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General life history theory suggests a trade-off between somatic maintenance and reproduction. However, in the honey bee, and other social insects, reproduction increases lifespan. The mechanisms for this positive relation between antagonistic demands are unclear but may be related to vitellogenin (Vg), a reproductive protein that has adopted other important survival functions, such as oxidative stress resistance and immunity. To study the role of Vg in survival functions, the susceptibility of reproductive and non-reproductive honey bee workers to Israeli Acute Paralysis Virus (IAPV) and pesticide-related oxidative stress was compared. Workers in the absence of a queen exhibited more active ovaries than workers in the presence of a queen at older (25d) but not younger (15d) ages. Survival measures of paraquat stress and IAPV infections, complemented with an assessment of gene expression patterns, indicated not only the predicted changes in survival and Vg titers but also correlated alterations in the differential expression of other functional domains: Toll-6 and Argonaute-2. These results support the relevance of non-reproductive functions of Vg in worker bee defense against stressors, demonstrating that social manipulations can alter worker physiology and improve resistance to viral and pesticide stressors of queenless workers. Data from this study has not only interesting implications for honey bee immunity and health but also understanding honey bee caste differences.

Keywords: Honey bee, queenless workers, vitellogenin, stress resistance, caste determination, immunity, health

INCREASED STRESS RESISTANCE IN SOCIALLY MANIPULATED HONEY BEE (*APIS MELLIFERA*) WORKERS

by

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CHAPTER I

INTRODUCTION

The disposable theory of aging suggests a trade-off between somatic maintenance and reproduction. However, in the case of the honey bee, reproduction increases lifespan. Although vitellogenin (Vg) is best known as a reproductive protein, it has been shown to be a multifaceted protein in the honey bee. Studies have shown Vg as a key component in increasing resistance to oxidative stress. There are implications for Vg having influence on immunity by increasing immune activity. However, the role of Vg in oxidative stress and immune activity has not been established in reproductive workers. I predict that workers in a queenless colony will undergo reproductive activation. The reproductive activation in honey bee workers will result in a greater production of Vg. Consequently, honey bee workers under queenless conditions will exhibit a greater resistance to induced stress. My goal is to confirm that social conditions regulate the expression of Vg by establishing queenless and queenright colonies. Secondly, I seek to relate the differential expression of Vg to the stress resistance of workers under these social conditions by inducing oxidative stress through paraquat injections and viral infection through inoculation with Israeli Acute Paralysis Virus (IAPV).

Importance of Honey Bees

Honey bees are thought to be one of the most similar insects to vertebrates. They share a similar circadian rhythm, RNA interference, DNA methylation, and more (Whitfield, Behura et al. 2006, Zemach, McDaniel et al. 2010). Based on their biological characteristics, honey bees have contributed to a variety of research topics, including but not limited to: learning and memory, division of labor, caste differentiation, and immunity. Honey bees have contributed to our understanding of genetic regulation of behavior, mental disorders such as Alzheimer's disease, and aging (Münch and Amdam 2010). Honey bees have also evolved complex social behavior, dance communication, and mutualistic interactions with flowering plants (Rehan and Toth 2015). Most research to date has been performed on honey bee workers, because they exhibit the highest behavioral complexity and constitute the majority of the colony make-up. Workers have to maintain their cognitive ability to exchange information, learn, make decisions, and navigate. Further, honey bee workers are relatively easy to rear and can be used to answer both basic and applied research questions. The knowledge gained from such studies contributes to understanding basic biological process, beekeeping practices, and ecosystem functioning.

The honey bee, *Apis mellifera*, is an essential organism to many agro-ecosystems, largely through pollination. It is estimated that a single honey bee can visit approximately 2,000 flowers per day (Dafni 1992). Nearly $15 billion dollars annually is contributed to the United States economy through pollination services (Calderone 2012). Their pollination services are required for vegetables, fruits, and nuts, especially almonds.

California's almond industry relies almost exclusively on honey bee pollination. Nearly 800,000 acres of almond groves require the annual services of 1.5 million hives (Aizen, Garibaldi et al. 2009).

Honey bees have received recent attention in many media outlets due to their continuing health problems. Honey bees and other pollinators are declining in health, and beekeepers across the world are suffering from colony losses (Lee, Steinhauer et al. 2015). Colony collapse disorder (CCD) has caught the interests of scientists across the world who are trying to explain the increased colony losses. However, CCD is defined by specific symptoms and is now considered just one of the reasons for overall colony losses. Some studies indicate that the health decline is the result of multiple factors such as parasites like mites, infections such as viruses and bacteria, availability and quality of food sources, climate and weather, pesticides, and beekeeping practices (Neumann and Carreck 2010).

