The Neurogenomic State Underlying Selfish and Altruistic Aggression in Honey Bees (*Apis mellifera*)

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Abstract

I studied aggression using the eusocial Western honey bee, *Apis mellifera*, as it displays aggression in altruistic and selfish contexts, allowing comparisons that improve our understanding regarding the molecular biology and neurogenomics underlying this ecologically important behaviour. Workers exhibit altruistic aggression to protect their colony while queens exhibit selfish aggression to gain reproductive control within their colony. I hypothesized that the neurogenomic state associated with altruistic aggression of worker bees is more evolutionarily derived than that associated with selfish aggression of queens. Using RNAseq I compared the neurotranscriptome of at-rest and aggressive workers and queens. Classifying the differentially expressed genes (DEGs) of workers and queens as taxonomically-restricted or conserved as well as comparing these DEGs to that of solitary species both revealed that worker altruistic aggression DEGs are not more evolutionary derived than queen selfish aggression DEGs. These comparative analyses help reveal the molecular underpinnings of aggression in honey bees.

Dedication

This thesis is dedicated to my true companion; I choose you every day.

Acknowledgements

Thank you to all those who have heard the word ‘aggression’ more times in the past two years than they’ll hear in a lifetime.

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Introduction

Questions about the genetics and molecular biology underpinning behaviour continue to intrigue scientists. Studying the genetics of behaviour in social and solitary animals, including humans, may offer insight into the long-term relationship between individual behaviour and how it is influenced by genetics, the environment, and social interactions. Behavioural neurogenomics is the study of how genetic and genomic mechanisms contribute to behaviour (Binder and Klengel, 2019). This field of study is especially useful for understanding how gene regulation influences behaviour over short (plastic response), intermediate (differences in animal personalities), and long (differences between species) time scales (Zayed and Robinson, 2012). This approach has been used to study behaviour in a wide variety of animals, from how songbirds learn to sing (Clayton et al., 2009; Johnson and Whitney, 2005) to novelty-seeking behaviour in scouting honey bees (Liang et al., 2012). Behavioural neurogenomics has also been used to study the gene-brain-behaviour linkages in fragile X syndrome in humans, a syndrome that originates from the disruption of a single gene, results in the loss of a specific protein, and is associated with emotional, cognitive, and behavioural issues (Reiss and Dant, 2003).

Motivation is an important aspect of animal behaviour. An individual's motivation can be viewed through a variety of lenses, one of which is the behaviour's effect on the fitness of both the actor and recipient (Table 1). The term motivation here is used in reference to McClelland et al.'s (1992) implicit motivational system; implicit motivation being a biological variable that drives behaviours (McClelland et al., 1992; Schultheiss et al., 2008). It has also been described as "the internal force that produces actions on the basis of the momentary balance between [… the individual's] needs and the needs of [… the] environment" (Dorman and Gaudiano, 1995).

Implicit motivation has been used to study simple decision-making processes, such as those made by bumblebees and other foraging insects when deciding whether or not to leave a patch (Louâpre et al., 2010).

Table 1: Categories of social behaviour (West et al., 2007).

	Decrease fitness of recipient	Increase fitness of recipient
Decrease fitness of actor	Spite	Altruism
Increase fitness of actor	Selfish	Mutual benefit

An individual's motivation can be classified as either selfish or altruistic. Behaviours motivated by selfishness are those that increase the fitness of the actor while decreasing the fitness of the recipient. Resource competition, whether for food, mates, or territory, is the most common type of selfish behaviour. Conflict over resources frequently results in a winner (the one obtaining the resource and increasing its fitness) and a loser (the one who relinquishes or yields to the other and forfeits the resource, thereby experiencing a decrease in fitness). For example, European rabbits (*Oryctolagus cuniculus*) are extremely territorial (Mykytowycz, 1968). Males (bucks) will fight each other to secure territory from other bucks, which includes access to females (does) within their territory (Rödel and von Holst, 2009; Vastrade, 1986), so male buck behaviour can be seen as selfish.

Behaviours driven by altruistic motivations are those that result in a benefit to another individual, or society as a whole, but come at a cost to the actor. These behaviours are common within groups of genetically related individuals. An example of altruism can be seen in vampire bats. A typical vampire bat roost contains 8-12 females and their pups; males roost alone or in small groups separate from the females (Wilkinson, 1990). If a vampire bat has an unsuccessful hunt one night, it will solicit others for some of their blood meal; vampire bats will die if they do not feed for two nights in a row (Wilkinson, 1990). Successful hunters are more likely to regurgitate and share their blood meal if the solicitor is familiar and/or genetically related (Wilkinson, 1990). Another example occurs in meerkats. A mob of meerkats is comprised of up to 50 individuals in which a dominant mating pair largely monopolize reproduction (Sharp and Clutton-Brock, 2011). The remaining adult females of a mob are mostly offspring of the dominant pair and therefore also full siblings of the pups they help raise (Sharp and Clutton-Brock, 2011). All members of a mob partake in baby-sitting, pup feeding, social digging, and raised guarding (Tatalovic, 2010). In the case of raised guarding, a behaviour where sentries look out for ground and above ground predators while other members of the mob forage underground, sentries forgo their own chance to forage underground (Tatalovic, 2010). Both these examples demonstrate instances of altruistic behaviour in the animal kingdom in which one individual incurs a cost to itself while benefiting another. In both examples, the actor directly gives away or sacrifices the opportunity to obtain sustenance in order for its kin to feed as well (e.g. vampire bats) or instead of (e.g. meerkats). While the evolution of altruistic behaviour may appear paradoxical, Hamilton (1964) showed that such behaviours can evolve so long as the recipient is genetically related to the altruistic individual. Hamilton's kin selection theory states that individuals are more likely to perform an altruistic act when $rB > C$ in which r is the genetic relatedness between the actor and the recipient, B is the reproductive gain the recipient experiences, and C is the cost to the actor (Hamilton, 1964). Simply put, individuals are more likely to perform a behaviour, at cost to themselves, when the recipient is related to them. This is because although the fitness of the actor may decrease, there is a net increase in fitness for the actor and recipient combined, called inclusive fitness.

Sociality exists on a spectrum ranging from solitary to advanced eusocial (Table 2). Eusocial behaviour is defined based on the presence of three features: cooperative brood care, overlapping generations, and division of labour (Crespi and Yanega, 1995). Eusocial societies display both altruistic and selfish behaviours. Examples of extreme altruistic behaviours from eusocial societies include foregoing one's own reproduction to help raise the offspring of their mother and sacrificially defending their colony to provide an opportunity for their kin to survive (Winston, 1987). While selfish behaviours may be less obvious in eusocial societies, they do occur. Aggression is a behaviour that occurs in both selfish and altruistic contexts in social insects. For the purposes of this research, aggression is the behaviour, or set of behaviours, associated with an attack or escalating behaviours occurring before an attack (Hinde, 1970; van Staaden et al., 2011).

Table 2: Stratification of sociality (Shell and Rehan, 2017).

Stage	Social class	Cooperative brood care	Overlapping generations	Division of labour
Early	Solitary	No	No	No
	Subsocial	No	Some	No
	Incipiently social	Yes	Some	Some
Late	Primitively eusocial	Yes	Some	Yes
	Advance eusocial	Yes	Yes	Yes

In solitary insects, aggression is clearly a selfish behaviour. *Drosophila melanogaster,* a model organism for studying aggression in solitary insects, displays aggression in both females and males (Zwarts et al., 2012). Males fight other males for access to food or female courtship and females fight over access to food, especially yeast, which has been suggested to enhance egg laying (Zwarts et al., 2012). Female cicada-killer wasps, *Sphecius speciosus*, have been shown to be more aggressive towards female conspecifics when a paralyzed cicada is present inside the resident's burrow than when a cicada is not present (Pfennig and Reeve, 1989). This supports the conclusion that female cicada-killer wasps are aggressive in order to protect the nutritional source for their larvae. In Darwin's beetles, *Chiasognathus grantii*, males grow abnormally large horns that are used to fight other males in order to obtain reproductive access to females (Emlen et al., 2007). The use of horns to fight male competitors can also be seen in scarab beetles. Larger horns have been reported to correlate with increased success in these inter-male fights (Rasmussen, 1994). In the examples above, the actor is aggressive for the sole purpose of obtaining a resource that directly increases its own fitness while decreasing the fitness of the recipient; this is the definition of a selfish act (Table 1).

In contrast to solitary insects which display selfish aggression, social insects display aggression in both altruistic and selfish contexts. An interesting example of altruistic aggression is worker policing (Ratnieks and Helanterä, 2009). For example, in wild colonies of the tree wasp, *Dolichovespula sylvestris*, workers have been observed to behave aggressively towards laying workers and selectively destroy worker-laid eggs (Wenseleers et al., 2005). These behaviours include grasping the egg-laying worker and attempting to sting and/or push her off the cell. Given the finite number of eggs and larvae a worker can rear, she would rather invest her energy into rearing queen-laid eggs (sisters) over worker-laid egg (nephews). This aggression can be explained genetically as non-laying workers are, on average, more related to their sisters than their nephews (Queller and Strassmann, 2002). Therefore, for any individual worker, it is more beneficial for eggs to be a sister than a nephew.

Another example of altruistic aggression in social insects is colony defense. Termites have a specialized caste for colony defense, the solider caste. Solider castes specialize in defense and protection of the nest. They are also sterile and therefore not capable of directly increasing their own fitness. Instead, they protect their colony to ensure the queen can continue producing offspring, which are siblings to the soldiers, and therefore at least 50% related to them (Ratnieks and Helanterä, 2009). Protecting the nest does not impact the direct fitness of a soldier as it is already zero because they are sterile, but it increases its indirect fitness because its genetic material can be passed on by its mom and/or siblings. Colony defense can also be found in wasp species (e.g. *Parachartergus colobopterus;* Strassmann et al., 1990), as well as ant species (e.g. *Solenopsis invicta;* Jerome et al., 1998). Non-reproductive members of these species will attack an intruder using their stinger, venom, and/or mandibles (Jerome et al., 1998; Strassmann et al., 1990). Thus, worker policing and colony defense are clear examples of altruistic aggression in social insects.

