48. Kotrschal, A., A. Corral-Lopez, M. Amcoff, and N. Kolm. 2014. A larger brain confers a benefit in a spatial mate search learning task in male guppies. Behavioral Ecology 26:527–532.

49.Kotrschal, A., S. D. Buechel, S. M. Zala, A. Corral-Lopez, D. J. Penn, and N. Kolm. 2015. Brain size affects female but not male survival under predation threat. Ecology Letters 18:646–652.

50. Kotrschal,A.,Rogell, B., Bundsen,A., Svensson,B., Zajitschek,S., Brannstrom,I., Immler, S., Maklakov,A., and Kolm, N., 2013. Artificial Selection on Relative Brain Size in the Guppy Reveals Costs and Benefits of Evolving a Larger Brain. Current Biology 23, 168–171

51.Kotrschal, A., N. Kolm, and D. J. Penn. 2016. Selection for brain size impairs innate, but not adaptive immune responses. Proceedings of the

Royal Society B 283:1826.

52. Eran Eden*, Roy Navon*, Israel Steinfeld, Doron Lipson and Zohar Yakhini. “GOrilla: A Tool For Discovery And Visualization of Enriched GO Terms in Ranked Gene Lists”, BMC Bioinformatics 2009, 10:48.

53.Eran Eden, Doron Lipson, Sivan Yogev, Zohar Yakhini. “Discovering Motifs in Ranked Lists of DNA sequences”, PLoS Computational Biology, 3(3):e39, 2007.

54. Fauvarqye, M. et al.(2002). Role and activation of type III secretion system genes in Pseudomonas aeruginosa-induced Drosophila killing Microbial Pathogenesis; 32: 287±295. doi:10.1006/mpat.2002.0504

55. Stern, R. Go Fly a Chitin: The Mystery of Chitin and Chitinases in Vertebrate Tissues. Front Biosci (Landmark Ed). 2017 Jan 1;22:580-595.

56. Varki, A. 1996. Does DG42 synthesize hyaluronan or chitin?: A controversy about oligosaccharides in vertebrate development. Proc. Natl. Acad. Sci. USA

Vol. 93, pp. 4523-4525, May 1996

57. Semino, C.E. and Robbins, P.E., 1995. Synthesis of “Nod”-like chitin oligosaccharides by the Xenopus developmental protein DG42. Proc. Natl. Acad. Sci. USA. Vol. 92, pp. 3498-3501

58.Turano, E. *et al.* (2015) Neurotoxicity and synaptic plasticity impairment of N-acetylglucosamine polymers: implications for Alzheimer’s disease. Neurobiology of Aging, 36. 1780-1791

59. Castellani, R.J., Perry, G., Smith, M.A., 2007. The role of novel chitin-like

polysaccharides in Alzheimer disease. Neurotox Res 12, 269e274.

60. Sotgiu, S., Musumeci, S., Marconi, S., Gini, B., Bonetti, B., 2008. Different content of chitin-like polysaccharides in multiple sclerosis and Alzheimer's disease brains. J. Neuroimmunol 197, 70e73

61.Gong, Y. H. et al. 2017. Functional characterization of carboxylesterase gene

mutations involved in Aphis gossypii resistance to organophosphate insecticides. Insect Molecular Biology (2017) 26(6), 702–714.

62. Kamath, D., O'connor, M. 2016. An investigation into the effects of alterations in ribosomal proteins S4 and S5 on translational accuracy, ribosomal RNA processing, and ribosome assembly. University of Missouri-Kansas City, MO. USA.

63. Agarwal D, Kamath D, Gregory ST, O'Connor M. 2015. Modulation of decoding fidelity by ribosomal proteins S4 and S5. J Bacteriol 197:1017–1025.

doi:10.1128/JB.02485-14.

64. Aranda, J. et al 2014. The Catalytic Mechanism of Carboxylesterases: A Computational Study. Biochemistry, 53 (36), pp 5820–5829

DOI: 10.1021/bi500934j

65. Kühnlein, R. P. et al. 2005. Brummer lipase is an evolutionary conserved fat storage regulator in Drosophila. Cell Metabolism Vol. 1. Elsevier.

DOI:10.1016/j.cmet.2005.04.003

66. Olson, P.F. et al. 1990. Glutactin, a novel Drosophila basement

membrane related glycoprotein with sequence similarity to serine esterases. The EMBO Journal vol.9 no.4 pp. 1 219 – 1227.

