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RNA interference of worker sterility genes: Testing mechanisms of reproductive regulation in Apis mellifera

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ABSTRACT

Social insects such as the honey bee (*Apis mellifera*) are well known for their reproductive division of labour and eusocial colony systems. Honey bee workers forgo reproduction in favour of being selfless in directing reproductive benefits towards their queen mother. This altruistic tendency is a result of both behavioural and physiological changes under control of the presence of the queen's mandibular pheromone. The genetic mechanism that underlies this response is, however, not well understood. Here, this study used RNA interference to knock down the expression of single genes (*fruitless* and *ftz-f1*) that have been previously identified as influential in regulating this response to the queen's pheromone. Cage experiments were performed to monitor changes to worker behaviour, gene expression and physiology. This study could not conclusively confirm that the target genes were indeed knocked down (due to small sample size); however, it was determined that certain *ftz-f1* siRNA treatments caused a significant impact on the ovarian development of the worker bees. Future studies would need to confirm that this is due to that fact that the gene was knocked down.

Keywords

Honey bees, social genetics, worker sterility, RNA interference, eusociality, altruism

SUMMARY FOR LAY AUDIENCE

Honey bees (*Apis mellifera*), as other social insects, display a distinct division of labour within their colony. This eusocial system is divided between reproductive (queen and drone) and nonreproductive (worker) castes. These castes coexist within a single colony and for this system to be maintained the workers behave altruistically, directing reproductive benefits towards their queen. Workers completely forgo their own reproduction and behave in such a way to help their queen to be as reproductively successful as possible, including behaviours such as caring for the queen and her larvae, cleaning the hive and collecting food. This has led to an interesting evolutionary problem in the eyes of biologists, how does this behaviour evolve if the workers do not reproduce and cannot pass on their own genes? Interestingly, the workers are maximizing their “inclusive fitness” instead of directing reproducing on their own, they are indirectly passing on their genes through a related individual, their queen mother. The queen helps to facilitate this behaviour by giving off a pheromone that causes repression of workers ovaries leading workers to have underdeveloped ovaries. Certain genes have been identified that are differentially expressed between ovary-active and ovary-inactive workers as well as between bees in the presence of queen pheromone versus its absence. These analyses have led to lists of potential genes, with few being tested functionally, to see if they are responsible for causing ovary

inactivation. This research selected two genes, *fruitless* and *fushi tarazu transcription factor 1* (*ftz-f1*) as they have been identified as potential "hub" genes for worker sterility behaviour. To test function of these genes in workers, a technique called RNA interference was used, which lowers expression of target genes, to test if workers' ovaries have a different response to queen pheromone in the absence of the products of these genes. Specifically, this involved seeing if the treated workers would have more developed ovaries than the control workers. This research found that certain siRNA treatments for *ftz-f1* affected ovary development in workers; however, gene knockdowns did not work for *fruitless* and there was insufficient data to determine if *ftz-f1* was knocked down.

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List of Abbreviations

AIC: Akaike information criterion

CRISPR: clustered regularly interspaced short palindromic repeats

DEG: differently expressed gene

***dlg*:** *discs large homolog*

d.f.: degrees freedom

***Dsp*:** *dorsal switch protein*

dsRNA: double-stranded RNA

***ftz*:** *fushi tarazu*

***ftz-f1*:** *fushi tarazu* transcription factor 1

GI: gastrointestinal

GLM: general linear model

gRNA: guide RNA

HDA: 9-hydroxy-(E)-2-decenoic acid

HDR: homology directed repair

His: *Histone*

HOB: methyl p-hydroxybenzoate

***hsp*:** *heat shock protein*

HVA: 4-hydroxy-3-methyoxyphenylethanol

LPA: Lysophosphatidic acid

mRNA: messenger RNA

mrjp1*:major royal jelly protein 1*

ncRNA: noncoding RNA

NHEJ: non-homologous end joining

ODA: 9-oxo-(E)-2-decenoic acid

***Or* :** *odorant receptor*

PFC-NPs: perfluocarbon-nanoparticles

***Pdk*:** *Phosphoinositide kinase*

Qe: queen equivalent

QMP: queen mandibular pheromone

RISC: RNA-induced silencing complex

RNAi: RNA interference

RP: ribosomal protein

SE: Standard error

SID-1: *systemic RNA interference defective protein 1* gene

siRNA: short interfering RNA

TALEN: transcription activator-like effector nucleases

TRN: transcriptional regulatory network

UAS: Upstream activation sequence

UTH: *unable-to-hatch* gene

Ulk: *Unc-51 like kinase*

WC: water control

1 Introduction

1.1 Evolutionary genetics of reproductive altruism

Any behaviour that decreases direct reproductive fitness seems unlikely to spread within a population under Darwinian selection. For this reason, reproductive altruism, whereby individuals forego reproductive opportunities to help others reproduce, has long been considered an evolutionary puzzle. Altruistic behaviours can make evolutionary sense, however, by considering the fitness consequence to those affected by the altruist's actions. As initially explained by Hamilton (1964; 1972), if the effect of directing altruism towards relatives is to increase their reproductive output, then genes for altruism can potentially evolve indirectly through the production of non-descendent kin (Bourke 2011; Marshall 2015). The precise conditions for the genetic evolution of altruism are captured in the metric known widely as Hamilton's Rule (Charnov 1977). The rule specifies the conditions under which a gene for altruism will increase in frequency – namely, when the reproductive cost (c) of altruism to the actor is lower than the sum of benefits (b) conveyed to a recipient times the degree of relatedness (r) between actor and recipient or $rb > c$. The rule is a heuristic simplification of an idea captured within the more generalized framework of inclusive fitness theory. Here, Hamilton essentially generalized Darwin's notion of personal fitness to include both a

direct and indirect component, the latter of which is realized by an actor's effect on another's fitness. Such indirect fitness effects, when combined with direct effects, sum to an individual's 'inclusive fitness'. Unknown to Darwin and underappreciated by even those who most developed Darwin's theory in-line with explicit genetic thinking, such examples include: Mayr (1982), Fisher (1930), Haldane (1931) and Wright (1942), it is inclusive fitness that selection tends to maximize (Okasha and Martens 2015).

Since Hamilton's original papers, our understanding of the evolutionary genetics of altruism has become more sophisticated. For example, the theory has become widely applied to non-conventional forms of altruism outside of the social insects, including social behaviour of micro-organisms (West *et al.* 2007), birds (Krams *et al.* 2008), primates (Seyfarth and Cheney 1984) and humans (Summers and Crespi 2013). Beyond explaining how classic eusociality and altruism can evolve, its seminal ideas have now become fully specified in mathematical models (Lehmann and Keller 2006) and intertwined with the notion of multi-level selection (Lehmann *et al.* 2007) and of major evolutionary transitions (Queller 2000). Despite this conceptual growth and expansion of inclusive fitness theory, however, much less is known of the specific genes that are under indirect selection (Akçay *et al.* 2015). To bridge

this gap, it will be important to identify the relevant genes using molecular biological tools.

Most progress has come from the study of eusocial insects (Thompson and Richards 2016). Molecular studies on ants, bees, wasps and termites have begun to document the genetic basis of caste and division of labour (Schwander *et al.* 2010). These studies have identified conserved pathways that are enriched for genes associated with metabolism and reproduction, supporting the idea that social evolution co-opts pathways already present in solitary ancestors (Smith *et al.* 2008). Arguably, the most well-studied eusocial insect model is the European honey bee (*Apis mellifera*). This single species has long been a target of research in both applied (Grozinger and Robinson 2015) and more fundamental aspects of behavioural genetics (Oldroyd and Thompson 2007). For honey bees and other so-called eusocial taxa (Batra 1968; Michener 1974; Wilson 1971), reproduction is coordinated among different task-specialists within the colony. Where once a single female cared for her brood, the cycle of reproduction is now divided into two separate components, which are: egg-laying and brood care (Toth and Robinson 2007). For highly eusocial insects, in which this division of labour among females for egg-laying versus brood-caring roles is most pronounced

(Michener 1990), there are obvious morphological differences between the female castes.

The genetic structure of male-haploid species is not straightforward. In singly-mated colonies, honey bee workers are related by as much as 75% (Crozier 1977). This is due to diploid female offspring inheriting the full complement of paternal genes (rendering true sisters already 50% related) plus half of the maternal complement, for a total of 75% of their genes shared identical by descent. However, since a queen can mate with multiple drones during her mating flight (range 1-28, mean ~12; Palmer and Oldroyd 2000), many of her worker offspring are in fact half-sisters with no shared common paternal genes. The more matings, the less related the workers are on average. Even in polyandrous species, whereby workers are a mix of full- (r ~ 75%) and half- (r ~25%) sisters, genetic similarity among workers can still average about 30% (Estoup *et al.* 1994), which is higher than the average level of relatedness any one worker has to a half-sisters' son (~ 12.5%; Figure 1.1). Even under polyandry, selection may therefore still favour worker investment into the altruistic rearing of their queen mother's female offspring over their half-sister's sons, as evidenced by worker 'policing' (killing) of worker-laid eggs (Ratnieks and Visscher 1989).

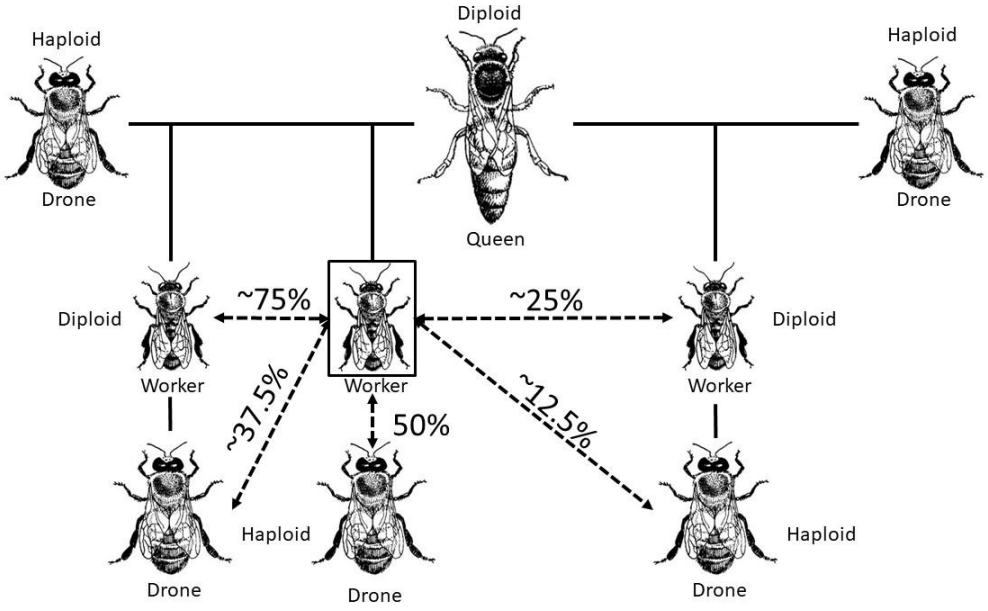

Figure 1.1 Average levels of genetic relatedness between a focal worker to various classes of her relatives. The worker of focus is boxed within this figure. Full sisters are approximately 75% related whereas half-sisters are related by 25%. A worker is related to her direct offspring by 50% and a nephew by 37.5%. A worker is related to her half-sister's son (i.e. a half nephew) by 12.5%. Modified from Barron *et al.* 2001.

1.2 *Apis mellifera* as a model in sociogenomics

Finding genes associated with reproductive altruism has often focussed on caste differences (Smith *et al.* 2008). In the honey bee, early studies used genomic screens to uncover genes associated in their expression with queen-worker caste differentiation (Evans and Wheeler 2001). After the honey bee genome was sequenced in the mid-2000s (Honey Bee Sequencing Consortium 2006), gene screens became more frequent and revealed broad-

scale patterns of gene expression in honey bee caste differentiation (Dolezal and Toth 2014; Humann and Hartfelder 2011), as well as differences in gene expression associated with worker age (Tsuchimoto *et al*. 2004), task (such as nurses or foragers, Whitfield *et al*. 2003) or fungal infection (Azzouz-Olden *et al*. 2018).

The sociogenomic era also ushered in studies that examined differences in gene expression more directly associated with worker reproductive altruism and sterility, including genome-wide differences in expression associated with exposure to ovary-inhibiting queen pheromone (Grozinger *et al*. 2003) or with level of worker ovary activation (Cardoen *et al*. 2011). Within honey bee colonies, workers can potentially activate their ovaries, but in queenright colonies, colonies that contain an egg-laying queen, workers are under indirect selection to forego this option and behave altruistically. The cue that workers use as a signal of queen fecundity is queen mandibular pheromone or 'QMP' (Butler 1957). This semio-chemical, which consists of 9-oxo-(E)-2-decenoic acid (ODA), two enantiomers of 9-hydroxy-(E)-2-decenoic acid (HDA), methyl p-hydroxybenzoate (HOB) and 4-hydroxy-3-methyoxyphenylethanol (HVA) (Slessor *et al*. 2005), has the effect of de-activating worker ovaries such that workers are effectively sterile (Hoover *et al*. 2003). At a physiological level, workers respond to QMP with programmed

oocyte death, rendering queenright workers with a small number of small egg cells and they, relative to a queen, are barren (Ronai *et al.* 2015). Despite the effectiveness of QMP in queenright colonies, a small proportion of workers may still attempt to lay unfertilized eggs that develop into haploid males (Visscher 1989). This reproductive polymorphism within colonies between ovary-active and inactive workers creates experimental opportunities to control for caste within a common-colony environment and screen for genes differentially expressed as a function of ovary activation. Some of the factors that affect the proportion of workers within queenright colonies having activated ovaries include relatedness (i.e., number of queen mates) and the effectiveness of worker policing (Wenseleers *et al.* 2004). Queen age and mating status (Peso *et al.* 2012) and worker genotype (Oldroyd *et al.* 1994) can also affect a worker's disposition to activate or suppress her ovaries in the presence of a queen.

By varying these factors experimentally, it has been possible to create sets of same-aged workers that vary in their level of ovary activation and screen for genes associated with this polymorphism. Any genes differentially expressed as a function of worker ovaries being switched 'ON' or 'OFF' are candidate genes for sterility (Thompson *et al.* 2006). Presumably, these sterility genes would be responsive to QMP and meet other qualifications as candidate

“genes for altruism” (Mullen and Thompson 2015). One characteristic of genes for altruism is that they ought to be differentially expressed between castes - that is, for them to evolve under indirect selection they need to be expressed in the altruist but remain unexpressed in the altruist's reproducing relatives (Charlesworth 1978; Queller and Strassmann 1998; Seger 1981). Microarrays and other genomic technologies that identify genes based on differential expression have therefore proven useful for identifying original short-lists of genes that are functionally associated with queen-induced worker altruism.

Controlled studies that apply QMP to induce worker sterility and reveal gene-expression differences include Grozinger *et al*. (2003, 2007), which found 1607 and 94 differentially expressed genes (DEGs), respectively. Likewise, Thompson *et al*. (2006, 2008) compared the gene expression profiles of ovary-active and inactive workers to reveal a total of 40 and 12 genes differentially expressed in brain and abdomen tissue, respectively. Finally, Cardoen *et al*. (2011) compared ovary-active and inactive workers on a custom-made microarray to reveal 1292 DEGs in whole-body samples. These studies were among the first to capitalize on genomic tools made available following the honey bee genome project and provided a backbone to help identify genes that are responsive to QMP and functionally associated with the sterile

worker phenotype. The mere association of single genes identified from microarrays with pheromone-induced sterility is useful but not in itself compelling evidence of 'genes for sterility' as might originally have been implied by Hamilton (Thompson *et al.* 2013). To help to prioritize genes identified from these first-generation screens, we need to add more functional information - for example, we need to position them within gene regulatory networks (Faragalla et al. 2018). Genes that occupy central positions within networks are more likely to be critically important to the gene regulatory control of the underlying phenotype (Junker and Schreiber 2011). For genes underlying worker sterility, we can then convert gene lists into gene networks.