Social insects can also display selfish aggression. In the eusocial black garden ant, *Lasius niger*, pleometrotic queens will fight to the death. Following their nuptial mating flight, foundresses may cooperate with one or more other foundresses during nest initiation and form pleometrotic nests (Sommer and Hölldobler, 1995). This cooperation breaks down and turns into a fight for reproductive control when workers begin to emerge (Aron et al., 2009). Another example occurs in *Harpegnathos saltator* ant colonies. Following the loss of its queen, *Harpegnathos saltator* workers will engage in aggressive antennal dueling tournaments to determine which few workers will become gamergates (a mated worker that can reproduce sexually) (Opachaloemphan et al., 2021). Finally, in the Southeast Asian ant, *Cardiocondyla sp.*, queens have been observed to act aggressively towards other sexual females with the presumed goal to prevent the other females from shedding their wings and laying eggs (Yamauchi et al., 2007). These three examples demonstrate selfish behaviours as the winning queen is then able to pass on her own genetic material to the nest.

Enabled by the advancements in biotechnology in the early 2000s, researchers started analyzing the molecular biology and neurogenomic states underlying natural behaviours such as aggression in a wide range of solitary and social species. Neurogenomic state is defined as the gene expression pattern in the brain. The first studies of the molecular biology underlying aggression focused on biogenic amine neurotransmitters in vertebrates (Anholt and Mackay, 2012; Edwards et al., 2006). Two of these biogenic amines, serotonin and dopamine, are the most widely studied across the animal kingdom. The relationship between these two neurotransmitters and aggression is complex and nuanced because both transmitters have been shown to increase and decrease aggressive behaviours depending on the context. For example, serotonin's effect on aggression depends on the receptor in question. Saudou et al. (1994), a study showed that male mice without a functional 5-HT1B receptor show high levels of aggression while a different study showed that male mice given a 5-HT1A receptor antagonist displayed reduced aggression (Bell and Hobson, 1993). Both studies effectively removed the receptor's activity, but yielded contrasting results. Dopamine appears to have a less nuanced relationship. Researchers, for the most part, agree that dopamine and aggression have a positive relationship (Anholt and Mackay, 2012; Seo et al., 2008; Zhang-James et al., 2018). While these genetic manipulation studies are informative, vertebrate genetic manipulation has its limits. Invertebrates, however, specifically *Drosophila melanogaster*, have a larger and more diverse genetic tool-kit than vertebrates, allowing for more detailed studies of the genetics and molecular biology underlying aggressive behaviours.

Studies of the *fruitless* gene provide an excellent example of behavioural genetics in *Drosophila melanogaster*. *Fruitless* is a sex-specific gene encoding a zinc-finger transcription factor that was initially found to play a critical role in male courtship and was subsequently investigated for its potential role in aggression and dominance (Billeter et al., 2006; Manoli et al., 2006; Vrontou et al., 2006). Vrontou et al. (2006) generated three versions of f*ruitless*; fru^M (the translated male version expressed in both sexes), *fruF* (the non-translated female version expressed in both sexes), and fru^C (a non-manipulated version subject to sex-specific translation). They found that flies, regardless of their sex, fought in a manner that correlated with the version of *fruitless* they had. Males with fru^F would fight like females and females with fru^M would fight like males. This study is an example of the greater repertoire of genetic modification available in fruit flies.

The studies discussed above use a reverse-genetic approach, by altering genes and examining the resulting change in behaviour. These studies establish causal relationships between genes and behaviour. While this approach is very powerful, it is not easily applicable to non-model organisms (Ekblom and Galindo, 2010; Gurumurthy et al., 2016). In such species, behavioural transcriptomics can be useful in understanding the genes and gene networks associated with natural behaviour (Cahais et al., 2012; Oppenheim et al., 2015). Using a behavioural transcriptomics approach, researchers can study either natural or experimentally-induced changes in behaviour and then quantify changes in gene expression associated with the behavioural changes. Techniques used in transcriptomic studies include microarrays (Ekblom and Galindo, 2010) and, more recently, RNAseq (Kravitz and Huber, 2003). RNAseq allows us to quantify gene expression of thousands of genes before and after exposure to a stimulus. For example, aggression can be induced in an animal, and the neurogenomic state can be analyzed to determine which genes are up- or down-regulated in response, allowing researchers to link gene expression in neuronal tissues with changes in behaviour such as aggression.

Honey bees have served as a model organism for sociogenomics to understand the genetic and molecular bases of social behaviours including aggression (Dolezal and Toth, 2013). A worker honey bee is aggressive towards intruders, such as other Hymenopteran insects or large vertebrate predators, to protect the hive but this comes at the cost of her own life. This is due to the barbed nature of her stinger causing it to become permanently lodged in the tissue of the intruder (Hermann, 1971; Nouvian et al., 2016). Unable to retract her stinger, the worker bee becomes eviscerated after stinging, leaving behind her stinger and poison gland in the intruder which ultimately results in her death (Hermann, 1971; Nouvian et al., 2016). The intensity with which workers attack an intruder varies and is heritable on both the colony and individual level. The most aggressive subspecies, nicknamed the killer bee, is the Africanized honey bee (AHB). AHB are a hybrid between *A. mellifera ligustica* and *A. mellifera scutellata* and their extreme aggression resulted from genetic interactions between alleles from each of their two ancestral lineages (Harpur et al., 2020). At the opposite end of the spectrum, known for their gentle nature, is the Italian bee, *A. mellifera ligustica.* These juxtaposing temperaments make the honey bee an ideal group to study the genetics and molecular biology of aggression.

Various approaches have been used to study altruistic aggression in eusocial honey bee workers. Early honey bee research, using quantitative trait locus analysis, identified three loci associated with worker defense; appropriately (although unimaginatively) named *sting-1, sting-2,* and *sting-3* (Hunt et al., 1998, 2007). *Sting-1* was found to have significant association with guarding behaviour, colony stinging response, and individual stinging response. Guarding behaviour is when a bee stands at the hive entrance with her forelegs off the ground and holds her antennae forward to inspect incoming bees; colony stinging response is how many bees sting a leather patch waved in front of a hive for two minutes; and individual stinging response is how many milliamps of voltage are required for an individual honey bee to sting in a laboratory analysis (Guzmán-Novoa et al., 2002; Hunt et al., 2007; Shorter et al., 2012). *Sting-2* and *sting -3* had significant associations with guarding and colony stinging response, but are less well studied (Hunt et al., 1998, 2007).

RNAseq was used by Alaux et al. (2009a) to investigate how environmental pressures on aggression might have evolved into inherited differences by comparing various worker stages of Africanized honey bees (AHB) to the corresponding worker stage in European honey bees (EHB) (Alaux et al., 2009a). The AHB versus EHB comparison was done as AHB are extremely aggressive. AHB and EHB were co- and cross-fostered to determine the effects of genetics and environment on neurogenomic state. Using microarray analysis, differentially expressed genes (DEGs) were obtained for the following comparisons; AHB vs EHB guard, AHB vs EHB soldier, AHB vs EHB forager, and before and after alarm pheromone exposure. Principal component analysis revealed that individual genotype accounted for ~30% of the variation in brain gene expression while host colony genotype accounted for ~25% of the variation. *Cyp6Q1* and *moody* were two genes found to be differentially regulated in AHB, alarm pheromone (AP) exposure, and older EHB (Alaux et al., 2009a). These three conditions, genetic and environmental, are all associated with increased aggression. Furthermore, *Cyp6Q1* and *moody* are also associated with aggression in rodents (Soma et al., 2008) and reproductive competition in *Drosophila* (Drnevich et al., 2004). This supports the claim that the neurogenomic state of worker honey bees is heavily influenced by both genetic and environmental factors.

More recently, a study compared the neuromolecular response to a territory intruder among honey bees (*Apis mellifera*), stickleback fish (*Gasterosteus aculeatus*), and mice (*Mus musculus*) using RNAseq in order to understand if there are common molecular mechanisms in similar behavioural responses (Rittschof et al., 2014). That is, do these three species, separated by ~650 million years, have common machinery that results in an aggressive response when their territory is challenged? Rittschof et al. (2014) found that genes associated with hormone-mediated signal transduction, neurodevelopment, changes in chromosome organization, and energy metabolism were differentially expressed in all species. Additionally, homologous transcription factors typically associated with neuronal development changed in response to the intruder in all three species (Rittschof et al., 2014). While there is great interest in understanding the molecular biology associated with altruistic forms of aggression, comparing the neurogenomic basis of altruistic aggression in worker honey bees to that associated with aggression in solitary insects and vertebrates involves making comparisons over very large evolutionary time scales. Comparing aggressive worker honey bees to aggressive queen honey bees may shed more insight on the neurogenomic differences underlying altruistic versus selfish aggression.

Selfish aggression in honey bee queens may be a better comparator to understand the neurogenomic state(s) involved in worker altruistic aggression. Queen honey bees are aggressive during the first week of their lives as they attempt to become the hive’s sole reproductive member (Jackson and Robinson, 2018; Winston, 1987). When a hive must re-queen itself, workers rear up to 20 queens to ensure at least one healthy queen survives to her reproductive stage (Harano and Obara, 2004a; Winston, 1987). Newly emerged virgin queens can: a) establish a new swarm if there are a large number of workers in her natal colony (Simpson, 1958); b) inspect the hive and destroy capped queen cells, thereby eliminating potential competitors (Harano and Obara, 2004b); and/or c) engage in a duel to the death with any adult queen present in the colony (Gilley and Tarpy, 2005). A queen duel is characterized by rapid movement within the hive, shaking, tooting or piping, physical contact, and a deadly sting (Gilley, 2001). These mechanisms of queen elimination continue until only one queen remains. The royal battle to be the hive's sole reproductive member is an example of selfish aggression as the winning queen gains a large increase in her fitness, and it comes at the complete reduction in fitness of the loser. This selfish aggression sharply contrasts that of the altruistic aggression seen in workers who sacrifice themselves to protect their hive.

Research into queen aggression has focused on the set of behaviours displayed before and during duels and the effect of winning previous duels on subsequent ones (Butz and Dietz, 1994; Gilley, 2001; Gilley and Tarpy, 2005; Harano and Obara, 2004a; Jackson and Robinson, 2018; Tarpy and Fletcher, 2003). However, there is a paucity of studies investigating the genetic or molecular basis for queen aggression. Using queen aggression as the selfish comparator against altruistic worker aggression is advantageous in that it controls for evolutionary distance, allowing the identification of genes uniquely associated with altruistic and selfish aggression within the same species.

