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Differential Gene Expression of Minnesota (MN) Hygienic Honeybees (Apis mellifera) Performing Hygienic Behavior

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By

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Abstract

Hygienic behavior is the ability to remove dead and diseased brood from the comb early as to limit the detrimental impact of the parasite or pathogen. Minnesota (MN) Hygienic bees are generalists of hygienic behavior with the ability to remove several brood infected with several pathogens including the *Varroa* mite. This study explored the mechanisms of MN Hygienic behavior by comparing the transcriptome of MN Hygienic bee brains to non-hygienic bee brains via cDNA microarray. The results suggest that the brains of MN Hygienic bees may have a greater number of dendritic connections or are more sensitive to neurotransmitters. Quantitative trait loci studies of MN Hygienic bees indicated three regions on chromosomes two, five and thirteen which may be responsible for the behavior. Genes from these quantitative trait loci were isolated based on olfaction as MN Hygienic bees have been linked to greater ability to discriminate between olfactory cues. However, none of the olfactory related genes indicated the quantitative trait loci were found to be differentially expressed in MN Hygienic bee brains. These results bring new understanding of the role the brain plays in MN Hygienic behavior. Results provide insight on potential candidate genes, including *XM_393199*, *XM_001120874*, *XM_*392202 and *XM_624940* to utilize for the breeding of bees hardy enough to handle a variety of invading parasites and pathogens.

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Acronyms and abbreviations

Absorbance.....A

Africanized honey bee.....AHB

Base pair.....bp

complementary Deoxyribose Nucleic Acid.....cDNA

Cycle threshold.....Ct

cyclic adenosine triphosphate.....cAMP

Deoxyribose Nucleic Acid.....DNA

Diacylgylcerol.....DAG

Ethylenediaminetetraacetic acid.....EDTA

gamma-Aminobutyric acid.....GABA

Gene ontology term.....GO Term

Hygenic.....Hyg

Inositol triphosphate.....IP3

Melting temperature.....Tm

Minnesota.....MN

National Center for Biotechnology Information.....NCBI

Number.....no.

quantitative real time Polymerase Chain Reaction.....qPCR

Quantitative trait loci.....QTL

Reporter fluorescence.....R

Ribose Nucleic Acid.....RNA

Ribose Nucleic Acid sequencing.....RNA seq.

The slope of a line.....m

Varroa sensitive hygienic.....VSH

Introduction

The worldwide economic value of insect pollination services has been estimated at $174 billion and accounts for 9.5% of the total value of the world's agricultural food production (Gallai et al. 2009). Because insect pollination services are primarily conducted by *Apis mellifera* or honeybees (Gallai et al. 2009), the agricultural and economic significance of the honey bee is great, as are threats to their success, such as colony collapse disorder and other sources of bee loss (Martin 2001; Ratnieks & Carreck 2010; Watanabe 1994). Of particular concern, honeybees may be more susceptible to disease compared to other insects because they are eusocial insect species (Johnson & Tsutsui 2011).

Eusocial insects have overlapping generations of adults; these adults are split into divisions by labor such as reproduction and cooperative brood care (Schmickl & Crailsheim 2007). Social life has consequences that are unique compared to solitary insects (Cremer et al. 2007; Wilson-Rich et al. 2009), for individual bees may benefit from efficient, collective acts like brood-care, foraging, and defense of the hive (Wilson-Rich et al. 2009; Fefferman et al. 2007). Conversely, the close living quarters potentially allow for infectious diseases and parasites to spread more easily (Perez-Sato et al. 2009).

Disease may spread more readily in social insects than solitary ones for reasons other than proximity and contact (Baalen & Beekman 2006; Cremer et al. 2007; Tarpy 2003). Individual bees have fewer immunity genes as solitary insects, rendering the immune system of each individual bee less capable of handling infections than solitary insects (Weinstock et al. 2006). Furthermore, the immunity genes present are likely to be

shared by highly related sisters, this decrease in gene diversity can lead to more infections within the colony (Shykoff and Schmid-Hempel 1991). Despite the increased chance of infection, the worldwide success of bees suggests that the benefits of social living outweigh the potential for disease transmission (Moller 1996).

Bees and other eusocial insects have evolved specific behaviors to combat parasites and pathogens called social immunity (Cremer et al. 2007; Perez-Sato et al. 2009; Schmickl & Crailsheim 2007). Social immunity behaviors are often expressed by castes or groups of individuals who remove infectious agents, as well as diseased or dead individuals from the colony (Rothenbuhler 1964; Spivak & Gilliam 1998a; Wilson-Rich et al. 2009). This behavior has been known to target brood infected with the pathogens that cause American foulbrood, and chalkbrood (Palacio et al. 2010; Spivak & Reuter 2001; Spivak and Gilliam 1993), and the parasite *Varroa destructor*, which has been implicated as one of the potential causes for colony collapse disorder (Bromenshenk et al. 2010; Ibrahim et al. 2007; Le Conte et al. 2010; Martin et al. 2012). The two step process of hygienic behavior begins when hygienic bees detect and uncap cells that contain diseased or dead brood. After uncapping the cells, bees remove the dead or diseased pupae by consuming them (Arathi et al. 2006; Rothenbuhler 1964; Spivak & Gilliam 1998a). Bees typically perform this behavior between fourteen and twenty days of age (Perez-Sato et al. 2009; Spivak and Gilliam 1998b).

Although hygienic behavior is a genetic trait, the genes involved in its expression are unknown. Rothenbuhler (1964), described hygienic behavior to be a homozygous recessive trait on two chromosomes. Mortiz (1988) was one of the first to dispute

Rothenbuhler's claim by utilizing Rothenbuhler's data and argued hygienic behavior fit a three loci model better than two. Since then, others have used quantitative trait loci to claim that six (Oxley et al. 2010) or seven loci (Lapidge et al. 2002) would best describe the genetic basis of hygienic behavior. The genes involved in hygienic behavior have been elusive for a number of reasons. First, not all bees within a hygienic colony are hygienic and second, there are several lines of hygienic behavior in existence that vary in expression.

The classification of the degree to which a colony is hygienic depends upon the colony's composition of hygienic individuals. Because queens mate with multiple males, it is possible to have varying ratios of hygienic bees in relation to the total population of a colony (Arathi & Spivak 2001; Panissiuk et al. 2008; Sherman et al. 1998). The ratio of hygienic bees within a colony determines the frequency and length of time spent per hygienic act for each hygienic individual. As such, a high ratio of hygienic individuals within the colony results in a lower frequency of performed hygienic acts and less time spent performing these acts per individual than a colony with a lower ratio of hygienic bees (Arathi and Spivak 2001; Perez-Sato et al. 2009). The resultant division of labor also means that colonies with low ratios of hygienic individuals tend have those individuals performing hygienic acts until later in their life when compared to colonies with higher ratios of hygienic individuals (Perez-Sato et al. 2009). Having a smaller ratio of hygienic individuals also means that fewer hygienic acts can occur, thus potentially preventing a colony with hygienic bees to be considered hygienic.

Today, there are several lines of honeybees bees that have been selected for hygienic behavior, including "Minnesota (MN) Hygienic," and *Varroa* sensitive hygienic (VSH) formerly called, "suppression of mite reproduction," or SMR (Ibraham & Spivak 2006; Le Conte et al. 2011). MN Hygienic stock was imported from Italy and bred to prevent a wide range of pathogens and parasites (Spivak and Rueter 2001). VSH bees were imported from Russia and were bred to resist the parasitic mite *Varroa* (Danka et al. 2011). Despite their geographical origins, the behaviors exhibited in VSH and MN Hygienic are quite similar. However, VSH bees have a higher removal rate of the *Varroa* mite than MN Hygienic bees, which in turn are better at removing the individuals infected with chalkbrood and American foulbrood (Ibrahim & Spivak 2006). Therefore, it is possible that the mechanisms for each hygienic strain to detect diseased brood differ from each other (Le Conte et al. 2011).

Further differences exhibited between VSH and MN Hygienic lines are greater olfactory senses in MN Hygienic bees relative to non-hygienic bees (Arathi et al. 2000; Gramacho & Spivak 2003; Masterman 2000). The heightened olfactory sense has been hypothesized to be the method of detection behind the behavioral trait (Masterman 2001; Swanson et al. 2009). Spivak et al. (2003) linked hygienic behavior to an increase in the receptors of octopamine, the primary excitatory neurotransmitter in the bee brain. Octopamine is a molecule involved in coincide detection in which excitatory neurotransmission occurs due to two different neurotransmitters being released upon a neuron from a single stimulus causes an action potential that would not exist from either neurotransmitter alone. This is believed to be the mechanism for olfactory learning, thus

reinforcing the idea that olfactory senses were at the root of hygienic behavior. However, Le Conte et al. (2011) found that the genes up-regulated for VSH bees are not yet linked to olfactory senses. Nor does the Le Conte et al. (2011) finding correspond with the quantitative trait loci findings of Oxley et al. (2010) which was conducted on MN Hygienic bees. MN Hygienic bees were found to have six quantitative trait loci by Oxley et al. 2010. Half of these were linked to uncapping, two were linked to all hygienic tasks and one to removing the brood. Quantitative trait loci performed upon VSH bees found two regions of interest, neither of which overlapped with the findings in Oxley et al. 2010.

The differences between quantitative trait loci of MN Hygienic bees and Le Conte et al.'s 2011 findings with VSH bees, paired with differences in removal efficiencies of various parasites, give cause to believe that they may have different genes involved in their regulation. To take an initial step in both understanding gene regulation underlying MN Hygienic bees and their differences with VSH bees, gene expression in the brains of bees from MN Hygienic and non-hygienic colonies was compared. Results from this comparison will then be compared to VSH bee brains (Le Conte et al. 2011). Due to differences of the behaviors, it is believed that the candidate genes for hygienic behavior between the lines are different.

Methods

Selection of hygienic hives

Selection of hygienic hives was determined via a series of freeze killed brood tests by Dr. Marla Spivak (University professor at University of Minnesota, St. Paul campus) and her associates during the fall of 2011. The criterion for hygienic hives was determined by how many sealed cells of brood comb were uncapped and or cleaned within 24 hours of applying liquid nitrogen to a 76.2 mm diameter circle of brood comb. Colonies were selected based upon their scores for hygienic behavior. Equal amount of both hygienic hives (>95% removed) and non-hygienic hives were needed (<80% removed).

Marking of bees for experimental research and placing them into observation hives

Brood frames of the desired bees were removed from their colonies, and the adult bees were removed from them. Frames were marked with the colony they originated from and placed into colony dependent incubators for eclosion. Day old bees from these frames were marked with a dot of enamel paint on their thorax to indicate their hygienic status and date eclosed according to standard protocol. (see Tables 1.1 and 1.2 for the painting schemes). Painted bees were moved directly to an observation hive which already had a hygienic colony present.

Table 1.1. Amount and date of non-hygienic bees marked and introduced into their respective observational hives.

Colony	Date of painting and adding to observation hive	Number of bees painted	Color painted	Colony paired with in observation hive
36	9/2/11	300	Green	85
	9/6/11	361	Light Blue	
	9/9/11	350	Purple	
86	9/2/11	400	Green	79
	9/6/11	350	Light Blue	
	9/9/11	297	Purple	
135	9/2/11	384	Green	17
	9/6/11	336	Light Blue	
	9/9/11	268	Purple	

Table 1.2. Amount and date of hygienic bees marked and introduced into their respective observational hives.

Colony	Date of painting and adding to observation hive	Number of bees painted	Color painted	Colony paired with in observation hive
85	9/2/11	328	Orange	36
	9/6/11	305	Yellow	
	9/9/11	266	Red	
79	9/2/11	400	Orange	86
	9/6/11	350	Yellow	
	9/9/11	350	Red	
17	9/2/11	316	Orange	135
	9/6/11	357	Yellow	
	9/9/11	339	Red	

Collecting hygienic and non-hygienic bees

The collections occurred on September 22nd, 25th, 27th, and the 28th 2011. Collections began via insertion of a 76.2 mm diameter plug of frozen brood into a hole cut out of the brood frame of the observation hive (Spivak and Downey 1998). The plug

was monitored for bees to perform a hygienic act such as a quick back and forth motion of the head used to remove the wax cap of the brood cell. If the marked bee was of appropriate hygienic age (greater than fifteen days) and from a hygienic colony, it would be selected to be flash frozen into liquid nitrogen via vacuum. After the plug of dead brood was completely uncapped, the same number of non-hygienic bees at the same age were collected from the same observation colony. Bees would be transferred and kept in a -20˚C freezer at the University of Minnesota until transported back to Minnesota State University, stored at -80˚C until brain dissection.

Brain excision and pairings

Bee brains were removed from frozen bee heads on a plate cooled by dry ice (Toma et al. 2000). The removal of bee brains was conducted utilizing a clean, cold micro-scalpel and a clean, cold pair of tweezers. Brains were stored in marked, and sterile micro-centrifuge tubes which had been chilled. Bee brains were paired in an effort to keep variables such as age, and observation hives consistent. Each pairing consisted of three brains from hygienic bees, and three brains from non-hygienic bees (Tables 1.3 and 1.4).

Table 1.3. Hygienic bee brains, age (in days) and colony of origin utilized in pairings.

Pair	Colony from which hygienic bee originated	Number of brains	Age of bee
1	17	3	15
2	85	3	20
3	17	3	18
4	85	3	17
5	85	3	21
6	85	2	17
	85	1	17
7	17	1	19
	17	1	21
	17	1	19
8	17	1	15
	17	1	20
	17	1	21

Table 1.4. Non-hygienic bee brains, age (in days) and colony of origin utilized in pairings.

Pair	Colony from which hygienic bee originated	Number of brains	Age of bee
1	135	3	15
2	36	3	20
3	135	3	18
4	36	3	17
5	36	3	21
6	36	2	17
	36	1	17
7	135	1	19
	135	1	21
	135	1	19
8	135	1	15
	135	1	20
	135	1	21

RNA extraction/RNA purification

RNA extractions were conducted utilizing the protocol of Qiagen's (Hilden, Germany) RNeasy kit. Quiagen's Rneasy kit is a multiple step process involving lysing cells in a solution of Buffer RLT containing guanidine isothiocycanate a choatropic agent

which supports Nucleic acids binding to the silica utilized in the spin column. Buffer RLT also contains β-mercapthanol, a protective agent for newly released RNA, as it inactivates RNases present in the solution. Next ethanol is added to the solution to aid the Nucleic acids in binding to the silica, it does so by causing the nucleic acids precipitate in solution. The resultant supernatant is passed through a spin column containing silica and the prepared nucleic acids bind to the surface along with choatropic salts and other byproducts of cell lysis. Next is the introduction of Buffer RW1 which contains a mixture of guanidine salts and ethanol that eliminates carbohydrates, proteins fatty acids and other impurities not specifically bound to the silica. Buffer RPE is also drawn through the silica spin column to eliminate any extra salts that may be present from the nucleic acid extraction process, allowing for easier amplification later on. Nuclease free water is then drawn through the silica to elute out the nucleic acid due to its stronger inter molecular force. This procedure was performed upon paired pools of bee brains listed in Tables 1.3 and 1.4. Immediately after RNA extractions were complete, any possible remaining DNA was removed by digesting with Turbo DNA-free from Ambion of Life Technologies (St. Austin, Texas).

RNA concentrations and potential contaminations were assessed via spectrophotometry at 260, 280 and 320nm on an Eppendorf (Hamberg, Germany) BioPhotometer. Conducted by placing 98.0µL nuclease free water in a cuvette, blanking it, and then mixing 2.0µl of sample RNA to obtain the data in Table 2.1. Samples also were assessed via agarose gel electrophoresis, as described below. Samples were frozen at -80°C.

Agarose gel electrophoresis

Agarose gel electrophoresis was used as a secondary measure to validate amplicon size and visually inspect the results of the RNA extraction. Gels are prepared via mixing agarose (IBI Pesota, IA) and 60ml of 0.5x Tris-Borate EDTA (TBE) at one and a half percent agarose (0.9g) for RNA extraction inspection and two percent agarose (1.2g) for PCR amplicon validation. This mixture is weighed, and microwaved (LG Yeouido-dong, Seoul, South Korea) for about three minutes on high until the light reflecting particles have fully dissolved. The mixture is then weighed again and any difference in weight is compensated for via distilled water. This solution is allowed to cool on a benchtop for five to seven minutes, so that it is still molten but will not warp the mold. 0.5µL of Ethidium Bromide (IBI Pesota, IA) is added and homogenized into the gel. The gel is then poured into a mold and a ten well comb (10µl) is inserted into the gel. After the gel solidifies the top of it is flooded with 0.5x TBE, the comb is removed, and the gel is positioned to be utilized in a gel electrophoresis box. The electrophoresis box is filled with 0.5x TBE until the surface of the gel has a centimeter of TBE covering it and two µl of Ethidium Bromide is inserted in the positive side of the box.

The gel is loaded 10 µL of the ladder mixture of four µl DNA ladder either New England Biosciences (Ipswich, MA) 1 Kilo-base (Kb) DNA ladder, or Bionexus (Oakland, CA) Hi-Lo, two µL of 6x loading dye New England Biosciences (Ipswich, MA) and six µL of nuclease free water. Samples are loaded the same way with four µL of Sample, two µL of 6x loading dye and six µl of nuclease free water. Gels are ran at 100V until the loading dye is approximately three quarters of the way down the gel. Gels

are carefully removed and placed upon a UV transilluminator (VWR Radnor, PA) set at 302 nanometers for interpretation.

cDNA microarray

cDNA microarrays were conducted by the Roy J. Carver Biotechnology Center at the University of Illinois. Each custom made Agilent microarray slide had 15,744 spots total, 459 positive controls, 77 negative controls and 15,208 spots interrogating bee genes. The 15,208 spots consisted of 11,730 bee genes tested once and 3,478 tested twice. They received eight hygienic and eight non-hygienic samples that were paired above as according to Tables 1.3 and 1.4.

Analysis of the microarray data was also conducted at Roy J. Carver Biotechnology Center. The data collected was analyzed utilizing limma (linear models for microarray data) as a statistical model (Ritchie et. al. 2015). The resultant raw p-values were adjusted using a false discovery rate correction to correct for multiple comparison testing.

Selection of genes for microarray validation via quantitative real time Polymerase Chain Reaction (qPCR)

Selection of candidate genes for microarray validation was determined by p-value ($p<0.05$), fold change, and knowledge of a specific gene, or by its usage in other studies

(Table 1.5). Two genes that were not differentially expressed between hygienic and non-hygienic individuals were *Elongation Factor 1α-F2* (*EF-1α*), a housekeeping gene and *β-actin*, a gene that was differentially expressed (Le Conte et.al 2011.) *EF-1α* was chosen as a control, and *β-actin* as a gene that was equally expressed in both. *Pteropsin* and *2 pore K pump* were chosen as they were two of the genes with the greatest positive fold change in the microarray.

Table 1.5. Genes utilized in verifying the microarray, their fold change, and their p-value.

Gene	NCBI reference sequence	Gene Symbol	Fold Change Hygienic vs. Non-hygienic	p-value
2 pore K pump	*XM*_394509.2	LOC411036	1.938563924	0.00107
Pteropsin	*NM*_001039968.1	LOC408985	1.445180415	0.01287
EF-1α	*NM*_001014993.1	EF1-alpha	-1.466110098	0.17043
β-actin	*NM*_001185145.1	Arp1	-1.040448396	0.95071

Primer designs

Primer designs were performed utilizing Applied Biosystems' Primer Express 3.0 (Foster City, CA) and gene sequences obtained from the National Center for Biotechnology Information's (NCBI) website. The primer pairs were given a penalty score based upon their length, composition, secondary structures, and potential dimers. Selected primers were chosen based upon having either the forward or reverse primer spanning an exon-exon junction, and having a penalty score of less than 200 if possible. The primers designed for the qPCR validation are indicated in Table 1.6. Primers were purchased from Sigma Aldrich (St. Louis, Missouri) or Integrated DNA Technologies (Coralville, Iowa) at Rp1 purification. Table 1.6 displays the sequences for each primer

and the melting temperature (Tm) as calculated by Sigma Aldrich Pure and Simple Primer designer at https://www.sigmaaldrich.com/.

Table 1.6. Forward and reverse primers for the genes selected for qPCR verification of the microarray.

Gene	Forward primer sequence 5' to 3'	Reverse primer sequence 5' to 3'	Tm in °C Forward / Reverse
2 pore K pump	AGGATGTCAGGCCTCAGAA	CGCAATCGTTAAGCAAGACAAG	66.6/66.0
Pteropsin	ATCCTGTCGCGATGGTCTCT	ACCAGGCGTACACACATTTTGT	66.1/65.3
EF-1α	CGTCTTCCTCTTCAGGACGTATATAA	AGGAGCGAATGTGACAACCAT	64.2/65.7
β-actin	AGGAATGGAAGCTTGCGGTA	AATTTTCATGGTGGATGGTGC	65.7/65.9

Reverse transcription

Reverse Transcription of the RNA was conducted via the standard protocol in Promega's (Madison, Wisconsin) GoTaq® 2-step RT-qPCR. For each sample, 10.0 ul of the GoScript™ reaction mix (Table 1.8), and 10.0 µl of the RNA and reverse transcription primer mix (Table 1.9) were combined and ran through the thermocycler conditions in Table 1.7. Random primers (Table 1.9) were utilized to transform experimental RNA in table into cDNA.

Table 1.7. Reverse transcription thermocycler protocol

Step	Temperature in ºC	Duration	Frequency
Anneal	25	5	1x
Extend	42	1 hour	1x
Inactivate	70	15	1x
Chill	4	∞	1x

Table 1.8. GoScript™ reaction mix

Component	Volume in µl
Nuclease free water	1.5
Goscript™ 5x reaction buffer	4.0
MgCl2	2.0
PCR nucleotide mix	1.0
Recombinant RNasin® ribonuclease inhibitor	0.5
Go script reverse transcriptase	1.0

Table 1.9. RNA and reverse transcription primer mix

Component	Volume in µl
RNA sample	5.0
Random primer	1.0
Nuclease free water	4.0

Primer validation

To ensure that the primers selected efficacious, we tested primer specific amplicons resulting from PCR via gel electrophoresis and comparison to known DNA ladders. (See Table 1.10, and 1.11, as well as Figures 1.1 and 1.2). The conditions of the PCR reaction and the thermocycler are in Tables 1.10 and 1.11. The resulting samples from this procedure were ran on a two percent agarose gel as described earlier. Hi-Lo DNA ladder from Bionexus (Figure 1.1) and the 1Kb DNA ladder from New England Biosciences (Figure 1.2) were used to validate amplicon size.

Table 1.10. PCR mix utilized to verify amplicon size

Component	Volume in µl	Concentration
Colorless GoTaq Reaction Buffer	10.0	5x
PCR Nucleotide mix	0.8	-
GoTaq DNA Polymerase	0.2	-
Reverse Primer	1.1	10µM
Forward Primer	1.1	10µM
cDNA Sample	1.0	50ng/µl
Nuclease Free Water	35.8	-

Table 1.11. PCR Size verification thermocycler conditions

Cycle	Temperature in °C	Duration in minutes	Frequency
Activation	95	10	1x
Amplification	95	1.0	40x
	60	0.25	
Chill	4	∞	1x

Figure 1.1. Hi-Lo ladder from Bionexus (Oakland, CA) on a 1.2% agarose gel (image provided by https://www.bionexus.net/product/BN2050.html)

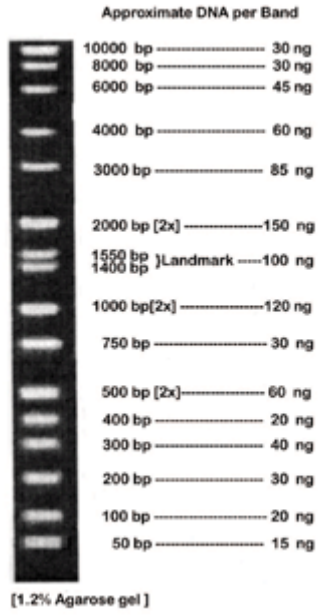
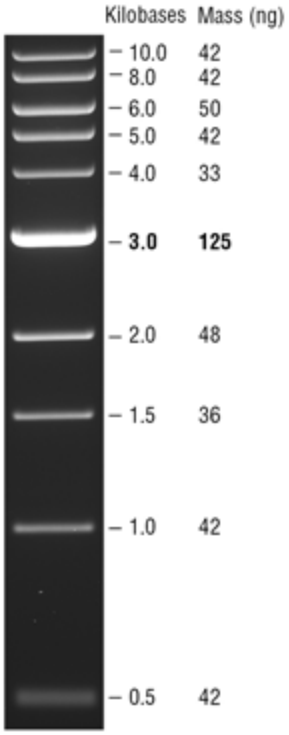


Figure 1.2. 1kb Ladder from New England Biosciences (Ipswich, MA) on a 0.8% agarose gel (provided by https://www.neb.com/products/n3232-1-kb-dna-ladder)



Quantitative polymerase chain reaction

qPCR was conducted via Promega's GoTaq® 2-step RT-qPCR, on Applied Biosystems One Step Plus system. Data obtained was analyzed by StepOne Software version 2.3. cDNA was prediluted into nuclease free water to aid in streamlining the process (Table 1.3). Each well was prepared as described in Table 1.13.

Table 1.12. cDNA Dilution scheme for qPCR

Pair	Hygienic	Initial concentration (ng/µl)	Volume of sample (µl)	Volume of nuclease free water (µl)	Final volume (µl)	Final concentration (ng)
1	H	552	2.7	12.3	15	100
	N	531	2.8	12.2	15	100
2	H	150	10.0	5.1	15	100
	N	631	2.4	12.6	15	100
3	H	367	4.1	10.9	15	100
	N	599	2.5	12.5	15	100
4	H	175	8.6	6.4	15	100
	N	170	8.8	6.3	15	100
5	H	260	5.8	9.2	15	100
	N	597	2.5	12.5	15	100
6	H	342	4.4	10.6	15	100
	N	160	9.4	5.3	15	100
7	H	260	5.8	9.2	15	100
	N	498	3.0	12.0	15	100
8	H	290	5.2	9.8	15	100
	N	388	3.9	11.1	15	100

Table 1.13. qPCR reaction components per well

Component	Volume in µl	Initial concentration	Final concentration
Promega MasterMix	10.0	2x	1x
Reverse Primer	1.0	4µM	200nM
Forward Primer	1.0	4µM	200nM
cDNA Sample	8.0	100ng	40ng/µl

Melt Curves

Melt curves were conducted via Applied Biosystem's StepOne software v2.3. The procedure requires that after qPCR amplification that the wells utilized are heated in in increments of three tenths of a degree Celsius from 60.0°C to 94.8°C. During each

incremental step the florescence of each well would be read. This data is transformed into the first negative derivative of fluorescence (Derivative reporter) and graphed to identify drops in fluorescence resulting from drops in double stranded DNA denaturation.