67.Chugani,S. et al. QscR, a modulator of quorum-sensing signal synthesis and virulence in Pseudomonas aeruginosa. Proc Natl Acad Sci U S A. 2001 Feb 27; 98(5): 2752–2757. doi: 10.1073/pnas.051624298

68. Apidianakis, Y., & Rahme, L.G., 2009. Drosophila melanogaster as a model host for studying Pseudomonas aeruginosa infection. Nature Protocols, Vol.4, No.9, 1285

68. Ponton, F. et al. 2011. Evaluation of potential reference genes for reverse transcription-qPCR studies of physiological responses in Drosophila melanogaster. Journal of Insect Physiology 57, 840–850

69. Behrman, E. et al. (2018). Rapid seasonal evolution in innate immunity of wild Drosophila melanogaster. Proc Biol Sci. Jan 10;285(1870). pii: 20172599. doi: 10.1098/rspb.2017.2599.

70. Behrman, E. L., Kawecki, T.J. and Schmidt, P. 2018.Natural variation in couch potato mediates rapid evolution of learning and reproduction in natural populations of Drosophila melanogaster. Biorxiv, doi:https://doi.org/10.1101/288696

71. Emborski, C. and Mikheyev, A. (2018). Ancestral diet leads to dynamic transgenerational plasticity for five generations in Drosophila melanogaster Bioarxiv, doi: https://doi.org/10.1101/273144

72. Crofton A.E., Cartwright E.L., Feitzinger A.A., Lott S.E. 2018. Effect of Larval Nutrition on Maternal mRNA Contribution to the Drosophila Egg. G3

(Bethesda). Apr 17. pii: g3.200283.2018. doi: 10.1534/g3.118.200283.

73. Dobin, A. et al. 2013. STAR: ultrafast universal RNA-seq aligner. Bioinformatics. Vol. 29 no. 1 , pages 15–21 doi:10.1093/bioinformatics/bts635

74. Trapnell, C. et al 2013. Differential analysis of gene regulation at transcript resolution with RNA-seq. Nature Biotechnology, Vol. 31, Iss. 1: 46-53.

75. Robinson, J.R. et al. 2011. Integrative Genomic Viewer. Nature Biotechnology Vol. 29, N.1.

76. Thorvaldsdottir,H., Robinson, J.T. and Mesirov, J.P. Integrative GenomicsViewer (IGV): high-performance genomics data visualization and exploration. Briefings in Bioinformatics. Vol 14. No 2. 178, 192 doi:10.1093/bib/bbs017

77.Supek F, Bošnjak M, Škunca N, Šmuc T. 2011. “REVIGO summarizes and visualizes long lists of Gene Ontology terms” PLoS ONE. doi:10.1371/journal.pone.0021800

78. Dunlap, A. S., & Stephens, D. W. (2014). Experimental evolution of prepared learning. Proceedings of the National Academy of Sciences of the United States of America, 111(32), 11750-11755. doi:10.1073/pnas.1404176111

79. Dunlap, A., Nielsen, M., Dornhaus, A., & Papaj, D. (2016). Foraging bumble bees weigh the reliability of personal and social information. Current Biology, 26(9), 1195-1199. doi:10.1016/j.cub.2016.03.009

80. Long, X. et al. 2017. Quantitative mRNA imaging throughout the entire Drosophila brain. Nature Methods, Vol.14, No.7. doi:10.1038/nmeth.4309

81. Jansen, S. et al 2000. Specific Gain- and Loss-of-Function Phenotypes Induced by Satellite-Specific DNA-Binding Drugs Fed to Drosophila melanogaster. Molecular Cell, Vol. 6, 1013–1024.

82. Adair, K. et al. 2018. Microbial community assembly in wild populations of the fruit fly Drosophila melanogaster. The ISME Journal 12:959–972 https://doi.org/10.1038/s41396-017-0020-x

83. Jiang, H. et al. 2015. A fully automated Drosophila olfactory classical conditioning and testing system for behavioral learning and memory assessment. Journal of Neuroscience Methods 261 (2016) 62–74.

84.Ekengrenab, S.and Hultmarka, D. (2001). A Family of Turandot-Related Genes in the Humoral Stress Response of Drosophila. Biochemical and Biophysical Research Communications. Vol. 284(4), 22: 998-1003 https://doi.org/10.1006/bbrc.2001.5067

85.Lang, M., Braun, C.L., Kanost, M.R., Gorman, M.J. (2012). Multicopper oxidase-1 is a ferroxidase essential for iron homeostasis in Drosophila melanogaster. Proc. Natl. Acad. Sci. U.S.A. 109(33): 13337--13342.