In this study, we conducted an experiment to compare the brain transcriptome of altruistic aggressive workers to that of selfish aggressive queens. We used RNAseq to understand the molecular biology and neurogenomic state associated with altruistic aggression (Figure 1). Johnson and Tsutsui (2011) posited that phenotypes of worker bees should be influenced by the largest number of taxonomically restricted genes as they display a number of behaviours unique to eusociality. They found that orphan genes are in fact disproportionally associated with workers, supporting their overarching hypothesis that taxonomically-restricted genes (genes unique to Insecta) play a role in the evolution of social behaviour. In other words, unique genes may give rise to unique behaviours. Therefore, I hypothesized that the neurogenomic state associated with altruistic aggression is more evolutionarily derived than that of the neurogenomic state associated with selfish aggression. Evolutionarily derived genes are those that have appeared recently in the organism's genome are therefore found only within a taxonomic group. I tested this hypothesis by comparing the proportion of conserved (non-taxonomically-restricted) to taxonomically-restricted differentially expressed genes (DEGs) in both workers displaying altruistic and queens displaying selfish aggression related behaviours (Figure 2B). I also tested this hypothesis using a different approach by comparing altruistic and selfish DEGs in honey bees to the DEGs associated with aggression in mostly solitary animals (Figure 2A, Table 2); here I predicted that selfish DEGs of queen honey bees should be more similar to DEGs associated with behaviour in solitary insects.

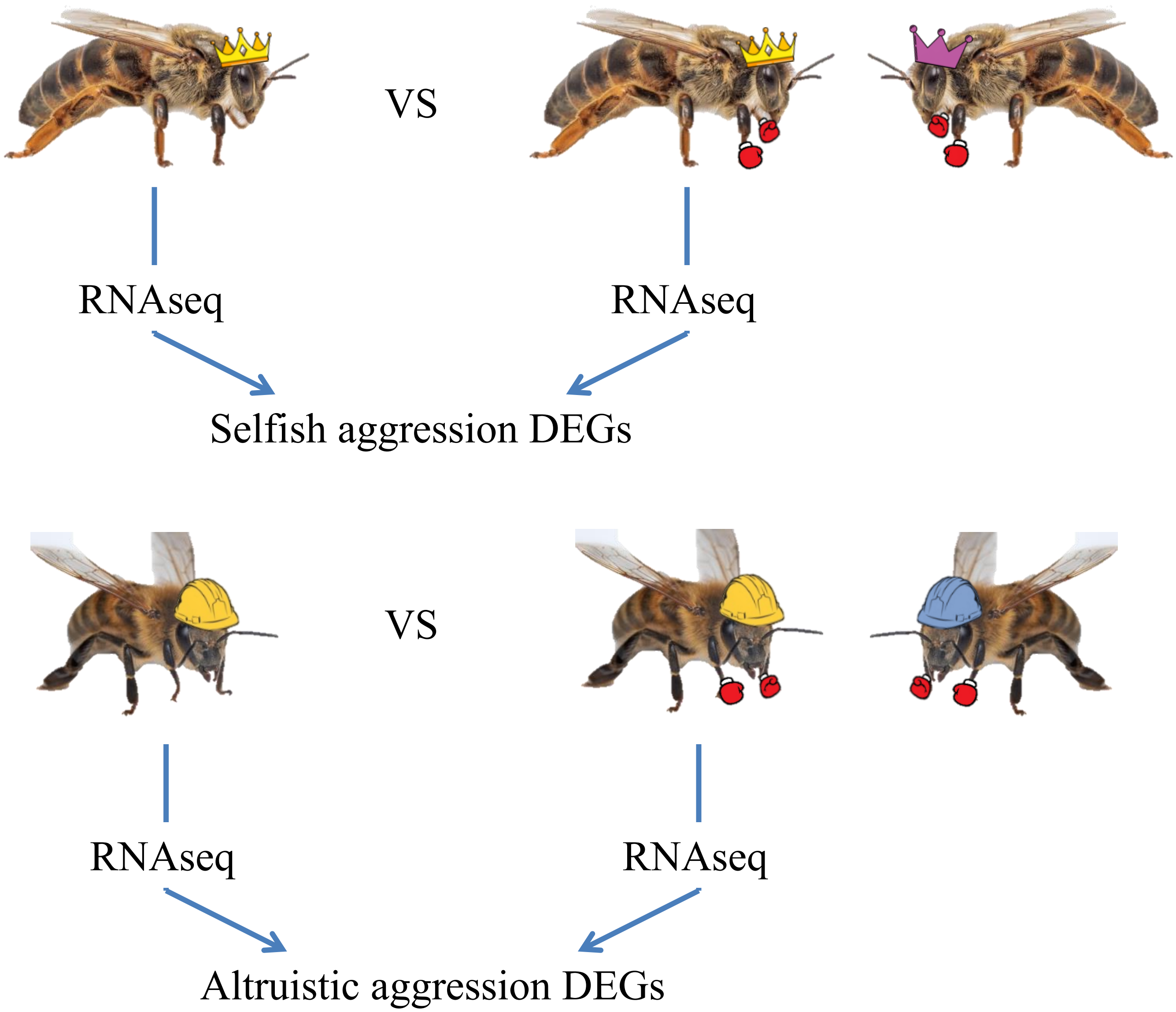

Figure 1: A schematic of my experimental design for how I obtained the differentially expressed genes (DEGs) of selfish queen aggression and altruistic worker aggression to understand their respective neurogenomic states.

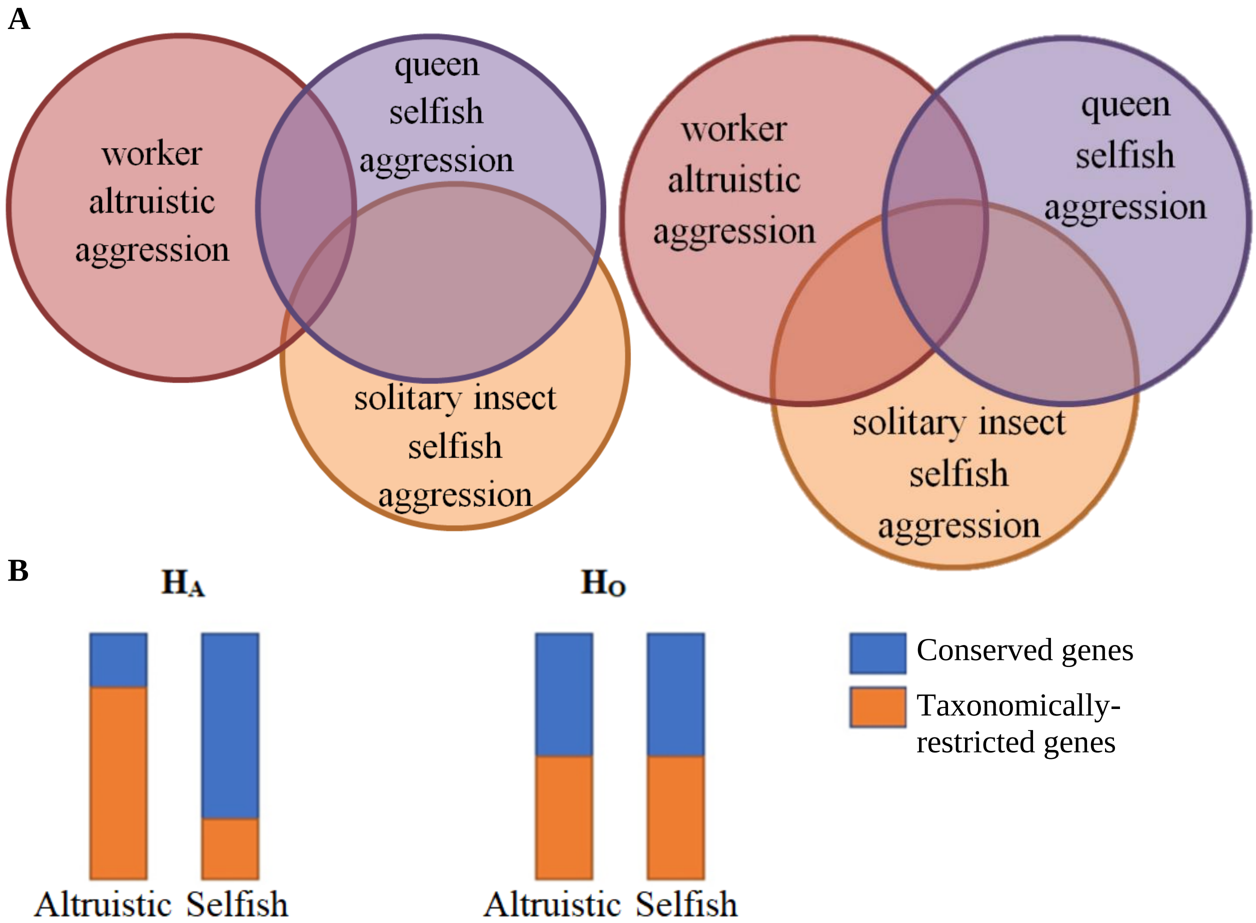

Figure 2: A schematic of hypotheses. If altruistic aggression is unique to eusocial species and has a unique neurogenomic state, it is expected worker altruistic aggression will share fewer genes with solitary insect selfish aggression than queen selfish aggression shares with solitary insect selfish aggression (Panel A, left). If altruistic aggression does not have a unique neurogenomic state, the number of genes shared between worker altruistic, queen selfish, and solitary insect selfish aggression will be non-significantly different from each other (Panel A, right). Panel B depicts the comparison of altruistic DEGs and selfish DEGs in terms of taxonomically-restricted vs conserved genes. If altruistic aggression's neurogenomic state is unique, it will have a higher proportion of taxonomically-restricted genes than selfish aggression.

Methods

Experimental Design – Worker Experiments

In order to explore the genetic components of honey bee worker aggression, I sequenced the brain transcriptomes of aggressive versus control workers to generate a list of differentially expressed genes (DEGs) associated with altruistic aggression. Guard bees were collected from two different rooftop colonies on the York University campus. Guards were identified using established methods (Alaux et al., 2009b; Hunt et al., 2003). Guards are workers at the hive entrance that made antennal contact with and/or lunged at other bees as they landed on the landing board. Once collected, they were transferred to the laboratory and incubated at 4°C for approximately five minutes to minimize movement and allow easy transfer into the petri dishes. Guards from the same colony were then placed in petri dishes, 10 bees per dish, along with two vials, one containing sugar water and the other honey. I placed the petri dishes in a well-ventilated area at room temperature for four hours to allow the bees to acclimate. This time also allowed for any alarm pheromone released during collection, transportation, or chilling to dissipate (Alaux and Robinson, 2007; Clayton, 2000). I used an established protocol known to elicit aggression in workers (Fan et al., 2010; Li-Byarlay et al., 2014; Richard et al., 2012). Briefly, I introduced an intruder, a forager from an unrelated colony, into a petri dish as well as a piece of filter paper saturated with alarm pheromone (2.5μL of isopentyl acetate, IPA, diluted in mineral oil at 1:10). I removed the filter paper after one minute while the intruder remained for one hour (Alaux and Robinson, 2007). I separated the intruder and focal bees using a mesh screen to avoid confounding the neurogenomic signal associated with potential injuries with changes in neurogenomic signal associated with aggression. I also recorded the intruder experiments for the first two minutes, during the one minute of alarm pheromone exposure and the one minute following.