1.3 Moving from gene lists to gene networks

Lists of candidate genes are important for identifying individual candidate loci that may underly phenotypic variation among individuals. Each entry in the list serves as an independent hypothesis, each amenable to functional genomic analysis. Gene lists do not in themselves, however, explain how genes interact with each other or with their broader environment to regulate a complex phenotype like sterility (Faragalla *et al.* 2018). Recent studies have therefore been transforming gene lists into graphical gene networks that better depict the functional relationships among genes (Civelek and Lusis

2014; Janky *et al*. 2014). Gene network analyses can potentially help to re-prioritize genes identified from genomic screens by revealing which genes are the most connected as 'hubs' in the network or that otherwise show evidence of functional or structural importance. Moreover, gene regulatory network analyses can reveal higher-order patterns in the organization of the transcriptome itself, in terms of how genes are clustered into sub-regulatory modules or motifs (Junker and Schreiber 2011). For example, Malik *et al*. (2015) performed a network analysis of DEGs to reveal that the gene that codes for cadherin-associated protein was the hub gene within the gene network characteristic of mouse muscle cells. This analysis also allowed for the identification of four functional sub-clusters or modules within this network.

A model of the honey bee brain transcriptional regulatory network (TRN) has been inferred by Chandrasekaran *et al*. (2011). The 2382-gene model is a computational prediction of a matrix that describes how transcription factors regulate the expression of downstream target genes. The model is provisional but has proven useful for predicting neurogenomic states associated with maturation, foraging and aggression in honey bee workers. As such, this model may serve as a template for inferring the regulation of candidate genes associated with worker sterility. Sobotka *et al*. (2016) plotted genes identified

by individual gene screens onto the network to test their distribution. Specifically, they tested whether these genes localized to a particular region of the 3D network, as can be expected if these genes evolved to coordinate the conditional expression of worker sterility. They found that many of the sterility gene sets did tend to localize to one particular region of the TRN. Examining the most well-connected genes within the cluster revealed two hub genes that predicted to be central to the integrity of the network and, by inference, integral to worker ovary activation and de-activation. These two genes are *fruitless* and *fushi tarazu transcription factor 1* (*ftz-f1*), which were connected in the network to 60 and 145 other genes, respectively. Both genes are well known transcription factors that regulate many genes within both *Apis* (Chandrasekaran *et al*. 2011) as well as within *Drosophila* (Ito *et al*. 1996 and Yu *et al*. 1997). In *Drosophila*, *fruitless* is an important gene that is required for mating and courtship behaviour, and for proper development of structures required for such behaviours (Ryner *et al*. 1996). *ftz-f1* is also important for development as well as maturation within insects and has been shown as important for regulating juvenile hormone levels which is one of the key controls for proper development and transitions from younger larval forms to adult forms (Riddiford 2008).

The network analysis of Sobotka *et al.* (2016) therefore helped to re-prioritize *fruitless* and *ftz-f1* as genes that are very well connected, a rank criterion that is not possible from the genes lists from which they originally came (Cardoen *et al*. 2011; Grozinger *et al*. 2003). However, to test if *fruitless* and *ftz-f1* are important to the regulation of pheromone-induced sterility in worker bees, we need functional information, ideally from gene modifications *in vivo*.

1.4 Testing the functional roles of networked genes via gene modification technology

One approach to testing the function of individual genes previously identified from gene lists or gene networks is to modify or alter their expression and monitor any effect on bee phenotypes. For some better-studied social insect taxa, gene-modification technologies are reliable, effective and in wide-spread use. Such gene modification techniques include the GAL4-Upstream activation sequence (UAS) system in *Drosophila* (Kaneko and Hall 2000), the use of transposable elements (e.g., Catteruccia *et al*. 2003), transcription activator-like effector nucleases (TALENs; Liu *et al*. 2012), zinc finger nucleases (Bibikova *et al*. 2002) and the clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9 system (Gratz *et al*. 2013).

Another gene modification technique that can be used with honey bees is the use of piggyBac vectors. piggyBac is a transposon-based system that utilizes a “cut and paste” mechanism (through use of transposase) to incorporate specifically designed DNA “cassettes” into genomic DNA by recognizing specific repeat regions (Zhao *et al*. 2016). These retro-transposable elements, originally shown to be successful in creating transgenic lines in Lepidoptera (Marcus *et al*. 2004; Tamura *et al*. 2000), have been modified to make them useful within honey bees (Schulte *et al*. 2014). This breakthrough has allowed researchers to develop transgenic lines of honey bees and it can potentially induce gene-expression changes within specific tissues (Ben-Shahar 2014). For honey bees, future application of this technology will need to overcome current limitations. Although successful, Schulte *et al*. (2014) showed only about 20-27% of queens stably transmitted the cassette (*6x-rubia* in this study) to their offspring, which is higher than most insect species (less than 10% for selected Diptera, Lepidoptera and Coleopteran species, Gregory *et al*., 2016). Other considerations are that the piggyBac retro-transposon vector inserts itself randomly (therefore not sequence specific or targeted), have a low efficiency of transfection and have a limited carrying capacity (Chen *et al*. 2016).

CRISPR-Cas9 has only recently been developed to introduce genome modifications in honey bees (Kohno *et al*. 2016). So far, this technique has been successfully used to test gene function in other social insects including ants (Trible *et al*. 2017) and wasps (Li *et al*. 2017). More recently, Hu *et al*. (2019) showed that CRISPR-Cas9 can successfully cause bi-allelic mutants within honey bees at efficiencies of over 70% (an increase over previous Kohno *et al*. 2016's 12.4%). CRISPRs are short DNA sequences that originally derive from viral infections in bacteria (Wiedenheft *et al*. 2012). The bacteria use the Cas9 enzyme, which is guided by RNA created from CRISPR regions, to protect themselves from future viral infections (Barrangou 2015). Researchers have been able to modify this prokaryotic defense pathway to strategically alter the genomes of a wide range of insect host organisms (Taning *et al*. 2017). CRISPR-Cas9 functions by having a guide RNA (gRNA) that directs the Cas9 nuclease to a target sequence within the genome where it creates a double-stranded break into the DNA (Ran *et al*. 2013). There are two methods that a cell can use to repair this damage; homology directed repair (HDR) requires the presence of a homologous sequence to repair the break, in the absence of homologous sequences, the error-prone non-homologous end joining (NHEJ) mechanism can be used, which often results in insertions or deletions of a few bases when connecting the strands (Cong

and Zhang 2015). This host-dependent repair mechanism can be exploited to engineer genomes. At the break, a nonsense mutation can be induced using NHEJ resulting in a gene knockout. Alternatively, insert specific mutations caused by HDR can result in differing protein product (Sander and Joung 2014). CRISPR technology while powerful and growing more prominent in the literature (Figure 1.2) has so far not been widely used with bees. Kohno *et al.* (2016) used it to create knockout mutant honey bee lines targeting *major royal jelly protein 1* (*mrjp1*).

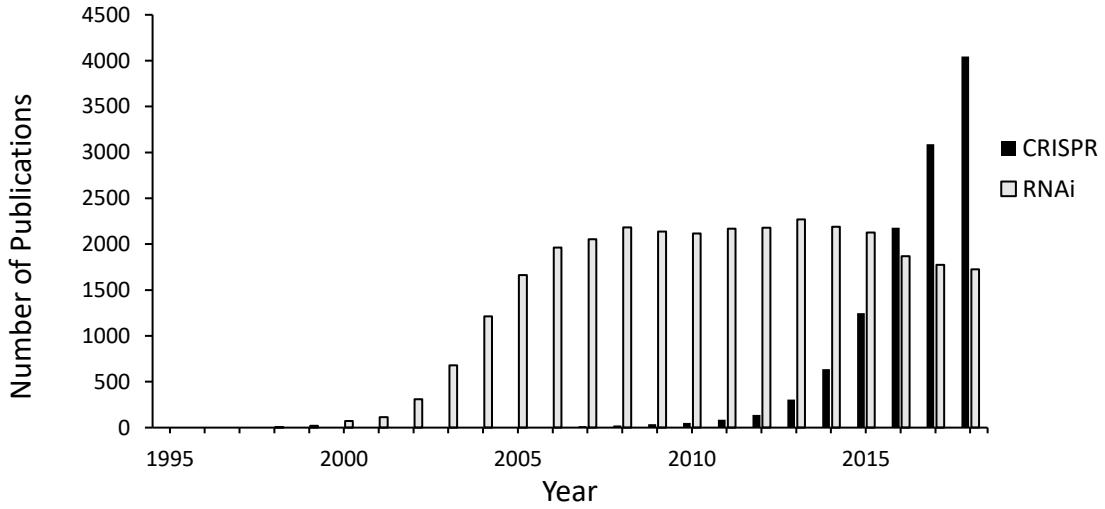

Figure 1.2 Comparison of the number of publications per year from 1995 to 2018, as determined by keyword searches on PubMed. For each year, the terms "CRISPR" ("CRISPR" in the title/abstract [tiab] AND "CRISPR" in the text words [tw]) and "RNA Interference" ("RNA Interference" [tiab] AND "RNA Interference" [tw]) were used for a search respectively and the number of responses were recorded.

Another technique that has been shown to be highly successful in honey bees and other social insects is RNA interference (RNAi). Originally discovered in the nematode worm *C. elegans* (Fire *et al.* 1998), RNAi, like CRISPR-Cas9, is an evolutionary conserved pathway that is used by cells to defend against viral infections (Stram and Kuzntzova 2006). RNAi plays another important role within eukaryotic cells, and that is to regulate gene expression of the cell's own genes (Szweykowsa-Kulińska *et al*. 2003). In *Drosophila* or *Anopheles*, RNAi is routinely used to induce gene knockdowns to test for effects on phenotype (e.g., Copeland *et al*. 2009). RNAi functions by targeting either the viral RNA or the cell's own messenger RNA (mRNA) for degradation. This occurs via a well-defined process, where firstly the enzyme DICER processes double-stranded RNA (dsRNA) and cleaves it into smaller fragments of RNA, around 20 to 25 base pairs in length (Bernstein *et al*. 2001), called short interfering RNA (siRNA). The siRNA fragments are the units that facilitate successful RNAi (Elbashir *et al*. 2001). These fragments then get incorporated into an enzyme complex called the RNA-induced silencing complex (RISC) (Hammond *et al*. 2000), which then keeps one strand of the siRNA (the guide strand) to target the complex to RNA that has high sequence complementarity with the guide strand, marking the RNA for degradation (Hutvagner 2005). Finally, the RISC complex contains an

ARGONAUTE protein that then degrades the RNA by cleaving the strand preventing the RNA from being translated and lowering gene expression (Pratt and MacRae 2009). This process has been used by researchers to target specific mRNAs for degradation, causing a reduction in the target gene's expression and by an overall reduction in protein production. This process is referred to as causing a "gene knockdown". This is different to a gene knockout as with a knockdown there can still be expression of a functional product but at a lower level than what is normal. Researchers have often used RNAi to identify gene function as a result of comparing the differing phenotypes and various physiological differences between wildtype and knockdown lines (as seen in Fraser *et al*. 2000). This can also extend to functional genomics studies to achieve a deeper understanding of the underlying functional or regulatory interactions of genes (Kamath and Ahringer 2003). RNAi has also been used as a medicinal treatment for certain disorders, from viral infections (i.e. Berkhout 2004; Khanna *et al*. 2015; McCaffery *et al*. 2002), to even attempts at cancer treatments (i.e. Cioca *et al*. 2003; Lapteva *et al*. 2005). This technology has also been shown to be effective in a large range of insect orders (Coleopterans: Kaplanoglu *et al*. 2017; Lepidopterans: Eleftherianos *et al*. 2006; Orthopterans: Dong and Friedrich 2005) and has even been modified to be used as an insecticide for

certain pest insects by targeting specific genes that are essential for the insects' viability or development (Baum *et al*. 2007; Sugahara *et al*. 2017). RNAi has also been successfully used in large-scale field applications as a pesticide for honey bee hives against the Israeli Acute Paralysis Virus which is transmitted to the bees by a common mite vector *Varrora destructor* (Hunter *et al*. 2010)

Analyzing the insect order Hymenoptera has also seen success with RNAi for example in sawflies (Yoshiyama *et al*. 2013) and more specifically for this study, the honey bee. Aronstein *et al*. (2006) showed that the *systemic RNA interference defective protein 1* (*SID-I*) gene is important for the cellular uptake of dsRNA in honey bees and that knockdown of different *TOLL*-related receptor genes (*AmTOLL8*, *AmTOLL6*, *AmTOLL10* and *AmTOLL1*) caused expression of *SID-I* to increase. Maleszka *et al*. (2007) used RNAi to test the role of genes encoding chemosensory proteins in embryonic development. This research found that injection of dsRNA for the *unable-to-hatch* (*UTH*) gene caused the embryos to fail to hatch. RNAi knockdown of genes has been successful in honey bees, either by injecting dsRNA into the abdominal haemolymph (Gatehouse *et al*. 2004) or by feeding dsRNA via mixture with diet for oral uptake (i.e. in royal jelly as in Patel *et al*. 2007). Alternatively, it is possible to deliver siRNA topically via an aerosol mist (Li-

Byarlay *et al*. 2013) or again via abdominal injection (Formesyn *et al*. 2014). Abdominal injection involves the use of a hypodermic needle to directly inject RNA into the honey bees' haemolymph (Amdam *et al*. 2003). This method is direct but may cause septic injury. Feeding is another method to supply the bees with RNA; however, most studies that use this method restrict the mobility of the insects (Wang *et al*. 2013) eliminating the "hive-like" aspect of the insects' life. Another consideration is that this method is less controlled for the total amount of RNA that the bees take in. Based on these considerations, aerosol delivery was chosen to test the effectiveness of the siRNA. The hope is that this application eliminates the invasiveness of the more physical methods, while also utilizing the hive-like environment of a cage experiment. The aim of this study is to knockdown both *fruitless* and *ftz-f1* within honeybee workers to determine the resulting phenotypic change on the workers' ovarian development.

1.5 A test of RNAi knockdown of *fruitless* and *ftz-f1* in workers

Both *fruitless* and *ftz-f1* are well characterised in *Drosophila*. The *fruitless* gene is a known BTB zinc finger transcription factor (Ito *et al*. 1996) that regulates a variety of essential genes and has been shown to directly impact behaviour in flies (Ryner *et al*. 1996; Vrontou *et al*. 2006). Likewise, in honey

bees, *fruitless* acts as a transcription factor (Chandrasekaran *et al*. 2011) that regulates genes affecting behaviour (Robinson *et al*. 2008). In at least one hymenopteran species, *fruitless* has sex-specific splice variants (Bertossa *et al*. 2009). Similarly, *ftz-f1* is a well-studied nuclear receptor and zinc finger transcription factor originally identified in *Drosophila* that regulates aspects of development including the homeobox segmentation gene *fushi tarazu* (Lavorgna *et al*. 1991). In honey bees, *ftz-f1* is a predicted transcription factor (Chandrasekaran *et al*. 2011) and may influence the development of worker ovaries (Cardoen *et* al., 2011). Similar to this study, Mello *et al*. (2019) used RNAi to modify the expression of the honey bee *ftz-f1* gene. It was found that *ftz-f1* expression co-varies with juvenile hormone titres and *vitellogenin* gene expression. *ftz-f1* expression peaked in the early fifth instar where juvenile hormone is also increased. Their work also showed that knockdown of *vitellogenin* expression also resulted in lower levels of *ftz-f1* expression, suggesting a positive molecular regulatory loop between these three components. Additionally, it was determined that *ftz-f1* shows a caste-dependent transcription profile as it also regulates *vitellogenin* which has caste-specific functions and expression.