86.Kausik Si, Raquel Barajas Azpeleta, Jianping Wu, Jason Gill, Ryan Welte, Chris Seidel. Antimicrobial peptides modulate long-term memory. bioRxiv 328286; doi: https://doi.org/10.1101/328286

87.Taylor, S. et al. (2010). A practical approach to RT-qPCR—Publishing

data that conform to the MIQE guidelines.Methods 50 (2010) S1–S5. doi:10.1016/j.ymeth.2010.01.005

88.Laver, J.D., Li, X., Ancevicius, K., Westwood, J.T., Smibert, C.A., Morris, Q.D., Lipshitz, H.D. (2013). Genome-wide analysis of Staufen-associated mRNAs identifies secondary structures that confer target specificity. Nucleic Acids Res. 41(20): 9438--9460.

89.Bean DM, Heimbach J, Ficorella L, Micklem G, Oliver SG, Favrin G. (2014). esyN: Network Building, Sharing and Publishing. PLoS ONE 9(9): e106035.

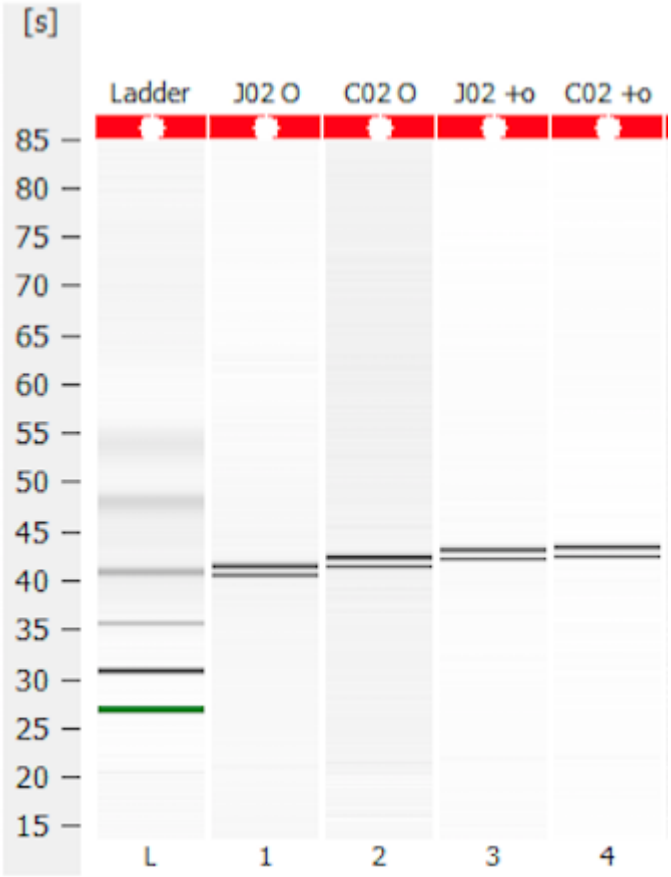
90. Lofdahl, K. L., Holliday, M., & Hirsch, J. (1992). Selection for conditionability in drosophila melanogaster. Journal of Comparative Psychology, 106(2), 172-183. doi:10.1037/0735-7036.106.2.172

91. Alghamdi, A., N. E. Raine, E. Rosato and E. B. Mallon (2009). No evidence for an evolutionary trade-off between learning and immunity in a social insect. Biology Letters 5: 55-57.

92. Kolss, M., Kraaijeveld, A. R., Mery, F., & Kawecki, T. J. (2006). No trade-off between learning ability and parasitoid resistance in drosophila melanogaster. Journal of Evolutionary Biology, 19(4), 1359-1363. doi:10.1111/j.1420-9101.2005.01068.x

2.7 Supplementary material

Supplementary Bioanalyzer Gel view of ribosomal bands in total RNA used for RNA Seq