Statistical validation of microarray

Data was tested for outliers after log transforming the experimentally obtained Cycle threshold (Ct) values (Bengtsson et al. 2005; Limpert et al. 2001). These log transformed values was separated into colony and gene based groups to biological groups (to limit the number of variables), and from here the data was evaluated for normality. Should the group be considered normal, a box and whisker plot was used to identify any potential outliers. These groups were tested via a Grubbs test for one potential outlier or a Teitjen-Moore test for more than one potential outlier (Burns et al. 2005; Grubbs 1969; Teitjen and Moore 1972). Outliers was displayed and excluded from the data used.

The relative gene expression of hygienic bees was compared between microarray findings and qPCR findings for the chosen genes described above. Calculating relative gene expression from qPCR was done utilizing a ΔΔCT values of the mean expression of technical replicates of each gene. Fold change was calculated utilizing the $10^{\Delta\Delta Ct/m}$ method. This method equates for differences in primer efficiencies. If technical replicates have less than two samples per set, the biological replicates was used. Microarray was validated if the fold change is in the same direction as the microarray.

FATIGO analysis

Drosophila melanogaster orthologues were determined for the differentially expressed genes utilizing microarray design A-MEXP-755. These were validated and updated via the Upload/convert IDs tool at FlyBase http://flybase.org (Santos et al. 2015). Analysis of these ID's at http://babelomics.bioinfo.cipf.es/ via the tool, FATIGO. FATIGO is utilized to minimize the effects of over-representation of Gene Ontology (GO) terms between two lists of genes (Al-Shahrour et al. 2007). Up-regulated genes, and down-regulated genes were run in separate analyses against the *Drosophila* genome. Duplicates from the differentially expressed genes were removed from the rest of the genome.

Comparisons between other studies

Comparisons between different microarray studies were performed to distinguish MN Hygienic behavior. Studies were chosen if they shared the same microarray design as this study (A-MEXP-755), and/or were chosen based upon Hygienic status such as for the Africanized bees. The studies utilized to help characterize MN Hygienic behavior were vibrating bees (Alaux et al. 2009a), VSH (Le Conte et al. 2011), Africanized Honey Bee (AHB) vs. European bees in the following roles: guard, soldier and forager, alarm pheromone bees (alarm) (Alaux et al. 2009b), as well as bees performing hygienic actions in a hygienic colony (uncappers), bees in a colony with hygienic and non-hygienic bees (genotype mixing), and uncappers from a mixed colony (genotype and uncapping) (Gempe et al. 2012). To compare the number of shared oligios that are differentially

expressed both the representation factors and hypergeometric probability were calculated using formulas found in Kim et al. 2001 and Halbritter et al. 2013 respectively.

A comparison to the quantitative trait loci findings involved genes and the confidence intervals found in MN hygienic bees specified in Oxley et al 2010. A list of genes within the confidence interval range was identified utilizing NCBI's website. Genes shared between Oxley et al. 2010 and our differential gene sets were noted.

Comparisons between hygienic stocks of bees involved utilizing the RNA sequencing data of VSH bee antennae (Mondet et al. 2015), the RNA sequencing data of hygienic bees in Canada (Boutin et al. 2015), and microarray analysis of differential gene expression present in mixing hygienic and non-hygienic bees in the same colony (Tsuruda et al. 2012). The data from these sets were translated by A-MEXP-755, and NCBI's website into NCBI reference sequences (if needed). These NCBI reference sequences were used to compare the microarray findings to find out if the differentially expressed genes in the studies matched any of the differentially expressed genes in our data, and if they shared direction of expression.

Results

To identify differentially expressed genes in MN Hygienic honeybees, the extracted RNA from bee brains has to be high yield. The primers selected for qPCR verification of the microarray need to be sufficiently specific in their binding. qPCR results need to be checked for multiple melting points and microarray data and qPCR data must be in concert with one another. Below are the results from the tests utilized to ascertain this information.

RNA extraction

RNA extractions were checked via spectrophotometry to ascertain the quantity of RNA as well as the levels of contaminants. Table 2.1 displays the concentration, as well as the Absorbance (A) at 260, 280, 260/280, and 320nm of our RNA extractions.

Table 2.1. Spectrophotometry data of RNA extraction including Concentration, absorbance at 260, 280 and 320 nm.

Pair	Hygienic	Concentration (µg/mL)	A260/280	A260	A280	A320
1	Hygienic	552	1.92	0.276	0.144	0.004
	Non-hygienic	531	1.92	0.265	0.138	0.003
2	Hygienic	150	1.9	0.076	0.040	0.000
	Non-hygienic	631	1.93	0.316	0.164	0.002
3	Hygienic	367	1.91	0.184	0.096	0.002
	Non-hygienic	599	1.90	0.300	0.158	0.007
4	Hygienic	175	1.93	0.085	0.044	0.003
	Non-hygienic	170	1.9	0.086	0.045	0.000
5	Hygienic	260	1.9	0.130	0.070	0.007
	Non-hygienic	597	1.90	0.299	0.157	0.003
6	Hygienic	342	1.82	0.171	0.094	0.010
	Non-hygienic	160	1.8	0.077	0.042	0.002
7	Hygienic	260	1.9	0.142	0.075	0.001
	Non-hygienic	498	1.92	0.214	0.111	0.001
8	Hygienic	290	1.9	0.145	0.076	0.001
	Non-hygienic	388	1.90	0.194	0.102	0.002

RNA extractions were also checked via one and a half percent agarose gel electrophoresis, to validate that the extraction didn't yield anything unexpected. Two bands and a smear (other charged nucleic material) are present in each of the samples displayed in Figure 2.1 and Figure 2.2.

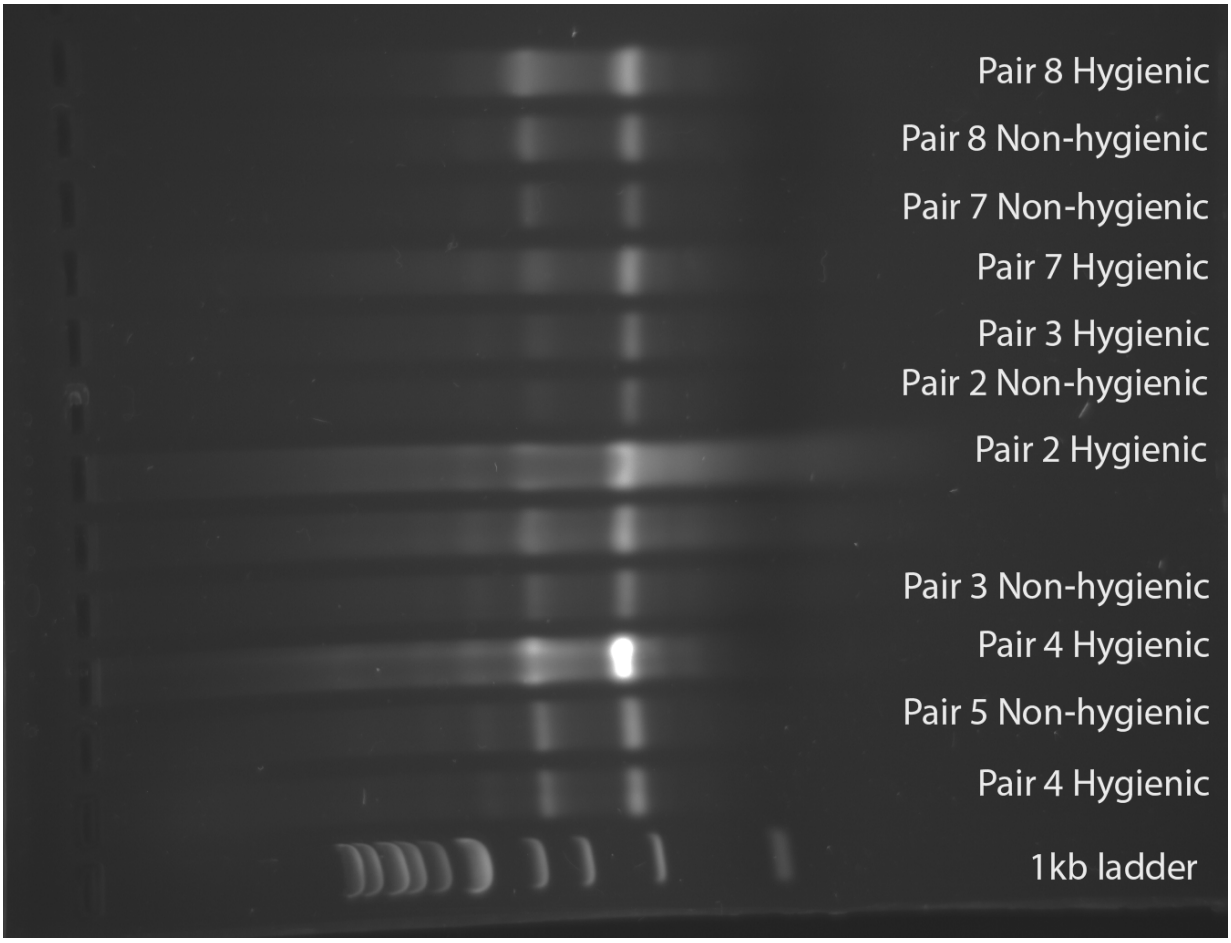


Figure 2.1. 1.5% agarose gel electrophoresis of samples and a 1kb DNA Ladder.

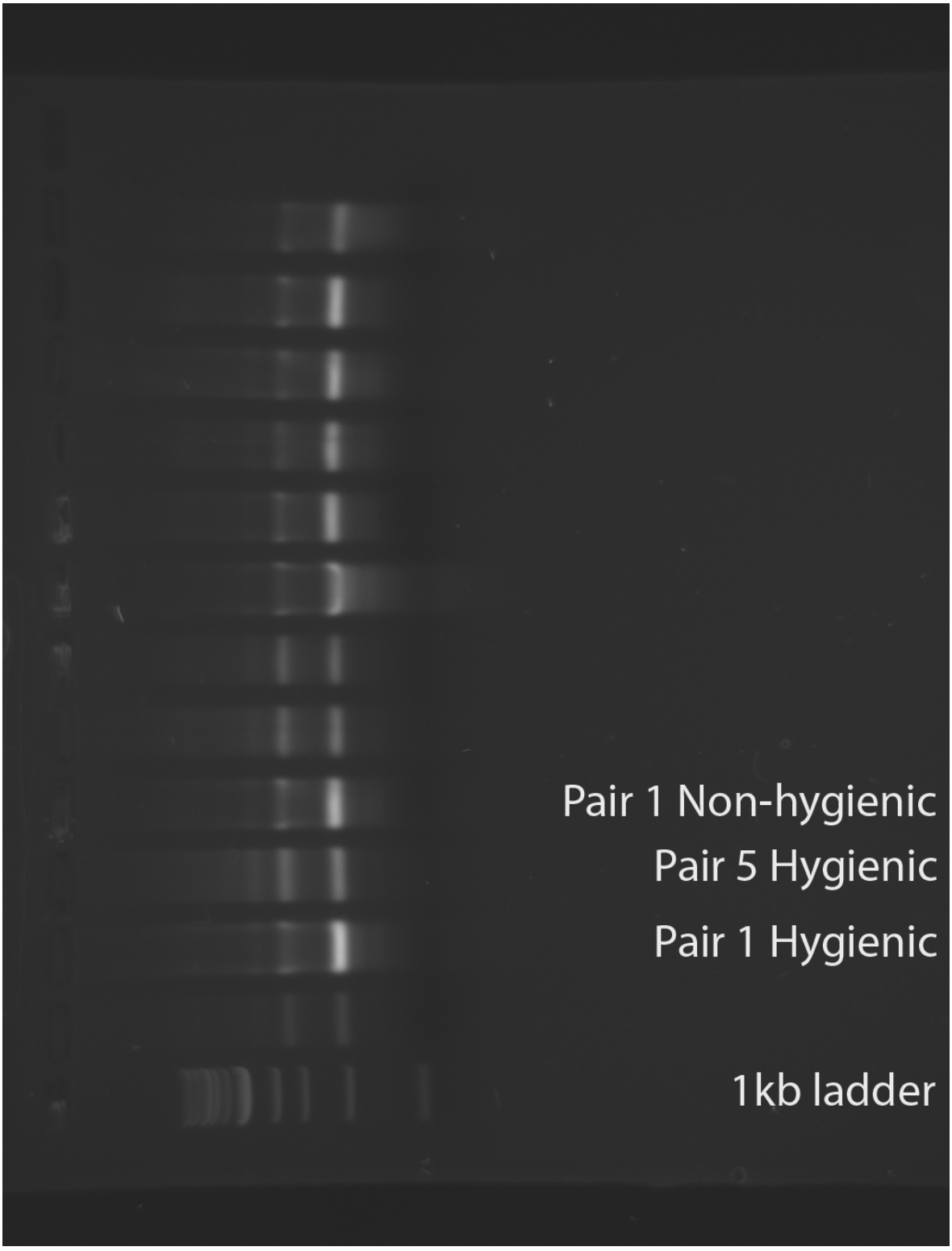


Figure 2.2. 1.5% agarose gel electrophoresis of samples and a 1kb DNA Ladder.

Amplicon size verification

Agarose gel electrophoresis was used to ensure that PCR amplification using our primers yielded the desired size of amplicon and no other visible amplifications. This was performed via two percent agarose gel electrophoresis (Figure 2.3). Amplicon sizes were compared to two ladders, the Promega 1 Kilo-base pair (Kb) ladder and the Bionexus Hi-Lo ladder. Table 2.2 displays the NCBI predicted amplicon length for the primers utilized.

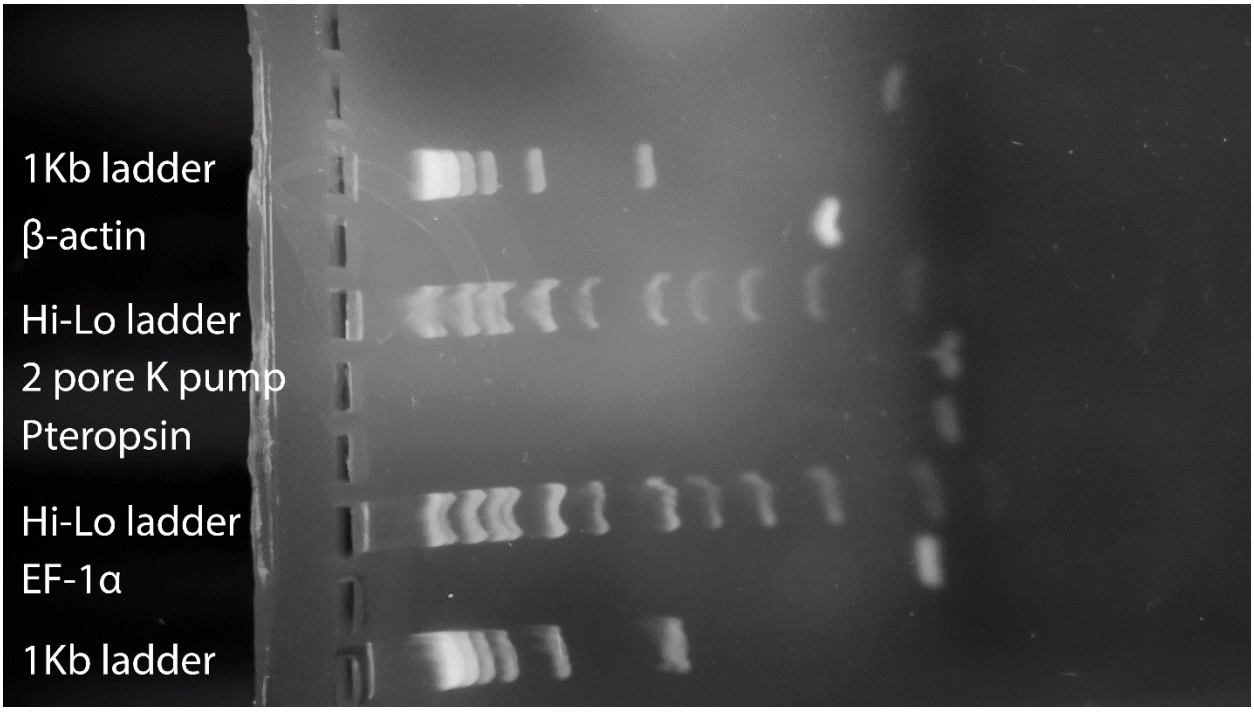


Figure 2.3. Two percent agarose gel electrophoresis PCR amplicon size validation.

Table 2.2. NCBI predicted amplicon size for the primers utilized.

Gene	NCBI Reference Sequence	Amplicon Length in bp
2 pore K pump	XM_394509.2	67
Pteropsin	NM_001039968.1	82
EF-1α	NM_001014993.1	108
β-actin	NM_001185145.1	181

MN Hygienic genes

616 of the 11,730 oligos tested in the microarray were differentially expressed between hygienic and non-hygienic bees at a false discovery rate of less than 0.05. Of these oligos, 335 were up-regulated and 281 were down-regulated. The span of up regulation was between 1.17 to 5.86 fold changes, while down-regulation spanned -14.31 to -1.14 fold changes. The findings of the microarray were verified before they are analyzed.

qPCR was used to verify the results of the microarray. Six data points were identified as outliers in the qPCR data and were removed (see Table 2.3). Removal of these outliers prevented utilizing samples within the *β-actin* gene set as a technical replicates as used for the *Pteropsin,* and *2 pore K pump,* instead it was handled as biological replicates for *β-actin*. Calculation of the corresponding fold changes was conducted via analyzing the cycle threshold data for *β-actin* (Figures 2.4 to 2.8), *EF-1α* (Figures 2.9 to 2.13 and Figures 2.30 to 2.45), *2 pore K pump* (Figures 2.14 to 2.29) and *Pteropsin* (Figures 2.46 to 2.61). The resulting fold changes displayed in Tables 2.5, 2.7 and 2.9 were calculated from mean cycle threshold values in Tables 2.4, 2.6 and 2.8. The resultant fold changes did not differ by more than one fold count for any of the genes tested between the microarray and qPCR data (Figure 2.62). 616 differentially expressed genes is unwieldy, to draw larger conclusions from this data grouping of genes needs to occur.

Table 2.3. Teitjen-Moore test for outliers.

Colony	Gene	Ct	Log of Ct	Shapiro-Wilk p value	Critical value	Calculated value	Number of outliers
135N	*β-actin*	14.83	2.70	0.332	0.097	0.038	6
		15.87	2.77				
		16.02	2.77				
		25.56	3.24				
		25.93	3.26				
		27.21	3.30				

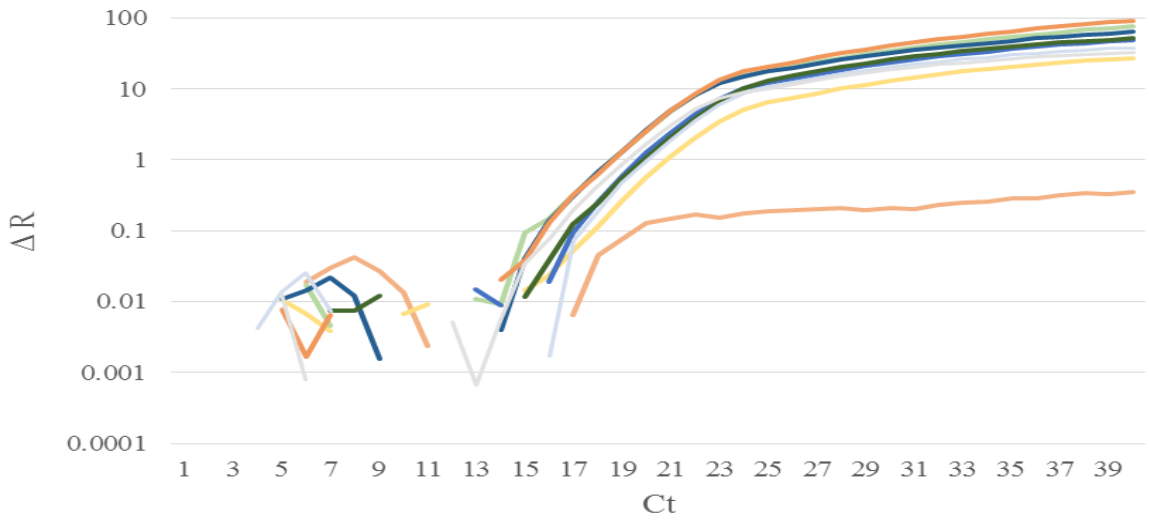


Figure 2.4. Cycle threshold vs. ΔR (reporter fluorescence) values of qPCR wells containing colony 135N bee brains with the *β-actin* primer.

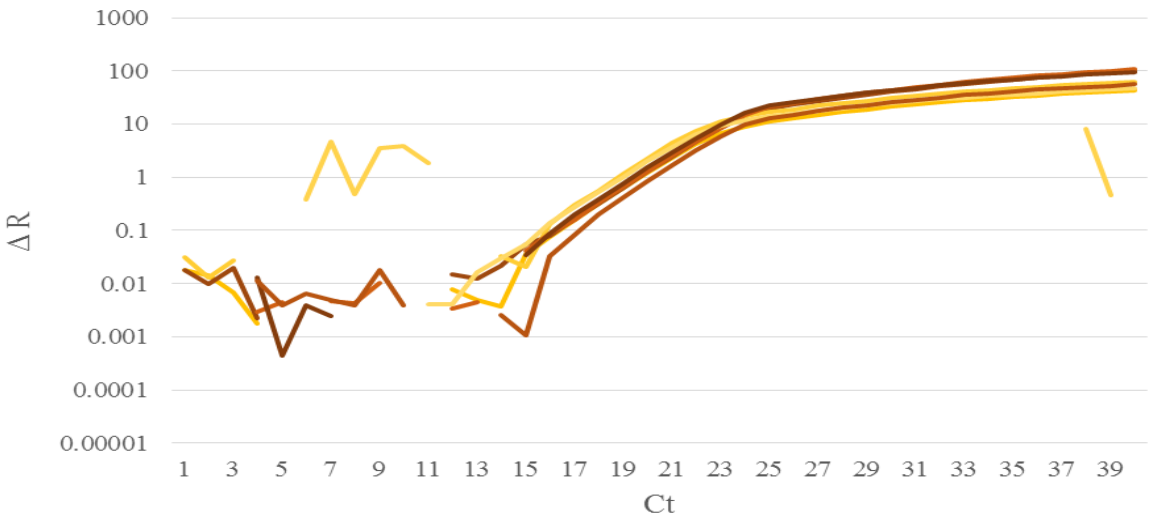


Figure 2.5. Cycle threshold vs. ΔR (reporter fluorescence) values of qPCR wells containing colony 36N bee brains with the *β-actin* primer.

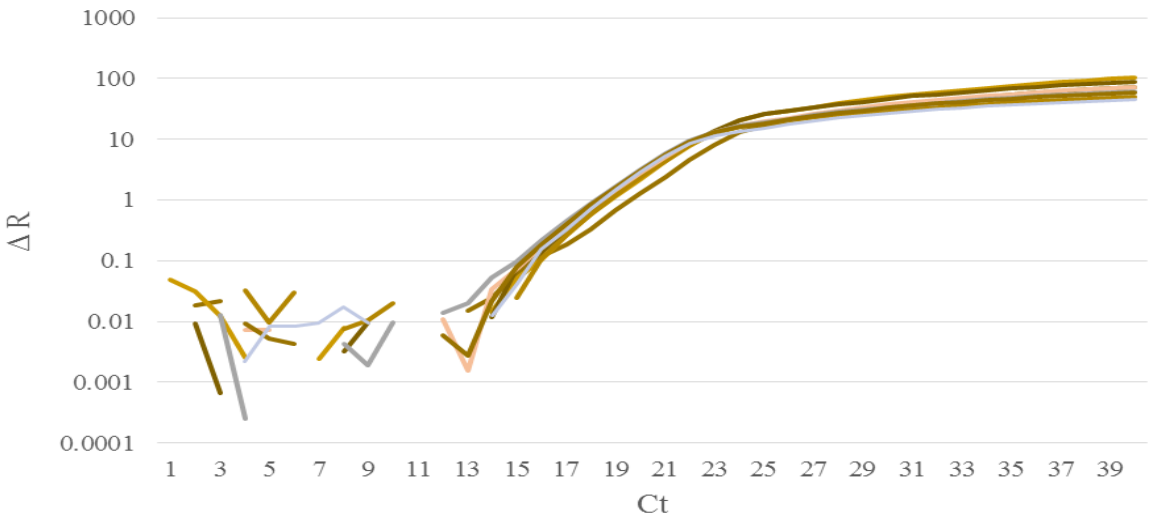


Figure 2.6. Cycle threshold vs. ΔR (reporter fluorescence) values of qPCR wells containing colony 86N bee brains with the *β-actin* primer.

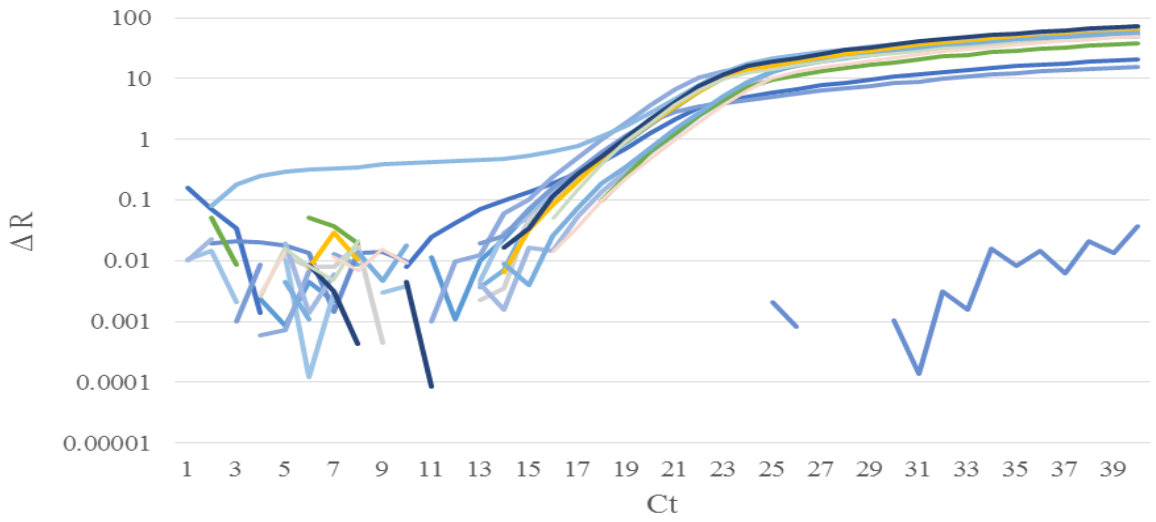


Figure 2.7. Cycle threshold vs. ΔR (reporter fluorescence) values of qPCR wells containing colony 17H bee brains with the *β-actin* primer.