Even though there are multiple candidate genes for worker sterility (Table 1.1) these two genes were focussed on for the following reasons. Firstly, from

Table 1.1 Genes derived from network or other analyses that are predicted to play an influential role in honey bee worker anti-ovarian response to QMP.

Gene Name	Basic Function	Citation
ftz-f1	Transcription factor, originally discovered to regulate the *fushi tarazu* (*ftz*) gene, nuclear hormone receptor and has been discovered to regulate many genes within honey bees.	Sobotka *et al.* (2016)
fruitless	Male courtship regulator in flies, and an important transcription factor regulating many genes.	
GAGA-Like	Transcription factor regulates chromatin structure and gene expression.	
Dorsal switch protein (*Dsp*) *1*	High mobility group protein potentially linked to caste differentiation.	
Heat shock protein (*hsp*) *83*	Molecular chaperone implicated in reproduction.	Mullen and Thompson (2015)
Discs large homolog (dlg)1	Disks large tumor suppressor protein, hub gene involved in cell signalling.	
eggless	Histone-lysine N-methyltransferase, important for gene expression and contributes to oogenesis.	
Histone (*His*) *2Av*	Histone protein involved in silencing gene expression via chromatin regulation.	
Odorant receptor (Or)56a	Odorant receptor linked to reproductive function in *Drosophila*. Is potentially receptive to queen bee pheromone	Camiletti *et al.* (2016)
Or98a, Or49b	Odorant receptors that mediate female *Drosophila* mating behaviour.	
AmOr2	Orthologue to *Orco* which is a major olfactory cofactor essential in olfaction in *Drosophila*.	
anarchy	Associated with pheromone regulation of oogenesis via programmed cell death.	Ronai *et al.* (2016)
Phosphoinositide kinase (*Pdk*)1, *S6 kinase, Unc-51 like kinase* (*Ulk*)*3*	Components of the mechanistic target of rapamycin signalling pathway, are weakly associated with ovarian state.	
Buffy	Regulates programmed cell death during oogenesis. When upregulated may buffer adult worker ovaries from programmed cell death.	
doublesex	Regulates ovary activation, most likely through the regulation of *vitellogenin* as well as pheromonal signalling in adult honey bee workers.	Velasque *et al.* (2018)

***** Modified from Faragalla *et al.* (2018).

the network identified in Sobotka *et al*. (2016), these genes had the highest number of interactions within the sterility sub-cluster (*fruitless* with 60 and *ftz-f1* with 145), so one can predict that interruption of one of these two genes will be most likely to cause a disturbance to the normal worker response to QMP affecting normal worker ovarian development. Secondly, by modifying the expression of either *fruitless* or *ftz-f1* and establishing a causal effect on worker ovaries, this research will validate (or refute) the utility of network analysis, at least specifically with respect to testing the functional significance of hub genes related to sterility. Finally, while both genes are well characterized in *Drosophila* as transcription factors, neither has been well studied in the honey bee. By using RNAi to knockdown *fruitless* and *ftz-f1* in *Apis mellifera* this study will be able to investigate the resulting impact on ovarian development.

2 Materials and Methods

2.1 Honey bee sampling

In this study, RNAi technology was used to knockdown two available candidate genes hypothesized to underlie worker sterility. Because honey bees are free-living animals and are not in-lab models (Camiletti and Thompson 2016), gene knockdowns were performed in the field using active single-queen colonies at the University of Guelph Honey Bee Research

Station. There, a Buckfast strain of honey bee were reared and maintained in colonies that each contained a single openly mated egg-laying queen. From each of n = 3 mature colonies, each containing ~20 thousand workers, a single brood and one honey frame were collected to generate a single nuclear colony (a small four frame colony, Figure 2.1).

Figure 2.1 A queenright nuclear colony containing four frames that was generated for the in-hive experiment.

Specifically, in the spring and early summer (May-June) of 2017 and 2018 field colonies were monitored for late stage pupae, by observing groups of workers beginning to emerge from their pupal cases (Figure 2.2). Whole frames were placed within a custom-made incubator (Kelly 1994) and were reared under hive-like conditions (32°C and 60% relative humidity) overnight,

Figure 2.2 Single brood frame repent with late stage worker pupae. Brown capped cells are pupating workers and an emerging worker is pointed out on this frame.

as described in Williams *et al*. (2013). The following morning, when a large cohort of same-aged workers had emerged (typically, more than three hundred per frame), they were brushed into Rubbermaid® bins (41 x 26 x 18 cm; Figure 2.3) and workers from the collected frames were able to mix freely amongst themselves. Finally, soft forceps were used to remove workers from the bin to either paint mark them for treatment (for the initial in-hive experiment) or place them into cages (Figure 2.4) in groups of n = 40 workers (for the in-cage experiment) via block randomization.

Figure 2.3 Frame of recently emerged workers prior to collection into the Rubbermaid® container.

Figure 2.4 Treatment cages with food and water vials, a glass front, mesh bottom and wooden sides. The cork stopper (top) can be used to add or remove bees from the cage.

2.2 RNAi treatment of worker bees

siRNA design

To design siRNAs, target gene sequences were retrieved by name from the on-line honey bee genomics resource BeeBase (Munoz-Torres *et al.* 2011). E-RNAi software tool (Horn and Boutros 2010) was used to generate siRNAs that met the following criteria: a minimum efficiency score of '90' and a homology cut-off value of 0.1. E-RNAi's efficiency score is a normalized score that combines two different scoring methods developed from previous siRNA optimization studies, the rational score (Reynolds *et al*. 2004) and the weighted score (Shah *et al*. 2007). In addition, siRNAs were designed such that they were 21 base pairs in length (as in Fakhr *et al*. 2016). For each candidate siRNA, its nucleotide sequence was used as a short query in on-line BLASTn (Madden 2003) homology searches against high-quality NCBI reference sequences (RefSeqs). Each siRNA was considered as a valid candidate if it was a one-to-one match (i.e., full sequence complementarity) against the intended honey bee target gene in the NCBI non-redundant RefSeq database and, further, if this match had an E-value less than 0.01 (Makarova *et al*. 1999; Clissold and Ponting 2000) for only the target gene of interest. Otherwise, it was rejected.

For siRNAs that passed both of these criteria, InvivoGen's siRNA Wizard v3.1 (https://www.invivogen.com/sirnawizard/scrambled.php) was used to design a matching 'sham' siRNA to be used as a technical control against any effects of the procedure itself (Evans *et al.* 2013). To generate shams, each siRNA sequence was computationally randomized to create a non-functional siRNA that otherwise matched each target siRNA's nucleotide length and composition. Shams were validated by BLASTn-searching each one with the expectation that there would be no significant (E-value < 0.01) matches to any honey bee gene in the RefSeq database. Finally, Eurofins Genomics Ltd. (Louisville, Kentucky) were employed to synthesize a total of four target and sham siRNA combinations (Table 2.1).

Table 2.1 siRNAs for the *ftz-f1* and *fruitless* genes and their associated sham controls ordered by efficiency score.

Gene	siRNA	Sequence 5'– 3'	Efficiency Score	Scrambled Sham 5' – 3'
ftz-f1	FTZ-F1:siRNA-1	CAGGCATCGCTTTTTGGATTA	97.75	GTATGTGTAAGCTGCCTTCAT
	FTZ-F1:siRNA-2	CAACAAGCTCTTTTGGATTAT	97.75	ACTTCTATATACGGATCTATG
	FTZ-F1:siRNA-3	GGCAGATCTTTTCAATGATTT	97.21	ATGTATCTTAAGGTCCTTGTA
	FTZ-F1:siRNA-4	CCTGCATATTAAACAGGAAAT	97.21	ATACTCATAACTCGGAATGAA
fruitless	FRU:siRNA-1	CCTTCAGCCTCCTCCTGAAAA	100.00	ATCATCCTACGTCTACCGACC
	FRU:siRNA-2	CTGCTACCGATGTTCCTTAAA	97.75	GCATCCGACTATAATTCCTGT
	FRU:siRNA-3	CACTGATAATAGTGTAAATAA	96.22	ATTAATATTAACGCAATGAGA
	FRU:siRNA-4	CCAGAAACGCAAACTGGTATT	94.96	GACAGGCTAACTCACTAAGTA

siRNA delivery method

To deliver individual siRNAs to living bees, the protocol of Li-Byarlay *et al.* (2013) was utilized with small modifications. First, the synthesized siRNAs were re-suspended in siRNA Dilution Buffer (Eurofins Genomics Ltd. Louisville, Kentucky) to a concentration of 20 µM, and then these stocks were further diluted with RNase-free water to a 1 µM working solution. A hand-held nebulizer (Aeroneb® Lab Micropump AG-AL7000SM; Kent Scientific) was used to treat whole groups of n = 50 bees for the in-hive experiment or n = 40 bees for the in-cage experiment with siRNA aerosol, sham control or, as a secondary control, with water. This hand-held nebulizer generated the aerosol at a rate of roughly 0.3 mL/minute, in order to deliver siRNA or water as a mist directly on the honey bees, as in Li-Byarlay *et al.* (2013). After the five-minute treatment, the mist was left to dissipate before introducing the treated workers to the hive or returning the cages to the incubator. For the in-cage experiments described in the following section, bees were treated starting on Day 0 using the nebulizer at approximately the same time (10 – 11:30 am), every other day, for a total of six treatments per group. After all treatments were complete (10 days), all surviving bees were collected, frozen in liquid nitrogen, and then stored by treatment in 15 mL conical tubes. For cages that had a high level of survival, this protocol was deviated from

slightly. Instead of sampling bees directly into liquid nitrogen, cages were first placed within a fridge for a few minutes to slow the individuals and make them easier to collect.

In-hive and in-cage experiments

For in-hive experiments, groups of n = 50 workers were treated with one of the four custom-made siRNAs or its corresponding sham. Groups of bees were paint-marked (Elmers© Painters Markers) by colour according to treatment and were fostered into a single queenright nucleus colony. Because paint-marked and handled bees can sometimes be rejected by workers from the foster colony (Harris 1985), especially if the foster colony is unrelated (Breed *et al*. 1988), the introduced bees were monitored for health twice daily. Specifically, the number of marked workers were counted across all four frames (both sides) as well as looked for signs of rejection, including dragging, removal from the hive, aggression towards the introduced workers and even dead treated individuals left outside of the colony.

For in-cage experiments, again groups of n = 25 (for the first trial) or 40 (for every subsequent trial) one-day old workers were treated with one of four custom-made siRNAs or its corresponding sham. Immediately the treated bees were transferred into standard bee holding cages (Huang *et al*. 2014, Figure 2.5A). To mimic the presence of a real queen within each cage, a third

of one 'queen equivalent' (Qe) strip of a commercial queen pheromone analogue (TEMPQueen, Intko Supply Ltd.) was fitted to the inside of each cage where needed (Beggs *et al*. 2006). One Qe is the amount a mated queen will produce in a 24-hr period (Pankiw *et al*. 1996). Finally, each cage was provided with two vials: one containing water and another with a 50% sugar syrup solution. These cages allowed for direct treatment by removing the water vial to fit the nebulizer during treatment (Figure 2.5B).

For *fruitless*, an in-hive experiment in summer 2017 was started where there were eight treatments (4 siRNA and 4 shams) and 50 bees paint marked for each treatment. All marked bees (n = 400) were placed into a single nuclear colony. In parallel, the in-cage experiment was initiated. For cages, the same eight treatments were utilized but used 25 workers per cage for the first trial. In the summer of 2018, the second *fruitless* trial was performed where a single water control (WC) cage was added (Figure 2.5C). The number of individuals per-cage was increased to n = 40. For *ftz-f1*, only the in-cage approach was utilized (no in-hive experiment) with the only difference from the second *fruitless* experiment being that for the first trial (June 2018) a new factor, the presence or absence of QMP, was introduced. In the *fruitless* experiment, all cages were in the presence of QMP. For the second trial (August 2018) of this same experiment, absence of QMP was dropped as a

factor and all cages were exposed uniformly to this pheromone. Therefore, all four of the caged gene knockdown experiments involving *fruitless* (Summer 2017, Summer 2018) and *ftz-f1* (June 2018, August 2018) were performed differently (Figure 2.6). For all cage experiments, survival was monitored by performing a census every other day just prior to treatment. A Mantel-Cox log rank test was performed on survival data.

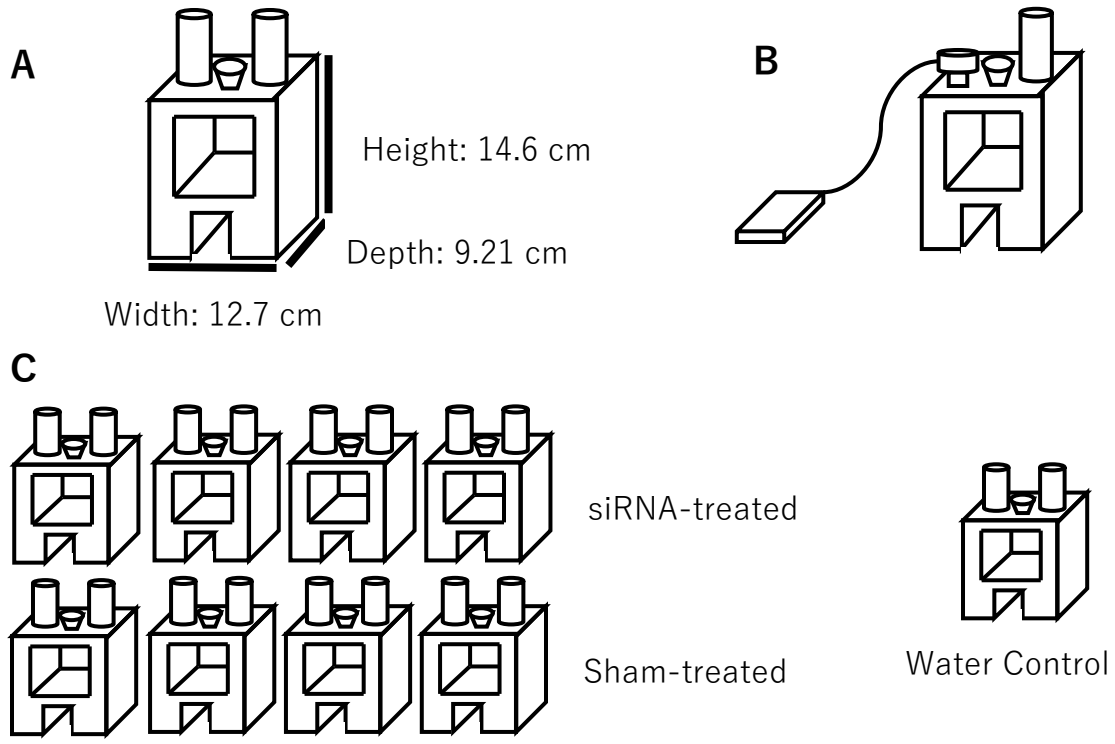

Figure 2.5 Experimental design for the RNAi experiments. **A**) Singular holding cage with dimensions. Each cage held 25 or 40 individual bees (depending on trial) and was provided with water and sugar syrup via canisters mounted on top of each cage. **B**) Schematic diagram of how the cages appeared when being treated with the nebulizer. The water vial was removed from each cage to fit the nozzle of the nebulizer inside the cage. **C**) RNAi experiment layout with total of eight cages (four siRNA-sham combinations), plus water control for the 2018 experiments, were used.