Prior studies introduced the intruder bee for three minutes, removed her, and then allowed one hour to pass for gene expression levels to change prior to flash freezing (Li-Byarlay et al., 2014; Rittschof and Robinson, 2013). For this study, I increased the time the intruder was in the dish to one hour before flash freezing the bees. This increase in exposure time was done to harmonize this protocol with that of the queen duel assays (described below).

Control bees were treated in an identical manner with the exception of having a nest mate introduced rather than an intruder and the filter paper containing only mineral oil (Alaux and Robinson, 2007). The introduction of a nest mate was not expected to elicit an aggressive response. These control conditions provided a baseline gene expression level while also controlling for any variation that may have been caused by the caged conditions (Rittschof and Robinson, 2013).

Of the ten bees in the petri dish, one was collected at random per condition for RNA sequencing. This assay was repeated a total of four times for each of the two colonies, resulting in four aggressive workers and four control workers per colony (16 in total). The brains were dissected for RNAseq extraction.

<u>Experimental Design – Queen Bee Studies</u>

I purchased 10-day-old queen cells from two different mothers from Tuckermore Bee Company in Puslinch, Ontario and placed each cell in a petri dish with the following: wax foundation; 0.6mL each of bee pollen, honey, and sugar water; and three or four newly emerged bees (Jackson and Robinson, 2018). Queen rearing dishes were kept in an incubator at 33°C with

~60% humidity, and no light. Queens from each source mother were divided equally between two incubators. Dueling pairs were comprised of sisters from separate incubators to ensure that the virgin queens did not have any interaction prior to the dueling assay. I created a wooden divider and placed it between the queen rearing dishes to prevent queens in the same incubator from seeing each other.

Two days later, when the queen emerged into the dish, I removed the queen cell and sealed off the hole with lab tape to prevent escape (Jackson and Robinson, 2018). I waited until the queens were four days old to conduct the dueling assays as this is halfway through the week long period when queen dueling happens in the field (Jackson and Robinson, 2018; Winston, 1987).

Queen duels were performed following established protocols (Jackson and Robinson, 2018). Duels were designed to occur between queens from the same mother as queen duels in nature only occur between sisters (Jackson and Robinson, 2018; Winston, 1987). Prior to the beginning of queen duel experiments, virgin queens were removed from their rearing dishes and transferred to 15 mL conical tubes, which acted as a holding area. These tubes were kept out of sight from each other, again to prevent any interaction between virgin queens prior to the dueling assay. Pairs of sister queens were introduced one after the other into the same petri dish arena, on opposite sides of a mesh divider, and allowed to duel for one hour. The mesh divider was used to prevent physical interactions between queens to avoid confounding changes in gene expression caused by behavioural states with those caused by physical interaction and possible injuries. The one hour time frame is the average length of time that queen duels lasted during prior studies (Jackson and Robinson, 2018). This timeframe has been used in previous studies on workers (Alaux et al., 2009a). Queen duels were conducted without workers to reduce variation and possible confounding variables. Current literature suggests that while workers do not directly participate in aggressive acts during queen duels (i.e., workers will not sting either virgin queen), they may play a supporting role that influences the outcome by immobilizing either opponent (Gilley and Tarpy, 2005; Jackson and Robinson, 2018). I thus excluded workers from queen duels as this study was concerned with the brain transcriptome during aggressive states, not the outcome of queen duels *per se*.

Control queens were treated in a similar manner but had a newly-emerged worker from an external colony introduced. Workers from the queen's origin hive were not used as queen grafting uses multiple hives, each with their own set of workers. Therefore, using a newly-emerged worker ensure that the control worker is not aggressive towards the virgin queen. Queen assays, control and duels, were video recorded for the entire hour. After the hour of dueling, both virgin queens were flash frozen on dry ice and then transferred to -80°C.

This assay was conducted a total of eight times for each condition, 16 overall. Two control queen samples were lost; one queen died prior to the dueling assay and the other was lost during dissection. Because virgin queen duels have two queens per assay, both queens in the duel were collected. One queen from each dueling pair was chosen at random to have sample sizes similar to the worker assays. Similar to the intruder assay, the brains of these queens were dissected for RNA extraction.

Behaviour Scoring

I scored recordings of the worker intruder and queen duel experiments for aggressive behaviour using an aggression index for honey bees in caged studies (Table 3). I did this in order to ensure that the bees were in fact displaying aggressive behaviour. Behaviours were scored in

15-second intervals with the most aggressive behaviour being recorded. For workers, scores were summed and averaged across all focal bees in the same dish. This resulted in one score per bee per minute. For queens, scores were summed and averaged for time only, giving a score per minute for each queen; no averaging was done between queens in the same dish. I compared the average aggression score between the control and experimental conditions for each caste separately using an ANOVA in RStudio (RStudioTeam, 2021).

Table 3: Behaviour scoring index used to quantify aggressive behaviour; adapted and modified from (Fan et al., 2010). The behaviour of 'open mandibles' was removed due to it not being detectable in the recordings.

Abbreviation	Description	Score
-	No interaction	0
A	Antennal contact	1
G	Grooming or licking	1
B	Biting	3
F	Biting with gaster flex	4
S	Stinging	5

<u>RNAseq and Analysis of Gene Expression</u>

I freeze-dried the bee heads to aid in brain dissection (60 minutes at -80 °C and 42 Pa). Once freeze-dried, I dissected the brains on dry ice in cold 100% ethanol in order to prevent RNA degradation. I isolated the RNA from brain tissue samples using Mag-Bind® Total RNA Kit. All 30 samples were sequenced in a single NovaSeq flowcell (2x150bp), resulting in an average of 96M reads per sample at Génome Québec's sequencing centre (Montreal, Canada). Worker and queen brain sample sizes, eight and six, respectively, both exceed the minimum sample size of five required for RNAseq experiments (Conesa et al., 2016). In order to remove adapters and low quality bases and reads, I processed the raw data using Trimmomatic (Bolger et al., 2014). I then performed quality control using FastQC and MultiQC (Andrews, 2010; Ewels et al., 2016). I mapped sequences to the most recent honey bee transcriptome assembly (Amel_HAv3.1) (Wallberg et al., 2019) using kallisto (Bray et al., 2016). I matched transcript names to their respective gene name. This information was obtained from the transcript FASTA file. Transcripts that did not meet the filtering criterion (sum of reads across all samples > 1) were removed. This resulted in a reduction from 27 887 to 26 804 transcripts for worker bees and 27 887 to 26 785 transcripts for queens. I transformed the data using rlog to stabilize variance (Love et al., 2014). DEGs were obtained using DEseq2 (Love et al., 2014) in RStudio (RStudioTeam, 2021). Each caste was analyzed separately. The design for DEseq2 accounted for the following variables: colony, treatment, and the interaction between treatment and colony. I used a false discovery rate [FDR; Benjamini-Hochberg method (Benjamini and Hochberg, 2000)] of 0.05 to generate lists of DEGs. I performed a hypergeometric test, one-tailed Fisher's exact test with a hypergeometric distribution (Johnson et al., 2005), to determine if the overlap in DEGs between castes was significant.

Gene Ontology

Apis mellifera genes were converted to their respective *Drosophila melanogaster* homologue using data from the Hymenoptera Genome Database 'HymenopteraMine v1.5' (organism → homologues template) (Walsh et al., 2021), which were then used for Gene Ontology (GO) analysis. Given the relatively small number of differentially expressed genes at FDR < 5% (see results), we decided to carry out the GO analysis using DEGs at FDR < 25%. This provided a larger gene list for exploring the molecular functions and biological processes associated with my DEGs. DAVID GO (Huang et al., 2009b, 2009a) was used to perform these analyses. GO terms with a Benjamini-Hochberg (Benjamini and Hochberg, 2000) FDR of < 0.05 were considered significant.

<u>Orthology of Differentially Expressed Genes</u>

I used OrthoDB (Kriventseva et al., 2019) to classify DEGS as 'taxonomically-restricted' or 'conserved'. Here, I defined taxonomically-restricted genes as those with orthologues only within the Hymenoptera order, while conserved genes are those with orthologues occurring in additional orders (Johnson and Tsutsui, 2011). Genes not found in OrthoDB were removed from the overall count when determining percentages. I used a Fisher's exact test (Fisher, 1992) to determine if the proportion of taxonomically-restricted genes in altruistic workers is significantly higher than that for selfish queens. Conducting this analysis with a more-recent definition of 'taxonomically-restricted' (i.e., bee-specific or honeybee-specific genes) was not possible because the altruistic and selfish gene lists did not contain any *Apis* only genes.

<u>Comparison to Other Research</u>

I compared my worker DEGs to those found in other studies that also measured changes in gene expression in *Apis mellifera* workers (Alaux et al., 2009a; Rittschof et al., 2014). I then compared my worker and queen DEG lists to comparable studies in other, non-eusocial species. In order to make these cross-species comparisons, gene lists from the literature were converted to their honey bee orthologues homologue. Orthologues were identified as the best BLAST result after I performed reciprocal tBLASTx between the species of interest and the honey bee (Figure 3). The species I used for comparison were: *Ceratina calcarata*, subsocial small carpenter bee

(Withee and Rehan, 2017); *Ceratina australensis*, incipiently social Australian small carpenter bee (Steffen and Rehan, 2020), *Mus musculus*, solitary house mouse, and *Gasterosteus aculeatus*, solitary three-spined stickleback fish (Rittschof et al., 2014).