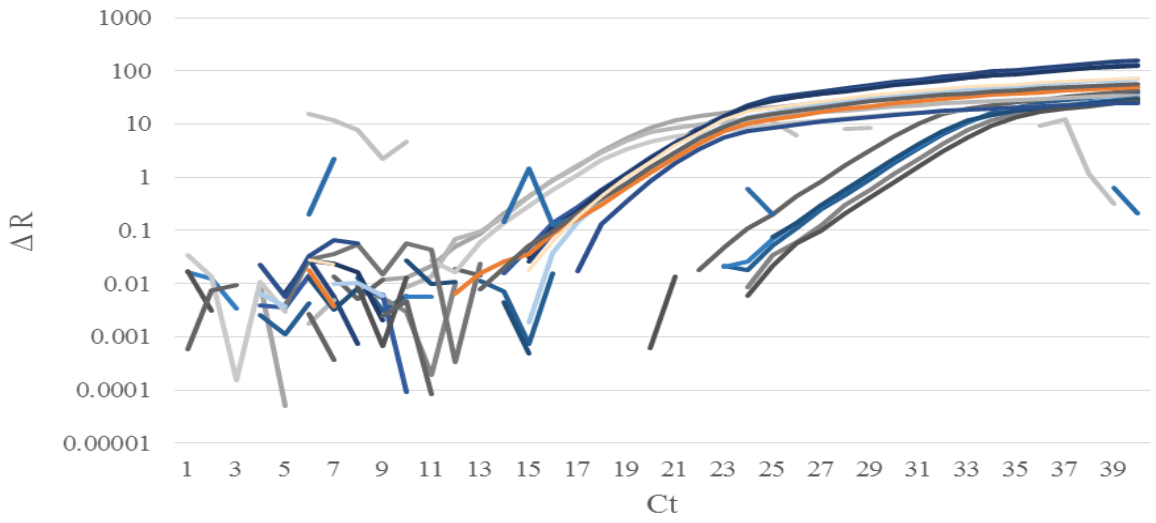


Figure 2.8. Cycle threshold vs. ΔR (reporter fluorescence) values of qPCR wells containing colony 85H bee brains with the *β-actin* primer.

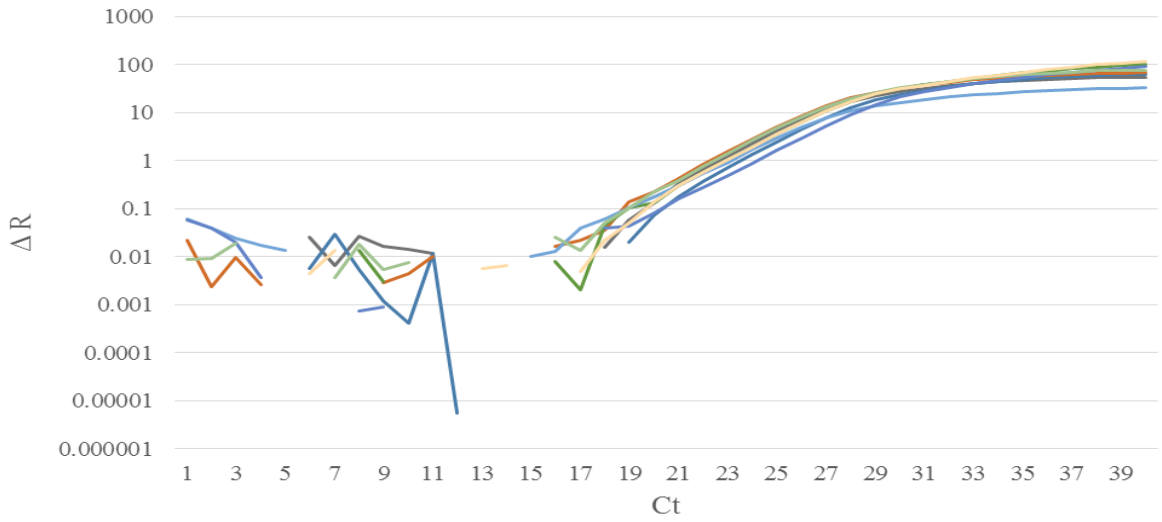


Figure 2.9. Cycle threshold vs. ΔR (reporter fluorescence) values of qPCR wells containing colony 135N bee brains with the *ELF1-α* primer.

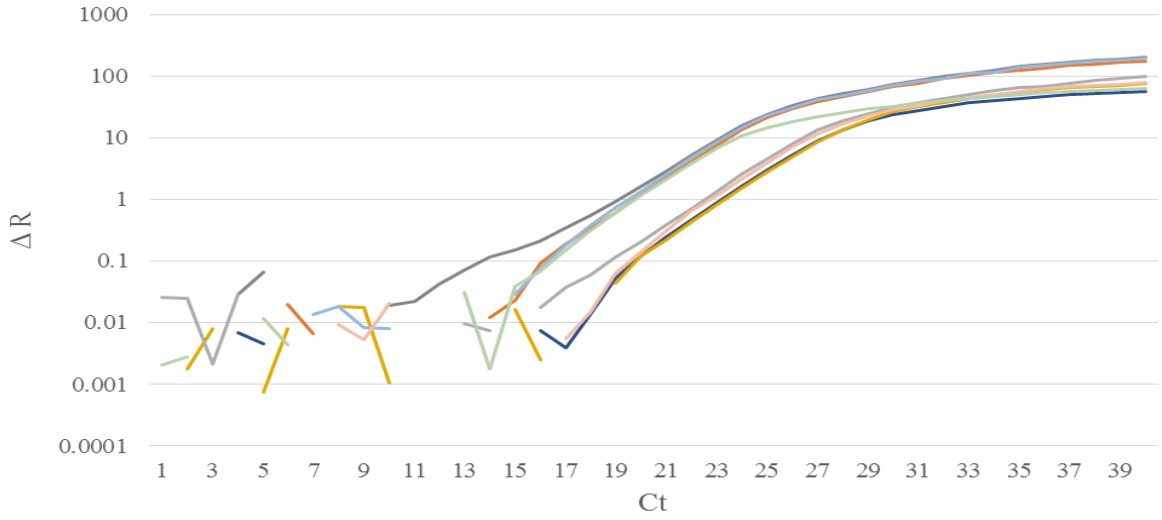


Figure 2.10. Cycle threshold vs. ΔR (reporter fluorescence) values of qPCR wells containing colony 36N bee brains with the *ELF1-α* primer.

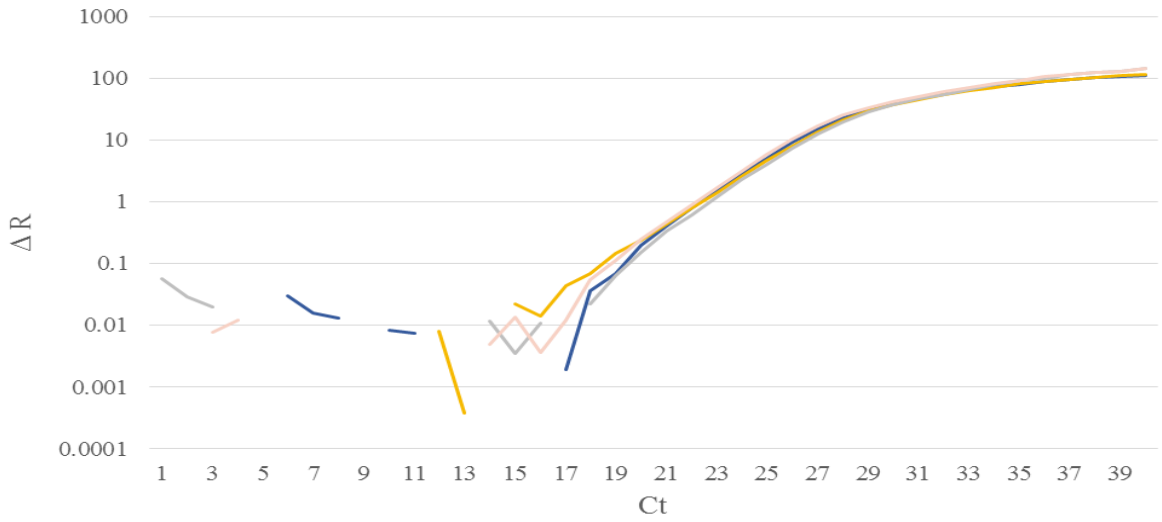


Figure 2.11. Cycle threshold vs. ΔR (reporter fluorescence) values of qPCR wells containing colony 86N bee brains with the *ELF1-α* primer.

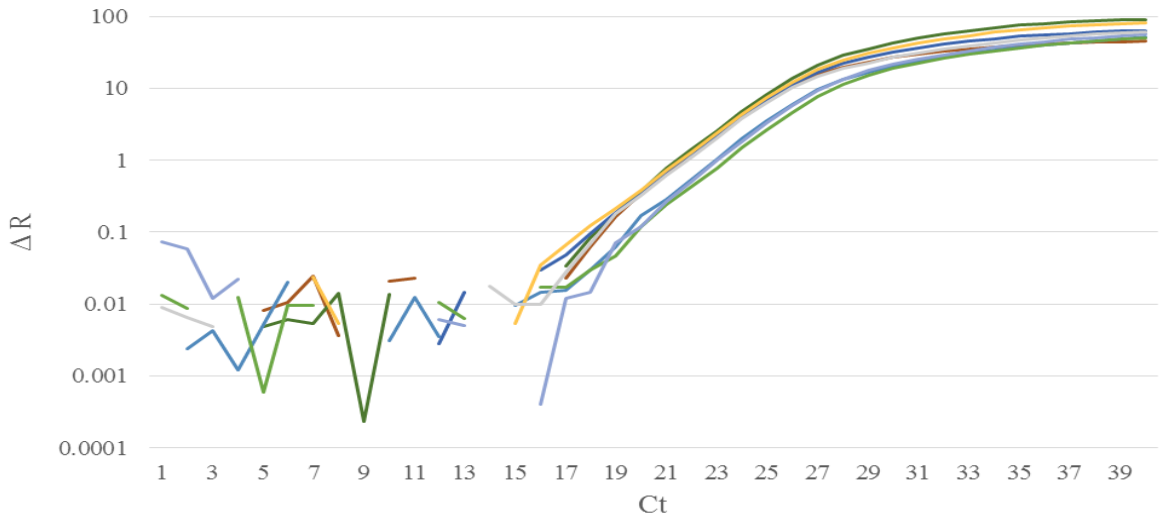


Figure 2.12. Cycle threshold vs. ΔR (reporter fluorescence) values of qPCR wells containing colony 17H bee brains with the *ELF1-α* primer.

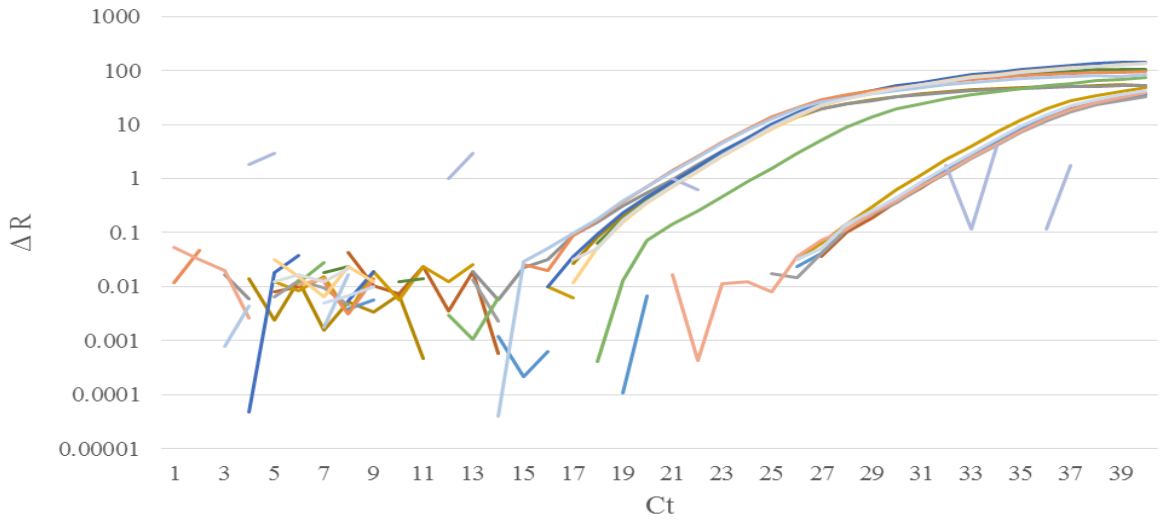


Figure 2.13. Cycle threshold vs. ΔR (reporter fluorescence) values of qPCR wells containing colony 85H bee brains with the *ELF1-α* primer.

Table 2.4. Mean cycle threshold of genes *β-actin* and *ELF1-α* by colony and pairing.

Pairing	Colony	Mean Ct of *β-actin*	Mean Ct of *ELF1-α*
1H	17H	18.45	22.79
1N	135N	20.34	23.39
2H	85H	21.19	24.55
2N	36N	16.22	22.33
3H	17H	18.45	22.79
3N	135N	20.34	23.39
4H	85H	21.19	24.55
4N	36N	16.22	22.33
5H	85H	21.19	24.55
5N	86N	17.55	21.77
6H	85H	21.19	24.55
6N	86N	17.55	21.80
7H	17H	18.45	22.79
7N	135N	20.34	23.39
8H	17H	18.45	22.79
8N	135N	20.34	23.39

Table 2.5. Fold change of *β-actin* and the mean hygienic and non-hygienic cycle threshold value for genes *β-actin* and *ELF1-α.*

Gene	Mean hygienic Ct value	Mean Non-hygienic Ct value	Fold Change
β-actin	19.820	18.615	-1.175
ELF1-α	23.669	22.727	

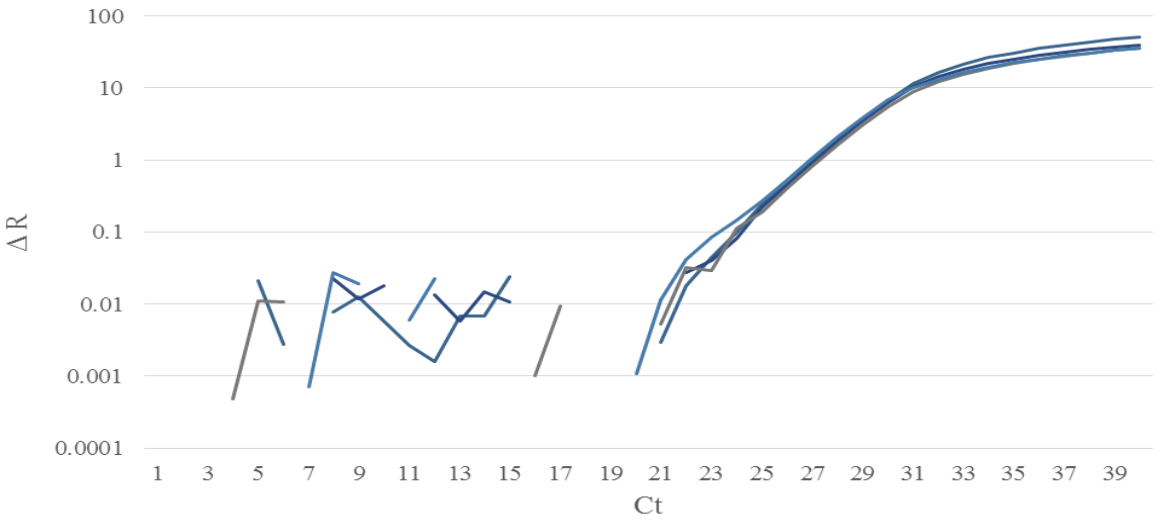


Figure 2.14. Cycle threshold vs. ΔR (reporter fluorescence) values of qPCR wells containing bee brains from the 1H pairing with the *2 pore K pump* primer.

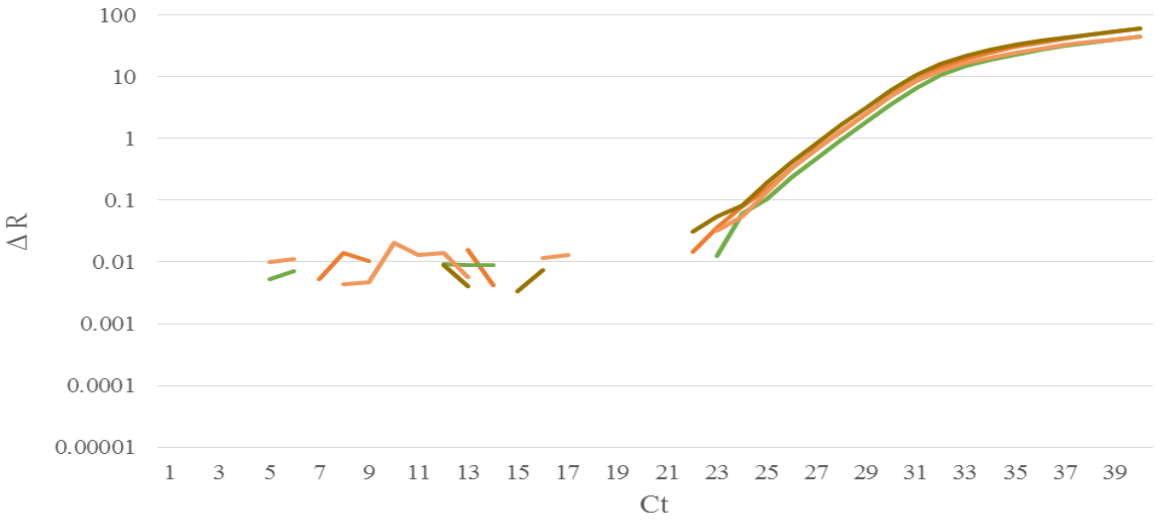


Figure 2.15. Cycle threshold vs. ΔR (reporter fluorescence) values of qPCR wells containing bee brains from the 1N pairing with the *2 pore K pump* primer.

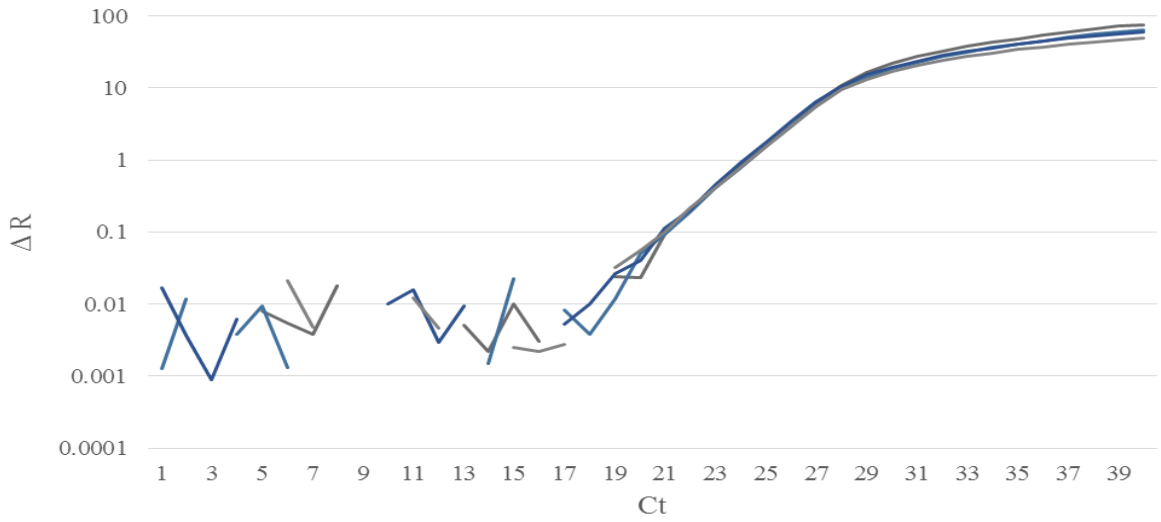


Figure 2.16. Cycle threshold vs. ΔR (reporter fluorescence) values of qPCR wells containing bee brains from the 2H pairing with the *2 pore K pump* primer.

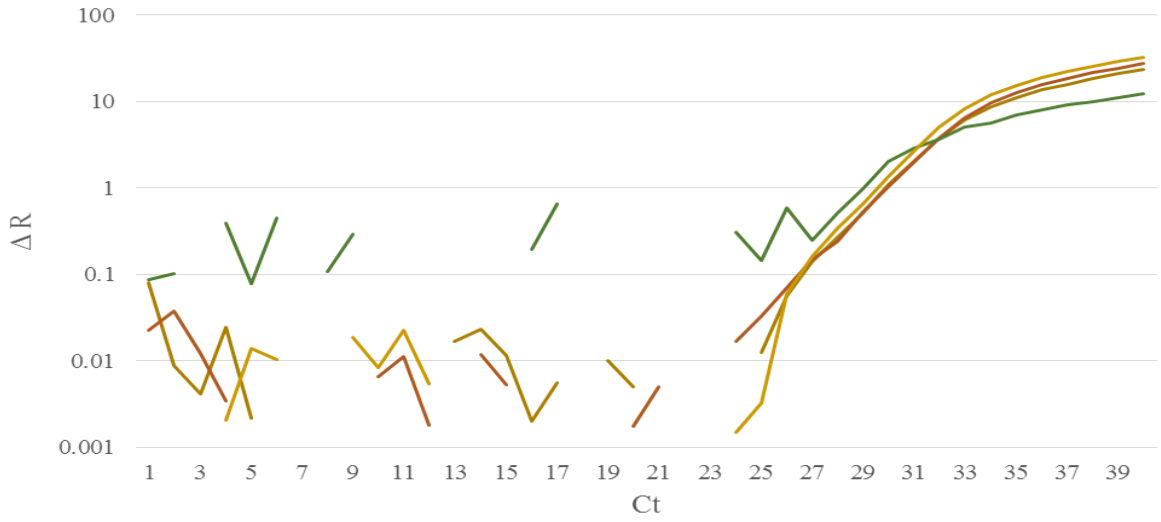


Figure 2.17. Cycle threshold vs. ΔR (reporter fluorescence) values of qPCR wells containing bee brains from the 2N pairing the *2 pore K pump* primer.

Figure 2.18. Cycle threshold vs. ΔR (reporter fluorescence) values of qPCR wells containing bee brains from the 3H pairing with the *2 pore K pump* primer.

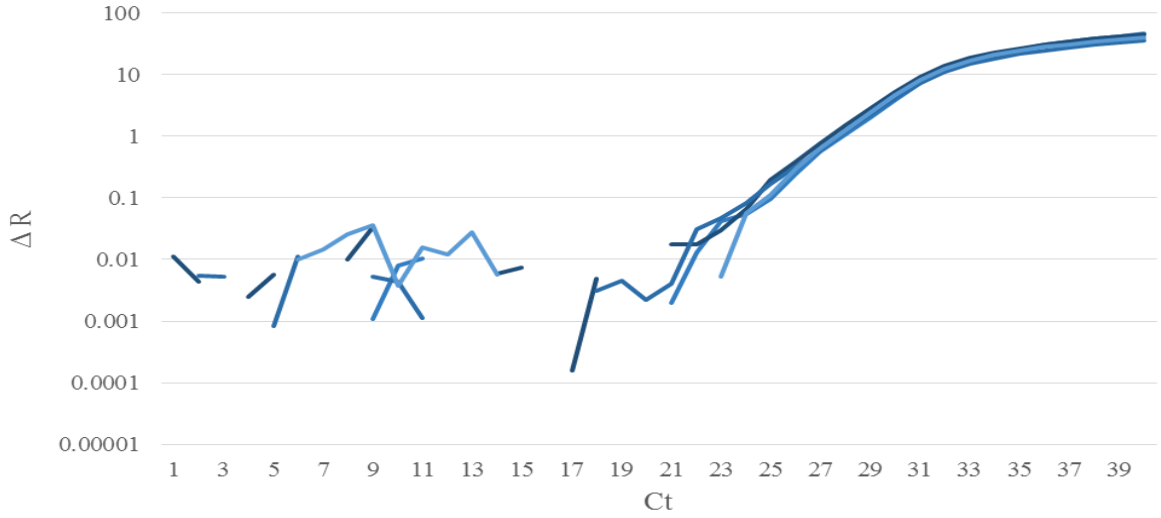


Figure 2.19. Cycle threshold vs. ΔR (reporter fluorescence) values of qPCR wells containing bee brains from the 3N pairing with the *2 pore K pump* primer.

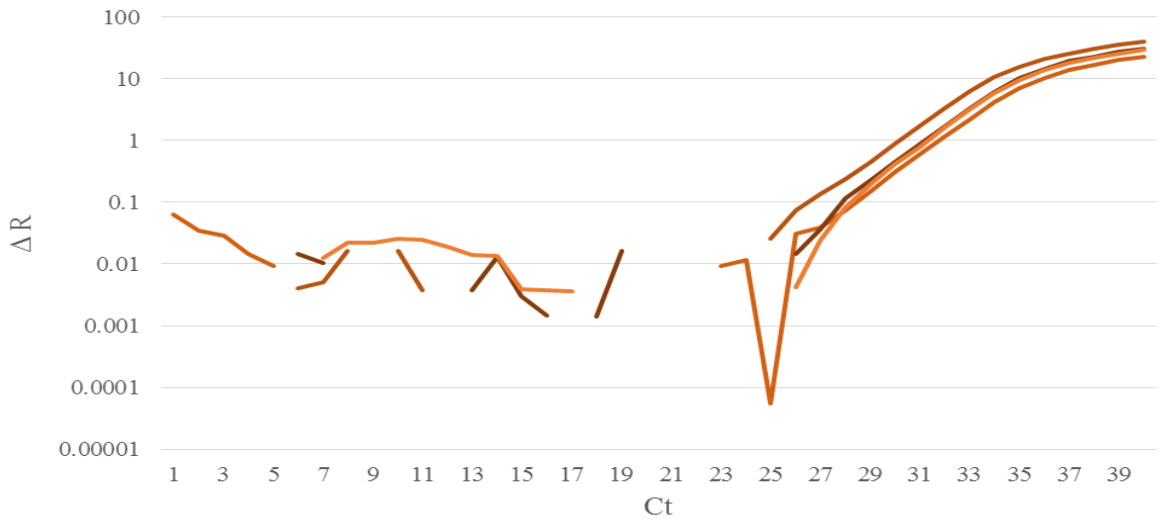


Figure 2.20. Cycle threshold vs. ΔR (reporter fluorescence) values of qPCR wells containing bee brains from the 4H pairing with the *2 pore K pump* primer.