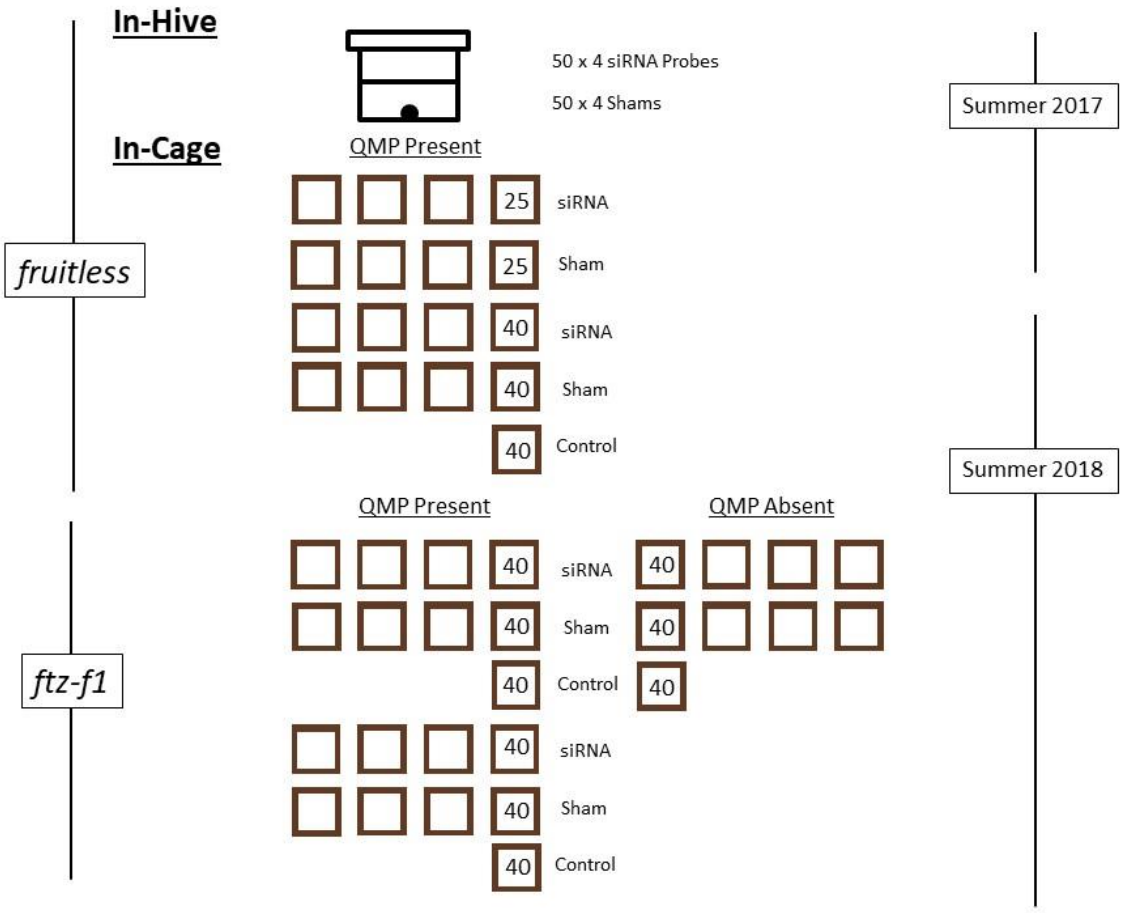

Figure 2.6 Experimental overview of the in-hive and in-cage experiments targeting two loci (*fruitless, ftz-f1*) across two field seasons (2017, 2018). Numbers inside cages (squares) represent the starting sample size of workers bees.

2.3 Quantifying the knockdown effect on target genes

First, total RNA was extracted from individual bees using a combined TRIzol™ /Qiagen protocol, as described in Thompson *et al.* (2008). Briefly, deep-frozen bees were removed from the -80 ˚C freezer and kept on dry ice while their abdomens were separated for nucleic acid extraction. Each abdomen was homogenized in 600 µl of TRIzol™ reagent (Thermo Fisher Scientific) using a

hand-held pestle. 200 µL of chloroform was added and vigorous inversions were performed for 20 seconds before centrifuging at 10,000 x g for 18 minutes. Following this homogenization step, Qiagen's protocol for the RNeasy Mini-Kit was followed. The final RNA product was eluted into 40 µL of RNase free water and a DNase treatment was performed using a TURBO DNA-*free*™ kit (ThermoFisher Scientific).

Following RNA extractions, each sample was tested for concentration and purity. Following Fleige and Pfaffl's (2006) recommendation and only samples with a high concentration [>100 ng/µl] and high quality (260:280 ratio of greater than 1.8 without exceeding a ratio of 2.2) of RNA were retained. From this total RNA, the iScript™ cDNA Synthesis Kit (Bio-Rad) was used to synthesize first-strand cDNA. For cDNA synthesis a standard 250 ng RNA was used as starting template and otherwise the iScript™ cDNA Synthesis Kit (Bio-Rad) protocol was followed. After cDNA synthesis a NanoDrop™ was used to check the concentration of samples ensuring that each sample had a minimum of 1000 ng/µL. Samples were then standardized to this amount. 1 µL (i.e., 1000 ng) of cDNA was used as template in qRT-PCR reactions using a CFX96 Real-Time PCR Detection System (Bio-Rad).

For both targets (*fruitless, ftz-f1*) amplicon primers were designed using the PrimerQuest Tool (Integrated DNA Technologies) that met the following

criteria: target an amplicon of approximately 100 base pairs, with primers having a Tm of at least 62˚C and a GC content of at least 50%. To choose possible endogenous reference genes, a literature search was performed to reveal previously-tested candidates that could then be screened for suitability in this own experiment (Table 2.2). For qPCRs, SYBR Green Supermix was used in a 20 µL reaction mixture that consisted of 10 µL of Supermix, 0.8 µL of 10 µM primers and 8.2 µL of MilliQ water plus 1 µL of cDNA template.

A temperature gradient PCR was performed to optimize the temperature for qPCR, whereby a range of annealing temperatures (56 – 66 ˚C) were simultaneously tested for each primer set. From this optimization step, the optimal annealing temperature for each primer pair was determined to be 60 ˚C. Finally, a standard curve analysis was performed to estimate 'efficiency values' for each primer pair (Taylor *et al*. 2010). Table 2.3 shows the primer sets and their associated efficiency values for both target and reference genes. For the actual qPCRs, a thermoprofile that consisted of an initial 3 minutes denaturation stage at 95 ˚C, followed by forty cycles of alternating denaturation at 95 ˚C (10 seconds) and annealing at 60 ˚C (30 seconds) was utilized. After the forty cycles, a melt curve analysis (Taylor *et al*. 2010) was performed, that consisted of an initial denaturation at 95 ˚C (10 seconds), followed by 5 seconds annealing stages starting at 60 ˚C and ascending in

***Table 2*.2** Primer sets and NormFinder stability values for eight reference genes tested for their suitability in the present study.

Gene Name		Sequence 5'-3'	Stability Value	Source
Actin	FWD	TGCCAACACTGTCCTTTCTG	0.215	Lourenço *et al.*, 2008
	REV	AGAATTGACCCACCAATCCA		
elf-alpha	FWD	GGAGATGCTGCCATCGTTAT	*	
	REV	CAGCAGCGTCCTTGAAAGTT		
tbp-af	FWD	TTGGTTTCATTAGCTGCACAA	*	
	REV	ACTGCGGGAGTCAAATCTTC		
RP49	FWD	CGTCATATGTTGCCAACTGGT	0.128	
	REV	TTGAGCACGTTCAACAATGG		
RPS18	FWD	GATTCCCGATTGGTTTTTGA	0.123	Scharlaken *et al.*, 2008
	REV	CCCAATAATGACGCAAACCT		
GAPDH	FWD	GATGCACCCATGTTTGTTTG	*	
	REV	TTTGCAGAAGGTGCATCAAC		
RPL13a	FWD	TGGCCATTTACTTGGTCGTT	*	
	REV	GAGCACGGAAATGAAATGGT		
RPS8	FWD	ACGAGGTGCGAAACTGACTGA	0.123	Kucharski and Maleszka 2005
	REV	GCACTGTCCAGGTCTACTCGA		

***** A lower stability value indicates the reference gene is more stably expressed. Primer pairs with an asterisk were rejected prior to the NormFinder test because they did not reliably amplify DNA.

two-degree increments to a final melt temperature of 95 ˚C. Melt-curve analysis was performed to test for proper amplification of target as well as test for presence of primer-dimers.

Based on their stable expression in prior studies (Kucharski and Maleszka 2005; Scharlaken *et al.* 2008), and likewise based on their stable expression

Table 2.3 Primers, amplicon size and efficiencies for both target and reference genes investigated in this study.

Gene Name		Sequence 5'-3'	Amplicon Size	Efficiency of Primers
fruitless	FWD	CTTCGGGCAGCTATGATGTT	105	96.2%
	REV	TCACAGGAGGGCTTGATTTG		
ftz-f1	FWD	CATTTAAGCCCTCAGGGTAGTC	101	107.4%
	REV	TACGGCGAAGAACCGTATTG		
RPS8	FWD	ACGAGGTGCGAAACTGACTGA	175	97.2%
	REV	GCACTGTCCAGGTCTACTCGA		
RPS18	FWD	GATTCCCGATTGGTTTTTGA	149	101.1%
	REV	CCCAATAATGACGCAAACCT		

between siRNA- and sham-treated bees in the present study (Table 2.2), *ribosomal protein* (*RP*) *S8* (NCBI Gene ID: 406126) and *RPS18* (NCBI Gene ID: 552726) were chosen as endogenous reference genes to normalize reactions against technical sources of variation. For each reference, the 'stability value' (a standardized measure of within- and between-group variation in a candidate gene's expression) was calculated using an Excel plug-in called Normfinder (Andersen *et al*. 2004).

Endogenous reference genes were chosen as they had most stable expression (in this case, a combined stability value of 0.109, which is within the recommended range, Andersen *et al*. 2004). Expression-fold changes

were calculated following MIQE guidelines (Bustin *et al.* 2009) by using the delta-delta CT method (Livak and Schmittgen 2001). Prior to any expression-fold analysis, gene expression log-fold change data was first tested to see if the data was normal, as evidenced by Shapiro-Wilk test. For data sets deemed to be normally distributed, a *t*-test or an ANOVA were performed to test for differences in relative gene expression between treatments. If at least one treatment set deviated from normality, a Mann-Whitney test or a Kruskal-Wallis test, were substituted respectively.

2.4 Quantifying biological effect of gene knockdowns on worker ovaries

To dissect each bee, first its abdomen was detached from the thorax, and pinned dorsal side up. The abdomen was stretched before pinning the thoracic end. Using a scalpel, the second, third and fourth tergites (abdominal plates) were lifted by cutting through the thin pleural membrane (Dade 1977). After pinning the tergites, ethanol was sprayed to clear out the abdomen and more easily visualize the organs. This allowed for the removal of the gastrointestinal tract as well as reveal the location of the ganglion, which connects to the ovaries (Figure 2.7). Ovaries were scored along a 4-point scale. Scoring was performed blind in order to avoid bias. To score ovaries, Formesyn *et al.* (2014) was followed, whereby ovaries scored as '1-3' were

considered 'active' (containing visible ova) and a score of '0' as not active. From these data, the effect of gene knockdowns by comparing the average ovary score per treatment group to their sham-treated controls could be gauged. Due to a relatively low level of ovary activation in the caged bees, the more widely-used four-point scoring scheme of Formysyn *et al.* (2014) was modified to a binomial scheme in which a worker with both ovaries scored at 0 (zero) to be inactive and at least one ovary scored a '1' or more to be active. A logistic regression was used to test for differences in ovary activation

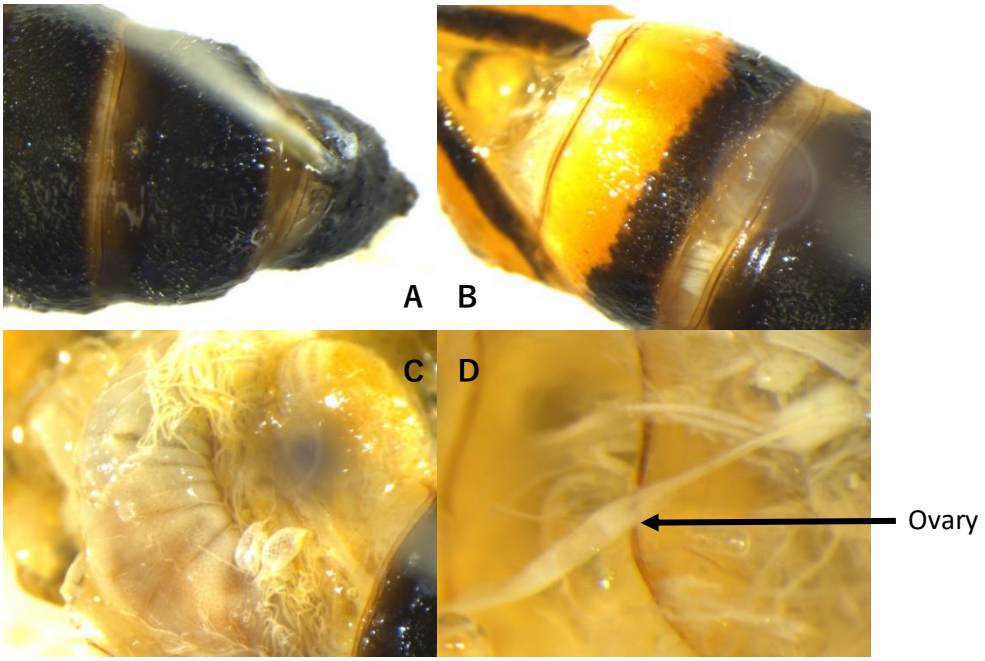

Figure 2.7 Images of worker ovary dissection protocol. **A)** Sting being pinned dorsal side down. **B)** Stretching the abdomen revealing the membrane between the tergite scales. **C)** Visualization of the gastrointestinal (GI) tract after the scales are pinned back. **D)** Ovary after removal of the GI tract with ethanol.

between RNAi- and sham-treated bees. A general linear model (GLM) was performed in R Studio in order to determine statistical significance of treatment on ovary score. The model used for the *ftz-f1* June 2018 experiment was “score ~ treatment * QMP” and then an ANOVA was performed. This model was selected due to Akaike information criterion (AIC) value being the lowest and hence the best model for this experiment (Akaike 1973). The model used for the *fruitless* Summer 2018 and *ftz-f1* August 2018 experiment was “score ~ treatment” since QMP was not a factor in those experiments.

3 Results

3.1 Survival of treated honeybees

To ensure that the siRNA treatments did not have a lethal affect on the honey bees, survival curves were generated from the censor data and investigated. For the in-hive experiment, survival of marked and treated bees within the single host colony was high. Only a single treated bee (of 400) was found dead outside of the hive on the first day and there were no obvious signs of marked bees being socially rejected from their un-related foster colony. Survival was high enough that at least 37 individuals (of 50) were recollected for each of the eight treatment groups (Table 2.4).

Table 3.1 Number of recaptured bees for each treatment from the in-hive *fruitless* experiment from Summer 2017 out of 50 original paint marked bees.