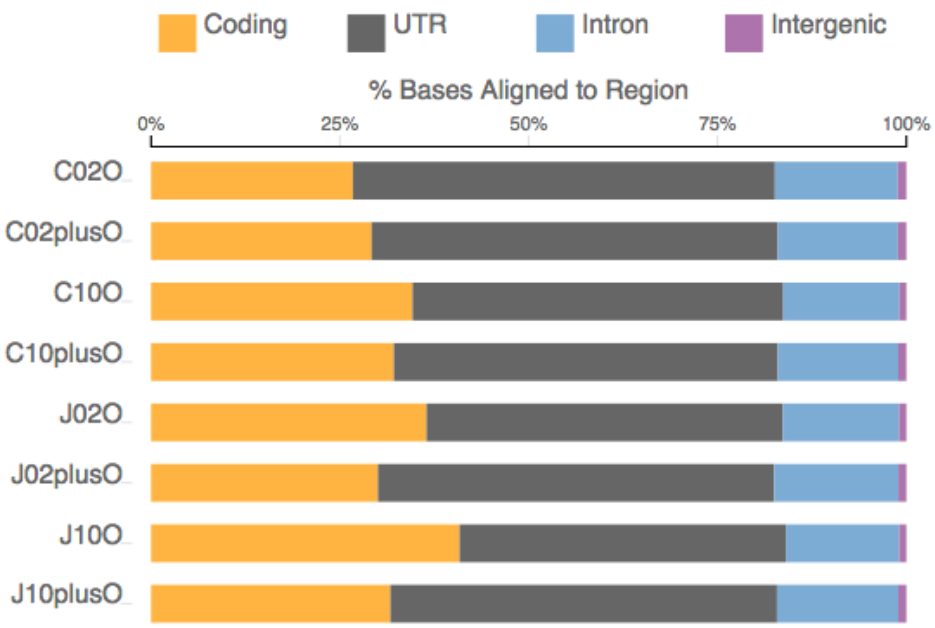


Suplemental Alignment Report:

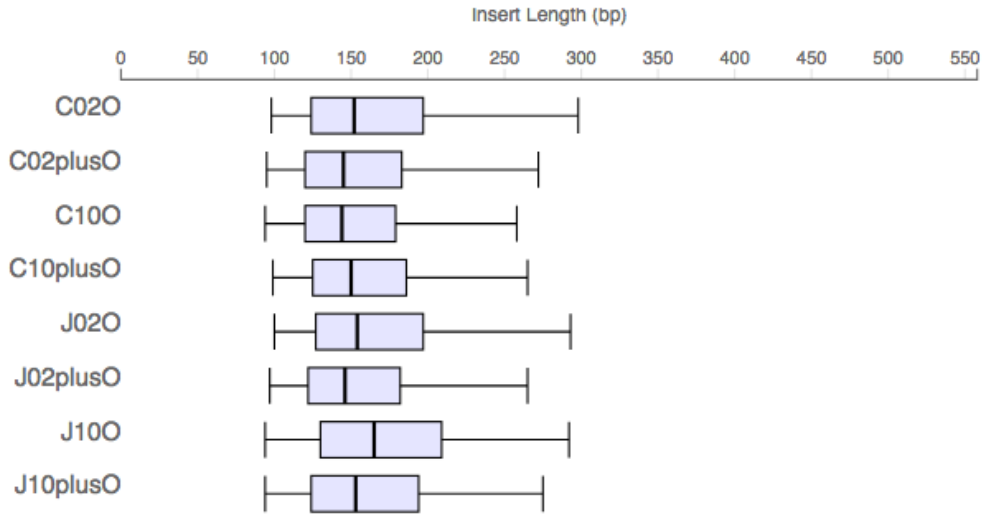
SUMMARY i

Sample ID	Read Length	Number of Reads	% Total Aligned	% Abundant	% Unaligned	Median CV Coverage Uniformity	% Stranded
C02O	76/76	44,142,946	95.85%	36.92%	4.15%	0.98	99.51%
C02plusO	76/76	35,168,937	97.28%	13.58%	2.72%	1.06	99.54%
C10O	76/76	53,030,716	96.35%	8.91%	3.65%	0.74	99.58%
C10plusO	76/76	57,139,811	97.19%	6.83%	2.81%	0.89	99.55%
J02O	76/76	44,717,805	96.55%	10.27%	3.45%	0.85	99.51%
J02plusO	76/76	47,453,978	97.53%	12.73%	2.47%	0.92	99.50%
J10O	76/76	45,819,952	95.86%	4.46%	4.14%	0.74	99.59%
J10plusO	76/76	41,802,798	96.62%	5.93%	3.38%	0.96	99.57%