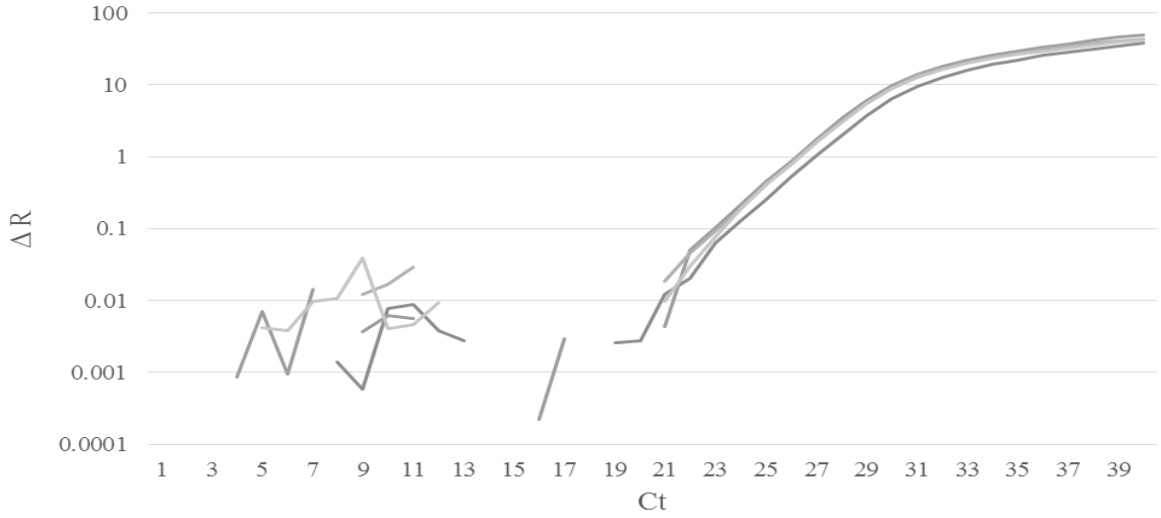


Figure 2.21. Cycle threshold vs. ΔR (reporter fluorescence) values of qPCR wells containing bee brains from the 4N pairing with the *2 pore K pump* primer.

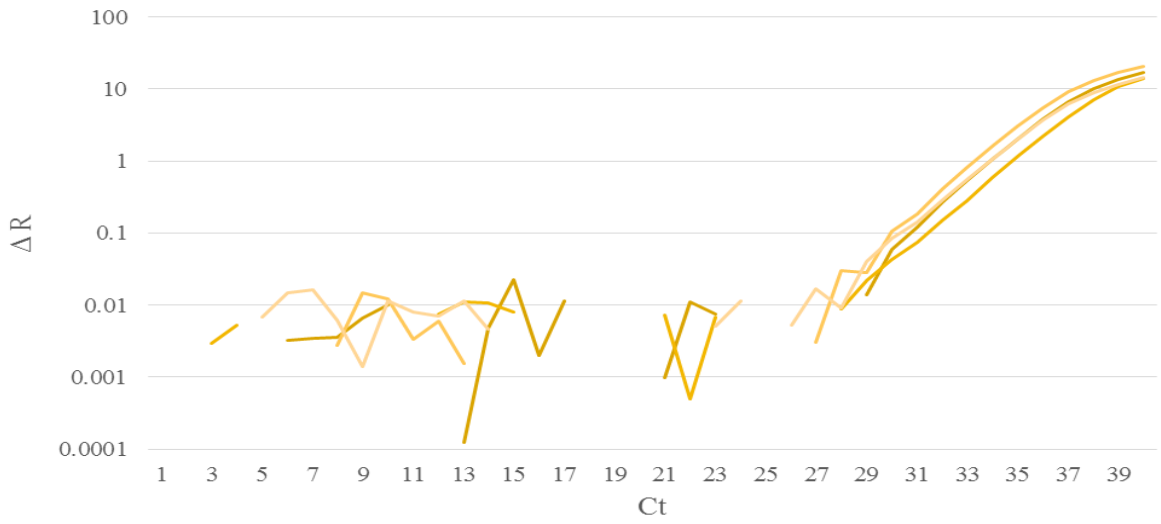


Figure 2.22. Cycle threshold vs. ΔR (reporter fluorescence) values of qPCR wells containing bee brains from the 5H pairing with the *2 pore K pump* primer.

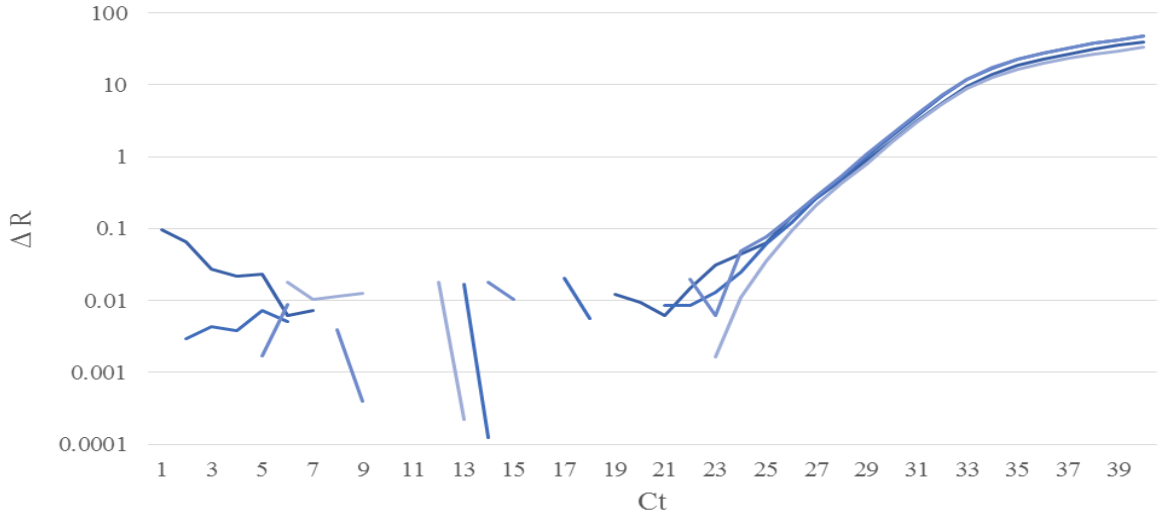


Figure 2.23. Cycle threshold vs. ΔR (reporter fluorescence) values of qPCR wells containing bee brains from the 5N pairing with the *2 pore K pump* primer.

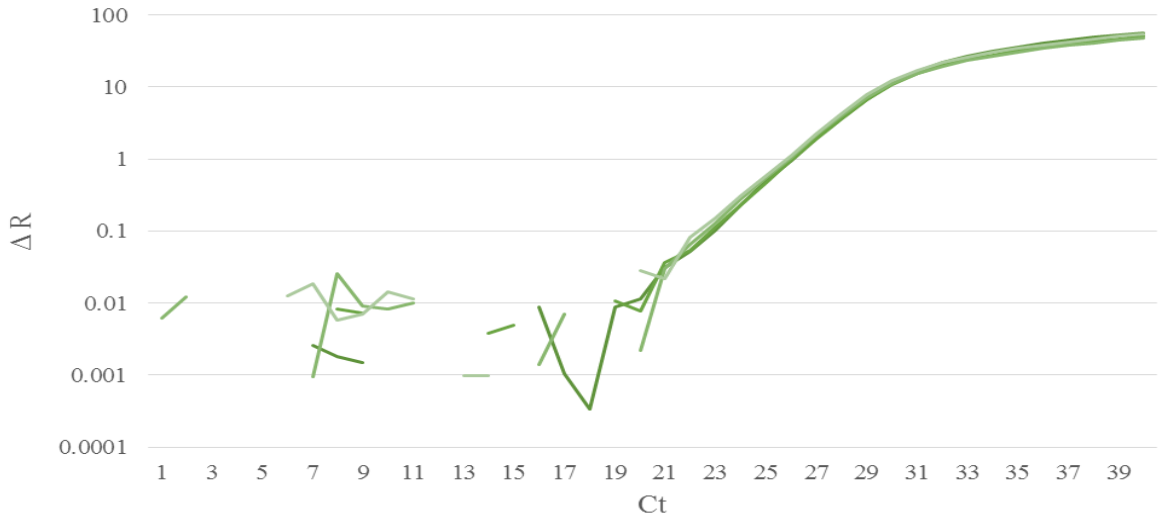


Figure 2.24. Cycle threshold vs. ΔR (reporter fluorescence) values of qPCR wells containing bee brains from the 6H pairing with the *2 pore K pump* primer.

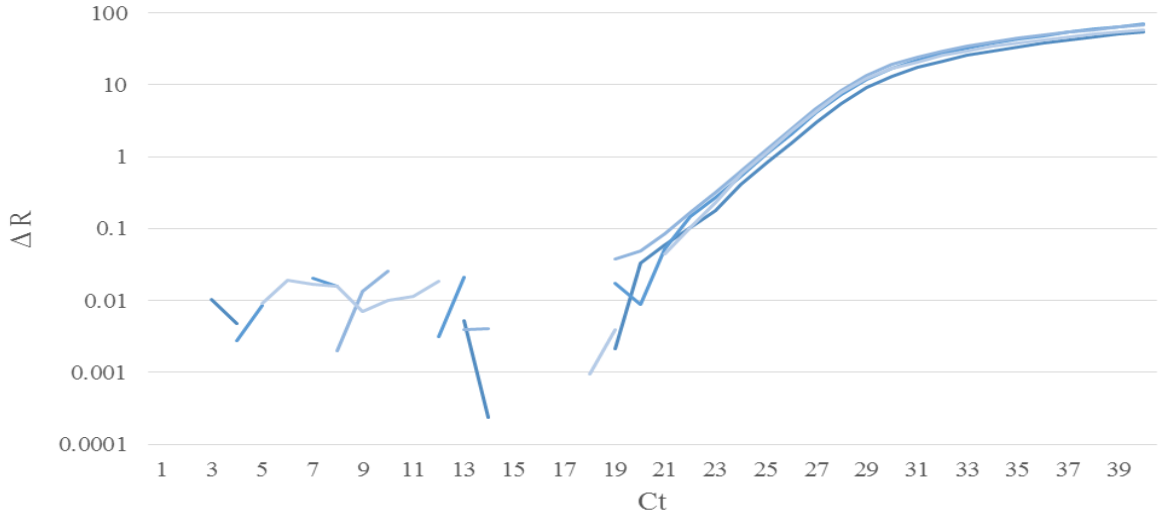


Figure 2.25. Cycle threshold vs. ΔR (reporter fluorescence) values of qPCR wells containing bee brains from the 6N pairing with the *2 pore K pump* primer.

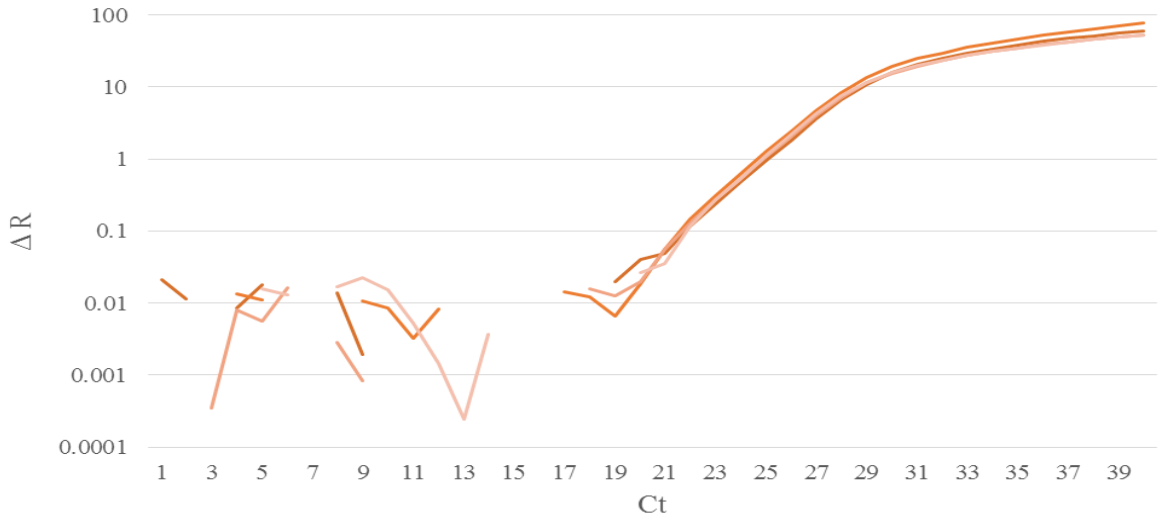


Figure 2.26. Cycle threshold vs. ΔR (reporter fluorescence) values of qPCR wells containing bee brains from the 7H pairing with the *2 pore K pump* primer.

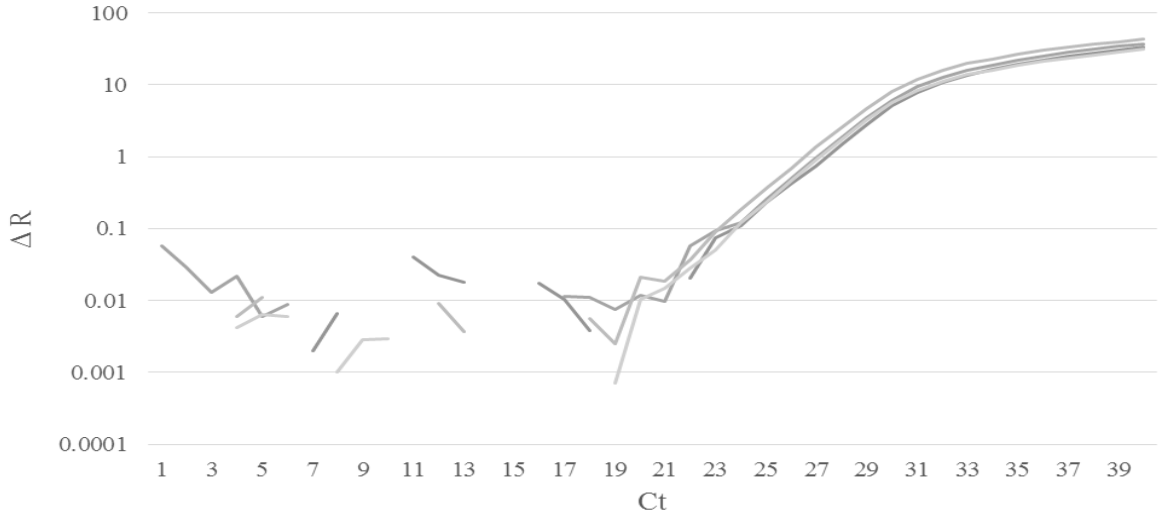


Figure 2.27. Cycle threshold vs. ΔR (reporter fluorescence) values of qPCR wells containing bee brains from the 7N pairing with the *2 pore K pump* primer.

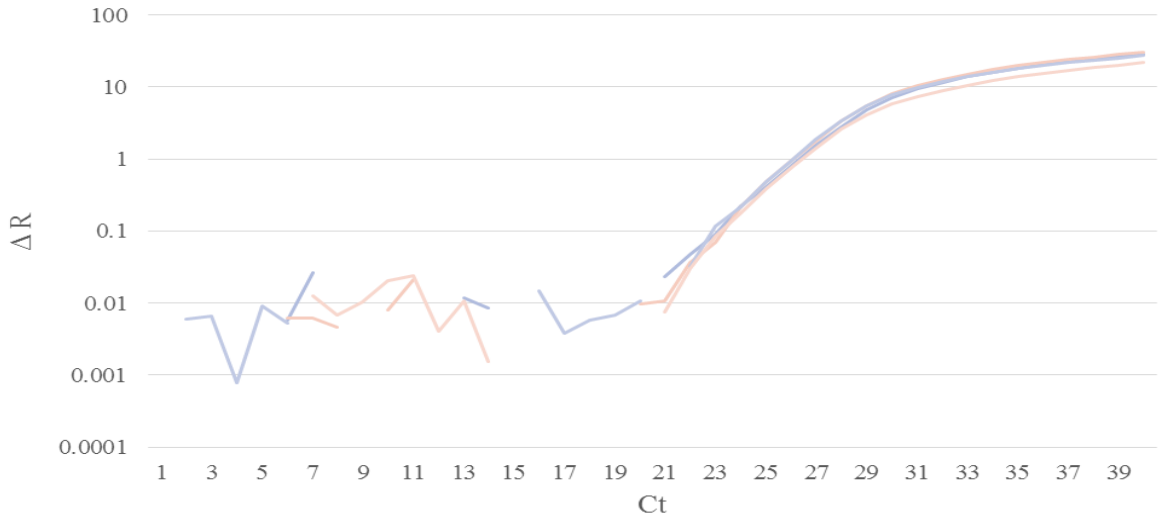


Figure 2.28. Cycle threshold vs. ΔR (reporter fluorescence) values of qPCR wells containing bee brains from the 8H pairing with the *2 pore K pump* primer.

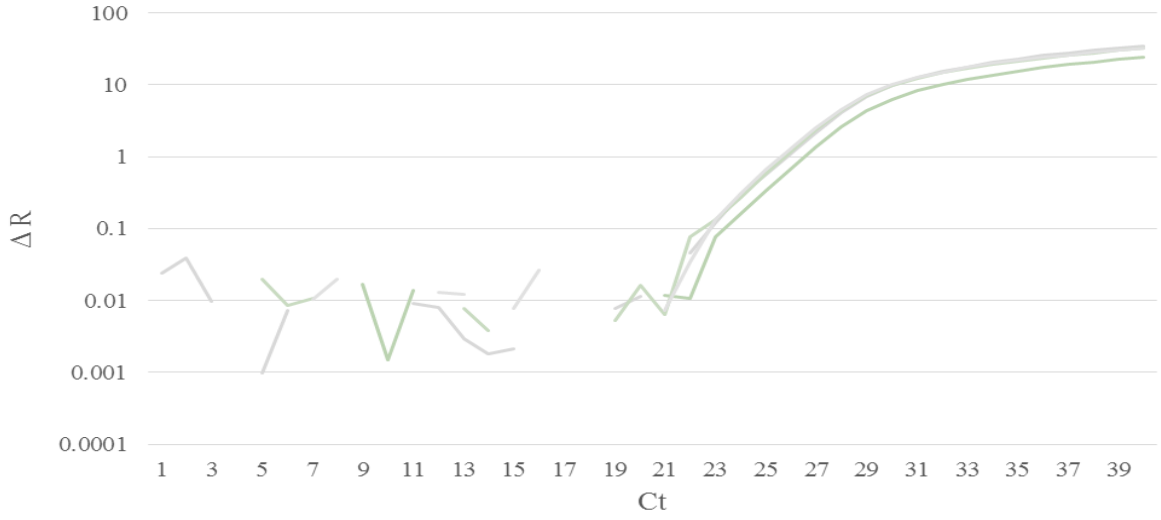


Figure 2.29. Cycle threshold vs. ΔR (reporter fluorescence) values of qPCR wells containing bee brains from the 8N pairing with the *2 pore K pump* primer.

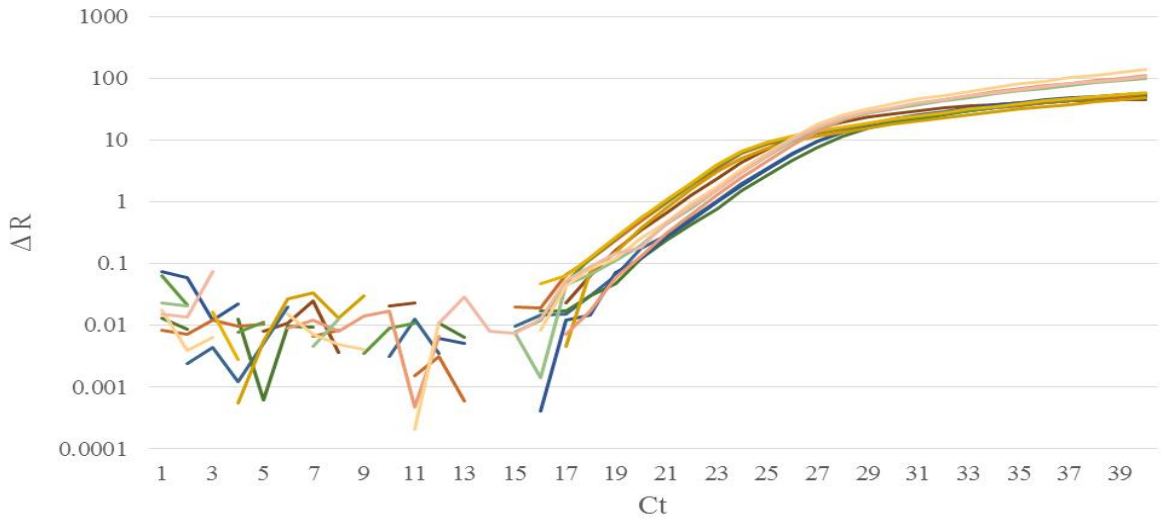


Figure 2.30. Cycle threshold vs. ΔR (reporter fluorescence) values of qPCR wells containing bee brains from the 1H pairing brains with the *ELF1-α* primer.

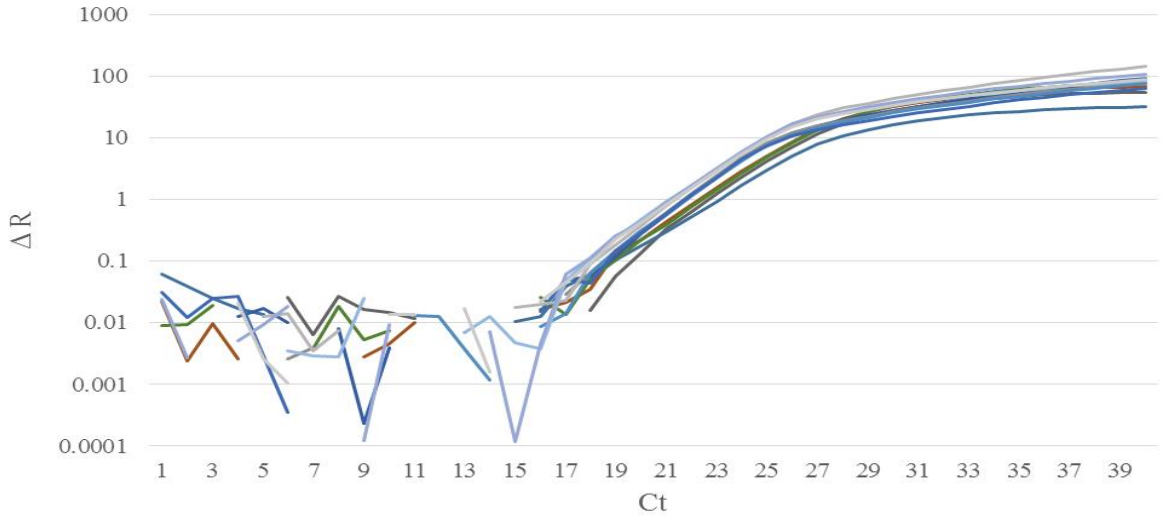


Figure 2.31. Cycle threshold vs. ΔR (reporter fluorescence) values of qPCR wells containing bee brains from the 1N pairing with the *ELF1-α* primer.

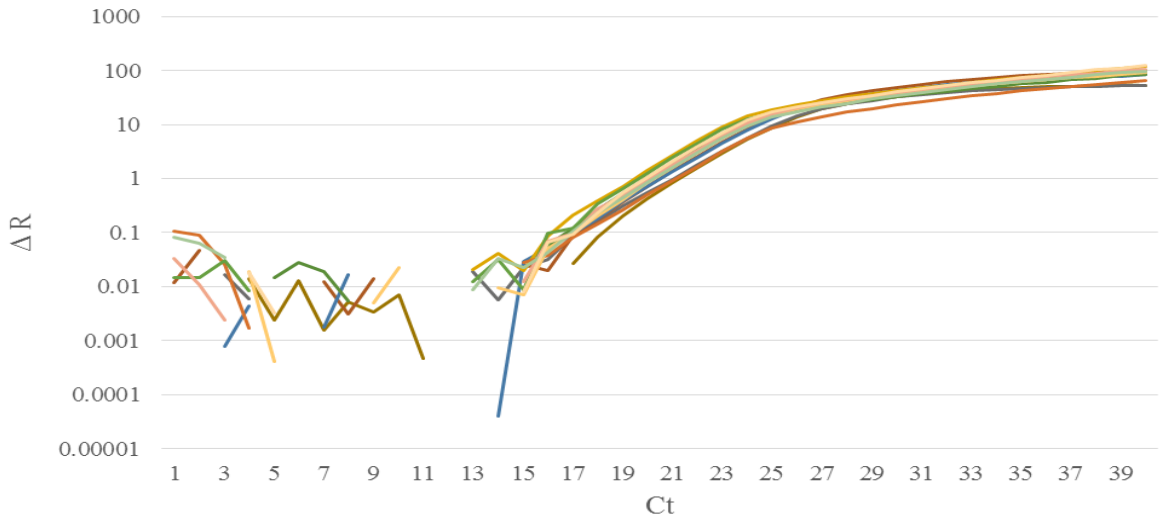


Figure 2.32. Cycle threshold vs. ΔR (reporter fluorescence) values of qPCR wells containing bee brains from the 2H pairing with the *ELF1-α* primer.

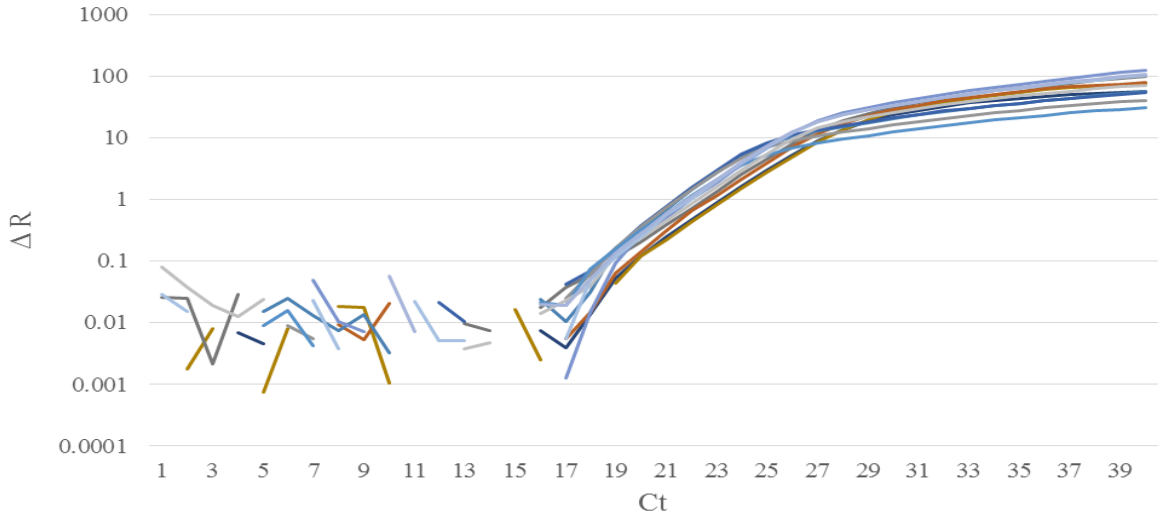


Figure 2.33. Cycle threshold vs. ΔR (reporter fluorescence) values of qPCR wells containing bee brains from the 2N pairing with the *ELF1-α* primer.