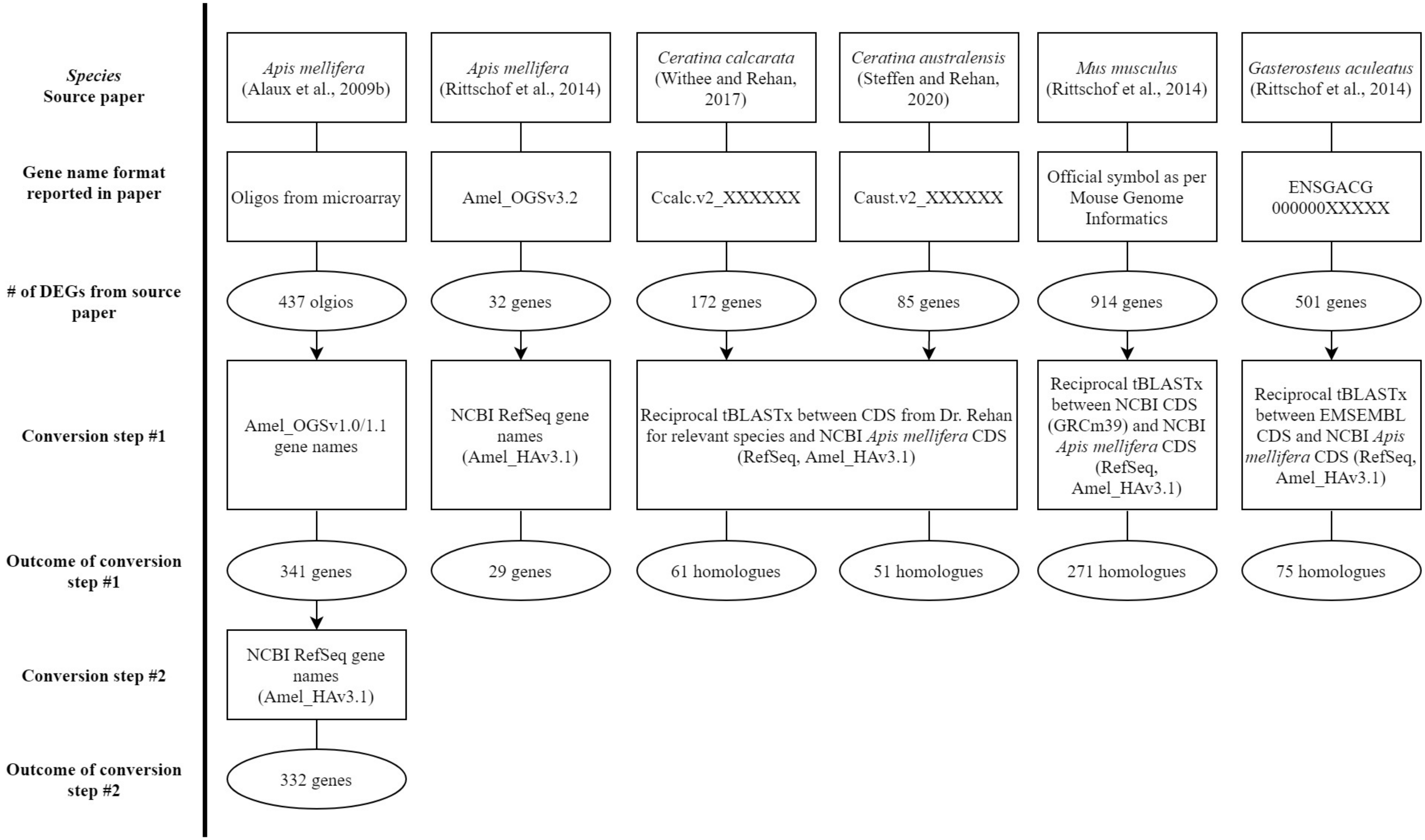

Figure 3: Data processing pipeline for converting DEGs datasets from existing literature to the NCBI's RefSeq gene name, Amel_HAv3.1. For non-honey bee species, homologues were identified as the best BLAST result after reciprocal tBLASTx between species.

Results

Intruder Introduction Leads to Increased Aggression

The introduction of an intruder increased aggression scores in both queens and workers (Figure 4). Workers were significantly more aggressive when exposed to an intruder + alarm pheromone than when exposed to a nest mate + mineral oil ($F(1,14) = 5.094$, $p = 0.0405$). Similarly, virgin queens were more aggressive when exposed to a sister virgin queen than to a newly-emerged worker ($F(1,12) = 12.79$, $p = 0.0038$).

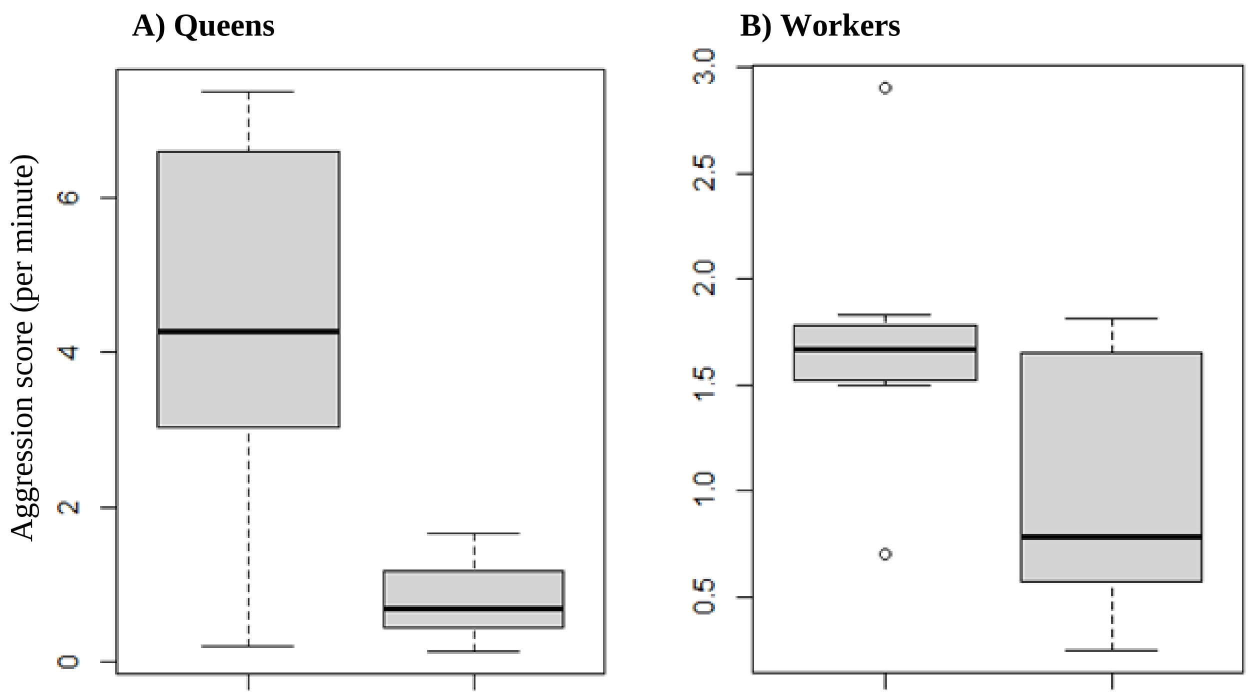

Figure 4: Aggression increased significantly during virgin queen duels and worker intruder assays. Box plots of aggression score per minute for queens ($F(1,12) = 12.79$, $p = 0.0038$) and workers ($F(1,14) = 5.094$, $p = 0.0405$). N.E.W. = newly-emerged worker, AP = alarm pheromone, MO = mineral oil.

Neurogenomic State Differs Between Aggressive Workers and Queens

I carried out analysis of DEGs to identify genes associated with altruistic aggression in workers and selfish aggression in queens. Using a false discovery rate 0.05, I found 93 DEGs when comparing aggressive workers against control workers (herein referred to as altruistic DEGs) and 90 DEGs when comparing aggressive virgin queens to their control (herein referred to as selfish DEGs). I constructed a Venn diagram to visualize the overlap in selfish and altruistic DEGs (Figure 5) which revealed 13 shared genes between selfish and altruistic DEGs. That is, 13 genes are significantly differentially expressed in both aggressive workers and aggressive queens. I then performed a hypergeometric test which determined that the overlap was significant ($p < 0.001$). These 13 overlapping genes, herein called the core aggression genes, are described in Table 4 and Table 7. Of these 13 genes, one gene was upregulated in both castes, five genes were downregulated in both castes, five genes were upregulated in workers but downregulated in queens, and two genes were downregulated in workers but upregulated in queens (Table 4).

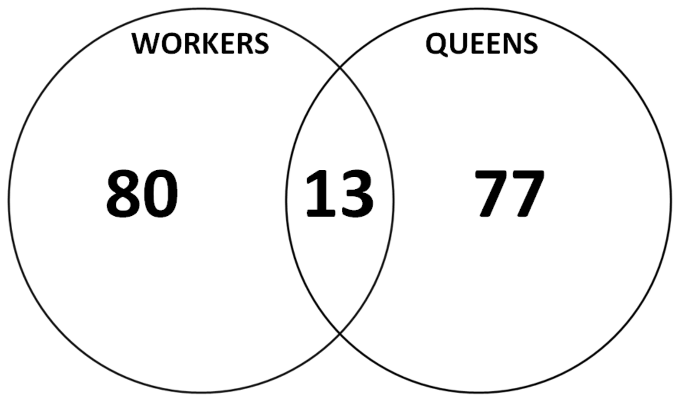

Figure 5: Workers and virgin queens had 80 and 77, respectively, genes differentially expressed in only their caste. 13 genes, called the core aggression genes, were differentially expressed in both castes. We used a FDR-corrected p-value of 0.05 to determine significance. Table 4 described the 13 overlapping core aggression genes.

Table 4: 13 core aggression genes along with their expression direction in aggressive individuals relative to their control.