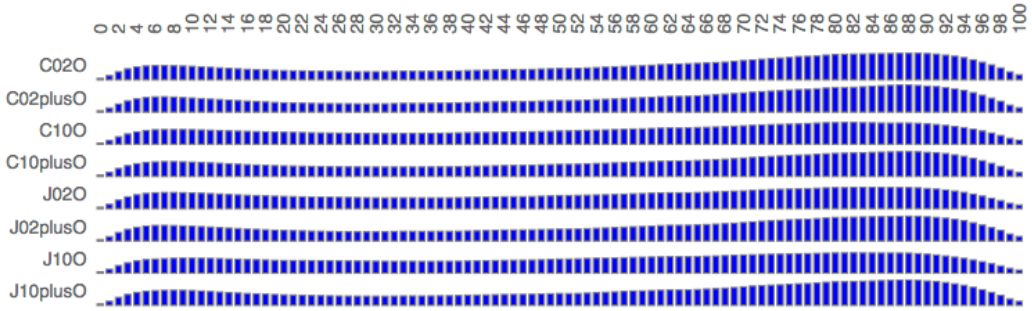
ALIGNMENT DISTRIBUTION



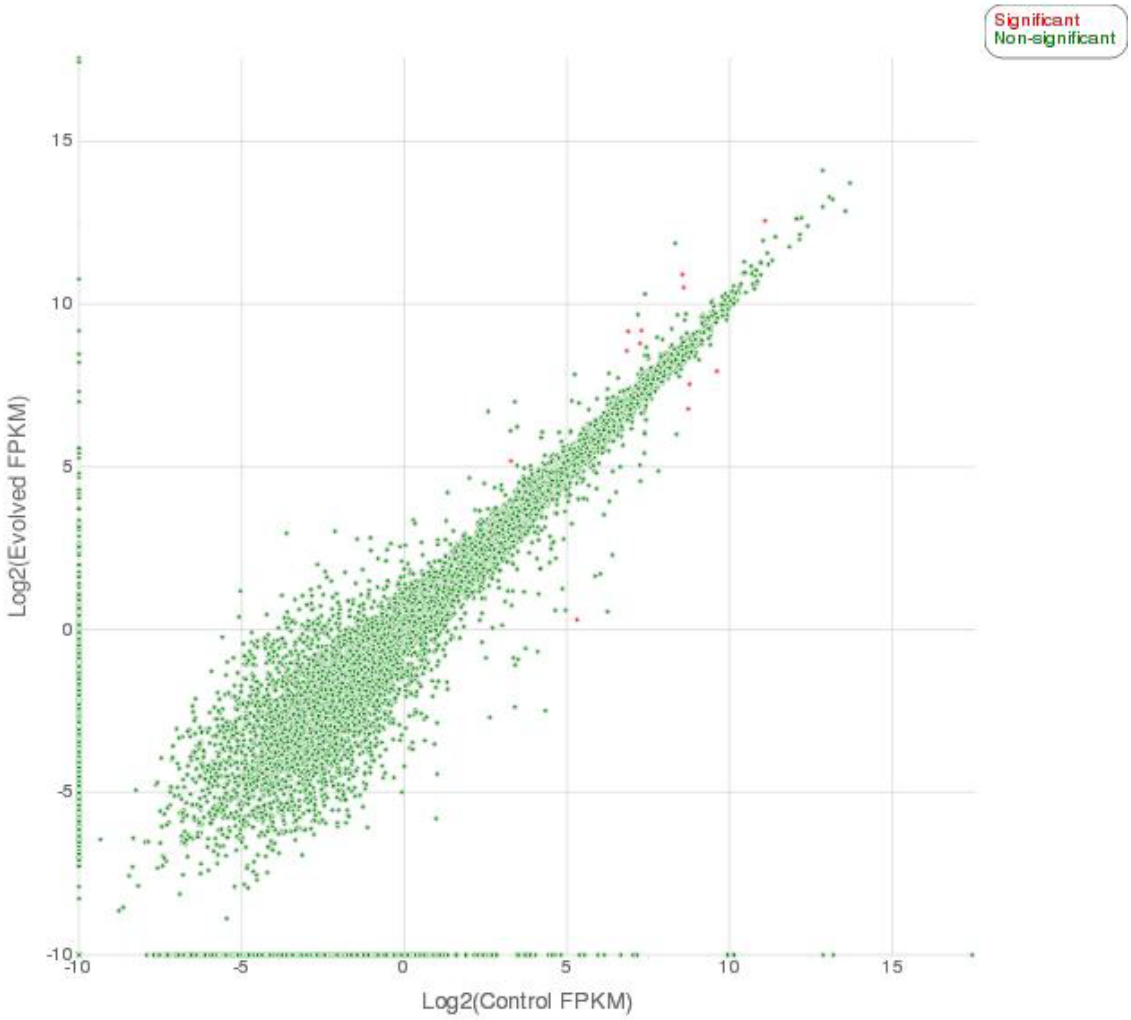
INSERT LENGTH DISTRIBUTION



TRANSCRIPT COVERAGE i



Supplemental RNA Seq Report (Evolved vs Control) Aligned using STAR



Differential Expression

Annotation Gene Count	17,175
Assessed Gene Count	11,278
DE Gene Count	18
Annotation Transcript Count	44,412
Assessed Transcript Count	27,968
DE Transcript Count	0

Assembly

Relation to reference transcripts: http://cole-trapnell-lab.github.io/cufflinks/cuffcompare/#transfrag-class-codes
.