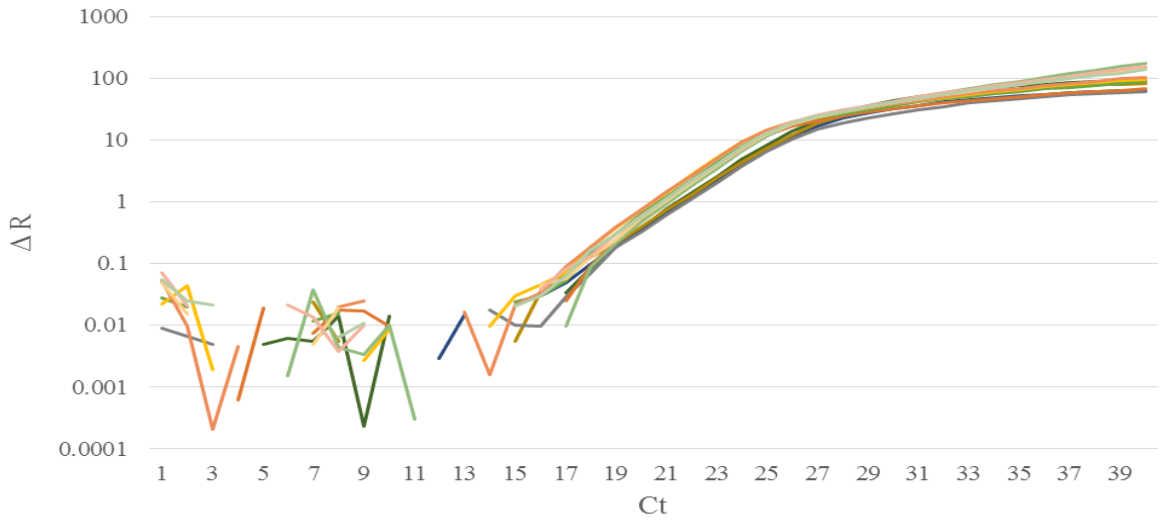


Figure 2.34. Cycle threshold vs. ΔR (reporter fluorescence) values of qPCR wells containing bee brains from the 3H pairing with the *ELF1-α* primer.

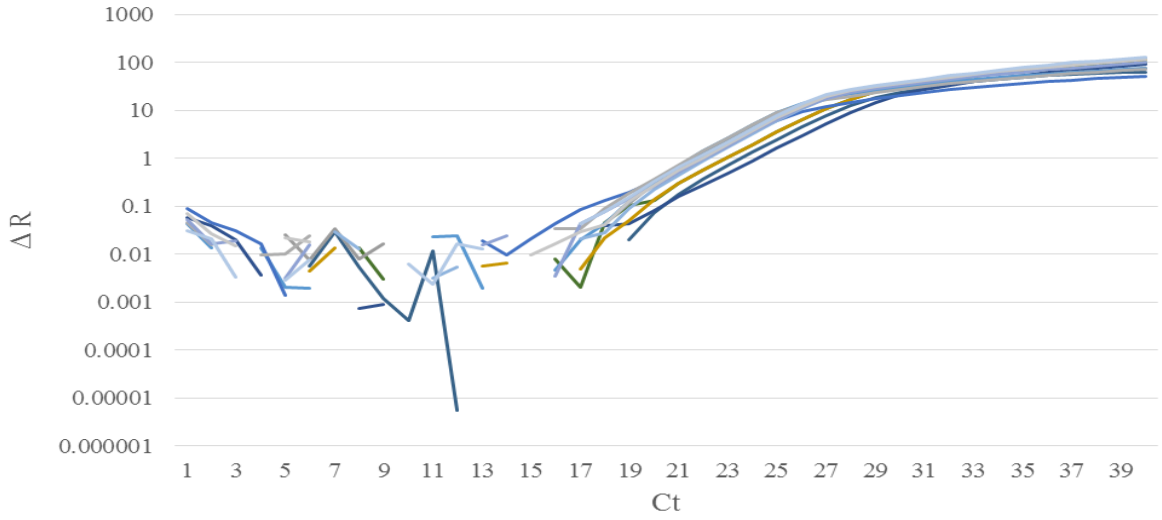


Figure 2.35. Cycle threshold vs. ΔR (reporter fluorescence) values of qPCR wells containing bee brains from the 3N pairing with the *ELF1-α* primer.

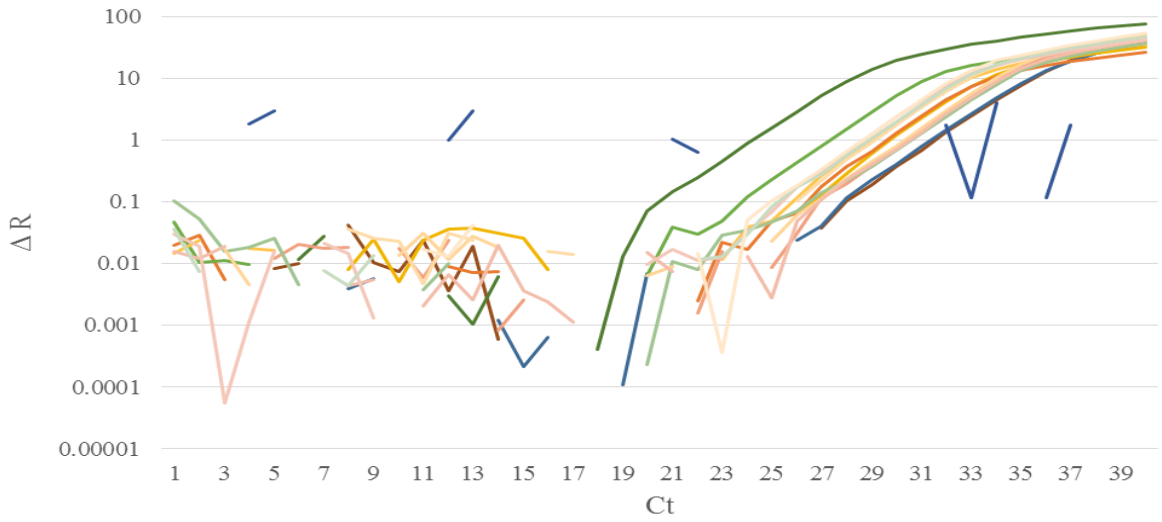


Figure 2.36. Cycle threshold vs. ΔR (reporter fluorescence) values of qPCR wells containing bee brains from the 4H pairing with the *ELF1-α* primer.

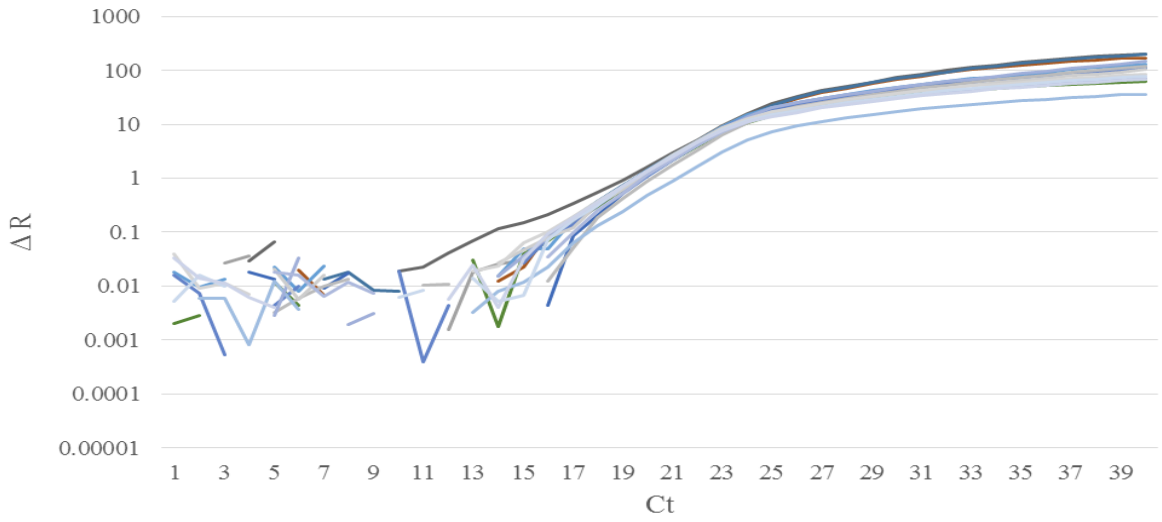


Figure 2.37. Cycle threshold vs. ΔR (reporter fluorescence) values of qPCR wells containing bee brains from the 4N pairing with the *ELF1-α* primer.

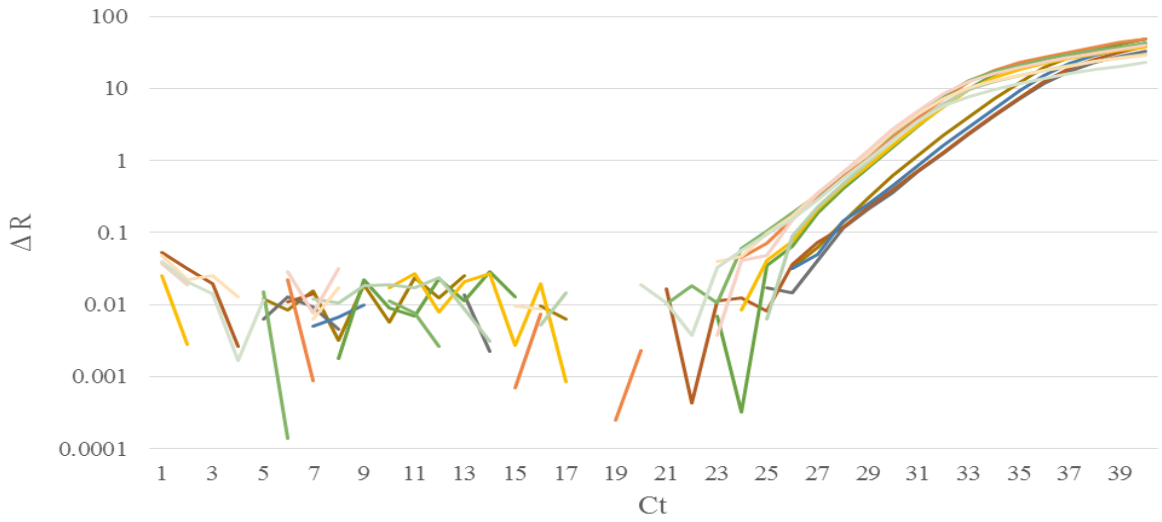


Figure 2.38. Cycle threshold vs. ΔR (reporter fluorescence) values of qPCR wells containing bee brains from the 5H pairing with the *ELF1-α* primer.

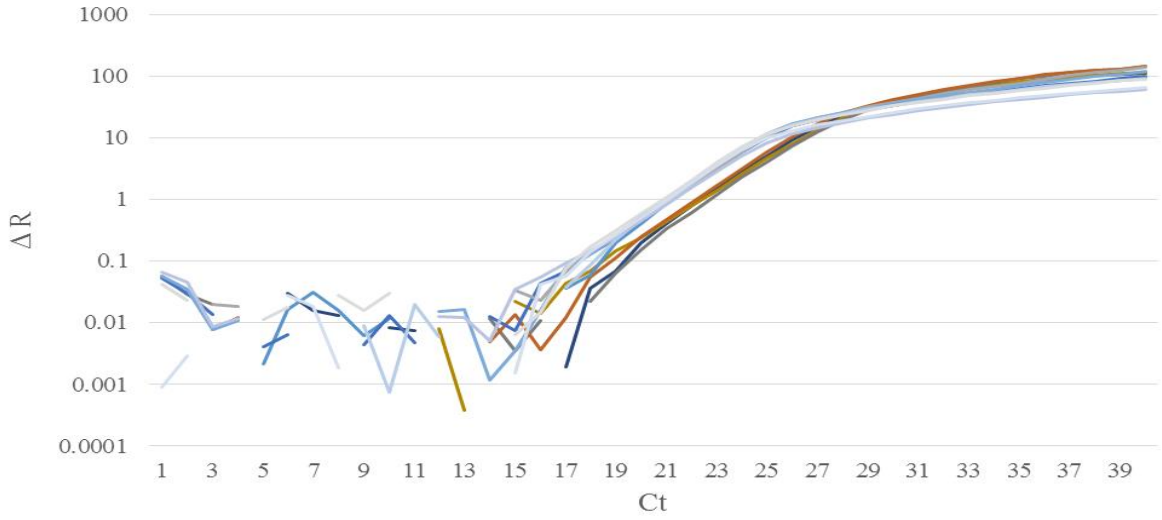


Figure 2.39. Cycle threshold vs. ΔR (reporter fluorescence) values of qPCR wells containing bee brains from the 5N pairing with the *ELF1-α* primer.

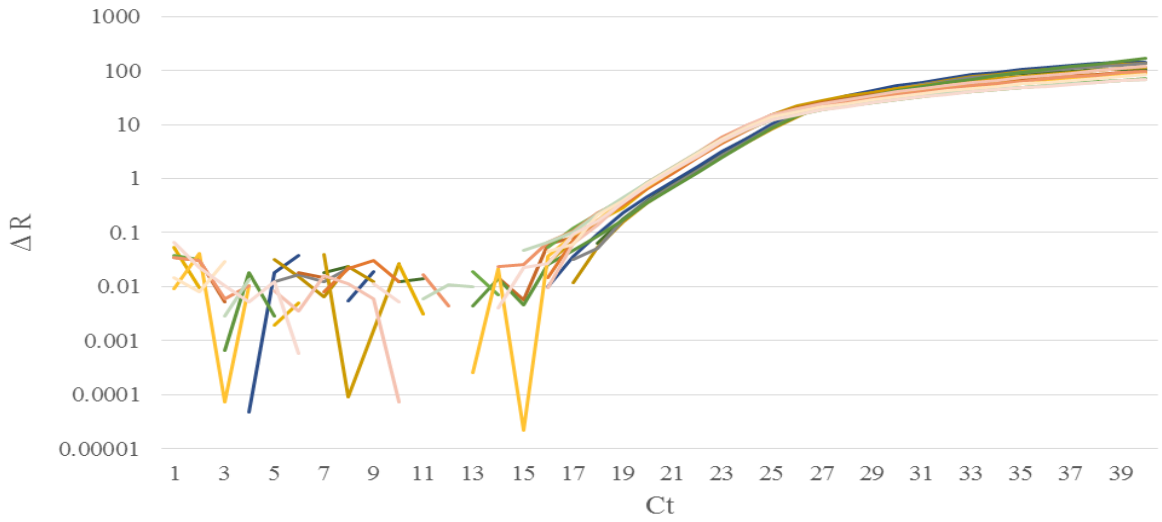


Figure 2.40. Cycle threshold vs. ΔR (reporter fluorescence) values of qPCR wells containing bee brains from the 6H pairing with the *ELF1-α* primer.

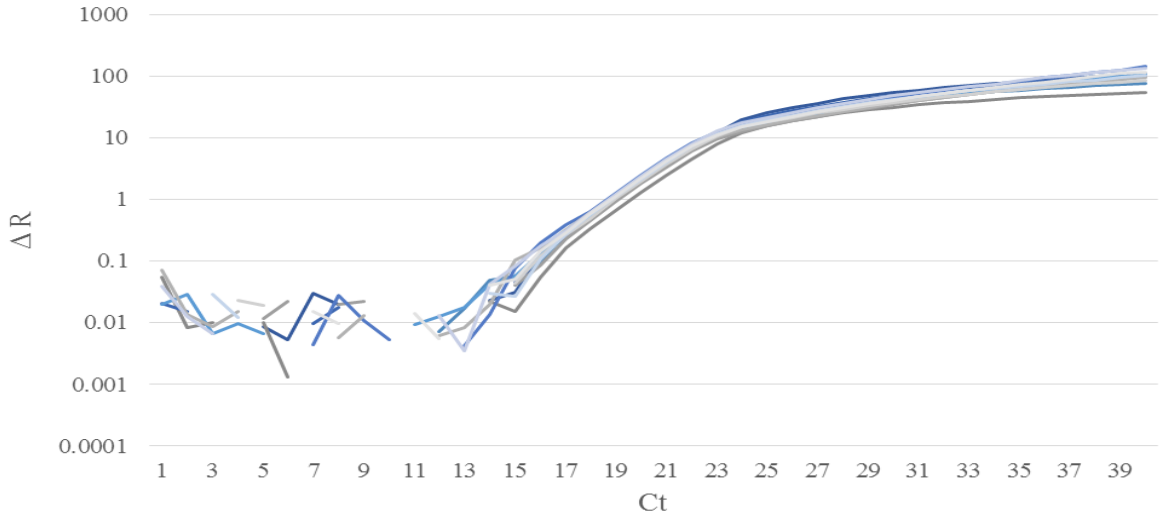


Figure 2.41. Cycle threshold vs. ΔR (reporter fluorescence) values of qPCR wells containing bee brains from the 6N pairing with the *ELF1-α* primer.

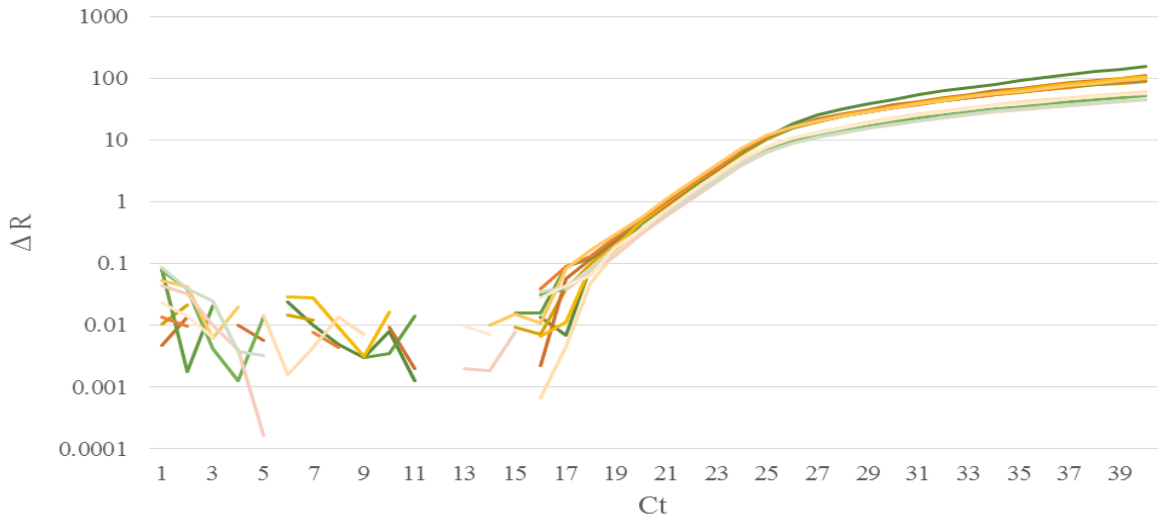


Figure 2.42. Cycle threshold vs. ΔR (reporter fluorescence) values of qPCR wells containing bee brains from the 7H pairing with the *ELF1-α* primer.

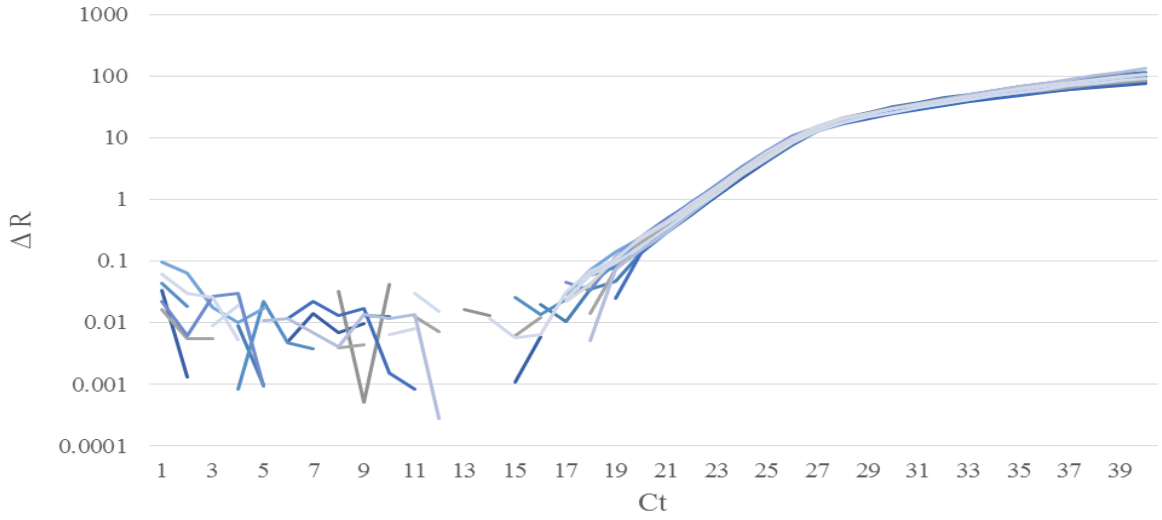


Figure 2.43. Cycle threshold vs. ΔR (reporter fluorescence) values of qPCR wells containing bee brains from the 7N pairing the *ELF1-α* primer.

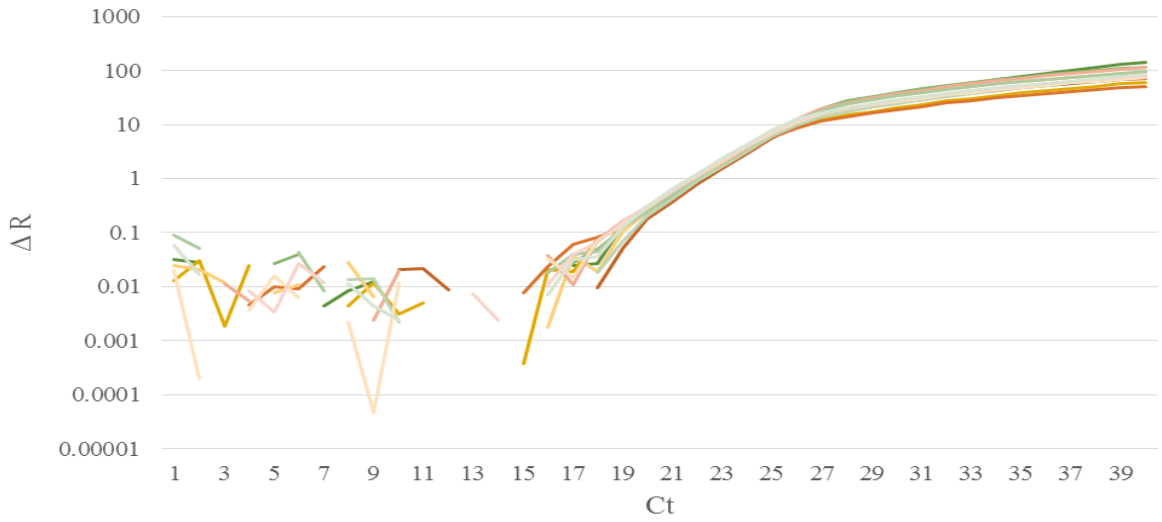


Figure 2.44. Cycle threshold vs. ΔR (reporter fluorescence) values of qPCR wells containing bee brains from the 8H pairing with the *ELF1-α* primer.

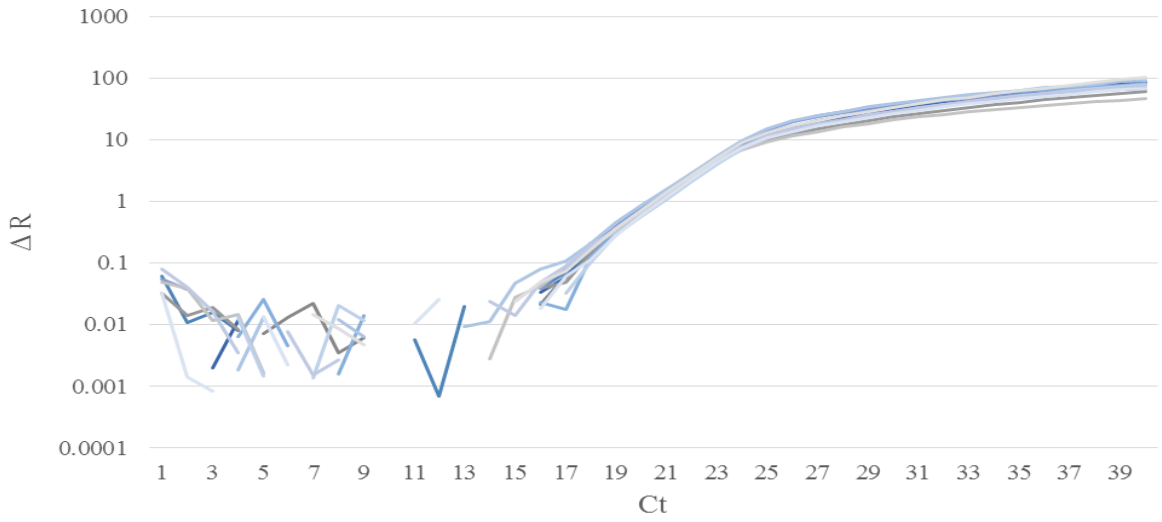


Figure 2.45. Cycle threshold vs. ΔR (reporter fluorescence) values of qPCR wells containing bee brains from the 8N pairing with the *ELF1-α* primer.

Table 2.6. Mean cycle threshold of genes *2 pore K pump* and *ELF1-α* by colony and pairing.

Pairing	Colony	Mean Ct of *2 pore K pump*	Mean Ct of *ELF1-α*
1H	17H	28.14	23.16
1N	135N	28.68	23.02
2H	85H	25.30	21.48
2N	36N	30.66	23.29
3H	17H	27.88	21.99
3N	135N	29.44	23.28
4H	85H	32.86	29.36
4N	36N	28.19	20.85
5H	85H	35.75	30.71
5N	86N	30.86	22.57
6H	85H	27.71	21.64
6N	86N	26.66	20.00
7H	17H	26.60	22.42
7N	135N	28.81	23.58
8H	17H	26.71	23.34
8N	135N	26.37	21.97

Table 2.7. Fold change of *2 pore K pump* and the mean hygienic and non-hygienic cycle threshold value for genes *2 pore K pump* and *ELF1-α*.

Gene	Mean hygienic Ct value	Mean non-hygienic Ct value	Fold Change
2 pore K pump	28.87	28.71	2.815
ELF1-α	24.26	22.32	

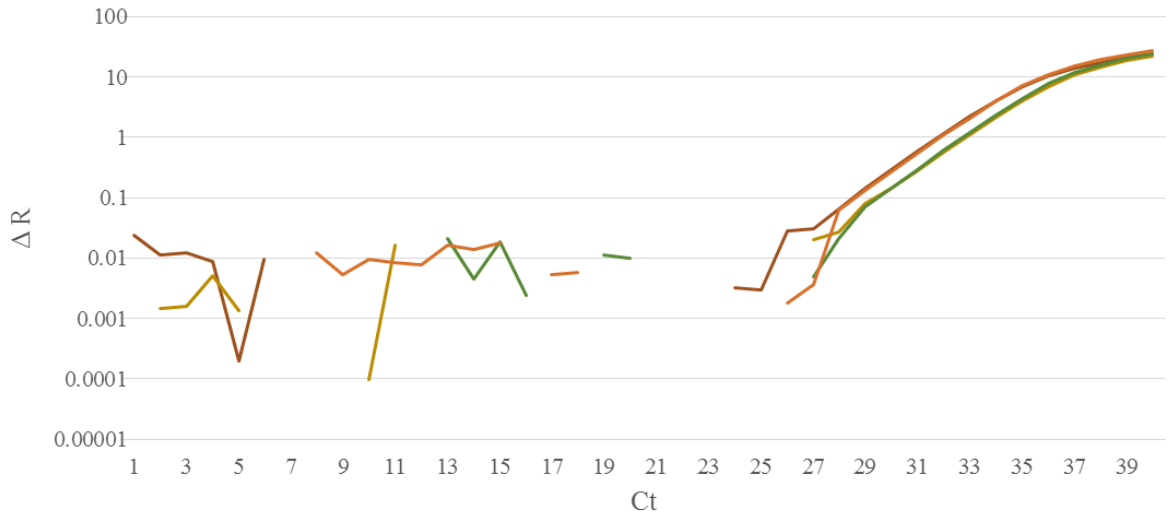


Figure 2.46. Cycle threshold vs. ΔR (reporter fluorescence) values of qPCR wells containing bee brains from the 1H pairing with the *Pteropsin* primer.