Gene symbol (*A. mellifera*)	Gene description	Regulated up or down?	
		Workers	Queens
Dscam	Down syndrome cell adhesion molecule	Up	Down
LOC408693	Epidermal growth factor receptor kinase substrate 8	Down	Up
LOC408779	Short stop	Up	Up
LOC408826	Dystroglycan	Down	Down
LOC409697	Filamin-like	Down	Down
LOC409821	Twitchin	Down	Down
LOC410041	Uncharacterized LOC410041	Up	Down
LOC410904	Formin-like protein CG32138	Up	Down
LOC411756	Bromodomain adjacent to zinc finger domain protein 1A	Down	Down
LOC552202	Monocarboxylate transporter 9	Up	Down
LOC724344	Probable Ras gtpase-activating protein	Up	Down
LOC726544	Serine/threonine-protein phosphatase 4 regulatory subunit 1	Down	Down
LOC100577174	Ras-responsive element-binding protein 1	Down	Up

To increase my understanding of the role of the DEGs, I performed a Gene Ontology (GO) analysis with a focus on biological processes (BP) and molecular function (MF). GO analysis was done on the DEG lists at FDR <25% as it provided a large enough gene list to explore the associated molecular functions and biological processes. GO analysis was FDR <5% resulted in no significantly enriched GO terms. Selfish DEGs were enriched for BPs associated with neuron movement and cytoskeleton structures. Four of the 11 enriched terms for selfish DEGs are child terms of the following two parent terms: GO: 0009605 (response to external stimulus) and GO: 0040011 (locomotion). For altruistic DEGs, I found several enriched annotation terms associated with transmembrane transport; 6 of 7 enriched terms fall under GO: 0022857 (transmembrane transporter activity). I then examined the core aggression genes and found no significant enriched GO terms. To further explore the core aggression genes, I examined all associated biological process and molecular function GO terms using DAVID GO's functional annotation table (Table 7). I found several annotation terms associated with neurobiology and behaviour among the GO terms. Of note is LOC724344 as its *Drosophila* homologue, *CG42684* (also referred to as *CG42270*), has been previously implicated in aggressive behaviour (Edwards et al., 2009; Shorter, 2015).

Genes Underlying Altruistic Aggression Are Not More Derived Relative to Selfish Aggression

To test if altruistic aggression is more derived at the gene level, I classified selfish and altruistic DEGs as taxonomically-restricted or conserved. Here, I defined taxonomically-restricted genes as those that have orthologues found only within the Hymenoptera order. Over 85% of genes for both castes are found across the eukaryote domain. While queens were found to have a slightly higher proportion of Hymenoptera specific genes (Figure 6), this difference was not statistically significant, Fisher's exact test, p = 0.37.

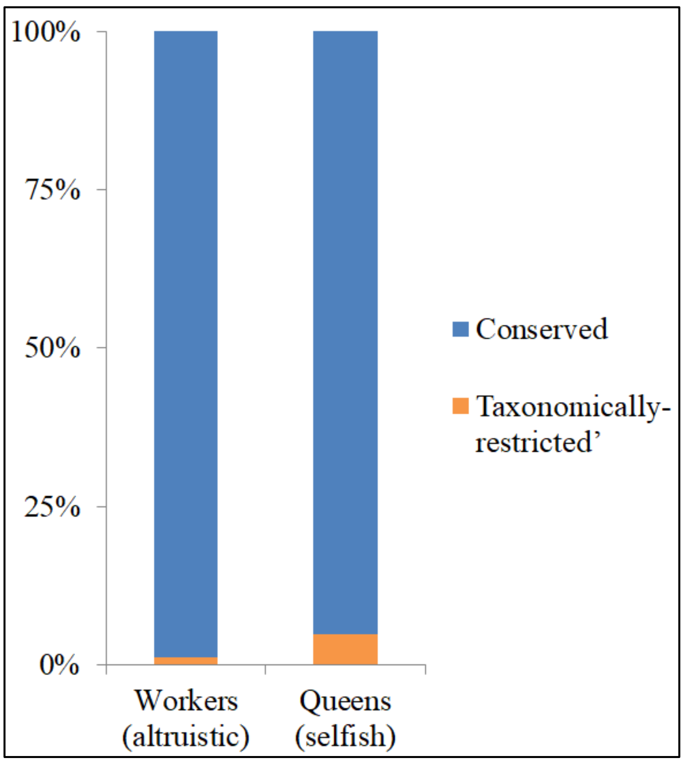

Figure 6: Altruistic aggression is not more derived at the gene level, p = 0.37. Taxonomically-restricted genes are those found only within the Hymenoptera order whereas conserved are genes found in other orders as well.

Honey Bee Aggression Genes Are Not Also Differentially Expressed in Other Aggressive Species

I compared the honey bee aggression DEGs identified here to aggression DEGs identified in other species along the solitary-eusocial spectrum (Table 2) to test if altruistic aggression is more derived at the species level. The species used for comparison were: *Ceratina calcarata*, subsocial small carpenter bee (Withee and Rehan, 2017); *Ceratina australensis,* incipiently social Australian small carpenter bee (Steffen and Rehan, 2020), *Mus musculus,* solitary house mouse, and *Gasterosteus aculeatus,* solitary three-spined stickleback fish (Rittschof et al., 2014). No significant overlap between the altruistic DEGs and selfish DEGs were found relative to other studies that examined the neurogenomic state associated with aggression in other solitary, subsocial, and incipiently social (Figure 7) (Hypergeometric test, $p > 0.05$ for all comparisons).

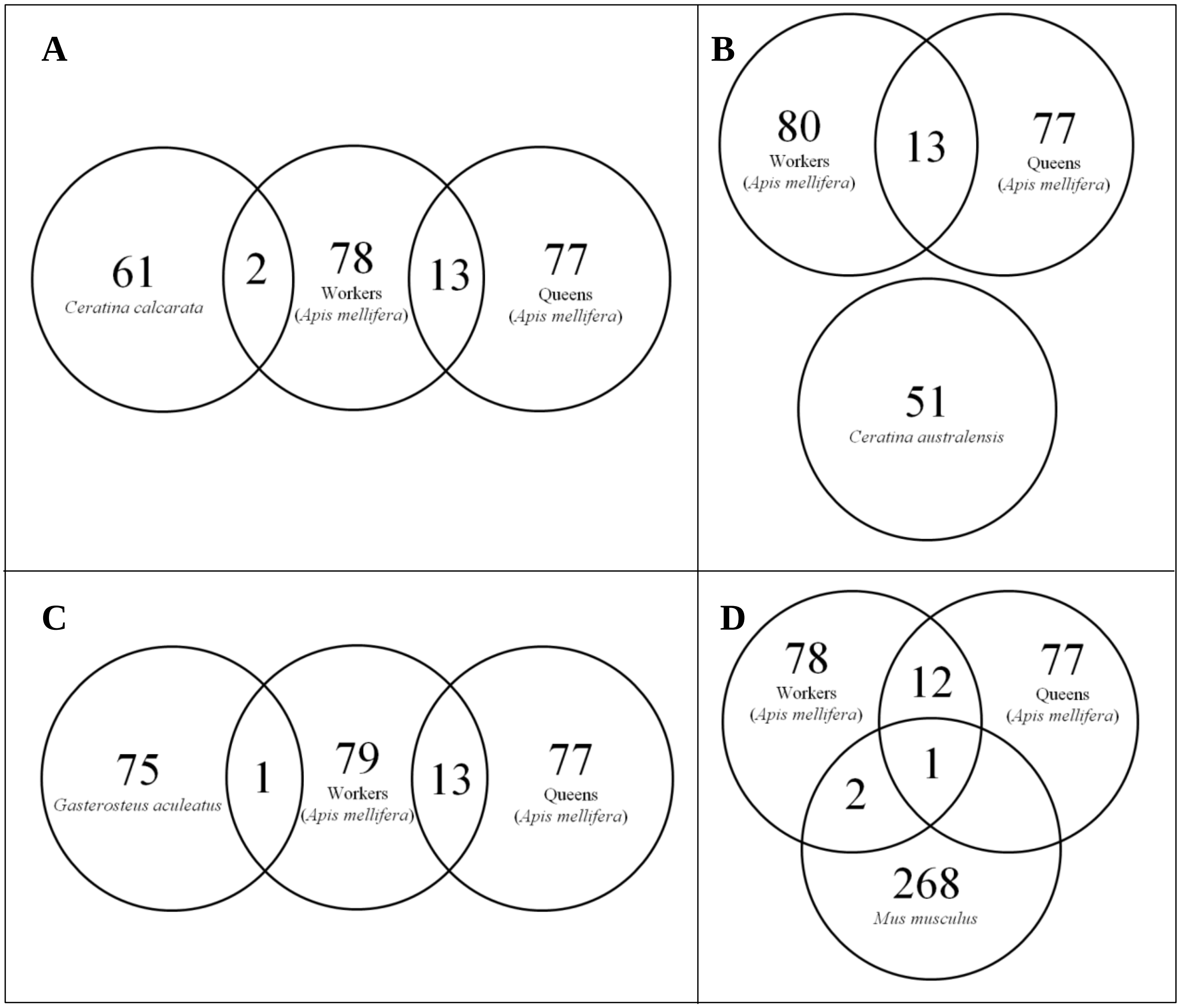

Figure 7: Aggressive honey bees do not share a significant number of differentially expressed genes with other aggressive species. *C. calcarata* is subsocial (panel A), *C. australensis* is incipiently social (panel B), *G. aculeatus* is solitary (panel C), and *Mus musculus* is solitary as well (panel D). Hypergeometric test; *C. calcarata* VS workers = 0.17, *C. calcarata* VS queens = 1; *C. australenesis* VS workers = 1, *C. australenesis* VS queens = 1; *G. aculeatus* VS workers = 0.60, *G. aculeatus* VS queens = 1; *M. musculus* VS workers = 0.63, *M. musculus* VS queens = 0.60.

Discussion

Intruder Experiments Lead to Aggression

My experimental manipulation was successful in eliciting aggressive behaviours in workers and queens (Figure 4). It is worth noting that worker and queen aggression levels cannot be scored using the same aggression index as they each have a unique repertoire of behaviours for their respective outcome of aggression (Butz and Dietz, 1994; Gilley, 2001; Gilley and Tarpy, 2005; Nouvian et al., 2016). My analysis here was only meant to confirm if my manipulations elicited aggression in workers and queens, not to directly compare aggression in queens versus workers.

Neurogenomic State Differs Between Aggressive Workers and Queens

Both castes have a similar number of differentially expressed genes, but they differ in enriched gene ontology terms. Workers have 93 differentially expressed genes while virgin queens have 90 differentially expressed genes (Figure 5). I found that altruistic DEGs were enriched for GO terms related to transmembrane transport (a molecular function) whereas selfish DEGs were enriched for GO terms related to cytoskeleton and neural response to stimuli, both biological processes (Table 5 and Table 6).

Table 5: Enriched GO terms for queen differentially expressed genes, FDR ≤ 0.05. If multiple terms within the same cluster had the same GO ID, the most general term was used. Terms with an asterisk are child terms to GO: 0009605 (response to external stimulus) and GO: 0040011 (locomotion). BP = biological process, MF = molecular function.