	Control (Control)	Comparison (Evolved)	Merged
Gene Count	16,705	17,120	17,175
Transcript Count	40,909	41,065	44,473
Gene Models	control/cuffmerge/Control.merged.gtf	comparison/cuffmerge/Evolved.merged.gtf	differential/cuffmerge/Control_vs_Evolved.merged.gtf
Equal (=)	33,621	33,552	33,792
Potentially novel (j)	5,733	5,597	8,260
Unknown, intergenic (u)	1,013	1,248	1,495
Overlap with opposite-strand exon (x)	372	467	620
Other	170	201	306

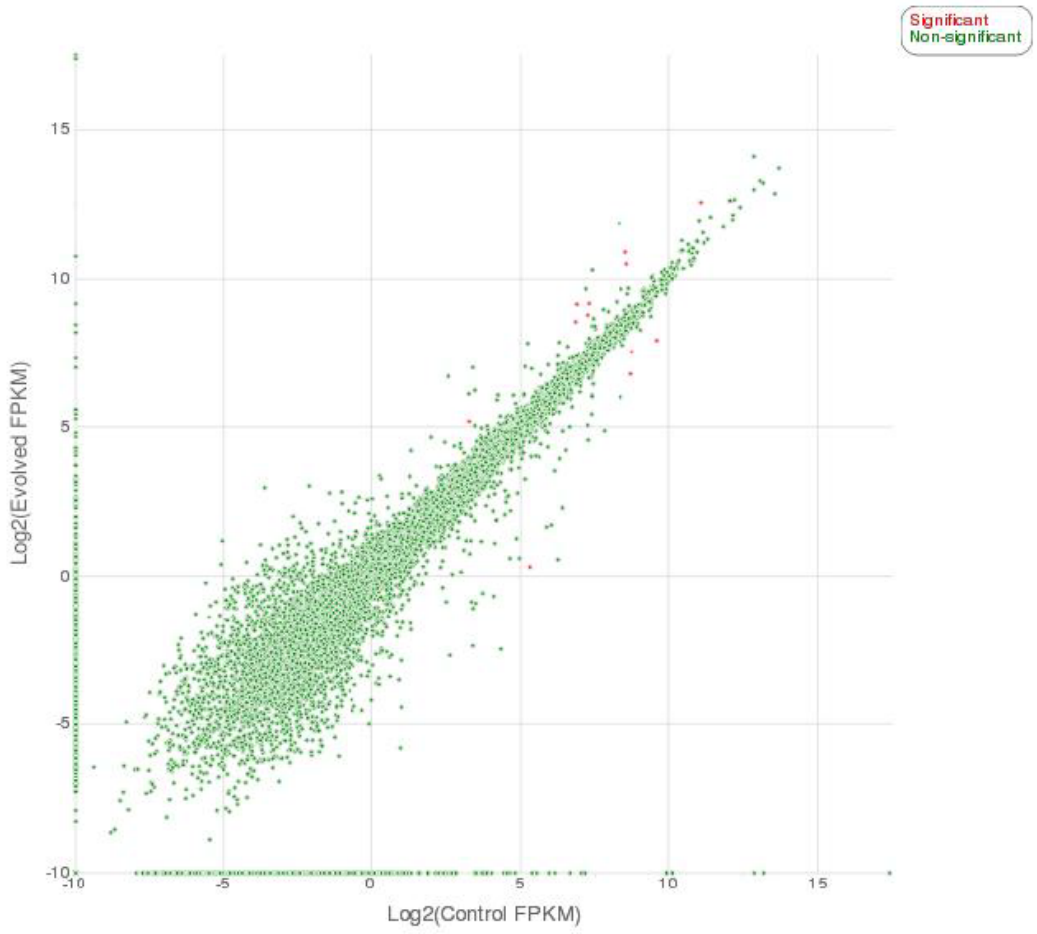
Analysis Details

Settings

Setting Name	Value
Reference Genome	Drosophila melanogaster (UCSC dm3)
BAM Summary Method	BamStats