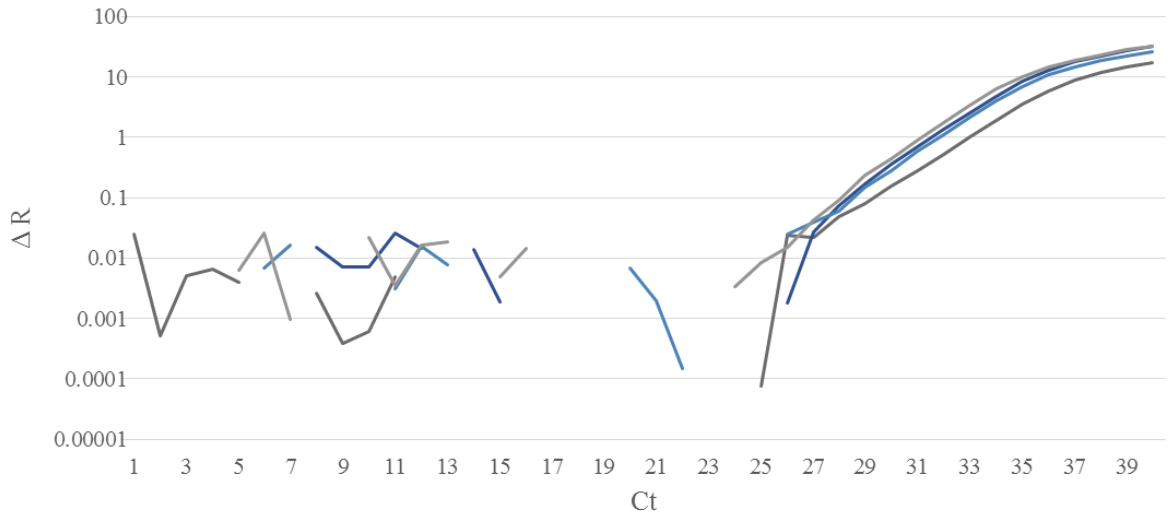


Figure 2.47. Cycle threshold vs. ΔR (reporter fluorescence) values of qPCR wells containing bee brains from the 1N pairing with the *Pteropsin* primer.

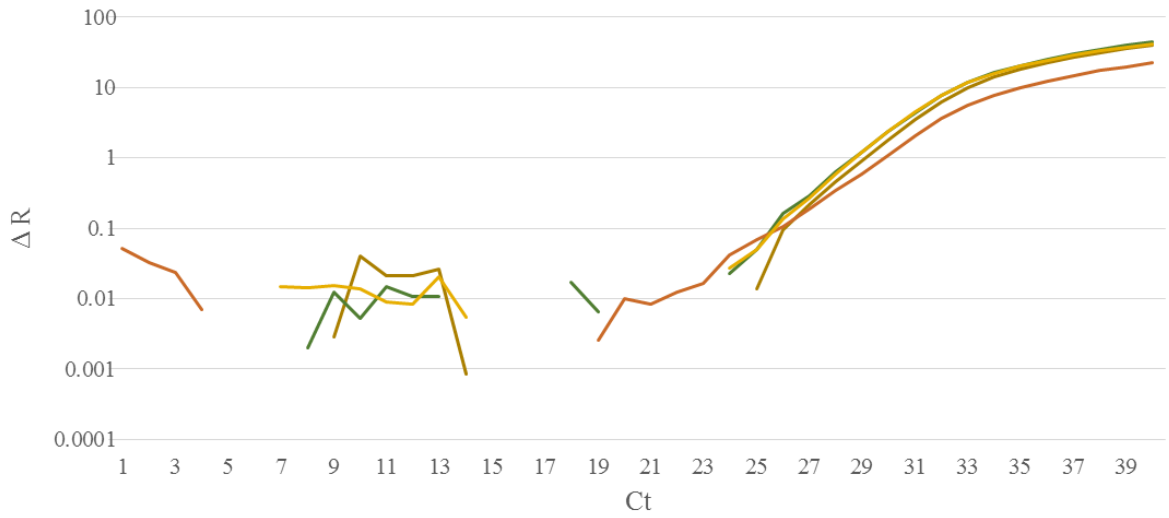


Figure 2.48. Cycle threshold vs. ΔR (reporter fluorescence) values of qPCR wells containing bee brains from the 2H pairing with the *Pteropsin* primer.

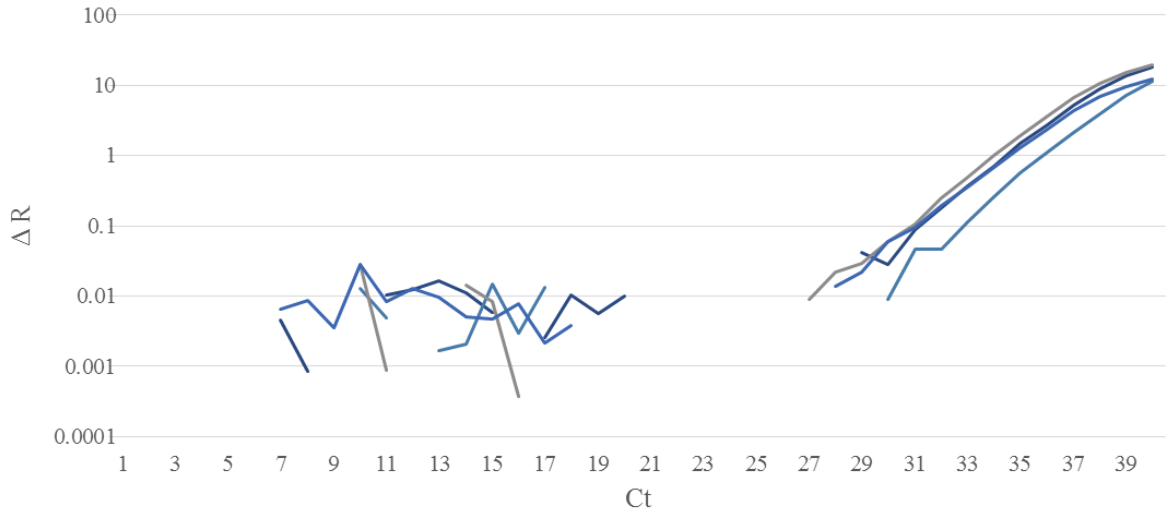


Figure 2.49. Cycle threshold vs. ΔR (reporter fluorescence) values of qPCR wells containing bee brains from the 2N pairing with the *Pteropsin* primer.

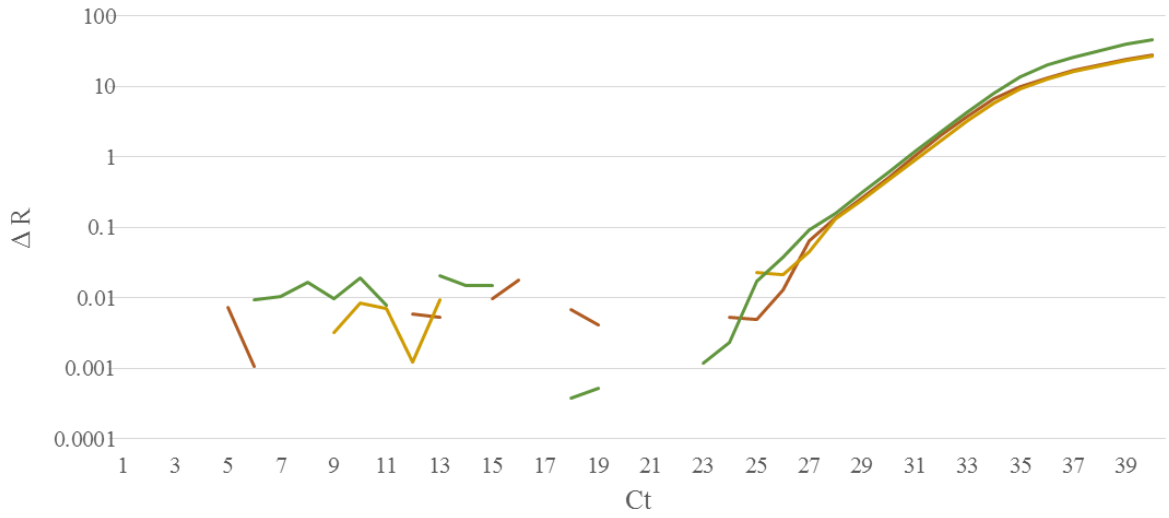


Figure 2.50. Cycle threshold vs. ΔR (reporter fluorescence) values of qPCR wells containing bee brains from the 3H pairing brains with the *Pteropsin* primer.

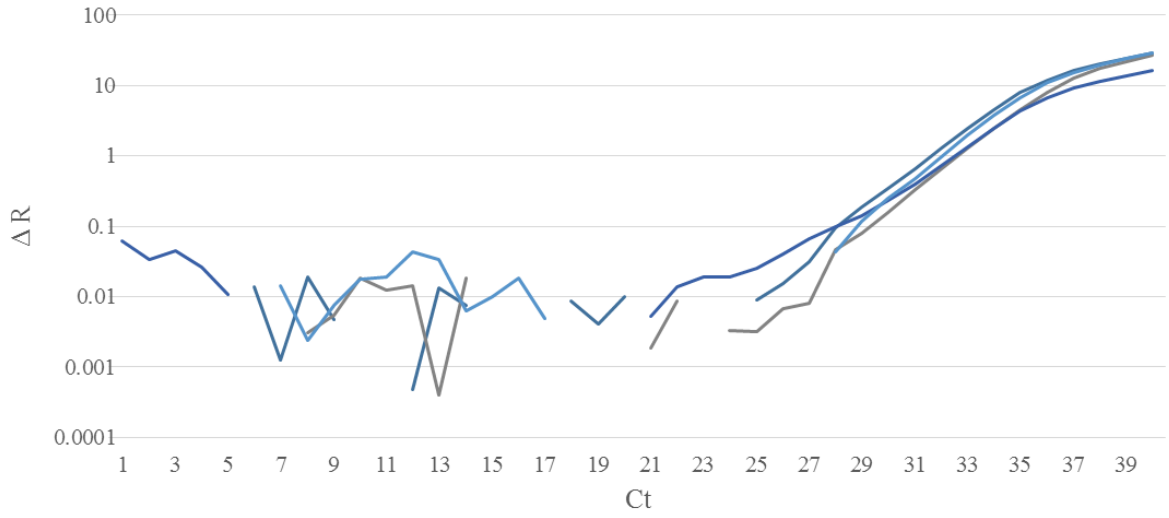


Figure 2.51. Cycle threshold vs. ΔR (reporter fluorescence) values of qPCR wells containing bee brains from the 3N pairing with the *Pteropsin* primer.

Figure 2.52. Cycle threshold vs. ΔR (reporter fluorescence) values of qPCR wells containing bee brains from the 4H pairing with the *Pteropsin* primer.

Figure 2.53. Cycle threshold vs. ΔR (reporter fluorescence) values of qPCR wells containing bee brains from the 4N pairing with the *Pteropsin* primer.

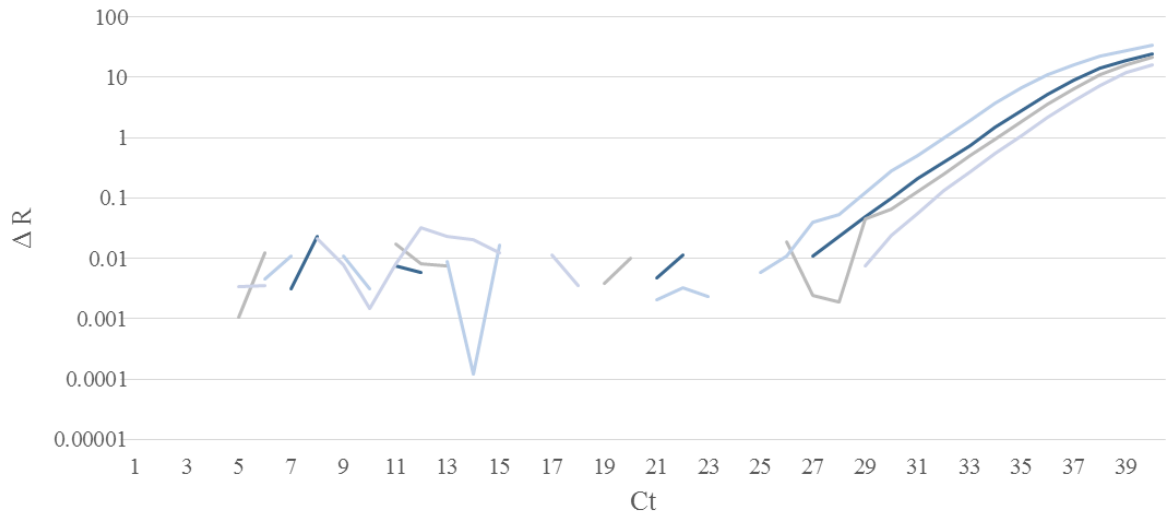


Figure 2.54. Cycle threshold vs. ΔR (reporter fluorescence) values of qPCR wells containing bee brains from the 5H pairing with the *Pteropsin* primer.

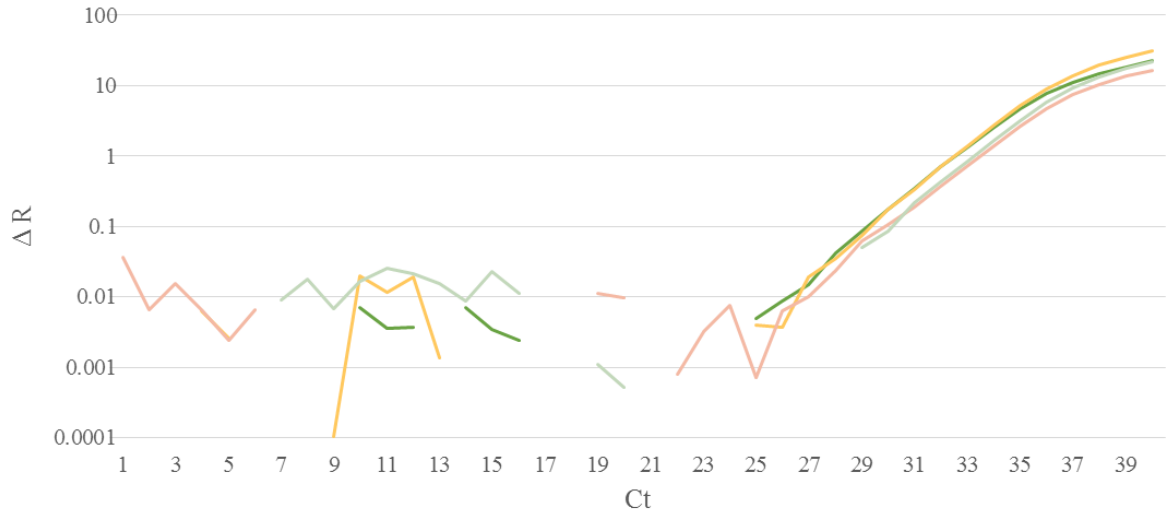


Figure 2.55. Cycle threshold vs. ΔR (reporter fluorescence) values of qPCR wells containing bee brains from the 5N pairing with the *Pteropsin* primer.

Figure 2.56. Cycle threshold vs. ΔR (reporter fluorescence) values of qPCR wells containing bee brains from the 6H pairing with the *Pteropsin* primer.

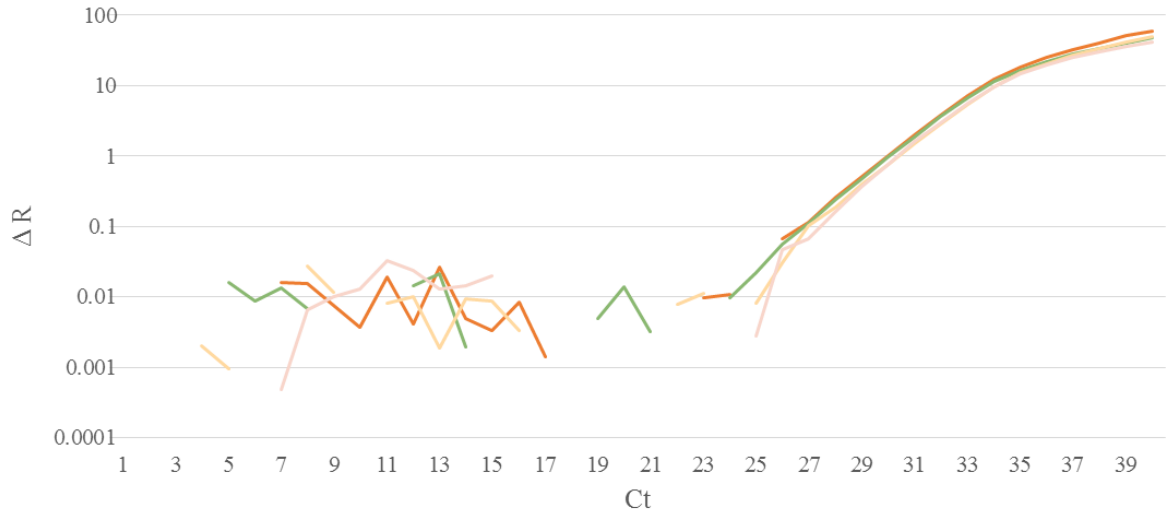


Figure 2.57. Cycle threshold vs. ΔR (reporter fluorescence) values of qPCR wells containing bee brains from the 6N pairing with the *Pteropsin* primer.

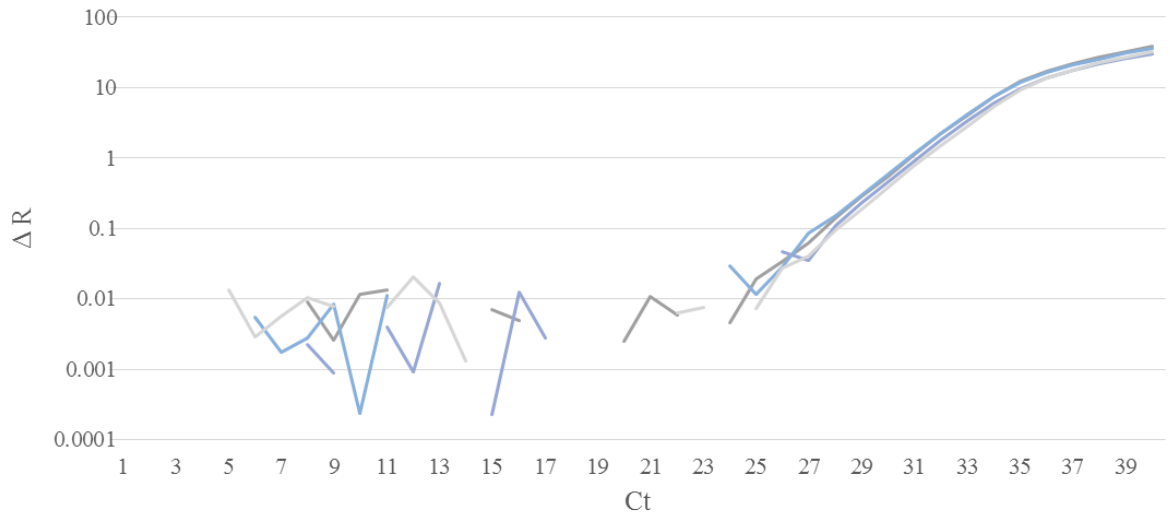


Figure 2.58. Cycle threshold vs. ΔR (reporter fluorescence) values of qPCR wells containing bee brains from the 7H pairing with the *Pteropsin* primer.

Figure 2.59. Cycle threshold vs. ΔR (reporter fluorescence) values of qPCR wells containing bee brains from the 7N pairing with the *Pteropsin* primer.

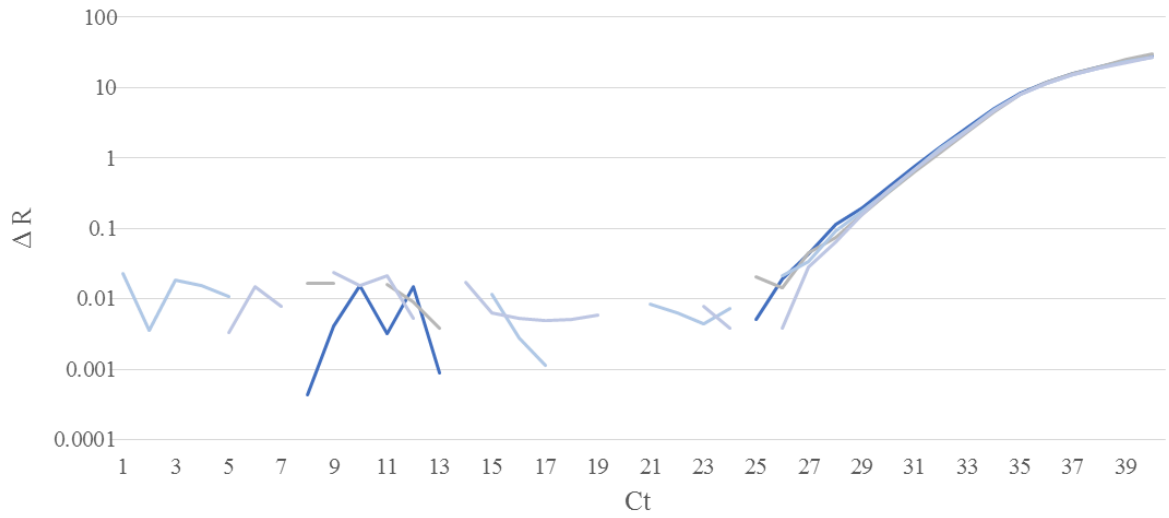


Figure 2.60. Cycle threshold vs. ΔR (reporter fluorescence) values of qPCR wells containing bee brains from the 8H pairing with the *Pteropsin* primer.

Figure 2.61. Cycle threshold vs. ΔR (reporter fluorescence) values of qPCR wells containing bee brains from the 8N pairing with the *Pteropsin* primer.

Table 2.8. Mean cycle threshold of genes *Pteropsin* and *ELF1-α* by colony and pairing.

Pairing	Colony	Mean Ct of *Pteropsin*	Mean Ct of *ELF1-α*
1H	17H	26.95	23.16
1N	135N	26.15	23.02
2H	85H	24.20	21.48
2N	36N	27.99	23.29
3H	17H	25.47	21.99
3N	135N	26.64	23.28
4H	85H	32.76	29.36
4N	36N	25.61	20.85
5H	85H	32.66	30.71
5N	86N	28.49	22.57
6H	85H	27.15	21.64
6N	86N	26.00	20.00
7H	17H	27.80	22.42
7N	135N	28.61	23.58
8H	17H	28.21	23.34
8N	135N	27.45	21.97

Table 2.9. Fold change of *Pteropsin* and the mean hygienic and non-hygienic cycle threshold value for genes *Pteropsin* and *ELF1-α.*

Gene	Mean hygienic Ct value	Mean non-hygienic Ct value	Fold Change
Pteropsin	28.15	27.12	1.853
ELF1-α	24.26	22.32	

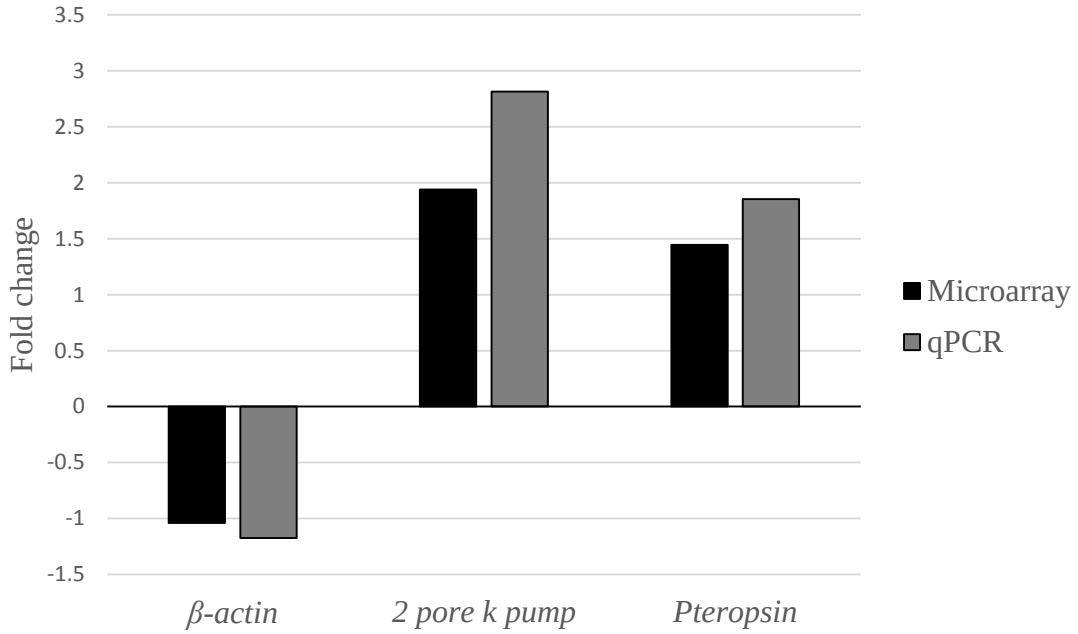


Figure 2.62. Comparison of fold changes between microarray and qPCR data of the three genes used to validate microarray data.

Melt Curves

Melt curves were conducted to ensure that the qPCR procedure yielded only one amplicon. Figures 2.63 through 2.66 display the melt curves of each of the primers utilized as well as the average Tm or the melting temperature for each primer. These melting curves yielded one peak each and show no signs of a shoulder. The average Tm for *Elf1-α,* β-actin, *2 Pore K pump , and Pteropsin* amplicons was respectively 79.5, 79.9, 78.3 and 81.6°C.