GO term	GO category	GO ID	# of genes	Benjamini FDR
Taxis*	BP	GO: 0042330	15	0.015
Cytoskeleton protein binding	MF	GO: 0008092	13	0.035
Anatomical structure morphogenesis	BP	GO: 0009653	35	0.041
Axon guidance*	BP	GO: 0007411	12	0.041
Neuron projection guidance*	BP	GO: 0097485	12	0.041
Axonogenesis	BP	GO: 0007409	13	0.041
Chemotaxis*	BP	GO: 0006935	12	0.041
Axon development	BP	GO: 0061564	13	0.041
Actin cytoskeleton organization	BP	GO: 0030036	12	0.041
Actin filament-based process	BP	GO: 0030029	12	0.041
Morphogenesis of an epithelium	BP	GO: 0002009	19	0.049

Table 6: Enriched GO terms for worker differentially expressed genes, FDR ≤ 0.05. If multiple terms within the same cluster had the same GO ID, the most general term was used. BP = biological process, MF = molecular function.

GO term	GO category	GO ID	# of genes	Benjamini FDR
Ion transmembrane transporter activity	MF	GO: 0015075	15	0.0027
Transmembrane transporter activity	MF	GO: 0022857	16	0.013
Ion channel activity	MF	GO: 0005216	9	0.013
Channel activity	MF	GO: 0015267	9	0.013
Passive transmembrane transporter activity	MF	GO: 0022803	9	0.013
Gated channel activity	MF	GO: 0022836	7	0.018
Regulation of biological quality	BP	GO: 0065008	30	0.021

When I searched the DEG lists for genes related to neurotransmitters, we found that 5-HT1, a serotonin receptor, was significantly downregulated in aggressive workers (FDR = 0.03, log2FC = -22.92). In non-social invertebrates, serotonin has been repeatedly shown to have a positive correlation with aggression (Kravitz and Huber, 2003). A study in honeybees, however, found that when workers were injected with a serotonin antagonist, effectively lowering the number of available and functioning receptors, they required a lower voltage shock to extend their stinger (Tedjakumala et al., 2014). In other words, workers with fewer functioning serotonin receptors are more likely to sting as their stinging threshold is lowered (Nouvian et al., 2016). My finding that aggressive workers had significantly fewer transcripts for a 5-HT1 receptor are thus consistent with Tedjakumala et al.'s findings. Interestingly, 5-HT1's *D. melanogaster* homologue, 5-HT1A, is associated with inter-male aggression (GO: 0002121). No serotonin related genes were significantly differentially expressed in queens.

I then shifted my focus to the genes that were differentially expressed in both castes; I called these the core aggression genes as they are associated with aggression regardless of implicit motivation. Thirteen genes are differentially expressed in both workers and virgin queens; six of which are expressed in the same direction in both castes, the remaining seven expressed in opposite directions (Table 4). These 13 genes account for approximately 14% of the DEGs of each caste.

LOC724344 stood out for its role its role in aggression. Mutations in its *Drosophila* ortholog, *CG42684* or *raskol,* have been implicated in aggressive behaviour in fruit flies (Edwards et al., 2009; Shorter, 2015). I conducted a Flybase search of the remaining 12 core aggression genes' fruit fly homologues to examine their function and relationship to behaviour

(Larkin et al., 2021). This search suggested that my core aggression genes are all known to be important for regulation of behaviour and neurobiology. For example, *Dscam1*, Down syndrome cell adhesion molecule 1, encodes a transmembrane immunoglobulin that regulates axon branching and guidance. *aru*, arouser, codes a SH3 adaptor protein that regulates memory formation and ethanol response. *shot*, short stop, encodes a cytoskeletal linker molecule that binds signalling factors to develop and maintain the nervous system. *Dg*, Dystroglycan, codes for a non-integrin extracellular matrix receptor that regulates animal survival and temperature preferences. *cher*, cheerio, encodes filamin, a protein that organize the F-actin cytoskeleton in migrating somatic cells and neuronal growth cones (Brody, 1999). In humans, mutations in the filamin protein cause periventricular heterotopia, a disease in which neurons fail to migrate properly during early fetal brain development (Reinstein et al., 2012). *Sqa*, spaghetti-squash activator, codes a myosin light chain kinase-like protein, a substrate for a kinase required for starvation-induced autophagy (Tang et al., 2011). *SP1173* is predicted to be active in the membrane; research into this gene is not yet substantial. *Frl*, Formin-like, codes for a protein that works with Cdc42 in eye development and is thus important for sensory perception. It can also work with Cdc42 and DAAM in neuronal development of the mushroom body. *Acf*, ATP-dependent chromatin assembly factor large subunit, plays a role in chromatin remodelling and nucleosome sliding. *CG*14196 encodes a protein that is predicted to be involved in monocarboxylic acid transmembrane transport, but not enough research has yet been done on this gene and its products to form a definitive link to neurobiology and behaviour. *peb*, pebbled, plays a role in retinal and respiratory system development. Honey bee gene *LOC726544*, serine/threonin-protein phosphatase 4 regulatory subunit 1, does not have a fruit fly homologue.

I examined the GO annotation terms associated with my core aggression genes, and discovered that most had annotation terms associated with neurobiology and behaviour (Table 7). For example, GO terms containing ‘axon’ and its variations appear 12 times among six core aggression genes. GO terms containing ‘neuron’ or ‘dendrite’ each appear four times among four and three genes, respectively. Axonogenesis is the *de novo* generation of the axon portion on a neuron (Binns et al., 2009). Genes associated with the axonogenesis GO term (GO: 0007409) have been found to be differentially expressed in other species, such as *Drosophila melanogaster* (Edwards et al., 2006), *Polistes metricus* (Toth et al., 2014) and *Ceratina calcarata* (Withee and Rehan, 2017), during aggressive acts. Of interest was Withee and Rehan’s (2017) finding that several genes overexpressed in repeat winners, those displaying more aggressive behaviours, in *C. calcarata* have axonogenesis as a putative gene function.

Table 7: Core aggression genes have annotations associated with neurobiology and behaviour (Huang et al., 2009b, 2009a).

(*A. mellifera*)	(*D. melanogaster*)		Biological process	Molecular function
Gene symbol	Gene Symbol	Gene description		
Dscam	Dscam1	Down syndrome cell adhesion molecule 1	Axon guidance, axonal fasciculation, neuron development, regulation of dendrite morphogenesis, axon extension involved in axon guidance, regulation of axonogenesis, dendrite self-avoidance	Calcium ion binding, protein binding, structural constituent of muscle
LOC408693	Aru	arouser	Olfactory behavior, response to ethanol, regulation of synapse structure or activity	Structural constituent of ribosome
LOC408779	shot	short stop	Axonogenesis, regulation of axon extension, dendrite morphogenesis, axon midline choice point recognition, positive regulation of axon extension	Actin binding, calcium ion binding, protein binding, microtubule binding, cytoskeletal protein binding
LOC408826	Dg	Dystroglycan	Regulation of glycolytic process, axon guidance, regulation of synaptic activity	Calcium ion binding, protein binding, structural constituent of muscle
LOC409697	cher	cheerio	Motor neuron axon guidance, behavioural response to ethanol	Actin binding, actin filament binding
LOC409821	Sqa	spaghetti-squash activator	Regulation of glucose metabolic process	Protein kinase activity, protein serine/threonine kinase activity, myosin light chain kinase activity, ATP binding

LOC410041	SP1173	CG10121 gene product from transcript CG10121-RC	n/a	n/a
LOC410904	Frl (CG32138)	CG32138 gene product from transcript CG32138-RC	Axon extension, neuron projection morphogenesis	Actin binding, Rho GTPase binding
LOC411756	Acf	ATP-dependent chromatin assembly factor large subunit	Sensory perception of pain, neuron development, dendrite morphogenesis	DNA binding, histone acetyltransferase activity, protein binding, zinc ion binding, transferase activity, transferring acyl groups
LOC552202	CG14196	CG14196 gene product from transcript CG14196-RA	Transmembrane transport	Monocarboxylic acid transmembrane transporter activity
LOC724344	raskol/CG42684	CG42684 gene product from transcript CG42684-RB	Inter-male aggressive behaviour	n/a
LOC726544	n/a	n/a	n/a	n/a
LOC100577174	peb	pebbled	Photoreceptor cell axon guidance	Nucleic acid binding, transcription factor activity, sequence-specific DNA binding, transcription regulatory region DNA binding, metal ion binding

Comparison to Other Honey Bee Aggression DEGs

I compared altruistic DEGs to aggression associated genes identified in a previous study examined the neuromolecular response to a territory-intruding conspecific, which should induce aggression (Rittschof et al., 2014). Rittschof et al. (2014) exposed their study organism (a mouse, stickleback fish, or a honey bee worker) to an intruder and used RNAseq to measure the change in gene expression that occurred in response to this social threat. This study was done on whole brain samples, the same tissue type used in this study.

Of initial interest was the possible relationship between Rittschof et al.'s honey bee gene list and my altruistic DEGs. All genes determined to be differentially expressed in honey bee workers in Rittschof et al.'s study were found to be present in my RNAseq dataset, two of which, *venom carboxylesterase-6-like* (LOC408395) and *troponin I* (LOC410815), were differentially expressed in both studies. This overlap was not significant (one-tailed Fisher's exact test with a hypergeometric distribution, $p = 0.39$). Differences in methodology, at least in part, can explain nonsignificant overlap of DEGs between my study and Rittschof et al.'s study. Rittschof et al. also used a similar intruder assay with 10 resident bee workers and a forager from an unrelated colony, but the length of exposure differed substantially between the two studies. Rittschof et al. introduced the intruder or control for 5 minutes, removed it, waited 25 minutes and then flash froze the residents for RNAseq. No alarm pheromone exposure was used. In my study, the introduced bee remained in the petri dish for one full hour, doubling the total time in which brain gene expression levels could change in response to the intruder. The change in gene express that occurs in response to alarm pheromone increases for one hour and then levels off (Alaux et al., 2009a; Breed et al., 2004). Rittschof et al.'s protocol used 30 minutes as this type of social challenge was new and 30 minutes is best practice for stickleback fish, a comparator species in their study. Therefore, it is possible that the difference in intruder exposure time between the two experiments led to this insignificant overlap between worker aggression DEGs.