Treatment	Number of Bees Recaptured
FRU:siRNA-1	38
FRU:siRNA-2	44
FRU:siRNA-3	44
FRU:siRNA-4	48
FRU:Sham-1	47
FRU:Sham-2	38
FRU:Sham-3	37
FRU:Sham-4	37

For cage experiments, however, survival was noticeably lower. Cage populations showed varying survival rates over the ten-day sampling period (range 5% – 92%). For example, Figure 2.8 shows that *fruitless* siRNA and sham treated workers showed similar survival, declining over the duration of the 2017 experiment from n = 25 individuals to roughly half this number, depending on the cage. In one case, survival of the siRNA treated workers was significantly lower than the sham treated controls (FRU:siRNA-3; Mantel-Cox Log Rank test statistic = 10.47, degrees freedom (d.f.) = 1, P = 0.0012). Similarly, in the following summer of 2018 survival generally declined over the census period from an initial n = 40 individuals to roughly half this number, depending on the cage (Figure 2.9). In one case, control bees had lower survival than either of the sham or siRNA treated bees (FRU:siRNA-2;

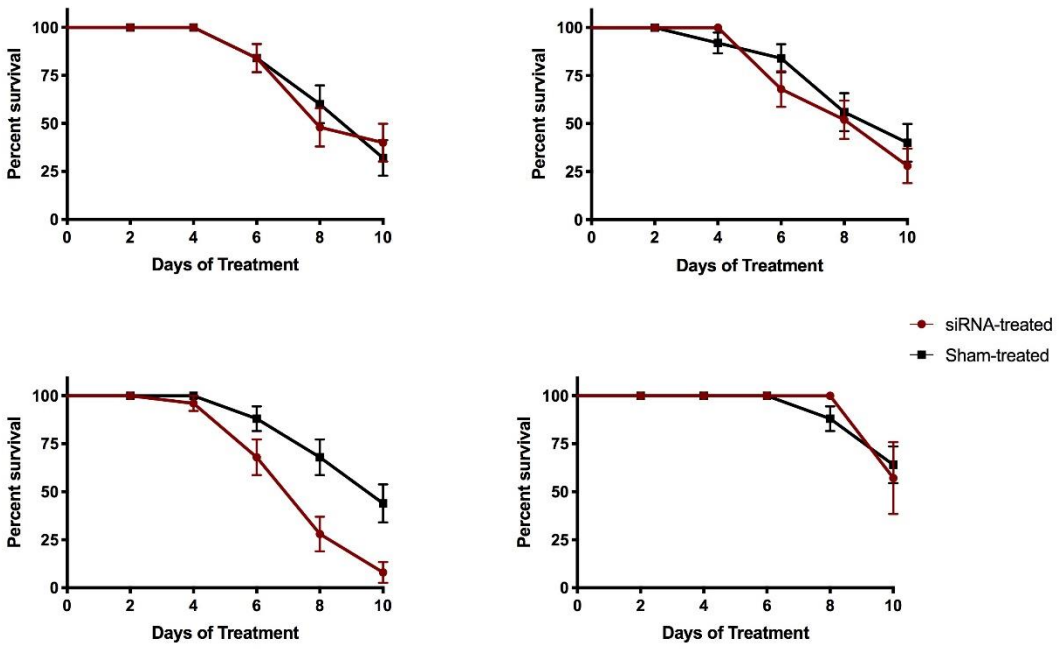

Figure 3.1 Survival of *fruitless* siRNA- and sham-treated workers held in cages in 2017. In this figure, the survival curves from paired siRNA treatments are combined from different cages for graphical purposes: Top left: FRU:siRNA-1 and FRU:Sham-1; Top-right: FRU:siRNA-2 and FRU:Sham-2; Bottom-left: FRU:siRNA-3 and FRU:Sham-3; Bottom-right: FRU:siRNA-4 and FRU:Sham-4. N=25 for all starting populations. Error bars represent standard error (SE). * Note that in one some of the caged bees escaped and the ending sample size is very small (FRU:siRNA-4, N=7).

Mantel-Cox Log Rank test statistic= 12.79, d.f.= 2, *P*= 0.0017). In another case, sham treated bees had higher survival when compared to siRNA or control treated bees (FRU:siRNA-3; Mantel-Cox Log Rank test statistic = 8.541, d.f. = 2, *P* = 0.0140). Finally, siRNA-treated bees had higher survival (FRU:siRNA-4; Mantel-Cox Log Rank test statistic = 12.40, d.f. = 2, *P* = 0.0020).

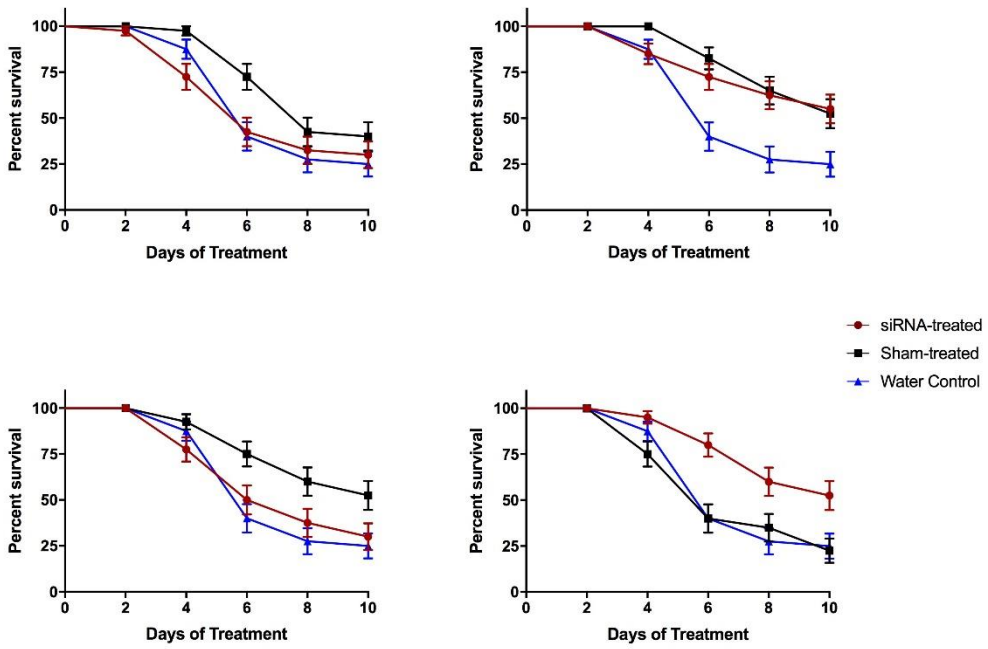

Figure 3.2 Survival of *fruitless* siRNA- and sham-treated workers held in cages in 2018. In this figure, the survival curves from paired siRNA treatments are combined from different cages for graphical purposes: Top left: FRU:siRNA-1 and FRU:Sham-1; Top-right: FRU:siRNA-2 and FRU:Sham-2; Bottom-left: FRU:siRNA-3 and FRU:Sham-3; Bottom-right: FRU:siRNA-4 and FRU:Sham-4. Error bars represent SE. N=40 for all starting populations.

For cage experiments involving the second candidate gene *ftz-f1*, survival was assessed in a slightly different manner – namely in the presence or absence of QMP (explained in discussion). Figure 2.10 shows that in the presence of QMP, siRNA-treated, sham-treated and water-treated cages had comparable survival (Mantel-Cox Log Rank test statistic = 0.67-2.71, d.f. = 2, $P > 0.05$ in all cases), which were generally higher than for either *fruitless* experiment (Mean survival *ftz-f1* = 69.91% versus mean survival *fruitless* = 39.11%).

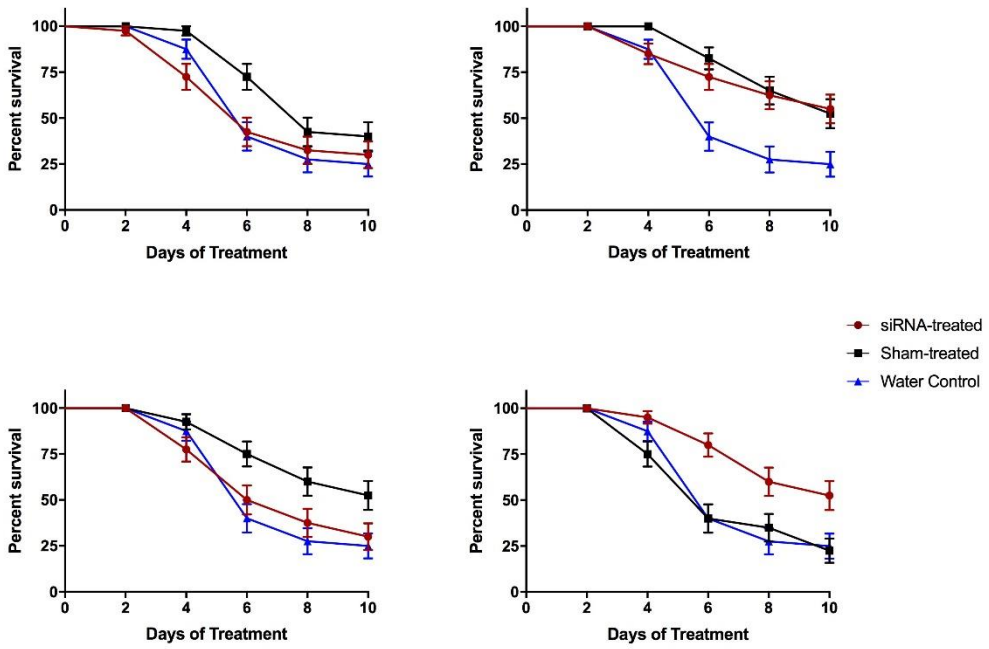

Figure 3.3 Survival of *ftz-f1* siRNA-, sham- and water-treated workers held in cages in the presence QMP from June 2018. The starting population of caged bees was N=40. The survival curves from paired siRNA treatments are combined from different cages for graphical purposes. Top left: FTZ-F1:siRNA-1 and FTZ-F1:Sham-1; Top-right: FTZ-F1:siRNA-2 and FTZ-F1:Sham-2; Bottom-left: FTZ-F1:siRNA-3 and FTZ-F1:Sham-3; Bottom-right: FTZ-F1:siRNA-4 and FTZ-F1:Sham-4. Error bars represent SE. N=40 for all starting populations. * Note that the single water control cage is shown across all graphs.

Similarly, in the absence of QMP, siRNA-treated, sham-treated and water-treated cages showed comparable, high-level survival with no significant differences between the three treatments (Mantel-Cox Log Rank test statistic = 0.072-3.26, d.f. = 2, $P > 0.05$ in all cases; Figure 2.11). In the final cage experiment, survival was again tested for siRNA-treated, sham-treated and water-treated cages in the presence of QMP. In this case, the majority of

comparisons yielded no difference between treatments (Figure 2.12). In one case (FTZ-F1:siRNA3), the siRNA-treated cage had significantly lower survival than did the sham- and water-treated cages (Mantel-Cox Log Rank test statistic = 24.73, d.f. = 2, $P < 0.0001$).

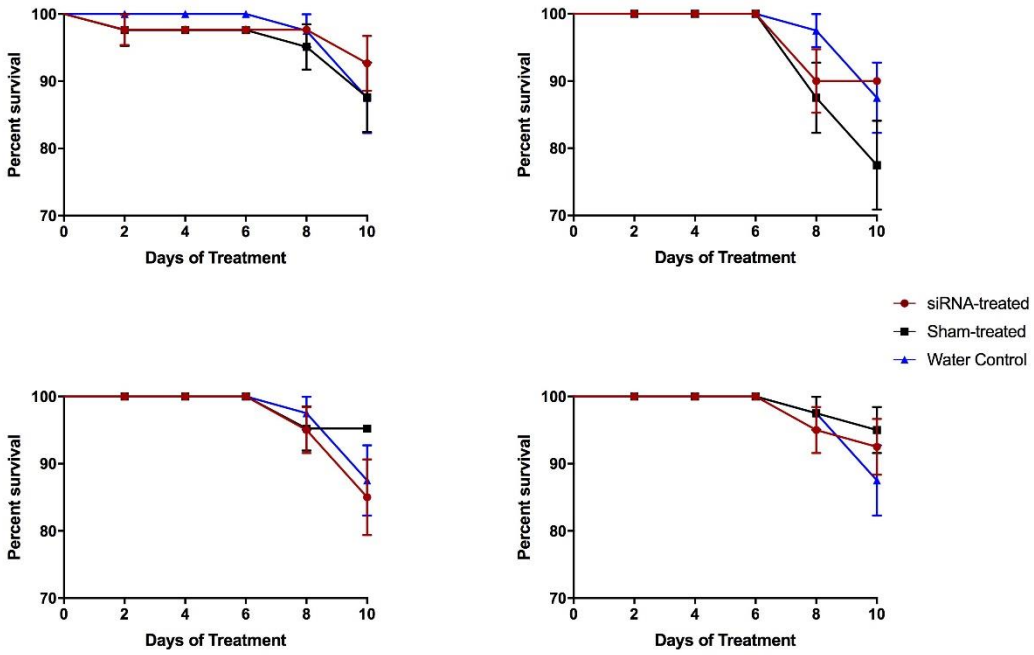

Figure 3.4 Survival of *ftz-f1* siRNA-, sham- and water-treated workers held in cages in the absence of QMP from June 2018. The starting population of caged bees was N=40. The survival curves from paired siRNA treatments are combined from different cages for graphical purposes. Top left: FTZ-F1:siRNA-1 and FTZ-F1:Sham-1; Top-right: FTZ-F1:siRNA-2 and FTZ-F1:Sham-2; Bottom-left: FTZ-F1:siRNA-3 and FTZ-F1:Sham-3; Bottom-right: FTZ-F1:siRNA-4 and FTZ-F1:Sham-4. Error bars represent SE. N=40 for all starting populations. * Note that the single water control cage is shown across all graphs.

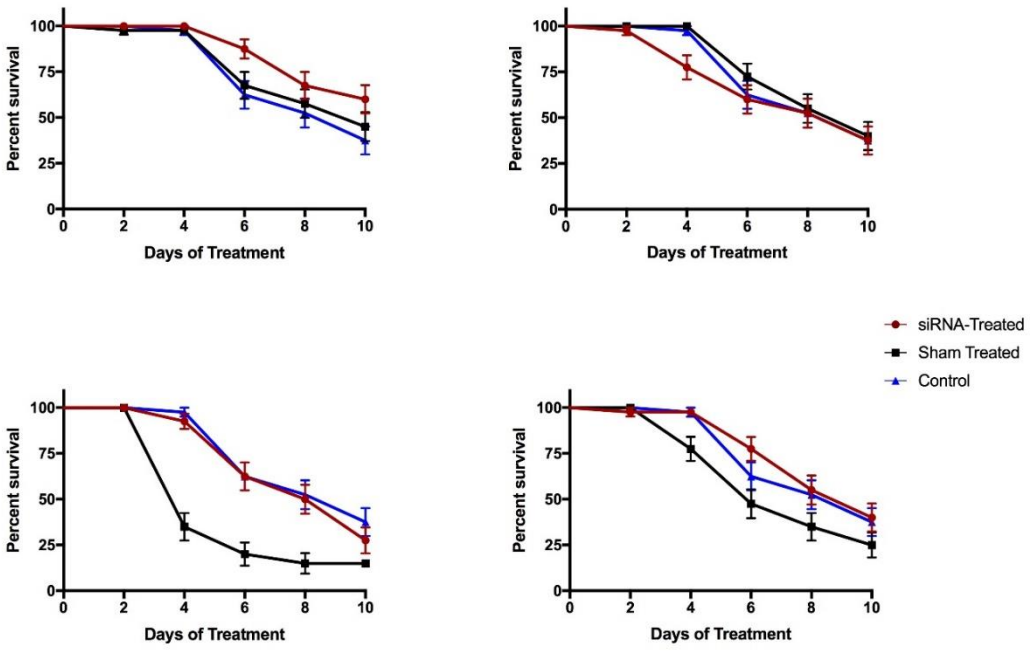

Figure 3.5 Survival of *ftz-f1* siRNA-, sham- and water-treated workers held in cages in the absence of QMP from August 2018. The starting population of caged bees was N=40. The survival curves from paired siRNA treatments are combined from different cages for graphical purposes. Top left: FTZ-F1:siRNA-1 and FTZ-F1:Sham-1; Top-right: FTZ-F1:siRNA-2 and FTZ-F1:Sham-2; Bottom-left: FTZ-F1:siRNA-3 and FTZ-F1:Sham-3; Bottom-right: FTZ-F1:siRNA-4 and FTZ-F1:Sham-4. Error bars represent SE. N=40 for all starting populations. * Note that the single water control cage is shown across all graphs.