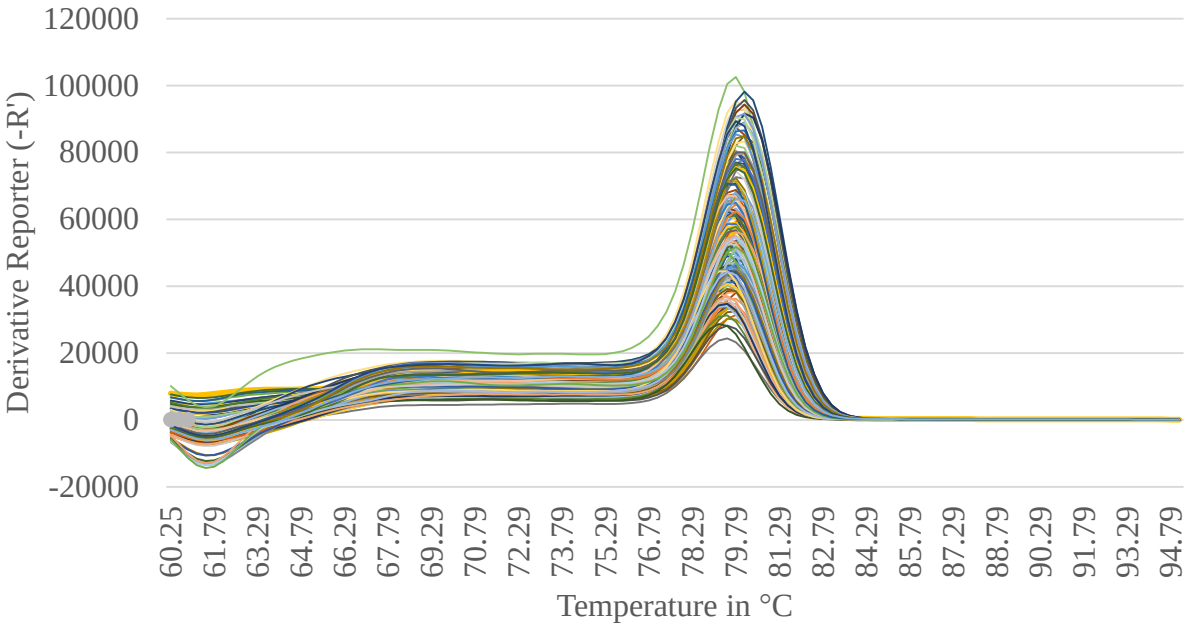


Figure 2.63. Melt curve for wells containing the *Elf1-α* primer.

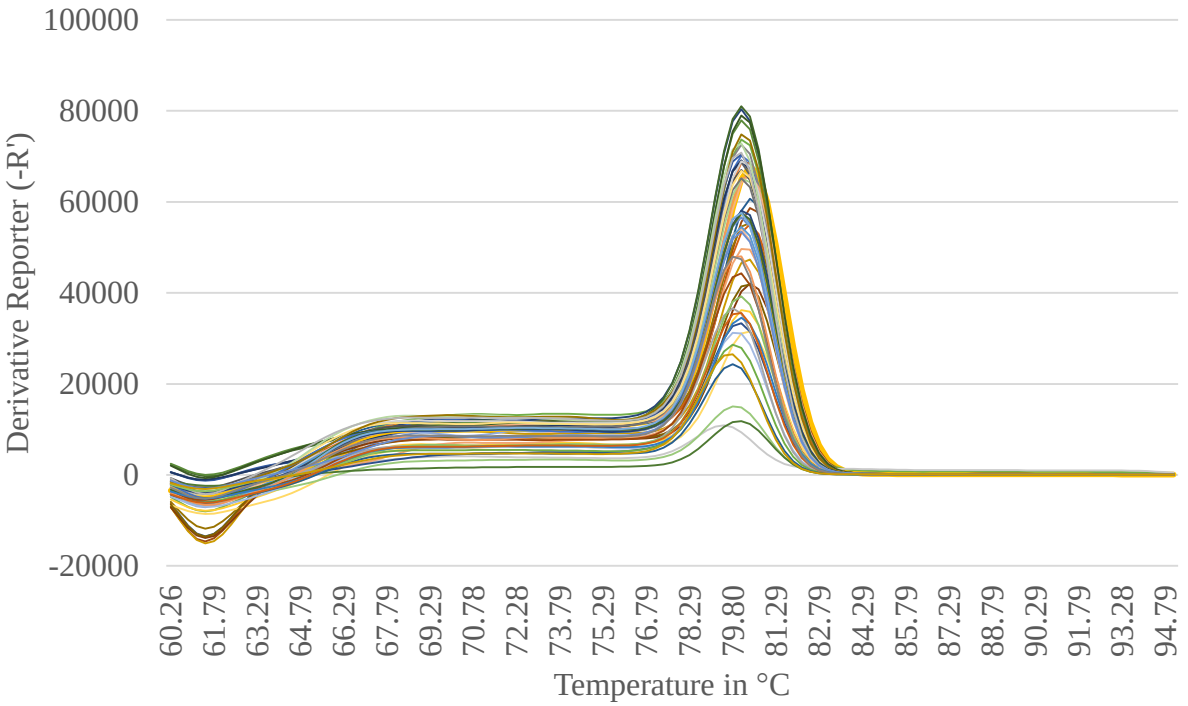


Figure 2.64. Melt curve for wells containing the *β-actin* primer.

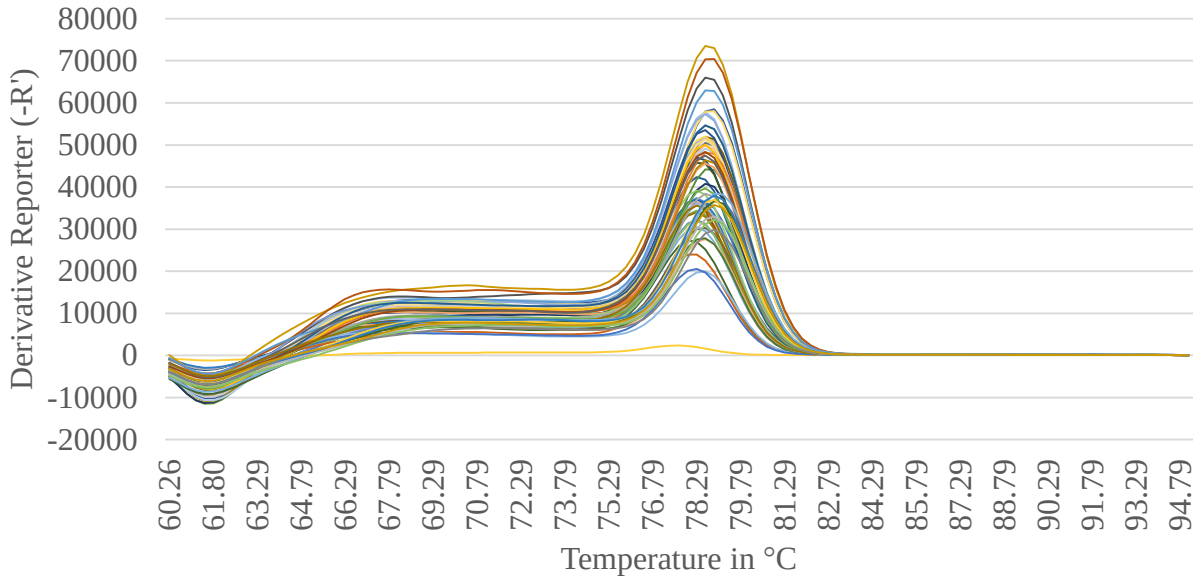


Figure 2.65. Melt curve for wells containing the *2 Pore K pump* primer.

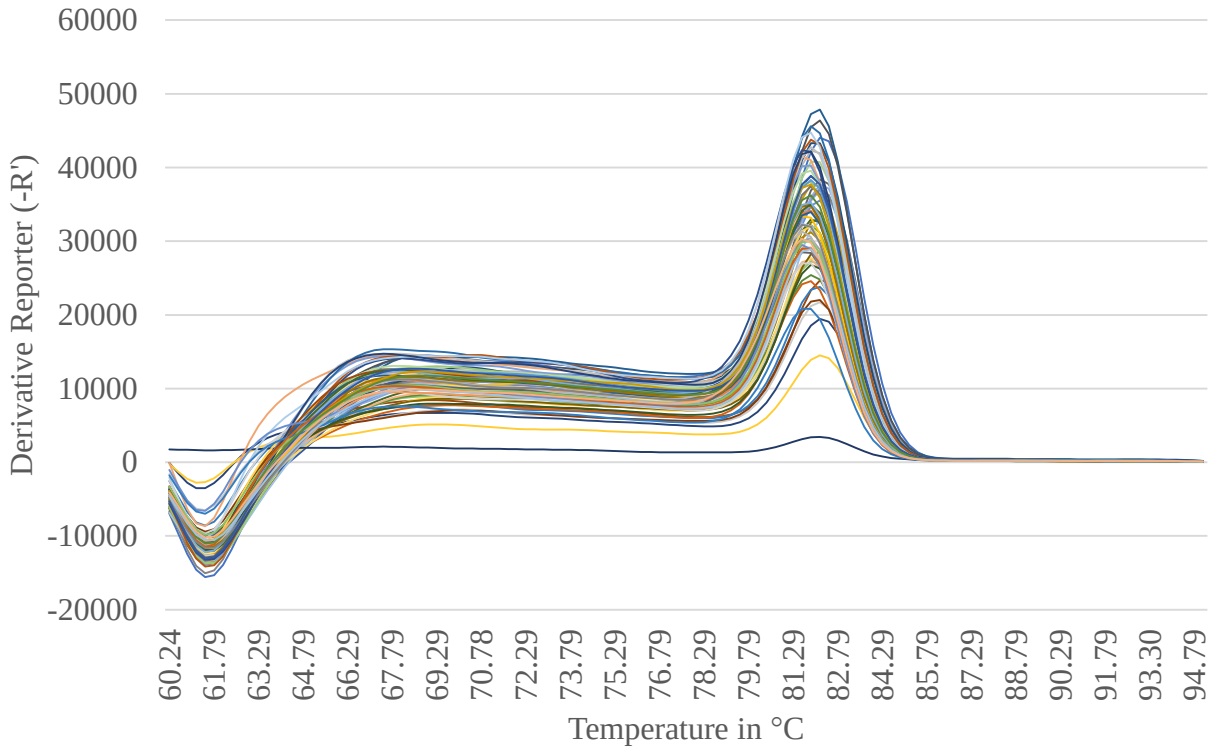


Figure 2.66. Melt curve for wells containing the *Pteropsin* primer.

FATIGO analysis

To make sense of the 616 differentially expressed genes, orthologues for these genes were found and then placed into functional groups called GO terms. Analysis via FATIGO of the frequency and proportion of genes in each GO term allows for greater insight on the patterns of gene expression. One GO term linked to 37 orthologues was found to be significant ($p < 0.05$) in the orthologues of down-regulated genes (Table 2.10). Indicating down regulation of structural constitutes of the ribosome. When comparing orthologues of up-regulated genes, seven GO terms linked to a span between two and twelve orthologues were found to be significant (Table 2.11). All seven GO terms were related to transport mechanisms across membranes.

Table 2.10. Related GO terms of down-regulated orthologues from FATIGO.

Name	Orthologues associated		NCBI Ref. Seq. of Associated orthologues	
Structural constituent of ribosome GO:0003735 p-value: 3.04E-36	*FBgn0010408*	*FBgn0064225*	*XM_392988*	*XM_624617*
	FBgn0010411	*FBgn0032987*	*XM_392726*	*XM_001120360*
	FBgn0086710	*FBgn0026372*	*XM_625101*	*XM_393135*
	FBgn0025286	*FBgn0260460*	*XM_394854*	*XM_392037*
	FBgn0010198	*FBgn0030616*	*XM_397314*	*XM_624868*
	FBgn0005593	*FBgn0015288*	*XM_003251102*	*XM_625006*
	FBgn0019936	*FBgn0031980*	*XM_393614*	*XM_394987*
	FBgn0032050	*FBgn0015521*	*XM_001120524*	*XM_623874*
	FBgn0010078	*FBgn0261602*	*XM_001122335*	*XM_393671*
	FBgn0002626	*FBgn0033912*	*XM_392812*	*XM_624648*
	FBgn0002622	*FBgn0261596*	*NM_001011587*	*XM_392330*
	FBgn0002607	*FBgn0014026*	*XM_623728*	*XM_393034*
	FBgn0002579	*FBgn0017579*	*XM_001119828*	*XM_392809*
	FBgn0039406	*FBgn0036825*	*XM_624256*	*XM_392059*
	FBgn0003942	*FBgn0013325*	*XM_001121176*	*XM_001121930*
	FBgn0003941	*FBgn0024733*	*XM_397323*	*XM_393092*
	FBgn0039757	*FBgn0011272*	*XM_001120521*	*XM_624893*
	FBgn0039713	*FBgn0034138*	*XM_624940*	*XM_624035*
	FBgn0039857		*NM_001011604*	

Table 2.11. Related GO terms of up-regulated orthologues from FATIGO.

Name	Orthologues associated		NCBI Ref. Seq. of Associated orthologues	
Secondary active transmembrane	*FBgn0026438*	*FBgn0037895*	*NM_001011597*	*XM_392977*
transporter activity	*FBgn0024315*	*FBgn0037807*	*XM_393759*	*XM_623909*
GO:0015291	*FBgn0040297*	*FBgn0037238*	*XM_391857*	*XM_624825*
p-value: 1.96E-03	*FBgn0036240*	*FBgn0034911*	*XM_001122134*	*XM_394655*
	FBgn0039915	*FBgn0033657*	*NM_001011643*	*XM_392656*
	FBgn0260795	*FBgn0028704*	*XM_392582*	*XM_396230*
Organic acid : sodium	*FBgn0026438*	*FBgn0037807*	*NM_001011597*	*XM_623909*
symporter activity	*FBgn0039915*	*FBgn0034911*	*NM_001011643*	*XM_394655*
GO:0005343				
p-value: 3.38E-03				
Sodium : amino acid	*FBgn0039915*	*FBgn0034911*	*NM_001011643*	*XM_394655*
symporter activity				
GO:0005283				
p-value: 1.00E-02				
Sodium ion transmembrane	*FBgn0024315*	*FBgn0032946*	*XM_393759*	*XM_624825*
transporter activity	*FBgn0040297*	*FBgn0037238*	*XM_391857*	*XM_394655*
GO:0015081	*FBgn0039915*	*FBgn0034911*	*NM_001011643*	*XM_396230*
p-value: 1.00E-02	*FBgn0037895*	*FBgn0028704*	*XM_392977*	*XM_623909*
	FBgn0037807		*XM_394381*	
Anion : cation symporter	*FBgn0024315*	*FBgn0037807*	*XM_393759*	*XM_623909*
activity	*FBgn0039915*	*FBgn0034911*	*NM_001011643*	*XM_394655*
GO:0015296	*FBgn0037895*		*XM_392977*	
p-value: 1.00E-02				
Solute : sodium symporter	*FBgn0026438*	*FBgn0037895*	*NM_001011597*	*XM_392977*
activity	*FBgn0024315*	*FBgn0037807*	*XM_393759*	*XM_623909*
GO:0015370	*FBgn0039915*	*FBgn0034911*	*NM_001011643*	*XM_394655*
p-value: 2.40E-02				
Anion transmembrane	*FBgn0024315*	*FBgn0037895*	*XM_393759*	*XM_623909*
transporter activity	*FBgn0036240*	*FBgn0037807*	*XM_001122134*	*XM_394655*
GO:0008509	*FBgn0039915*	*FBgn0034911*	*NM_001011643*	*XM_392656*
p-value: 4.67E-02	*FBgn0031064*	*FBgn0033657*	*XM_393071*	*XM_392582*
	FBgn0260795		*XM_392977*	

Overlap between MN Hygienic bees and other behavioral gene sets

Comparisons of the brain genomic profile of MN Hygienic bees and other behavioral gene sets were used to characterize the transcriptome of MN Hygienic behavior. These other behavioral genes sets were also run on microarray A-MEXP 755, which allowed for the usage of hypergeometric probabilities. Hypergeometric probabilities takes into account the number of genes differentially expressed in each experiment and then calculates an expected number of these genes that would overlap. The result of comparing this expected result with the observed frequencies of overlap can give us a p-value of sharing the number of genes between gene sets. Significant p-values ($p < 0.05$) would indicate that the number of genes shared between the two gene sets exceeds that of the likelihood of chance and could indicate a relationship between the gene sets. Between two and 72 genes were shared between other behavioral gene sets and MN Hygienic bees, only the Vibrating gene set was found to be significant (Table 2.12). Analysis of direction of expression of these genes or probe ID's from these other behavioral gene sets follows in Table 2.13.

Table 2.12. Significance of overlap between MN Hygienic and Vibrating (Alaux et al. 2009a), or Alarm (Alaux et al. 2009b), or AHB guard (Alaux et al. 2009b), or AHB soldier (Alaux et al. 2009b), or AHB forager (Alaux et al. 2009b), VSH (Le Conte et al 2011), Uncappers (Gempe et al. 2012), or Geneotype mixing (Gempe et al. 2012), or Genotype mixing and uncapping (Gempe et al. 2012).

Behavioral gene set	Differentially expressed genes	Expected no. of genes to overlap	Observed no. of overlapping genes	Representation factor	p-value from hypergeometric probability
Vibrating	903	47.417	72	1.5184	0.001
Alarm	437	22.947	18	0.7844	0.886
AHB guard	249	13.075	7	0.535	0.979
AHB soldier	538	28.251	20	0.708	0.964
AHB forager	58	3.046	2	0.657	0.816
VSH	39	2.0480	2	0.977	0.615
Uncappers	650	34.131	19	0.557	0.999
Genotype mixing	426	22.369	18	0.805	0.861
Genotype and uncapping	943	49.517	30	0.606	0.999

Table 2.13. Multiple shared probe IDs and their regulation of differentially expressed genes between MN Hygienic bees and other behavioral gene sets.

Probe ID	MN Hygienic	Vibrating	Alarm	AHB Guard	AHB Soldier	AHB Forager	VSH	Uncappers	Genotype mixing	Genotype and uncapping
AM05924	Up	Down	Down	-	Down	-	-	-	-	-
AM03995	Down	Down	Down	-	-	-	-	-	Down	-
AM07552	Down	Down	-	-	-	-	-	-	Down	Up
AM02847	Up	Up	Up	-	Up	-	-	-	-	-
AM08682	Up	Up	Up	-	-	-	-	Up	-	-
AM03333	Up	Up	-	-	Up	-	Down	-	-	-
AM05209	Down	-	Down	-	Down	-	-	-	Down	-
AM12302	Down	-	-	Down	Down	Down	-	-	-	-
AM07461	Up	-	-	-	Down	Down	-	-	-	-
AM01620	Up	Down	-	-	Down	-	-	-	-	-
AM03922	Down	Down	-	-	-	-	-	-	-	Up
AM05125	Down	Down	-	-	-	-	-	-	Down	-
AM05837	Down	Down	-	-	-	-	-	Down	-	-
AM06691	Down	Down	-	-	-	-	-	Down	-	-
AM07577	Up	Down	-	-	-	-	-	-	-	Up
AM08572	Up	Down	-	-	-	-	-	Down	-	-
AM08759	Up	Down	-	-	-	-	-	-	Down	-
AM10059	Down	Down	-	-	-	-	-	-	Down	-
AM10678	Down	Down	-	-	-	-	-	-	Down	-
AM04810	Down	Up	-	-	-	-	-	-	-	Up
AM05377	Up	Up	-	-	-	-	-	Up	-	-
AM06745	Up	Up	-	-	Up	-	-	-	-	-
AM07022	Up	Up	Down	-	-	-	-	-	-	-
AM07388	Up	Up	Up	-	-	-	-	-	-	-
AM08469	Up	Up	Up	-	-	-	-	-	-	-
AM08968	Up	Up	-	-	Up	-	-	-	-	-
AM11549	Up	-	Up	-	-	-	-	Up	-	-
AM06519	Down	-	-	Down	Down	-	-	-	-	-
AM04560	Down	-	-	Down	Down	-	-	-	-	-
AM06729	Up	-	-	-	Down	-	-	-	-	Down
AM09569	Down	-	-	-	-	-	-	Down	-	Down
AM12635	Down	-	Up	-	-	-	-	-	-	Up
AM03355	Up	-	-	-	-	-	-	-	Up	Down

Tables 2.13 through 2.16 display the direction of expression for genes or probe ID's shared between the behavioral gene sets utilizing microarray A-MEXP 755 and MN Hygienic bees. Analysis of these tables is taken on a per paper basis for each paragraph. Starting with the Vibrating bees of Alaux et al. 2009a and ending with VSH (Le Conte et al. 2011). Vibrating bees shared a significant number of differentially expressed genes with MN Hygienic bees. Of the 72 shared genes (Tables 2.13 and 2.14), 51 of them were regulated in the same direction.

There were a total of 67 genes were shared between our findings and those of Gempe et al. 2012. Tables 2.13 and 2.15 displays nineteen genes shared between uncappers, eighteen genes via genotypic mixing and 30 genes via the interactions between both uncapping and genotypic mixing. Amongst the shared genes, 35 of the 67 genes shared the same regulation direction as MN Hygienic.

Analysis of behavioral groups in Alaux et al. 2009b is conducted in Tables 2.13 and 2.16. Where alarm pheromone bees shared eighteen overlapping genes with MN Hygienic, seven of which were regulated in the same direction. Africanized honey bee guards shared five of their seven overlapping genes. AHB Soldiers shared the same directional regulation of twelve of their twenty overlapping genes and AHB foragers shared regulation in one of the two overlapping genes with MN Hygienic.

Le Conte et al. 2011 (VSH) had two genes overlap with MN Hygienic. Only one is present in Table 2.13. The other shared gene, probe ID AM03972, or NCBI reference

sequence *XM_624537*. Neither of these genes are regulated in the same direction as MN Hygienic.

Table 2.14. Shared probe IDs of differentially expressed genes between MN Hygienic and Vibrating bees (Alaux et al. 2009a).

Probe ID	MN Hygienic	Vibrating	Probe ID	MN Hygienic	Vibrating
AM02647	Up	Down	AM03527	Up	Up
AM02722	Down	Down	AM03993	Up	Up
AM02880	Up	Down	AM04434	Up	Up
AM02983	Down	Down	AM04457	Up	Up
AM04327	Up	Down	AM04459	Up	Up
AM04761	Down	Down	AM04626	Up	Up
AM04903	Down	Down	AM05193	Up	Up
AM05844	Down	Down	AM05415	Up	Up
AM06329	Down	Down	AM08227	Up	Up
AM06339	Down	Down	AM08330	Up	Up
AM06412	Down	Down	AM08958	Up	Up
AM06714	Down	Down	AM09302	Up	Up
AM06721	Down	Down	AM10071	Up	Up
AM07733	Down	Down	AM11372	Up	Up
AM07889	Up	Down	AM11623	Down	Up
AM07994	Down	Down	AM11916	Up	Up
AM08218	Up	Down	AM12195	Up	Up
AM09052	Down	Down	AM12271	Down	Up
AM09128	Down	Down	AM12292	Up	Up
AM09491	Down	Down	AM12347	Up	Up
AM09826	Down	Down	AM12514	Up	Up
AM10242	Down	Down	AM12743	Down	Up
AM10325	Down	Down	AM07133	Up	Up
AM11993	Down	Down	AM11925	Up	Up
AM03116	Up	Up			

Table 2.15. Probe IDs and their regulation of shared genes between MN Hygienic, Uncappers, Genotype mixing, and Genotype and uncapping (Gempe et al. 2012).

Probe ID	MN Hygienic	Uncappers	Genotype mixing	Genotype and uncapping
AM03929	Up	Down	-	-
AM07443	Up	Up	-	-
AM11333	Up	Up	-	-
AM04773	Up	Down	-	-
AM12797	Down	Down	-	-
AM05180	Up	Up	-	-
AM07367	Up	Up	-	-
AM03374	Up	Up	-	-
AM02062	Down	Down	-	-
AM04461	Up	Up	-	-
AM08758	Up	Up	-	-
AM09471	Up	Down	-	-
AM12039	Down	-	Down	-
AM07814	Down	-	Down	-
AM09206	Down	-	Down	-
AM06183	Down	-	Down	-
AM11830	Down	-	Down	-
AM08221	Down	-	Down	-
AM12416	Up	-	Up	-
AM03225	Down	-	Down	-
AM11331	Down	-	Down	-
AM11298	Down	-	Down	-
AM08950	Up	-	-	Down
AM02972	Up	-	-	Down
AM03900	Down	-	-	Up
AM05497	Down	-	-	Down
AM08913	Up	-	-	Down
AM11104	Down	-	-	Down
AM08966	Up	-	-	Down
AM08419	Down	-	-	Down
AM08491	Up	-	-	Down
AM03657	Down	-	-	Down
AM11585	Down	-	-	Up
AM12282	Down	-	-	Up
AM10730	Down	-	-	Up
AM09993	Down	-	-	Up
AM08101	Up	-	-	Up
AM06196	Up	-	-	Up
AM02746	Up	-	-	Down
AM09067	Up	-	-	Down
AM11973	Up	-	-	Up
AM10203	Down	-	-	Down
AM05178	Up	-	-	Down
AM02013	Up	-	-	Down

Table 2.16. Shared probe IDs and their regulation of differentially expressed genes between MN Hygienic, Alarm, AHB Guard, AHB Soldier, and AHB Forager (Alaux et al. 2009b).

Probe ID	MN Hygienic	Alarm	AHB Guard	AHB Soldier	AHB Forager
AM04114	Up	Down	-	-	-
AM10977	Up	Up	-	-	-
AM11260	Up	Up	-	-	-
AM06556	Up	Up	-	-	-
AM12445	Up	Up	-	-	-
AM09120	Up	Up	-	-	-
AM06655	Down	Up	-	-	-
AM12055	Up	Up	-	-	-
AM05668	Down	-	Up	-	-
AM08800	Up	-	Up	-	-
AM03361	Up	-	Down	-	-
AM07861	Down	-	Down	-	-
AM08372	Down	-	-	Down	-
AM11870	Up	-	-	Down	-
AM10728	Up	-	-	Down	-
AM07820	Down	-	-	Down	-
AM09798	Down	-	-	Up	-
AM09740	Up	-	-	Up	-
AM09591	Up	-	-	Up	-
AM10873	Down	-	-	Down	-

RNA sequencing

RNA sequencing (RNA-seq) is used to determine differential gene expression of a transcriptome, much in the same way microarrays have been used. In Tables 2.17 and 2.18 explored the shared genes between MN Hygienic bees and two other hygienic gene stocks specifically the bee brains of Hygienic bees in Canada (Boutin et al. 2015) as well as the antenna of VSH bees in Mondet et al. 2015. Table 2.17 shows the RNA-seq on brains of hygienic bees in Canada and MN Hygienic bee brains where eleven genes overlapped with MN Hygienic bees and seven of these genes are expressed in the same direction. Table 2.18 displays the shared genes of the MN Hygienic, and VSH antenna,

as well as three other microarray studies including VSH (Le Conte et al. 2011), Uncappers and Genotype and Uncapping (Gempe et al. 2012) . In the comparison between MN Hygienic and VSH antennae seven of the thirteen overlapping genes share the same direction of regulation. However, only one gene *XM_313199* is up-regulated in three different studies.

Table 2.17. Shared genes between Boutin hygienic (Boutin et al. 2015) and MN Hygienic.