The worker-intruder experiments in this study used a 'natural' setup to elicit aggression in honey bee workers by exposing workers to an intruder (i.e. non-nestmate) along with alarm pheromone. To investigate if the worker aggression DEGs we identified were mostly driven by exposure to alarm pheromone alone, I compared the DEGs to those from Alaux et al. (2009b) who identified DEGs associated with the alarm pheromone exposure in honey bee workers. Alaux et al. presented a filter paper soaked in alarm pheromone (2.5µL diluted in mineral oil at a 1:10 ratio) at an outdoor hive entrance for 60 seconds, collected the most-aroused workers, caged them for 60 minutes and then flash froze them for gene expression analysis. Brain gene expression of these aroused bees were compared to that of nestmates at the hive entrance prior to presenting the pheromone to identify DEGs associated with alarm pheromone exposure. I compared the list of altruistic DEGs to Alaux et al.'s AP DEGs and found no significant overlap (Hypergeometric test, p = 0.91). These two studies share only two DEGs: *afadin* (LOC410161) and *mothers against decapentaplegic homolog 4* (LOC409321). This suggests that the altruistic DEGs cannot be attributed solely to alarm pheromone exposure.

Finally, I examined the overlap between Alaux et al.'s (2009a) and Rittschof et al.'s study (2014). Five genes were differentially expressed in both studies (Table 8*); alanine--glyoxylate aminotransferase 2-like* (LOC408817), *zinc finger protein 182* (LOC100576293*), long wavelength sensitive opsin 2* (LOC768250), *apidermin 1* (LOC551367), *actin clone 205* (LOC551176), and *uncharacterized LOC724644*. This overlap is not significant (hypergeometric test, p = 0.34). Both of these studies, as well as the current study, used different methodology to induce aggression in honey bees. Therefore, the non-significant overlap between all three studies suggests that the neurogenomic changes associated with aggression are context dependent.

Table 8: No genes are differentially expressed across workers in all three honey bee aggression studies.

Gene symbol	Gene description	Differentially expressed in study?		
		My study	Alaux et al. 2009	Rittschof et al. 2014
LOC410161	Afadin	Yes	Yes	-
LOC409321	mothers against decapentaplegic homolog 4	Yes	Yes	-
LOC408395	venom carboxylesterase-6-like	Yes	-	Yes
LOC410815	troponin I	Yes	-	Yes
LOC408817	alanine--glyoxylate aminotransferase 2-like	-	Yes	Yes
LOC100576293	zinc finger protein 182	-	Yes	Yes
LOC768250	long wavelength sensitive opsin 2	-	Yes	Yes
LOC551367	apidermin 1	-	Yes	Yes
LOC551176	actin, clone 205	-	Yes	Yes
LOC724644	Uncharacterized LOC724644	-	Yes	Yes

Genes Underlying Aggression, Selfish or Altruistic, are not Taxonomically-Restricted

In 2011, Johnson and Tsutsui classified approximately 700 of the 10 700 honey bee genes as taxonomically restricted (Johnson and Tsutsui, 2011). They classified genes as taxonomically restricted if they fell into one of the following categories: orphan (182 genes), Hymenoptera (69 genes), social insect (103 genes), Hymenoptera-conserved (133 genes), or insect specific (209 genes). Orphans are those with no homologue in any species, Hymenoptera genes have at least one homologue in another Hymenopteran species but no others, social insect genes have homologues in the three other social insect species used in their study but no others, Hymenoptera-conserved are those with homologues in all Hymenoptera species but no others, and insect specific are genes with homologues in all insect genomes but none in any non-insect species (Johnson and Tsutsui, 2011). They suggested that unique genes give rise to unique behaviours and phenotypes found in the eusocial honey bee. This was supported by the finding that orphan genes were overrepresented and overexpressed among worker bees, who display a number of behaviours unique to eusociality.

Based on Johnson and Tsustui's work, I hypothesized that if altruistic aggression is a novel behaviour unique to eusociality, then the genes that are differentially expressed during altruistic aggression should have a higher proportion of taxonomically restricted genes than the genes differentially expressed in virgin queens displaying selfish aggression (Figure 2B). For the purposes of this study, I defined taxonomically restricted genes as honey bee genes with orthologues [as defined by OrthoDB (Kriventseva et al., 2019)] found only within Hymenoptera. When the altruistic and selfish DEGs were categorized, both castes had a low percentage of their total DEGs classified as taxonomically restricted: 1.22% and 4.76%, respectively (Figure 6). The difference in taxonomically-restricted genes between selfish and altruistic DEGs was not statistically significant ($p = 0.37$). This means that altruistic aggression does not have more taxonomically-restricted genes than selfish aggression and is therefore not more derived at the gene level.

It should be noted that Johnson and Tsutsui used OGSv2 which was assembled circa 2006 (Consortium, 2006), whereas I used Amel_HAv3.1, assembled 13 years later in 2019 (Wallberg et al., 2019). OGSv2 had missing sequences and incorrect gene orientations that were vastly improved in the latest honey bee assembly. Amel_HAv3.1 not only corrected these orientation errors but also contains more genes than OGSv2, therefore providing me with additional information to determine which honey bee genes are taxonomically restricted or not. Nevertheless, it is important to note that perhaps the size of the altruistic and selfish DEGs was a factor in this analysis. Orphan genes, *Apis*-specific and bee-specific genes are rare, and my DEGs contained almost no genes in these categories. The most exclusive group of taxonomically-restricted genes I found in my DEGs was Hymenoptera-specific. It can be argued that this classification is not exclusive enough to test for the involvement of taxonomically-restricted genes in honey bee worker aggression because the self-sacrificing aggression of honey bee workers is a derived feature within the genus (Johnson and Tsutsui, 2011) and therefore it may be logical to assume that only *Apis*-specific genes should show enrichment within the altruistic DEG list. My gene expression experiments are obviously not appropriate for identifying the 'genetics' of workers aggression – genes and DNA sequences that are causal influences on worker aggression. As such, it may be possible, and probable, that *Apis*-specific genes are still involved in the genetic architecture of worker aggression in honey bees, but that they are upstream of the plastic transcriptional changes we observed in worker brains when exposed to an intruder. Nevertheless, the results suggest that plastic changes in worker neurogenomic states associated with short term shifts in aggression are associated with conserved genes.

Genes Underlying Altruistic Aggression are not More Derived Relative to Selfish Aggression

The previous section tested my hypothesis that altruistic aggression is 'neurogenomically' derived by examining if altruistic and selfish DEGs differ with respect to this origin within the insect phylogeny. I also tested this hypothesis using a different approach by comparing altruistic and selfish DEGs with the aggression DEGs identified in other non-eusocial species. If altruistic aggression is novel and derived, the aggression DEGs of non-eusocial species should have minimal overlap with the altruistic DEGs from workers and instead should overlap more with selfish DEGs from queens (Figure 2A). This is based on assumption that solitary insects and queen bees are aggressive for selfish reasons and that altruistic aggression is more derived.

The ideal comparator gene list would have been *Drosophila melanogaster* as it is a solitary insect with a large repertoire of aggression-related research. Unfortunately, I could not find a suitable comparable transcriptomic study on brain tissues of aggressive and control flies. Therefore, I compared my DEGs to several studies that have used RNAseq to quantify the neurogenomic state associated with aggression in several solitary and subsocial species, including: *Ceratina calcarata*, subsocial small carpenter bee (Withee and Rehan, 2017); *Ceratina australensis,* incipiently social Australian small carpenter bee (Steffen and Rehan, 2020), *Mus musculus*, solitary house mouse, and *Gasterosteus aculeatus,* solitary three-spined stickleback fish (Rittschof et al., 2014).

I found minimal but not statistically significant overlaps between my DEGs and these four species (Figure 7). *Ceratina calcarata* share two genes with workers, none with queens. *Ceratina australensis* shares no genes with either workers or queens. Stickleback fish share one gene with workers but none with queens. Mice share three genes with workers, one of which is a core aggression gene, and no genes with queens. Hypergeometric tests for all 8 comparisons were not significant. The small overlap between both carpenter bee studies and my work can be attributed, in part, to differences in biology. Both *Ceratina* species and *Apis mellifera* deviate from each other in terms of their nesting biology, colony size, social environment, as well as the evolutionary origin of their social behaviour (Rehan and Richards, 2010; Wenseleersa and Van

Zweden, 2017). Any, or all, of these biological differences may explain the small overlap. Overall, the hypothesis that solitary or non-eusocial species share more of their aggression DEGs with virgin queens was not supported.

The finding of the 13 core aggression genes suggests an underlying set of genes that are required for aggressive behaviour in both queen and worker honey bees (Figure 7). My overarching hypothesis that the neurogenomic state of altruistic aggression, as displayed by worker honey bees, is more evolutionary derived than that of selfish aggression, as displayed by virgin queen bees, was not supported by my findings in this study (Johnson and Tsutsui, 2011) (Figure 6). The lack of taxonomically-restricted genes among the altruistic DEGs as well as the lack of overlap in DEGs between altruistic, selfish, and solitary aggression DEGs suggest that the neurotranscriptomics of queen honey bees are too different from that of their worker counterparts and therefore are not the ideal comparator for understanding the neurogenomic state involved in altruistic aggression (Evans and Wheeler, 1999; Grozinger et al., 2007). Repeating this experiment in a primitively eusocial species could provide interesting results as individuals from such societies are capable of displaying both selfish and altruistic behaviours based on the situation. Additionally, while our study is helpful for highlighting gene expression patterns correlated with aggression, we can not directly infer causal associations between gene expression and behaviour (e.g. changes in gene expression that cause aggression vs. aggression / aggressive encounters leading to changes in gene expression). Follow up RNA interference (RNAi) experiments can be used to establish a causal connection between genes and behaviour (Walton et al., 2020). For example, RNAi can be used to knock down *LOC408779* (short stop) which was found to be up-regulated in both workers and queens. If there is a causal relationship between *short stop* and increased aggressive behaviours, then bees with reduced *short stop* expression should show a decrease in aggressive behaviours when exposed to an intruder or sister virgin queen

Conclusion

My study was successful in inducing aggressive behaviours in guard and virgin queen honey bees. RNAseq analysis of the brain of these aggressive bees revealed a number of genes that were differentially expressed in aggressive workers and/or aggressive queens. Hypothesis testing revealed that while altruistic DEGs of worker honey bees are relatively unique when compared to the selfish DEGs of virgin queen bees, they are not more unique than the selfish DEGs when compared to a third, non-eusocial insect. Aggressive worker honey bees do not display a neurogenomic state that is more evolutionarily derived than that of aggressive virgin queens. I also identified a core set of differentially expressed genes associated with both altruistic and selfish aggression; these genes may be helpful in understanding the molecular biology of aggression in this species. By examining the relationship between differences in implicit motivation and differential gene expression, this study helps us better understand aggression the molecular and evolutionary level, thereby advancing the fields of genetics, behaviour, and evolution.

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