3.2 Gene expression of *fruitless* and *ftz-f1*

To establish whether the application of Li-Byarlay *et al.* 's (2013) protocol for siRNAi-mediated gene knock down was effective, gene expression at target loci between treated and control groups was compared. Gene expression was normalized for technical variation against the mean of the expression of two endogenous reference genes. To compare gene expression between treated and control groups a quantitative PCR experiment was performed.

The application of the Li-Byarlay *et al*. (2013) method for gene-knock down showed mixed results. In 2017, siRNAs appeared to be associated with knockdown of *fruitless* in living workers, relative to shams, at least for three of the four attempts (FRU:siRNA-1, FRU:siRNA-2 and FRU:siRNA-4; Figure 2.13). In only one case, however, was this difference statistically significant: the relative expression of FRU:siRNA-2 was lower than its sham control, as expected ($t = 3.33$, d.f. = 4, $P = 0.03$). In other cases, siRNAs were not different from controls (FRU:siRNA-1, FRU:siRNA-4; Mann-Whitney tests; U-Stat = 2, $n_1 = n_2 = 3$, $P > 0.05$ in both cases) or no test was possible owing to small sample size (FRU:siRNA-3).

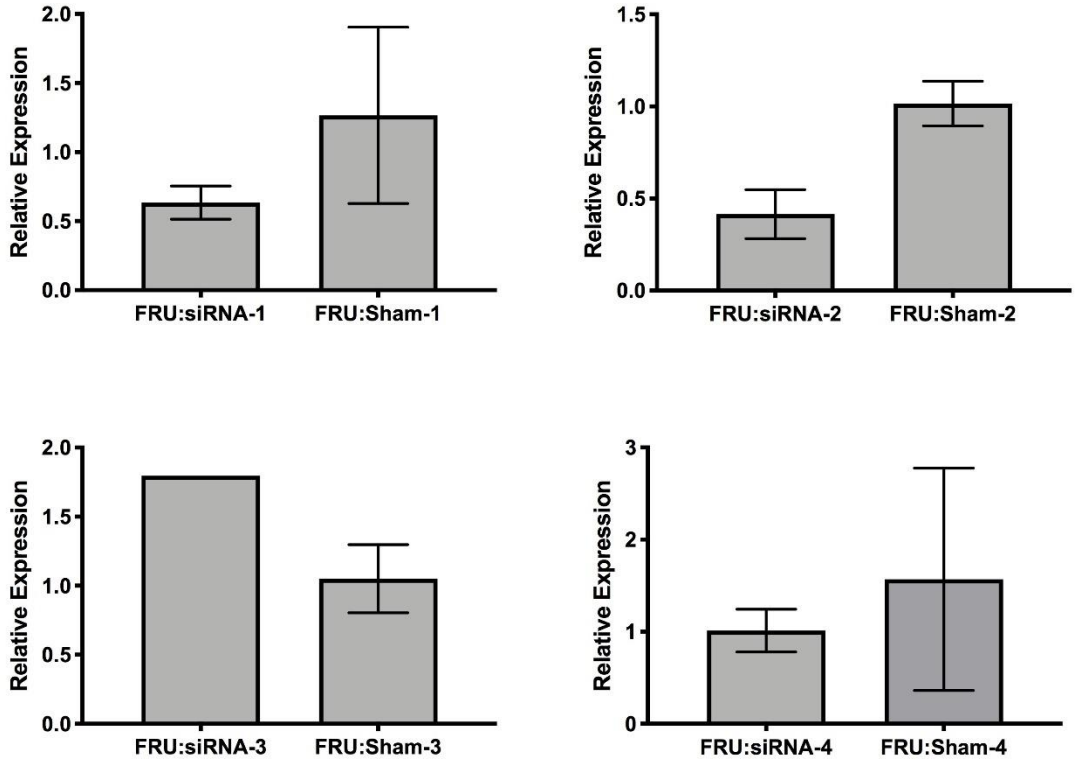

Figure 3.6 Relative gene expression of *fruitless* after treatment of one of four siRNAs or an associated sham control from the Summer 2017 experiment. Error bars represent SE.

In 2018, there were again suggestive differences in expression at the *fruitless* locus between siRNA-treated and sham-treated workers, and in the direction expected, but in no case were these differences significant (Fru-1,Fru-3,Fru-4; ANOVA analyses; F=0.4968, 0.3349, 0.6339, d.f.=2, $P > 0.05$; Fru-2; Kruskal-Wallis test; H-stat=4.032, $P = 0.1338$, Figure 2.14). Moreover, the inclusion of the WC sample permitted an all-in-one test for gene expression differences across all eight treatment groups. No overall effect of treatment was found on *fruitless* expression.

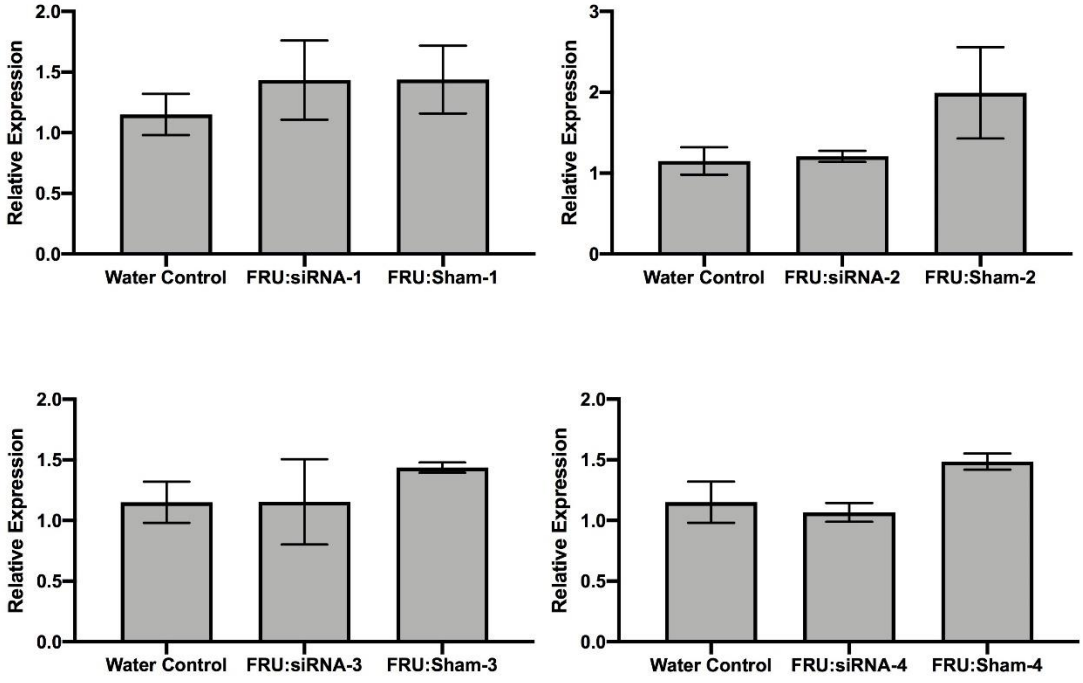

Figure 3.7 Relative gene expression of *fruitless* from the Summer 2018 experiment after treatment of one of four siRNAs, associated sham control or a water control. Error bars represent SE.

At the *ftz-f1* locus, the application of the Li-Byarlay *et al*. (2013) method again produced inconclusive results. FTZ-F1:siRNA-1, FTZ-F1:Sham-1 and the WC had no significant difference in expression in the presence (Kruskal-Wallis test; H-stat = 1.16, $P = 0.63$; Figure 2.15A) or absence (ANOVA, F = 1.32, $P = 0.33$; Figure 2.15B) of QMP. Similarly, FTZ-F1:siRNA-2, FTZ-F1:Sham-2 and the WC did not show a significant difference in expression in the presence of QMP (ANOVA, F = 1.02, $P = 0.4176$; Figure 2.15C). qPCR analyses for FTZ-F1:siRNA-2 under no-QMP, or for the remaining two siRNAs (FTZ-F1:siRNA-3,FTZ-F1:siRNA-4) were not performed.

Finally, with the August experiment 2018 trial, no significant difference in expression in the presence of QMP at *ftz-f1* was found (ANOVA, $F = 1.68$, $P = 0.26$; Figure 2.15 D). qPCR analysis for FTZ-F1:siRNA-1 under no-QMP, or for the remaining three siRNAs (FTZ-F1:siRNA-2, FTZ-F1:siRNA-3 and FTZ-F1:siRNA-4) were not performed.

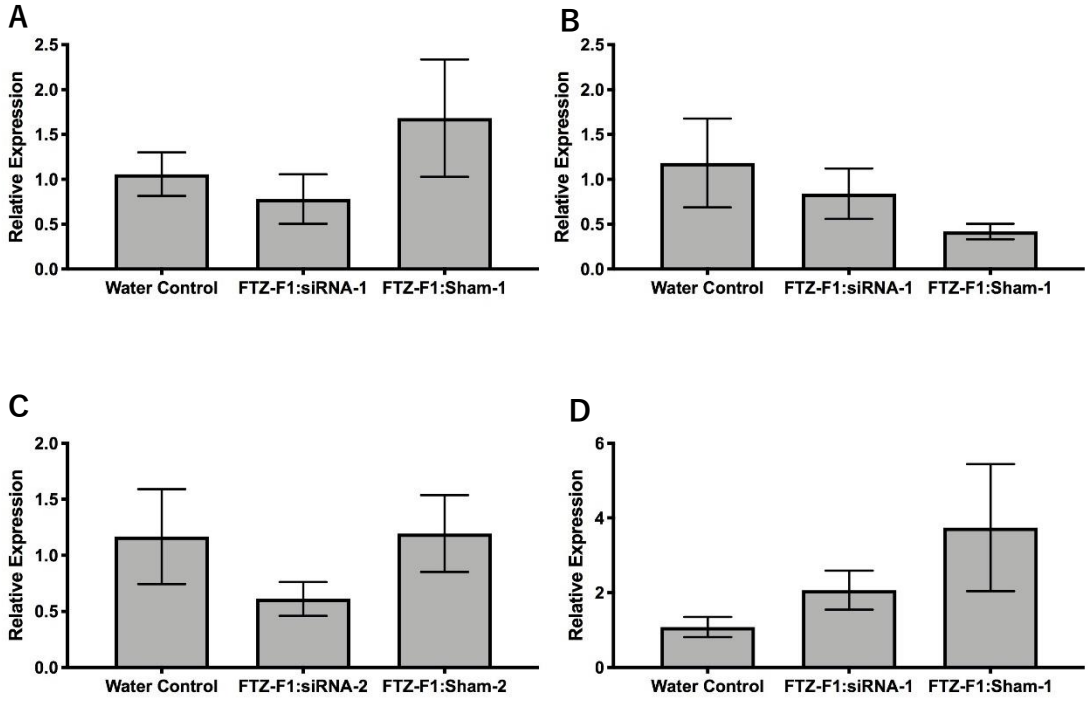

Figure 3.8 Relative expression of *ftz-f1* after treatment with FTZ-F1:siRNA-1, FTZ-F1:siRNA-2, an associated sham control or water control. Error bars represent SE. **A)** Gene expression of *ftz-f1* after treatment of FTZ-F1:siRNA-1, FTZ-F1:Sham-1 or water control in the presence of QMP from June 2018 experiment. **B)** Gene expression of *ftz-f1* after treatment of FTZ-F1:siRNA-1, FTZ-F1:Sham-1 or water control the absence of QMP from June 2018 experiment. **C)** Gene expression of *ftz-f1* after treatment of FTZ-F1:siRNA-2, FTZ-F1:Sham-2 or water control in the presence of QMP from June 2018 experiment. **D)** Gene expression of *ftz-f1* after treatment of FTZ-F1:siRNA-1, FTZ-F1:Sham-1 or water control in the presence of QMP from August 2018 experiment.

3.3 **Ovary scoring**

A majority of dissected caged-bee ovaries had inactive ovaries. In order to investigate the results, a GLM was fitted to the dissection data. Due to low survival in 2017, there is no dissection data from bees for that year. However, in 2018 the following tests were able to be performed. For the *fruitless* experiment, no significant effect of treatment on ovary score was found

(ANOVA, df= 8, $P = 0.98$; Figure 2.16). In the June 2018 *ftz-f1* trial there was a significant effect of 'siRNA treatment' on ovary score (ANOVA, df = 8, $P = 0.0001999$; Figure 2.17) as well as a significant interaction of treatment and QMP (ANOVA, df=8, $P < 0.001$). The GLM indicated that FTZ-F1:siRNA-2 ($P = 0.0291$), and FTZ-F1:Sham-1 ($P = 0.0291$) each contributed to this effect, regardless of the presence or absence of QMP (ANOVA, df= 1, $P = 0.124$). In the August 2018 trial for *ftz-f1* no significant effect of siRNA treatment on ovary score was determined (ANOVA, df =8, $P = 0.095$; Figure 2.18).

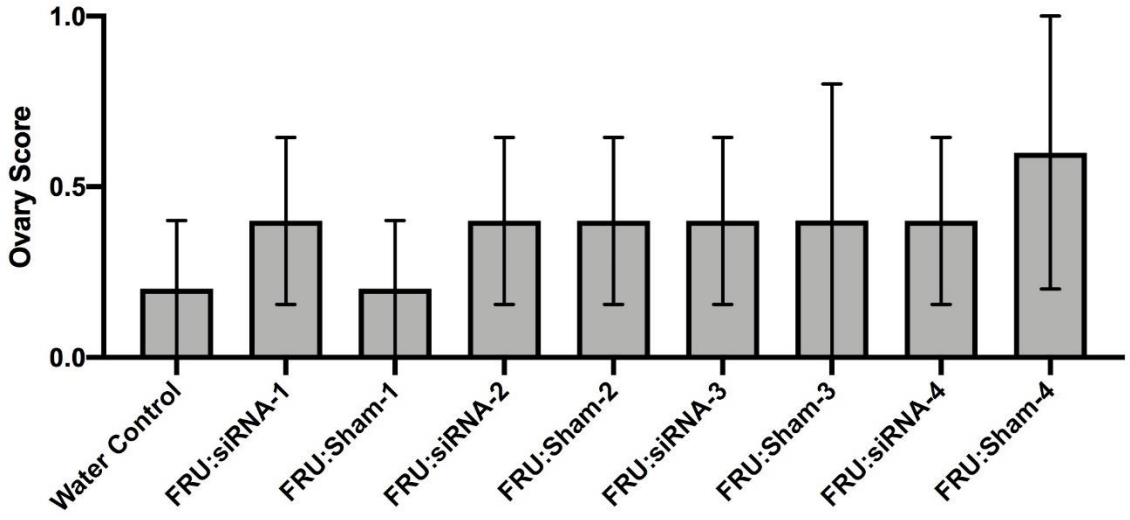

Figure 3.9 Dissected ovary scores for the *fruitless* 2018 experiment. Error bars represent SE.