Software	Version
Cufflinks Assembly & DE (BaseSpace Workflow)	2.1.0
Isis (Analysis Software)	2.6.25.17
STAR (Aligner)	STAR_2.5.0b
BEDTools	2.17.0
Cufflinks	2.2.1
BLAST	2.2.26+

Supplemental RNA Seq Report (Evolved vs Control) Aligned using Bowtie



Differential Expression

Annotation Gene Count	16,681
Assessed Gene Count	11,487
DE Gene Count	19
Annotation Transcript Count	42,150
Assessed Transcript Count	27,036
DE Transcript Count	0

Assembly

Relation to reference transcripts: http://cole-trapnell-lab.github.io/cufflinks/cuffcompare/#transfrag-class-codes
.

	Control (Control)	Comparison (Evolved)	Merged
Gene Count	16,315	16,644	16,681
Transcript Count	39,687	39,542	42,192
Gene Models	control/cuffmerge/Control.merged.gtf	comparison/cuffmerge/Evolved.merged.gtf	differential/cuffmerge/Control_vs_Evolved.merged.gtf
Equal (=)	33,575	33,517	33,689
Potentially novel (j)	5,094	4,828	6,910
Unknown, intergenic (u)	716	925	1,158
Overlap with opposite-strand exon (x)	196	191	282
Other	106	81	153

Analysis Details

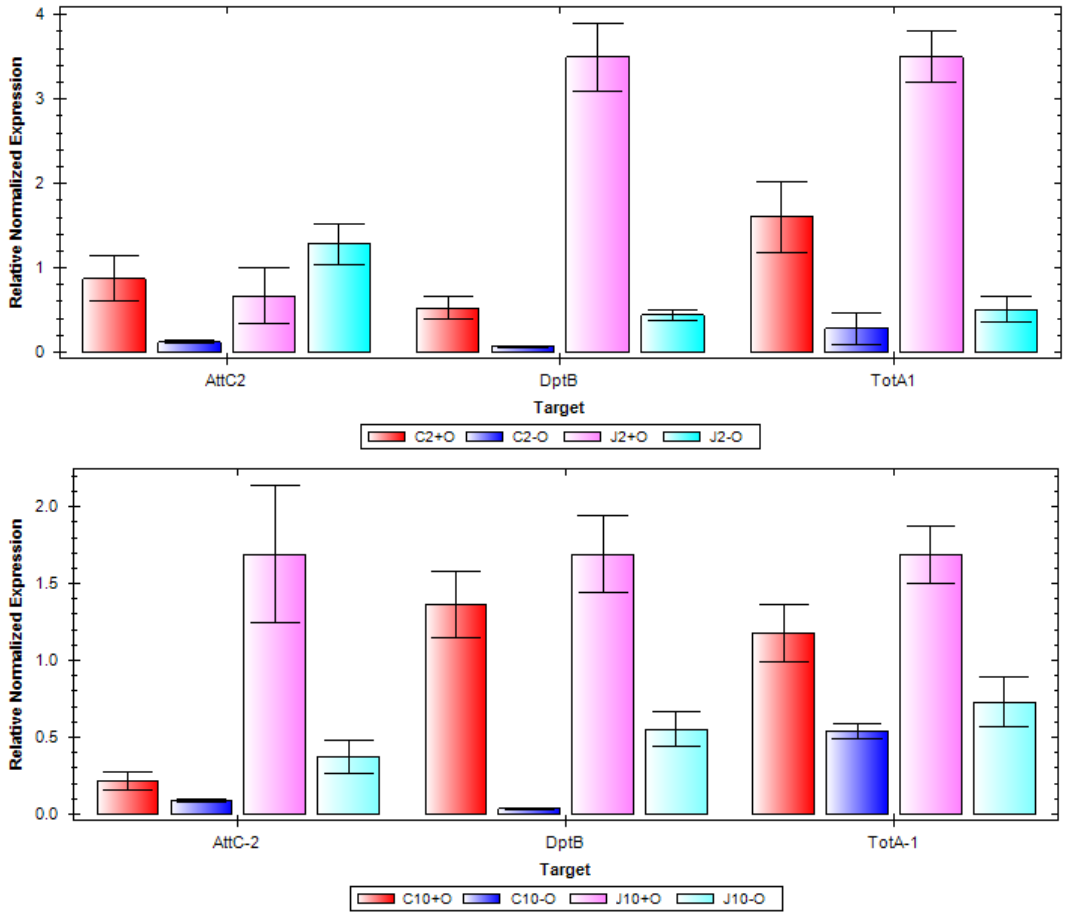
Settings

Setting Name	Value
Reference Genome	Drosophila melanogaster (UCSC dm3)
BAM Summary Method	BamStats