	MN Hygienic	Boutin hygienic
NM_001114198	Down	Down
XM_003250529	Down	Down
XM_623785	Down	Down
XM_397346	Up	Down
XM_001120535	Up	Down
XM_001120947	Down	Down
XM_001123279	Down	Down
NM_001134934	Up	Up
XM_003250089	Down	Up
XM_625264	Down	Up
XM_624499	Up	Up

Table 2.18. Regulation of shared differentially expressed genes between MN Hygienic bee brains, VSH antenna (Mondet et al. 2015), VSH brains (Le Conte et al. 2011), Uncappers and Genotype and uncapping (Gempe et al. 2012).

	MN Hygienic	VSH Antenna	VSH	Uncappers	Genotype and uncapping
*XM*_001121886	Up	Down	-	-	-
*XM*_397526	Up	Down	Down	-	-
*XM*_393500	Up	Down	-	-	-
*XM*_001120535	Up	Down	-	Up	-
*XM*_001119981	Up	Up	-	-	-
*XM*_395180	Down	Up	-	-	-
*XM*_393199	Up	Up	-	-	Up
*XM*_394041	Up	Up	-	-	-
*XM*_001119829	Up	Up	-	-	-
*XM*_394655	Up	Up	-	-	-
*XM*_394671	Up	Up	-	-	-
*XM*_394509	Up	Up	-	-	-
*NM*_001011578	Down	Up	-	-	-

Quantitative Trait Loci

Quantitative trait loci (QTL) have often been used to attempt to identify the location of vital genes required for a specific trait. Oxley et al. 2010 and Tsuruda et al. 2012 utilized QTL to attempt to identify the genes responsible of MN Hygienic and VSH behavior respectively. Of the six QTL found in Oxley et al. (2010), none of the genes singled out for olfaction in the paper were found to be differentially expressed in our microarray. We looked for differentially expressed genes within the given confidence intervals of the three Hygienic QTLs. Table 2.19 displays these genes for all three confidence intervals available for each QTL. Tsuruda et al. 2012 identified two QTLs for

VSH bees, and only one of the genes identified as candidate genes in those locations was differentially expressed in MN Hygienic bees, *XM_001120826.*

Table 2.19. Differentially expressed genes present in differing confidence intervals found representing quantitative trait loci in MN Hygienic bees (Oxley et al. 2010).

QTL	95% CI Bootstrapping	80 % CI	95 % CI
Hyg1	*XM*_623667	*XM*_003249764	*XM*_392202
	*XM*_003249640	*XM*_394358	*XM*_392201
	*XM*_394822	*XM*_001122514	*NM*_001114198
	*XM*_392251	*XM*_003250707	*XM*_397515
	*XM*_395235	*XM*_003250708	*XM*_394277
	*XM*_624856	*XM*_003250709	
	*XM*_392566	*XM*_003250672	
	*XM*_001120195	*XM*_392617	
	*XM*_003249764	*XM*_003250724	
	*XM*_394358	*NM*_001011597	
Hyg2	*XM*_623140	*XM*_003250379	*XR*_120381
	*XM*_392588	*XM*_001120874	*XM*_001120826
	*XM*_396579	*XM*_001120289	*XM*_395694
	*XM*_003249990	*XR*_015011	*XM*_394859
	*XM*_392359	*XM*_001119888	*XM*_394588
	*XM*_394955	*XM*_003250634	
	*XM*_001123290	*XR*_120381	
		*XM*_001120826	
		*XM*_395694	
		*XM*_394859	
		*XM*_394588	
Hyg3	*XM*_003249113	*XM*_003249113	*XM*_003249113
	*XM*_001119916	*XM*_001119916	*XM*_001119916
	*XM*_001122652	*XM*_001122652	*XM*_001122652
	*NM*_001142561	*NM*_001142561	*NM*_001142561
	*XM*_624909		*XM*_624909
	*XM*_001122006		*XM*_001122006
	*XM*_623643		*XM*_623643
	*XM*_001120278		*XM*_001120278
	*XM*_394125		*XM*_394125
	*XM*_623560		
	*XM*_003249431		
	*XM*_395282		
	*XM*_624940		

Discussion

To identify differentially expressed genes in MN Hygienic honeybees, the extracted bee brain RNA was tested for purity, and for quantity, before it was sent to be transcribed into cDNA and used for a microarray. Primers selected from the microarray were then tested for specific binding their ability to produce one amplicon. qPCR results from these primers then needs to be tested if it is producing one amplicon as well. Below we discuss the results of these tests and the comparisons to other bee populations.

RNA extraction

The RNA extraction was tested by spectrophotometry and gel electrophoresis. Table 2.1 displays the concentration, as well as the Absorbance (A) at 260, 280, 260/280, and 320nm of our RNA extractions. Absorbance at 260nm indicates nucleic acid, and absorbance at 280nm indicates protein. Absorbance at 320nm indicates the presence foreign materials or scratches on a cuvette. None of the samples used had an A260/A280 below 1.75 as they aren't high yield RNA (Warburg and Christian 1942, and Glasel 1995).

The gel electrophoresis of the RNA in Figures 2.1 and 2.2, displays two bands and a smear. When comparing the bands to the 1kb DNA ladder, we see that the larger band is somewhere between 2,000 and 1,500 base pairs (bp) indicative of an intact 28S and 5.8S ribosomal units while the smaller band is between 1,500 and 1,000 bp indicative of the 18S ribosomal subunit (Winnebeck et al. 2010). Two distinctive bands are

resultant of not heating the RNA and degrading it into multiple 18S like subspecies (Winnebeck et al. 2010). Whilst the smear is leftover contaminations (proteins, degraded nucleic acids), or potentially overloading the well with sample (Aaij and Borst 1975).

Primer validation

Agarose gel electrophoresis was used to ensure that PCR amplification using our primers yielded the desired size of amplicon and no other visible amplifications. This was performed via two percent agarose gel electrophoresis. Amplicon sizes were compared to two ladders, the Promega 1 Kilo-base pair (Kb) ladder and the Bionexus Hi-Lo ladder. The two percent gel in Figure 2.3 doesn't allow for discrimination between the larger bands however, the 1kb ladder ends at 500bp, as such when we compare this band to the 500bp band on the Hi-Lo ladder. Utilizing the Hi-Lo ladder we can ascertain that the *β-actin* primer set produces an amplicon between 200 and 100 bp in length (closer to 200bp). The *Elf1-α* primer set produces a band between 200 and 100bp (closer to 100bp). While the *2 pore K pump*, and the *Pteropsin* primer sets produce bands between 100 and 50bp with the *Pteropsin* amplicon being slightly larger than *2 pore K pump* amplicon. These single bands do not conflict with NCBI predicted amplicon lengths as displayed in Table 2.2.

Melt Curves

Melt curves were conducted to ensure that qPCR results were not skewed via unspecific primer binding yielding in many different and undesired products. Such an outcome would invalidate the assumption that we are measuring an analog of gene

expression rates. Figures 2.63 through 2.66 display the melting curves for each primer used. Each figure displays a single peak with no signs of a shoulder or plateau suggesting that there is a single amplicon (Ririe et al. 1997).

MN Hygienic bees and non-hygienic bees

MN Hygienic bees significantly differ in their expression of 616 genes when compared to non-hygienic bees based upon microarray and qPCR validation. To understand these differences, these genes should be characterized so that patterns within the expression of genes can be explored. Genes can be characterized via transcription factors which regulate the expression rate of multiple genes, via significant correlations of similar GO terms like that found in FATIGO or even within their capabilities of being a mechanism for MN Hygienic behavior.

Transcription factors

The broadest characterization of the 616 differentially expressed genes of MN Hygienic bee brains is via transcription factors. MN Hygienic bees have fourteen differentially expressed genes related to transcription factors.

The seven up-regulated (↑) transcription factors involve *run-like* (*XM*_001121886), *calmodulin binding transcription activator* (*XM*_001120489), *kruppel-like protein 1* (*NM*_001011566), homeotic protein *ocelliless* (*XM*_394161) and POU-domain motif 3 (*XM*_391982) as well as homeobox genes such as *NKx-2.4 like*

(*XM*_394578), and *labial* (*XM*_001120278). *Run-like* transcription factor is comparable to *runt* in *Drosophila melanogaster*. In the dipteran the primary pair-rule transcription factor *runt* has been implicated in neurogenesis (Hartmann et al. 1994; Kania, 1990). Calmodulin binding transcription activators in *Drosophila* have been implicated in regulating the long term feedback from calcium stimulating G coupled protein receptors. This could prevent the cell from being damaged from excitotoxicity (Han et al. 2006). *Kruppel* is a gap gene that in *Drosophila* that plays a role in axonal guidance and neuroblast fate determination (Abrell & Jackle 2001; Isshiki et al. 2001). *Ocelliless* or *Orthodenticle* is a gap gene in *Drosophila*, known for its role in helping define antennal segments, and regional head development (Royet and Finkelstein 1995). POU domain motif 3 is responsible for axonal guidance, axonal target recognition , axonogeneis, and sensory perception of smell (Chen et al. 2012; Tichy et al. 2008). *NKx-2.4 like,* also known as *Scarecrow* in *Drosophila* has been implicated in dendrite morphogenesis (Iyer et al. 2013). *Labial* is involved with brain development and segmentation in Drosophila (Meier et al. 2006 and Hirth et al. 1998).

The seven down-regulated (↓) genes include a *kruppel homolog* (*XM*_001121743), two homeobox proteins *B-H2* (*XM*_001121415) and *extradenticle* (*XM*_392297), *homeodomain interacting protein kinase* (*XM*_392202), transcription initiation factor *TFIID subunit 9 like* (*XM_001122736*) and transcription factors *IIA L* (*XM_003251780*) and *BTF3 homolog 4 like* (*XM_001122345*). Where the *Kruppel homolog* has been found to accompany metamorphosis in *Drosophia* (Shi et al 2007). In the honeybee expression of the *kruppel homolog* is thought to play a role in initiating

foraging behavior (Fussnecker and Grozinger 2008). *B-H2* in *Drosophila* plays a role in eye-antennal disk morphogeneis (Kojima et al. 2000). *Extradenticle* has been implicated as playing a role in brain development (Nagao et al. 2000). *Histone interacting protein kinase* affects positive regulation of the following gene expression, hippo signaling, and notch signaling pathway (Dewald et al. 2014; Chen and Verheyen 2012; Lee et al. 2009). *Transcription initiation factor TFIID subunit 9-like* or the *enhancer of yellow* in *Drosophila* has the following inferred functions from mutant phenotypes regulation of glucose metabolic process, and positive regulation of transcription of *Notch* receptor target (Xie et al. 2014; Ugrankar et al. 2015). *Transcription factor IIA L* in *Drosophila* plays a role in the positive regulation of transcription from RNA polymerase II promoter (Yokomori et al. 1994). *BTF3 homolog 4 like* or *bicauldal* in *Drosophila* is responsible for pole determination inside the cell for mRNA as well as regulation of the establishment of protein localization (Markesich et al. 2000). Transcription factors often interact with each other and can alter the rate of transcription for multiple genes. Therefore they may have been instrumental in the statistically significant groupings found via FATIGO.

FATIGO

Significant correlations between GO terms of orthologues were analyzed via FATIGO. 37 differentially down-regulated genes shared GO terms for the structural constituents of ribosomes. However, due to a discrepancy between orthologues and the predicted function of honey bee genes, there may be as many as 55 genes that are differentially down-regulated for ribosomal constituents in MN Hygienic bee brains (see

Tables 2.10 and Table 3.1). Large decreases in the ribosomal constituents have been found in neuronal differentiation, colony collapse, miRNA silencing, as well as vibrating bees (Alaux et al. 2009a; Bévort and Leffers 2000; Janas et al. 2012; Johnson et al. 2009). However, none of these scenarios share the exact gene expression pattern as in MN Hygienic bees.

FATIGO analysis of up-regulated genes yielded seven groups of GO terms that were related to transmembrane transport of amino acids, ions, and solute. Whilst some of these genes are present in several of the seven groups, most were related to utilizing the gradient of sodium to assist in the moving of molecules across the membrane. Further analysis of the microarray data reveals six up-regulated genes not identified via FATIGO that are related to ion channels or ion exchangers (*XM*_394509 ↑, *XM*_395235 ↑, *XM*_003250628 ↑, *XM*_391895 ↑, *XM*_393798 ↑, *XM*_003249711 ↑). These channels and exchangers help the neuron either maintain concentration gradients or allow the neuron to depolarize as well as repolarize more quickly, which would in turn allow neurons to fire more frequently.

Table 3.1. Down-regulated gene transcripts related to ribosomal constitutes.

NCBI Ref. Seq.	
NM_001135909	*XM_003250946*
XM_392726	*XM_392809*
XM_001123215	*XM_393614*
NM_001174143	*XM_393034*
NM_001011587	*XM_392988*
XM_394854	*XM_001119888*
XM_001119828	*XM_001121176*
XM_624256	*XR_120351*
XM_624940	*XM_394987*
XM_001120521	*XM_003249292*
XM_624035	*XM_003251109*
XM_001120524	*XM_392037*
XM_003250911	*XM_623926*
XM_393092	*XM_625006*
XM_624648	*NM_001011604*
XM_003251321	*XM_393671*
XM_001120360	*XM_625101*
XM_003249764	*XM_397323*
XM_003251102	*XM_396646*
XM_624081	*XM_624893*
XM_003251103	*XM_623521*
XM_624617	*XM_392059*
XM_003251119	*XM_001121930*
XM_393511	*XM_393135*
XM_397314	*XM_001120315*
XM_624868	*XM_392330*
XM_003251935	*XM_623728*
XM_392812	

Hygienic behavior

MN Hygienic behavior is believed dependent upon olfaction (Masterman 2000). Olfactory signals are converted into neurotransmitters in the flagella of the antennae and transmitted to the brain for interpretation. The role of a specific neurotransmitter in

olfactory memory is evidenced via proboscis extension reflex experimentation in conjunction with neurotransmitter receptor blocking (El Hassani et al. 2005, and El Hassani et al. 2008). MN Hygienic bees may have more sensitive neurons to olfactory signals as they have a greater amount of regulated genes for receptors than non-hygienic bees. MN Hygienic bees have nineteen differentially up-regulated genes related to receptors (*XM*_395235, *XM*_393375, *NM*_001011623, *XM*_396271, *NM*_001011625, *NM*_001171108, *XM*_394520, *XM*_625037, *XM*_397268, *XM*_392128, *XM*_003249170, *NM*_001011565, *XM*_003249172, *XM*_396348, *XM*_003250672, *NM*_001011573, *NM*_001011595, *XM*_003249169, and *XM*_003249573), and five down-regulated genes for receptors (*XM*_001122652, *XM*_003250875, *XM*_395004, *NM*_001143921, and *XM*_396085). Each of these receptors specifically binds to neurotransmitters in the brain. On an organismal level, each neurotransmitter has a different spatial-temporal effect upon olfactory memory formation as seen via proboscis extension reflex experimentations (El Hassani et al. 2009, and Stopfer et al. 1997). On a cellular level, the increased number of receptors either can affect the rate at which a cell polarizes or repolarizes and/or it causes interactions with the cell via secondary messengers such as cyclic adenosine monophosphate (cAMP) and inositol triphosphate (IP3)/ diacylglycerol (DAG) (Mustard et al. 2003).

MN Hygienic bees have differentially expressed genes in the cAMP pathway (*XM*_393859 ↑, *NM*_001077808 ↑, and *XM*_001122246 ↓) and the IP3/ DAG pathway (*XM*_003250942 ↑). In the IP3/DAG pathway there are two calmodulin genes which are up-regulated and potentially assist the influx of calcium ions emanating from the smooth

endoplasmic reticulum (*XM*_001120489 ↑, and *XM*_392226 ↑). Calcium may play a role in MN Hygienic behavior because they have seven transcripts for calcium channels as well as ion exchangers relating to calcium (*XM*_393500 ↑, *XM*_003249174 ↑, *XM*_624825 ↑, *XM*_396230 ↑, *XM*_003249711 ↑, *XM*_393798 ↑, and *XM*_003250302 ↑). However, the IP3/DAG, and cAMP pathways are not the only affected parties of neurotransmitter signaling. There are also the effects of each neurotransmitters on memory formation (Gauthier and Grünewald 2012).

Acetylcholine is the primary excitatory neurotransmitter in the brain and is directly involved in olfactory memory (Gauthier and Grünewald 2012; Grünewald 2012). Nicotinic acetylcholine receptors like those differentially expressed in MN Hygienic bees are implicated in acquisition and retrieval of long term memory (*XM*_625037 ↑, and *NM*_001011625 ↑). It is believed that acetylcholine is one of the coincidence detection molecules utilized in learning (Perez-Orive et al. 2002).

Gamma-Aminobutyric acid (GABA) is an inhibitory neurotransmitter in the bee brain. GABA gated chlorine channels have been linked to olfactory memory via chloride channel blocker experimentation paired with proboscis extension reflex conditioning (Gauthier and Grünewald 2012; Grünewald 2012). Three transcripts in MN Hygienic bees relate to GABA, one is a GABA gated ion channel (*XM*_397242 ↑), and the other two are transporters (*NM*_001011643 ↑, and *NM*_001134934 ↑).

Dopamine and serotonin's function in the brain are not yet understood (Gauthier and Grünewald 2012; Grünewald 2012). The roles played by dopamine tend to be

temporally and spatially based and have impact upon behavior, aging, maturity and aversive learning (Mustard et al. 2012). One transcript (*NM*_001171108 ↑) for dopamine receptor 1 was up-regulated in MN Hygienic bees. This receptor yields increasing levels of cAMP in its host cell. Serotonin has been implicated in phototactic behavior as well as associated learning. The 5-Ht 1 receptor (*NM*_001171108) up-regulated in MN Hygienic bees is believed to associate with phototactic behavior in bees (Thamm et al. 2010).

Octopamine is an excitatory neurotransmitter in the bee brain and it is related to reward processing (Gauthier and Grünewald 2012; Grünewald 2012). It is well understood to be an accompaniment of olfactory learning, memory, recall and consolidation (Mustard et al. 2012; Farooqui et al. 2003; Hammer and Menzel 1998). It is believed to be a parallel signal with acetylcholine that coincidence detection utilizes to create an action potential when paired together. Our microarray data supports Mastermann 2001 which found greater amounts of octopamine receptors in MN Hygienic bees as five of the differentially expressed transcripts related to octopamine receptors were up-regulated (*XM*_003249172 ↑, *XM*_396348 ↑, *XM*_003249170 ↑, *NM*_001011565 ↑, and *XM*_003249169 ↑).

In addition to neurotransmitters, neuropeptides may serve as a secondary format of neurotransmission. Neuropeptides are an ancient chemical messaging system used by neurons. It is believed that they are released after long spike trains, as there is high calcium influx at the presynaptic junction (Galizia and Kreissl 2012). This type of neurotransmission can travel to distal targets. MN Hygienic bees differentially express ten genes related to neuropeptides (*XM*_001122576 ↑, *NM*_001110712 ↑,

*NM*_001143921 ↓, *XM*_001122652 ↓, *XM*_001119829 ↑, *XM*_001120062 ↑, *XM*_397268 ↑, *XM*_395479 ↓, *XM*_393375 ↑, and *NM*_001167720 ↑). Neuropeptides of interest are pheromone biosynthesis-activating neuropeptide, neuropeptide F and neuropeptide Y (*NM*_001110712, *NM*_001167720, and *XM*_393375) as they have been implicated in foraging behavior as well as the motivation for food (Ament et al. 2011). These transcripts follow the expression patterns of foraging bees regardless of age in MN Hygienic bees.

Glutamate plays a role in appetitive olfactory memory, specifically in the formation of medium-term memory and early long term memory formation (Gauthier and Grünewald 2012; Grünewald 2012; Le Boulle 2012). MN Hygienic bees have ten genes differentially regulated that are related to glutamate transmission (*XM*_391979 ↑, *NM*_001011623 ↑, *XM*_001123051 ↑, *NM*_001011597 ↑, *XM*_396271 ↑, *NM*_001011573 ↑, *XM*_397242 ↑, *NM*_001011643 ↑, *NM*_001134934 ↑, and *XM*_395682 ↓). Three of them are for receptors (*XM*_396271, *NM*_001011573, and *NM*_001011623), three are GABA gated ion channels (*XM*_397242, *NM*_001011643 and *NM*_001134934), one is glutamate synthase (*XM*_391979) and two of them are glutamic transporters (*XM*_395682 and *NM*_001011597).

Overall, MN Hygienic bees have 32 differentially regulated genes related to neuropeptide and neurotransmitter signaling. This deviation from non-hygienic bees may signify a learned proponent of the behavior, as increased numbers of receptors, transporters, ion channels, ion exchangers may indicate increased number of dendrite connections. It is unclear if one or all of these chemicals is utilized or needed for MN

Hygienic behavior, as well as MN Hygienic bee's ability to distinguish between dead or alive brood (Gramacho and Spivak 2003).

MN Hygienic and other behavioral gene sets

Vibrating bees (Alaux et al. 2009a) are the only group of bees to share significant similarities to MN Hygienic bees (p-value ~ 0.001). Together, they share 72 differentially expressed genes. 51 of these genes are regulated in the same direction. Of these genes, one encodes for a known transcription factor (*XM*_391982), and seventeen encode for ribosomal constituents (*XM*_625101, *XM*_394854, *XM*_392059, *NM*_001011604, *XM*_001121176, *XM*_394987, *XM*_001121930, *XM*_392809, *XM*_001119888, *XM*_623521, *XM*_393614, *XM*_624035, *XM*_001119828, *XM*_393135, *XM*_001120315, *XM*_001120524, and *XM*_001120521). Due to the similarities in expression between MN Hygienic and vibrating bees, it is possible that the transcription factor may play a role in the regulation of the ribosomal constitutes as well as the other shared genes.

MN Hygienic bees and other hygienic bee studies

MN Hygienic bees differ by 616 genes from their non-hygienic counterparts. But, what do they share in common with other hygienic bee studies? Shared genes may serve many purposes, such as potential candidate genes and as marker genes for the trait. Such genes should be differentially expressed between hygienic and non-hygienic bees, and share a similar regulation of expression, see Table 3.2. Genetic mapping, such as quantitative trait loci, is often the first attempt to find candidate genes.

Quantitative trait loci (QTL) performed by Oxley et al. 2010 gave rise to three hygienic loci of interest for MN Hygienic bees. Of the genes indicated in Tables 2.13 and 2.15, it is worth noting that five of them are shared in Genotype mixing or Genotype and uncapping (Gempe et al. 2012). One gene is shared with Boutin hygienic (Boutin et al. 2015) and one with QTL study on VSH bees (Tsuruda et al. 2012). Within the three confidence intervals of Hyg1 (Oxley et al. 2010), the following genes are found in MN Hygienic bees as well as Genotype and uncapping bees (*XM_392202, XM_395235, XM_394277*) (Table 3.2). *XM_*392202 has been down-regulated in both MN Hygienic and Genotype and uncapping bees (Gempe et al. 2012). This gene is of interest as it encodes for histone interacting protein kinase which been implicated in transcription factor binding. Boutin hygienic shared down-regulation of *NM_001114198* which codes for an apolipophorin-III-like protein. Hyg2 shared the transcript *XM_001120874* with Genotype and uncapping and MN Hygienic bees both had the gene up-regulated. *XM_001120874* codes for tubulin polyglutamylase TTL6. Hyg2 also shares *XM_001120826* with VSH QTL (Tsuruda et. al 2012) and MN Hygienic bee brains have the gene significantly down regulated. Hyg3 shares down-regulation of *XM_*624940 with Genotype mixing and MN Hygienic bees. It encodes a ribosomal protein S7. It is important to note however that quantitative trait loci do not have to indicate loci of genes needed for a trait. Instead, it could be indicating a mutation or alteration of the genetic code which yields the behavioral phenotype. As these alterations could also change or alter gene expression patterns especially if found in promoter region of a gene.

Comparisons of hygienic genes stocks yielded three genes that were shared in three different studies (Table 3.2). *XM_001120535* was shared between VSH antenna (↓), MN Hygienic (↑), and Gempe Uncappers (↑) and it codes for a hypothetical protein which warrants attention for its role in hygienic behavior. *XM_397526* was differentially expressed in MN Hygienic bees as well as both studies of VSH bees, however the regulation between VSH and MN Hygienic bees differed. The other gene is Defective Proboscis extension Response 18 like (*DPR18 like*, *XM_393199*). It encodes a hypothetical protein which appears to be a transmembrane protein that has two immunoglobulin regions. Similar genes such as *DPR1* in *Drosophila* have been implicated in gustatory salt aversion (Nakamura 2002). *DPR18 like* was differentially up-regulated in MN Hygienic bees, VSH antennae, and in Gempe et al. 2012's genotype with uncapping bees. As it wasn't differentially expressed in the uncappers (Gempe et al. 2012), it is possible that it is a result of mixing the colony genotypes more than being a trait of hygienic bees.

Table 3.2. Genes shared between MN hygienic and other Hygienic bee studies.

	Study utilized						
Gene Name	MN Hygienic	Le Conte et al. 2011	Boutin et al. 2015	Mondet et al. 2015	Uncappers (Gempe et al. 2012)	Genotype and uncapping (Gempe et al. 2012)	Oxley et al. l 2010
XM_393199	Up	-	-	Up	-	Up	-
XM_001120874	Up	-	-	-	-	Up	Hyg2
*XM*_624940	Down	-	-	-	-	Down	Hyg3
NM_001114198	Down	-	Down	-	-	-	Hyg1
XM_392202	Down	-	-	-	-	Down	Hyg1
XM_001120535	Up	-	-	Down	Up	-	-
XM_395235	Up	-	-	-	-	Down	Hyg1
XM_394277	Up	-	-	-	-	Down	Hyg1
XM_397526	Up	Down	-	Down	-	-	-

Conclusion

With 616 differentially expressed genes, MN Hygienic bee brains share two with VSH bee brains. The genes (*XM_624537,* and *XM_397526)* are too few to represent a correlation between the two gene sets according to the hypergeometric probabilities (p-value = 0.6). This fails to reject the hypothesis that, despite the many similarities between the two behavior types, the candidate genes differ between groups. This difference may originate from where the sensitivity within the olfactory system takes place. From Mondet et al. 2015's research, it appears VSH bees may have more sensitivity in the antenna flagella than non-hygienic bees. MN Hygienic bee antenna have yet to be tested, however from this study, they appear to have greater sensitivity within the brain to neurotransmitters which could be transmitting olfactory signals.

Future research could see if any of the genes identified here make adequate candidate genes for Hygienic behavior. By testing them across different hygienic groups

and in the wild. Additionally maybe this pattern of expression is carried out into other tissues of the bee, which could be easier to obtain. *XM_393199* could be a good place to start as it has little to no research behind it. Further characterization of the protein or proteins resultant from this gene, could help direct future research. Currently, it appears that the transmembrane protein product from this gene could play an important role in cell signaling or cellular communication. If the current hypothetical product of this gene is correct, knock out studies or florescent marking of the protein could yield valuable information on the role of the gene, or a region of the brain it is differentially regulated in.

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