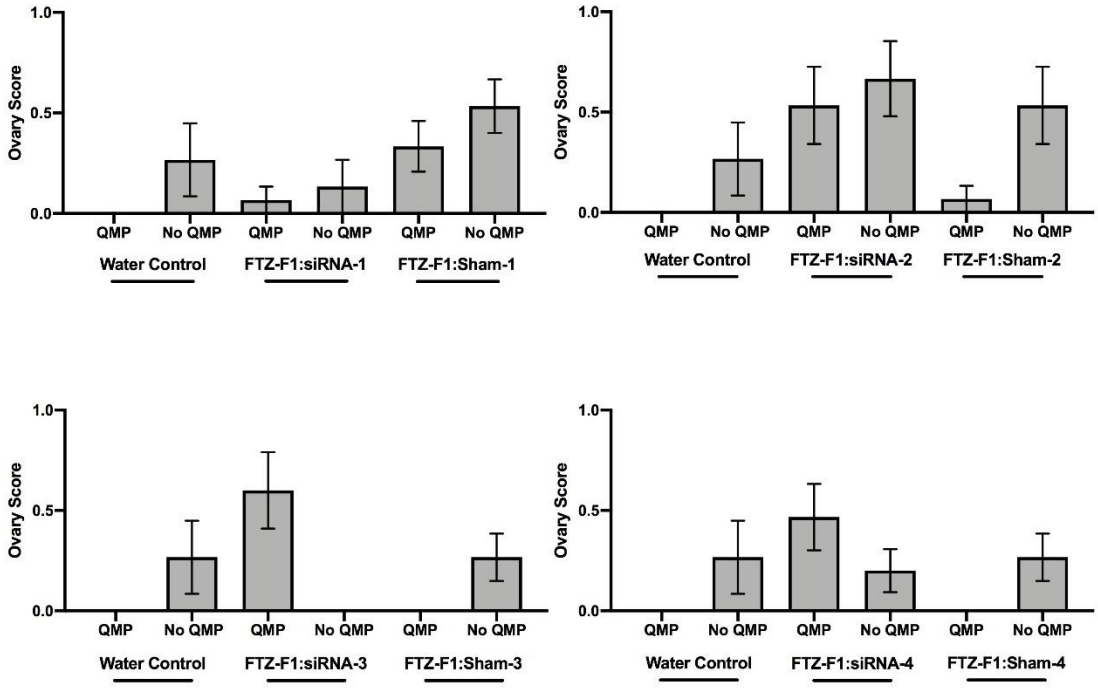

Figure 3.10 Ovary scores for the *ftz-f1* June 2018 experiment, sorted by treatment group. Error bars represent SE. N=15 individual abdomens for each treatment group. * Note that controls are the same across all charts.

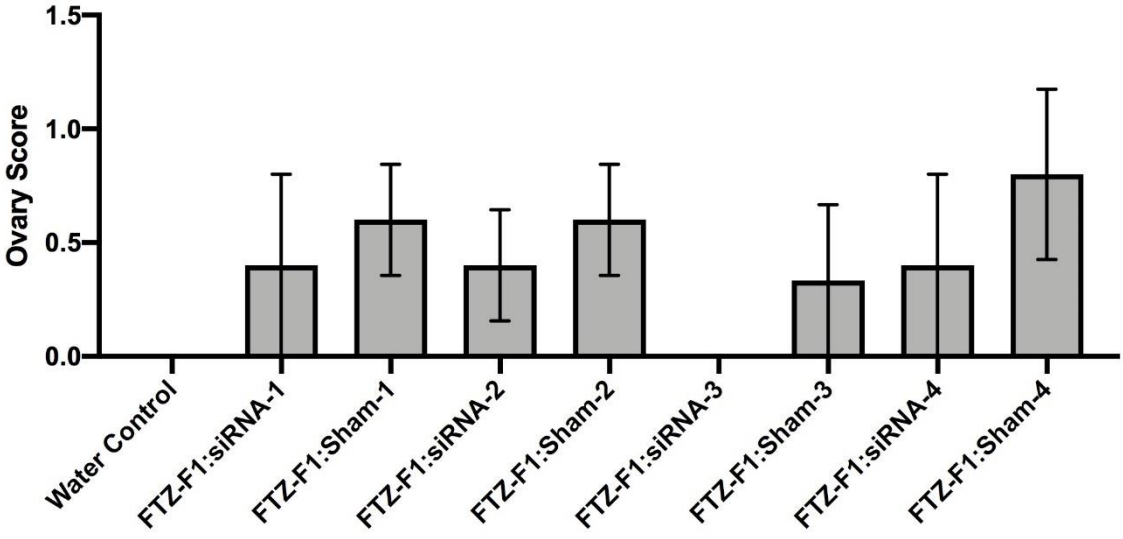

Figure 3.11 Dissected ovary scores for the *ftz-f1* August 2018 experiment. Error bars represent SE. N=5 individual abdomens for each treatment group.

4 Discussion

In this present study, the efficacy of siRNA technology on the knockdown of gene expression at two honey bee loci was tested: *fruitless* and *ftz-f1*. This study found that treatments using siRNA targeting the *fruitless* locus were not successful at reducing gene expression or changing ovarian response to QMP. Experiments targeting *ftz-f1* were inconclusive as to whether the siRNA treatments caused a gene knockdown due to that fact that analyses were incomplete. There does however appear to have been an effect on the ovary scores with respect to the siRNA treated bees. There was a deviation for the siRNA treated workers from the control workers with respect to ovary activation response in the presence or absence of QMP.

The honey bee *fruitless* gene (NCBI Gene ID: 409022) is a known transcription factor (Chandrasekaran *et al*. 2011) and has been shown to have highly conserved homology (Cristino *et al*. 2006) to *fruitless* (NCBI Gene ID: 42226) in *Drosophila melanogaster* (Ryner *et al*. 1996; Ito *et al*. 1996). In honey bees, this gene functions to affect behaviour (reviewed in Robinson *et al.* 2008; Chandrasekaran *et al*. 2011) and has been shown to be a target of other regulatory genes, such as *ultraspiracle* (Ament *et al*. 2012). *fruitless* has also been shown to be down-regulated in response to *vitellogenin* RNAi, which is interesting as *vitellogenin* acts in honey bee adult maturation by regulating gene expression within the honey bee brain (Wheeler *et al*. 2013).

fruitless has also been shown to have sex-specific splice variants within a related hymenopteran, a haplo-diploid parasitic wasp (Bertossa *et al*. 2009), a property previously shown in *Drosophila* (Demir and Dickson 2005). This sex-linkage potentially suggests that fruitless has evolved sex-specific roles in behaviour of insects.

Similarly, *ftz-f1* (NCBI Gene ID: 726450) which is a known transcription factor in *Drosophila* (NCBI Gene ID: 40045, Yu *et al*. 1997) has been shown to also be an important transcription factor related to behaviour in honey bees (Chandrasekaran *et al*. 2011), specifically regarding an individual's response to juvenile hormone. This gene-mediated response is important for development and maturation in insects (Riddiford 2008). Additionally, this gene has been shown to be up-regulated in low-strain workers (Wang *et al*. 2012), which are workers that have repressed and underdeveloped ovaries. *ftz-f1* is considered to be an important gene in ovary repression (Cardoen *et al*. 2011). Based on these facts, these genes were selected for silencing, with the expectation that knocking down one of them would likely cause an effect on the worker's ability to respond to QMP. Knockdown of gene expression might also interrupt development of sterile workers and therefore cause more developed ovaries.

4.1 Survival

Firstly, with respect to the in-cage experiments, a third of a QMP strip was used since these strips can maintain a queenless colony for about 21 days and this experiment was using cages for only ten days. Looking at the data for the *fruitless* Summer 2017 experiment only one siRNA (FRU:siRNA-3) was significantly different from its sham control. All other siRNAs did not differ in survival to their controls. In the 2018 trial of *fruitless*, the number of individuals was increased. This was done to promote an increase in the number of individuals that survived the entire experimental procedure. Honey bees, being colonial animals, do better in numbers than on their own (Winston 1991), and as such almost doubling the number of individuals makes it such that the cage becomes much more hive-like to the insects.

Similarly, in the Summer 2018 experiment FRU:siRNA-3 also showed a significant decrease in overall survival compared to its sham control. Additionally, in that experiment FRU:Sham-4 also had a significant decrease in overall survival compared to FRU:siRNA-4. Originally, this was thought to correlate to gene knockdown; however, since sham controls also shown a similar decrease, their deaths are more likely due to bad hygiene and poor health. Normally hygienic bees that keep their colonies clean by removing dead bees and other debris (Bigio *et al*. 2013) could not clean the cages out. This applied to the *ftz-f1* experiments as well, but for that experiment, to

increase the number of individuals that survived for the duration of the ten-day experiment, a total of 40 individual bees was started with. In the *ftz-f1* June experiment, a drastic increase in overall survival was seen (all trials showed upwards of 70% survival, whereas the *fruitless* experiment had roughly 50%). Since honey bees are not lab reared animals (Camiletti and Thompson 2016), experimental approaches should be done in hive if possible, or attempt to recreate a hive-like environment for the bees. That being said, honey bee holding cages are a suitable alternative and have been used in many honey bee studies (examples include Evans *et al*. 2009 and Thompson *et al*. 2007 among others). Similar to other cage studies, sample size was comparable, with the commonly used sample size of thirty individuals per cage (such as in Hoover *et al.* 2003 and Koywiwattrakul *et al.* 2005). The temperature and relative humidity were also comparable to previous studies. Chaimanee *et al*., (2012) had a cage temperature of 30 ± 2˚C whereas Grozinger *et al*., (2003) had a cage temperature of 33˚C and 50% relative humidity. The were no significant differences in survival for any of the trials in the presence or absence of QMP. This result is interesting as there has been much research that shows that certain pheromones in honey bees affect lifespan of the workers (Amdam *et al*. 2009), however this is mainly true of brood pheromone which is given off by the larvae (Démares *et al*. 2017; Smedal *et al*. 2009). Since these workers were incubated until they emerged,

they did not encounter brood pheromone. Another reason why QMP may not have affected survival in my study is that the trial lasted only for ten days, perhaps in a longer experiment the pheromone may have had some impact on worker survival.

Now looking at the *ftz-f1* experiment from August, the absence of QMP was dropped as a factor. The reasoning behind this was that with reading the literature (Cardoen *et al*. 2011), the research was more convincing to suggest that this gene is upregulated in workers that had inactive ovaries. In order to cause workers to have more inactive ovaries, QMP presence was the favoured state for this experiment. Cardoen *et al*., (2011) performed a large microarray study to identify differential gene expression based on the ovarian state in the presence of QMP and found that the expression ratio for *ftz-f1* was higher in ovary repressed workers than in those workers that had active ovaries.

This trial also had a lower overall survival, in fact it had dropped back to around 50%, even using 40 workers per cage. One possible explanation for this is that another student (from the University of Guelph) was also running an experiment in the same incubator that held this study's trials. This student was investigating the impact essential oils had on the survival of honey bees and one can suspect that it had an impact on this research as well. FTZ-F1:Sham-3 was found to be significantly different from both FTZ-F1:siRNA-3

and the WC in this experiment due to poor survival. Overall, the results of survival did not indicate a consistent difference between treated groups and controls. This either indicates that the target genes were not knocked down or that these genes are not as important to the honey bees with regards to survival after they have emerged as adults.

4.2 Gene expression

Firstly, when creating the siRNAs for this study, the BLASTn search was limited to RefSeq RNA in the honey bee in order to confirm that the designed siRNAs would not cause potential off-target effects in other honey bee transcribed genes. Since there is an element of stochasticity in siRNA function, alternate versions for each target gene were created to help ensure that there will be at least one that is effective at knocking-down the target gene (Evans *et al*. 2013). Additionally, with respect to reference gene selection one of the genes selected, *RPS18*, was recently identified as one of the two best reference genes to use in honeybee qPCR studies (Moon *et al*. 2018). With that said, with regards to the gene expression data, *fruitless* does not appear to have been successfully knocked down. The Summer 2017 trials only compared the siRNA treated samples with their associated sham controls, no WCs were used in that experiment. Only FRU:siRNA-2 had a significant decrease in *fruitless* expression, while the remaining siRNA treatments had no significant effect on gene expression, both FRU:siRNA-1

and FRU:siRNA-4 seem to follow the predicted pattern of lower expression but in a non-significant manner. FRU:siRNA-3 appears to have increased expression but statistical analysis could not be performed due to small sample size. Although there is a significant decrease in FRU:siRNA-2, there is no corresponding ovary dissection data due to small amount of survival, interestingly as there was also no significant difference in survival. Investigating the *fruitless* Summer 2018 gene study, there was no significant effect of the siRNA treatments on gene expression. The *fruitless* siRNA-treated bees, show expression similar to the WC bees while the sham-treated bees show slightly higher expression. In conclusion, it appears that the knockdowns did not work in this experiment. This could be due to a couple of factors, firstly *fruitless* is already downregulated in the presence of QMP (Grozinger *et al*. 2003) so attempts at knocking down a gene with low expression is difficult as target abundance is the limiting factor that gives RNAi, especially with siRNA, its efficiency (Hong *et al*. 2014). Another factor to consider is that the knockdown was present within the ectoderm but did not get transferred into other tissues. Systemic RNAi as seen in *C. elegans* (Timmons *et al*. 2003) is often not seen in insects such as *Drosophila* (Roignant *et al*. 2003) and as such perhaps there was a knockdown effect in the cells that took up the siRNA but did not transfer to other tissues (i.e. the abdominal tissues that were tested in qPCR) due to the absence of a

transitive RNAi pathway. Interestingly, honey bees do in fact show systemic RNAi (Maori *et al*. 2019); however, this is has been shown to be initiated through the ingestion of dsRNA not as with siRNA that were used in this study.

The *ftz-f1* experiment appears to tell a different story; however, limited data from this experiment is available. From the data gathered, in the presence of QMP, the knockdowns appear to be successful in reducing the expression of the *ftz-f1* gene. Both FTZ-F1:siRNA-1 and FTZ-F1:siRNA-2 (June) groups show a similar pattern in the presence of QMP with WC and sham controls showing similar levels of expression and a decrease in the siRNA-treated bees. This result is not a significant decrease due to a small sample size (n=3) for the qPCR experiments, and therefore repeated or future experiments could confirm this result with a larger sample size (most likely double or triple number of individuals or by using pooled samples of individuals). Interestingly in the absence of QMP, FTZ-F1:siRNA-1 does not appear to cause a knockdown when compared to FTZ-F1:Sham-1. This could be due to the fact that since *ftz-f1* has been shown to be upregulated in ovary-active bees, since it is predicted to be involved in ovary regression (Cardoen *et al*. 2011) and as such in the absence of QMP, more of the bees in the experiment would be expected to have some level of ovary development. This research's methodology was based on (Li-Byarlay *et al*. 2013), who had

used specific nanoparticles called perfluocarbon-nanoparticles (PFC-NPs) so that the siRNA would have an increased stability. This allows for the siRNA to have a better chance of invoking a knockdown in the insect. PFC-NPs were not used in this study as they are not commercially available. They were originally donated through a within University collaboration for Li-Byarlay *et al.*'s research. siRNA has recently been shown to function more effectively with the presence of stabilizing nanoparticles when using an aerosolized method (as seen in Thairu *et al.* 2017) as was performed in this research. Another additional consideration is that although this method was chosen due to it being non-invasive to the insect, this method has shown an RNAi silencing effect of around 30% (Li-Byarlay *et al.*, 2013) which is comparable to the more invasive dsRNA injection protocol (35% as seen in Ament *et al.* 2012).