Software Versions

Software	Version
Cufflinks Assembly & DE (BaseSpace Workflow)	2.1.0
Isis (Analysis Software)	2.6.25.17
TopHat (Aligner)	2.1.0
Bowtie2 (Aligner)	2.2.6
BEDTools	2.17.0
Cufflinks	2.2.1
BLAST	2.2.26+

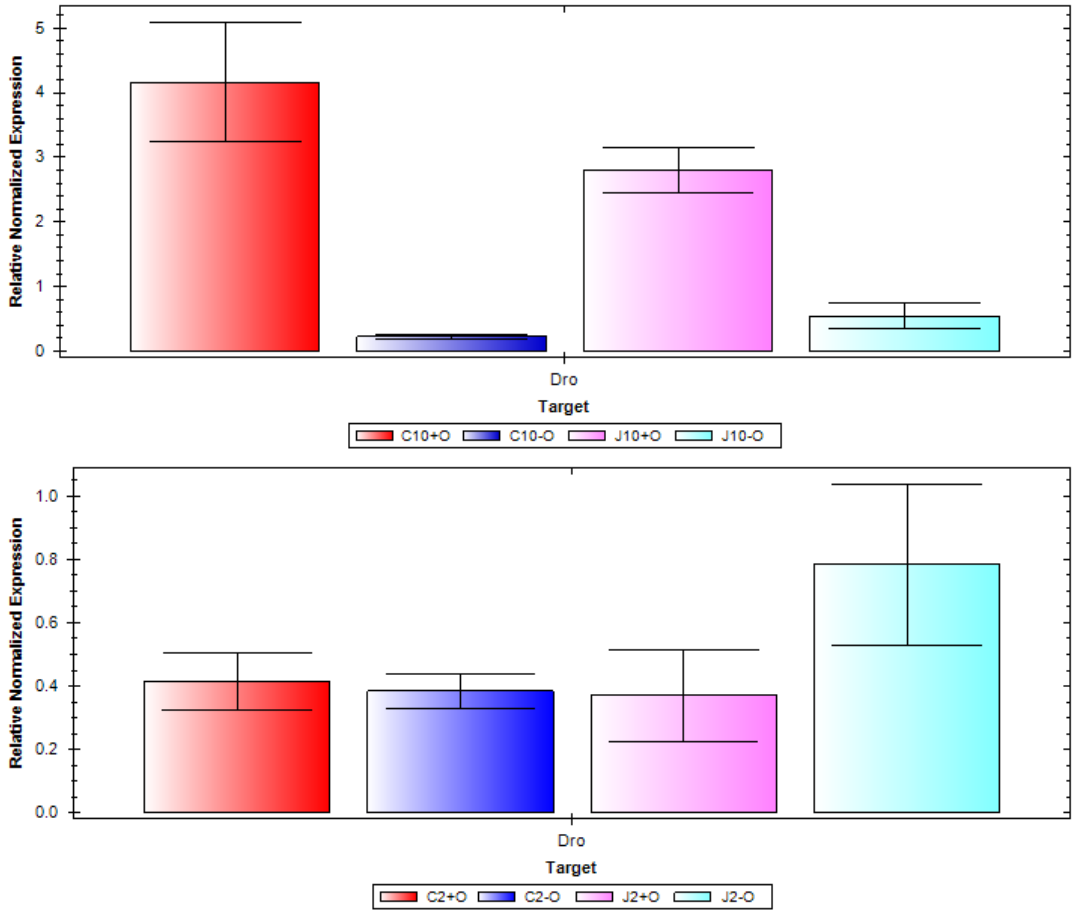
Supplemental qPCR



Two evolved populations (J2&10) were analyzed with their respective controls (C2&10), both before (-O) and after learning (+O). Controls before learning (Blue), Evolved (Teal), Evolved after learning (Pink) and Controls after learning (Red).

Innate immunity is confirmed to be an evolved trait alongside with greater learning, there is conflict with the immune energetic trade-off after learning.

Supplemental qPCR



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Innate immunity is confirmed to be an evolved trait alongside with greater learning, there is conflict with the immune energetic trade-off after learning.