The *ftz-f1* experiment from August appears to show very low expression for the WC bees. This is most likely due to a problem in generating the cDNA used in that run rather than an actual result of the siRNA treated bees having upwards of 2 to 4-fold increased expression compared to the WCs as no other qPCR run in the *ftz-f1* experiment shows this. In conclusion for this experiment, the results are inconclusive as to whether the siRNA treatments caused a knockdown. A larger sample size plus the completion of the analyses would have to be performed in order to confirm the results obtained.

4.3 Ovary scoring

Due to low survival in my single cage experiment in the Summer 2017, I did not have enough bees to dissect for that experiment. Investigating the ovary scores for the June *ftz-f1* experiment, the WCs acted as predicted, in the presence of QMP all the ovaries were scored as a 0 (i.e. no development); however, in the absence of QMP, some of the workers showed ovarian development. This is as expected as in queenright colonies with the presence of a healthy and fecund queen, there is a high level of QMP present in the colony and few (if any) workers reproduce (Barron *et al*. 2001). FTZ-F1:siRNA-2 which statistically impacted ovary score, show that in the presence and absence of QMP, the ovaries scored are both larger and more developed. All the sham controls except for FTZ-F1:Sham-1 show a developmental pattern that is similar to the WCs in that there is very little to no ovary development in the presence of QMP and in the absence there can be development. Interestingly, FTZ-F1:Sham-1 does not behave like the other sham controls and is statistically significant in impacting the ovary development.

One potential reason for this could be due to potential off-target effects that were not identified in the original BLASTn search. Upon investigation, this sham has some similarity to a few predicted noncoding RNA (ncRNA) sequences within the honey bee. ncRNAs play important roles in regulation of

gene expression and have been known to have both activating and repressing roles towards developmental processes (Amaral and Mattick 2008). There is also some slight similarity in sequence to two predicted transcripts for the honey bee lysophospholipase D an enzyme that catalyses a reaction that produces Lysophosphatidic acid (LPA) (Xie and Meier 2004). LPA has been identified as an important signalling molecule in both brain development and olfaction (Garrett and Grisham 2010), so if this pathway was interrupted, perhaps the ability of the bee to sense and respond to QMP was compromised.

Alternatively, a less likely explanation is that the FTZ-F1:siRNA-1 and FTZ-F1:Sham-1 were mistakenly switched at some point from the order of these siRNAs to the application to the bees. The August experiments did not show any significant difference in ovary development, this is attributed to the small sample size that had to be utilized due to lowered survival. Again, the WCs showed little development if any as expected; however, there was development in most of the other groups. Some other aspects should be considered, firstly, this study only lasted for ten days which is the minimum period in order to see development of the ovaries in workers (Hoover *et al*. 2006). If the study lasted for 14 days or longer instead, which is the peak time for workers to develop their ovaries (Velthuis 1970), perhaps there would be higher scores or more workers that showed development. Another

consideration to make is the low levels of survival. Over half of the individuals per cage died during the experiment (with the exception for the June *ftz-f1* experiments), so if these bees are in poor health, it would not be beneficial for them to invest their few resources that they have into their ovarian development when they are trying to survive.

4.4 Considerations for future experiments

Originally in the proposal assessment for this thesis, it was brought up that perhaps the in-hive experiment was most likely not going to be successful. The main concern was that due to siRNA having a short half-life *in vivo* (Strapps *et al*. 2010), the knockdown effect (if obtained) would not likely persist for the entire ten-day period. Being in hive, it would be disruptive to recollect the workers every other day for treatment, so holding cages were used for the remainder of my study.

Considering that the results are inconclusive as to whether there was a successful knockdown of the *ftz-f1* gene (*fruitless* knockdown did not appear to work), there are many improvements that could be made to any further research attempting to knockdown these genes. One of the first suggestions that should be taken is that if using siRNA, the use of nanoparticles should also be considered. As mentioned earlier, the nanoparticles used in Li-Byarlay *et al*., (2013), were not commercially available and therefore not used in this study.

Another consideration is the use of siRNA over dsRNA. Although dsRNA has often been used with regards to honey bee RNAi studies (Aronstein *et al.* 2006; Gatehouse *et al.* 2004; Hunter *et al.* 2010; Li *et al.* 2016 among others) and has seen great levels of success, siRNA was chosen for this study since the aerosolized method of application was utilized. dsRNA is often the choice due to the fact that that it is more effective in having an overall silencing effect on the gene of interest as well as having a longer transient effect within the organism (Wang *et* al. 2013); however, in honey bees it has been shown that dsRNA has the potential to cause off-target effects and therefore the design of the dsRNA sequences (with the consideration of the siRNA secondary sequences) is very important for both controls and for targets of interest (Jarosch and Moritz 2012).

Often in RNAi-based studies, the use of a non-expressed protein is used as a control for the experiment, the most common of these being green fluorescent protein (GFP) in insect related studies (Amdam *et al.* 2006; Li-Byarlay *et al.*, 2013 and Wang *et al.*, 2010 among others). One could then question why GFP-siRNA wasn't used as a control for this study, especially as the study that the methodology for this experiment was based off used GFP as a control. There are two reasons for this; firstly, recent research has shown that the use of GFP as a RNAi control, while not being present in honey bee genome, can affect over 1000 different genes' expression (Nunes *et al.* 2013).

This large amount of off-target effects could potentially give false results on gene expression data. Secondly, the standard methods for molecular research in *Apis mellifera* (Evans *et al*. 2013) suggests using the scrambled sham as a control and in theory makes sense to use as a baseline control; however, in this research, at least with regards to the *fruitless* experiment, appear to cause a change in expression in an unpredicted way. Perhaps the use of sham controls should be advised against as well or if used, include the proviso that there is very little complementarity to any gene within the study organism's genome (in this study all were checked to have less than a 60% match).

Other considerations to make moving forward are the difficulties working with honey bees as a study organism. As previously mentioned, honey bees are colonial animals and not in lab models, which increases difficulty when attempting to perform RNAi-based studies. Cage experiments provide the most "colony-like" effect for the bees but again death is very common in these kinds of studies (Milne Jr. *et al*. 2015). Another confounding factor considering the aerosolized method utilized in this research is that uniform distribution of the aerosolized siRNA treatments is not guaranteed, one individual could take up more of the treatment than another. An injection-based method would eliminate that effect but add other impacting factors.

Injection runs the risk of injuring the bees beyond the effect of the RNA knockdown impacting the survival of the bees.

Another factor that has been considered is that in this experiment only abdominal mRNA was extracted for cDNA synthesis, perhaps the use of head or whole bodies for RNA extraction should be considered or performed. Since QMP is recognized by odorant receptors from the antennae (Camiletti *et al*. 2016), which would then stimulate areas of the honey bee brain to have downstream effects on ovary development, full body extractions should be utilized in the future.

4.5 Conclusion

Ultimately, further research needs to be performed to elucidate the functional roles of both *fruitless* and *ftz-f1* within the realm of honey bee ovarian development and social biology. Although challenging, working with bees can provide a deep-rooted insight towards the development and maintenance of complex social structure and social networks. The underlying genetic mechanism of honey bee ovary suppression still needs to be identified, regardless of said challenges, as it could play a similar role in other social insects, from eusocial to semi-social, as well as affect or control individual overall behaviour with respect to varying social or environmental cues. The above considerations should be made if using honey bees as the model and deciding to chose RNAi as the methodology of choice with respect to gene

expression analyses. These future analyses could pave the way for a more complex and deeper insight in the realm of sociobiology.

Currently, the underlying genetic mechanism that controls the sterile worker phenotype in honey bees is for the most part unknown (Cardoen *et al*. 2012). The conditional expression for DEGs, with respect to worker sterility, continues to be a topic within insect sociobiology of utmost significance (Linksvayer 2015). The genetic basis of worker sterility has remained a focus of research as we are now becoming better able to identify the key players that are responsible for this phenotype (as seen in Ronai *et al*. 2016); however, more work needs to be done with respect to this area. The significance of this response to QMP to sociobiology is that by gaining an understanding towards the underlying genetics of this behaviour-mediating phenotype would allow us to expand our understanding of how and why such highly social insect social structures evolved.

Thompson *et al*. (2013) originally proposed criteria for “genes for altruism” and since then very few genes have been identified as such. It has long been understood that within honey bees, QMP plays a role in causing workers to forgo their own reproduction by inhibiting the growth and activation of the worker ovaries, to direct altruistic benefits towards their queen (Backx *et al*. 2012; Hoover *et al*. 2003; Naumann *et al*. 1991; Van Oystaeyen *et al*. 2014). Although there have been many genes identified as potential candidates

(Chandrasekaran *et al*. 2011, Sobotka *et al*. 2016), the genes responsible for this selfless response have, however, not yet been thoroughly analysed on a functional level to test if gene knockdowns or knockouts cause an interruption into the normal worker response and ovary activation pathway in the presence of QMP.

One approach for testing the function of candidate genes implicated in the social regulation of worker reproduction is RNA-interference, which shows remarkable potential as a functional genomic tool for blocking expression of single genes *in vivo*. Pioneering RNAi studies by Amdam *et al*. (2003) and Gatehouse *et al*. (2004) have shown that the use of dsRNA is efficient in causing honey bee gene knockdowns. Hunter *et al*. (2010) showed a "real-world" application for apiculture by performing an RNAi experiment in a large scale in-hive experiment whereby they fed the bees Israeli Acute Paralysis Virus (IAPV) dsRNA sequences and found they were successful in causing a decrease in pathogen loads and making the hives able to produce more honey. Marco Antonio *et al*. (2008) and Mustard *et al*. (2010) have expanded this application to include RNAi as a test of different behavioural phenotypes as a result of down-regulation of specific target genes. Further, Ronai *et al*. 2016 is an example of an RNAi-based study which confirms that a honey bee gene, *anarchy*, plays a central part in worker ovary activation using the programmed cell death pathway and by extension fulfills the criteria originally

laid out in Thompson *et al*. (2013) for being a “gene for altruism.” That study proved that it is possible to identify such a gene with RNAi and hopefully this technology will allow for the confirmation of other genes, specifically including *fruitless* and *ftz-f1*, as being considered “genes for altruism” and involved in the worker ovary activation state.

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Curriculum Vitae

Anthony Gallo

EDUCATION

Bachelor of Science **2016**

(*Honours Specialization in Genetics and Biochemistry*)

The University of Western Ontario (Western), London, Ontario

- Relevant Courses: Scientific Methods in Biology; Evolutionary Genetics; Genomics and Beyond: A Laboratory Course, Regulation of Gene Expression; Molecular Genetics of Human Cancer

RESEARCH EXPERIENCE

Volunteer Research Assistant **Summer/Fall 2014**

Singh Lab: Molecular Genetics of Complex Disease

- Analyzed methylation data for Schizophrenia patients and families using databases to identify potentially impacted genes.
- Performed Real time PCR analyzes in order to confirm if a gene was up-regulated in a patient compared to a control.
- Learned how to use Droplet Digital PCR, as a means to further experiment and investigate differences between two samples.
- Organized and collected data from past research, investigating relevant journal articles for the research that I was assisting with.
- Co-Authored an article based on the research and assisted with another article.

Graduate Student **Winter 2017 to Now**

Thompson Lab: Behavioural Genetics and Sociobiology

- Worked with the University of Guelph's Research Apiary in order to learn how to take care and maintain Buckfast honey bee hives.
- Currently working on an RNA interference project, performing gene knock downs targeting sterility genes within the honey bee and testing the impact on ovary development.
- Successfully generated siRNA to target genes of interest, using the online application e-RNAi.
- Learned how to successfully extract RNA from the abdomens of honeybees.
- Learned how to run a qPCR experiment including designing primers, running standard curves, testing effective primer annealing temperatures, setting up the plates and analysing the results.
- Became efficient at dissecting honeybee worker abdomens to extract and image ovaries at varying stages of development.

COMMUNICATION AND INTERPERSONAL SKILLS

Summer Camp Volunteer

Family Karate Centres, London Ontario **Summer 2009**

- Was part of a team that led a group of young children through martial arts training and physical activity.
- Interacted with children of various ages and solved disagreements and issues that arose.
- Led small teams through instruction during week long camps.

TEACHING EXPERIENCE

Assistant Instructor

Family Karate Centres, London Ontario **2008 to 2012**

- Taught students of various ages, races and learning styles and helped them to develop confidence, respect and self-discipline through martial arts.
- Learned to solve disputes, and disagreements between students and administer aid to those that got injured.

Head Instructor

Family Karate Centres, London Ontario **2012 to 2018**

- Lead classes and design class plans on a weekly basis that provide students with exercise and martial arts training.
- Lead adult classes in addition to children's classes.
- Run a recreational class once a week for younger students who need more attention.

Teaching Assistant

Western University, London, Ontario **2017 to Now**

- Assisted in the course, Biology 3596B: Genomics and Beyond.
- Ran one laboratory section every week, supervising the students through a total of four different modules including; forensic genotyping, gene expression assays, bacterial cloning and yeast complementation.
- Was nominated for a TA award for my teaching in this class
- Gave a lecture on my current research to the class, which appeared on the final exam.
- Gave the class my own assignment, that was graded by myself.
- Assisted in the course, Bio4583A: Molecular Genetics Laboratory
- Ran two laboratory sections every week, supervising the students through modules including differential gene expression and research-based assignment.
- Assisted in the course, Bio2290G: Scientific Methods in Biology.
- Facilitate undergraduate molecular biology rotation in this course
- Assisted in the course, Bio3222G: Synthetic Biology
- Was the first TA in this brand-new course for the 2019 Winter semester; duties included marking as well as helping to shape the course for next year

VOLUNTEER EXPERIENCE

- Volunteered at London Bug Day (September 9th, 2017 and September 8th, 2018) teaching children and parents about different aspects of honey bee social life. Monitored an observational hive and explained the inner workings to event goers.
- Visited Saint Andre Bessette Catholic High School, to give a guest talk to the Grade 12 Biology Class of Mr. Samuel J. Geddes. I lectured about my Undergraduate volunteer and Graduate research as well as answered questions regarding Western University and basic University life.
- Volunteered at Ontario Biology Day 2019 (Western University), where I was a chair of multiple talk sessions and judged these sessions as well. I also was one of the individuals present at the registration table to get the attending students ready for the conference.

PUBLICATIONS

- Castellani, C. A., Melka, M. G., Lui, J. L., **Gallo, A. J.**, O'Reilly, R. L. & Singh, S. M. Post-zygotic genomic changes in glutamate and dopamine pathway genes may explain discordance of monozygotic twins for schizophrenia. *Clin Transl Med* **6**, (2017).
- Faragalla, K. M., Chernyshova, A. M., **Gallo, A. J.** & Thompson, G. J. From gene list to gene network: Recognizing functional connections that regulate behavioral traits. *J. Exp. Zool. B Mol. Dev. Evol.* **330**, 317–329 (2018).

MEDIA OUTREACH

- Had a blog article published based on the Faragalla *et al*., 2018 article on Advanced Science News – "Bee Friendly: Network Biology Meets Sociobiology." Published November 29th, 2018.
- Volunteered on "GradCast" a weekly podcast that interviews Western University graduate students on their current research projects. Episode 207 – "To Bee or Not to Bee." March 19th, 2019.

SCHOLARSHIP AND ACADEMIC HONOURS

The University of Western Ontario Scholarship	**2012**
Christina A. MacKerracher Scholarship	**2012**
Dean's Honour List	**2013, 2015 & 2016**
Western Graduate Research Scholarship	**2017-18**
Nominated for a TA Award - Bio3596	**2017**
Nominated for a TA Award – Bio4583A	**2018**

CERTIFICATION AND TRAINING

Lab Safety – Hazardous Waste	**2014**
Accessibility in Service (AODA)	**2014**
Worker Health and Safety Awareness	**2014**
Safe Campus Community	**2014**
Comprehensive WHMIS Certification	**2016**
Accessibility in Teaching	**2016**