Colony Organization and Population Dynamics

Social insects are highly evolved and typically live in large groups or colonies. Social insects include ants, wasps, termites, bees, and some other, minor taxonomic groups. The colonies of these social species have three distinct characteristics: overlapping generations, cooperative brood care, and division of labor. A typical honey bee colony consists of a queen bee, thousands of female workers, and a few hundred male drones (Lindauer and Watkin 1953). Each caste is responsible for various hive tasks that contribute to the overall health, maintenance, and functioning of the colony (Huang and Robinson 1996). This normal hive make-up is referred to as a queenright colony.

Honey bee colonies display functional specialization among the colony members, which leads to division of labor known as polyethism. The various roles can be carried out by individuals of different morphologies (caste polyethism). Roles can also be performed by members of different ages (age polyethism). There is a single reproductive queen per colony and, under normal colony conditions, she is the sole reproductive member, laying approximately 2,000 eggs per day. The queen is responsible for releasing pheromones that help to maintain and regulate colony conditions. The workers constitute the majority of the colony and forgo reproduction to care for the queen, her offspring, and maintain and defend the colony.

When a queen lays an egg, it can be either fertilized (2n, diploid) or unfertilized (n, haploid). Fertilized eggs develop into females while unfertilized eggs will develop into males. After about three days, the eggs hatch into first instar larvae. The larvae will undergo a series of molts, and at the fifth instar the larvae will be capped over to transition into the pupal stage. The pupae will begin metamorphosis until complete maturation to adults. The developmental process is the shortest for queens, longest for drones, and workers have an intermediate development time.

Hormonal Regulation in the Female Caste

Development in honey bee females is largely mediated by hormones. The differentiation in development of queens and workers is based on the quantity and quality of the brood food. The total time for larval development takes between 5-6 days, a difference in feeding within the first three days can lead to physiologic differences in development and adult longevity (Page Jr and Peng 2001). Larvae chosen for queen rearing feed on a diet consisting of royal jelly, a substance rich in proteins and sugars. Worker-destined larvae feed primarily on honey and pollen during the later stages of development. These differences in diet contribute to the distinct behavior, physiology, and morphology between queens and workers (Evans and Wheeler 1999). Probably the most important difference between the two female castes is the physiology and anatomy of their reproductive system. The reproductive system of a honey bee female consists of two ovaries, oviducts, spermatheca, and vulva. The ovaries consist of a variable number of ovarioles, tubes that can produce eggs in parallel. Queens have large ovaries with approximately 160 ovarioles. Workers have smaller ovaries that can vary in size but have fewer ovarioles, ranging typically from 2 to 8 (Rueppell, Phaincharoen et al. 2011). Previous studies have concluded that more ovarioles per ovary result in a bias towards worker reproductive activation under queenless conditions (Makert, Paxton et al. 2006). Eggs mature in active ovarioles and exit through the oviducts. Queens selectively fertilize eggs passing through the oviducts by releasing small amounts of sperm from the spermatheca. The queen stores the sperm from drones during mating flights, but workers

are unable to mate and do not have a spermatheca. Therefore, all worker-produced eggs will result in drones (Velthuis 1970).

Juvenile hormone (JH) influences larvae development, reproduction, and age polyethism (Robinson 1987, Sullivan, Jassim et al. 2000). During the larval stage, juvenile hormone ensures larval growth and development while preventing metamorphosis. Once the larval development stage is complete, a transition to the pupal stage will be induced by a decrease in JH levels (Capella and Hartfelder 1998). JH will continue to be synthesized at lowered levels throughout the remainder of development (Rachinsky, Strambi et al. 1990). Newly emerged workers have reduced JH levels that increase with age according to a genetically determined pattern of development. There are also internal and external factors that regulate the expression of JH and regulation of the colony's division of labour, by altering the probabilities of response to tasks (Sullivan, Jassim et al. 2000).

Juvenile hormone is repressed by the protein vitellogenin (Barchuk, Bitondi et al. 2002). Vitellogenin(Vg) is a precursor protein to the egg yolk in the hemolymph of females. However, this glycolipoprotein is also associated with behavioral development within the worker caste system (Nelson, Ihle et al. 2007). It is the most abundant circulating protein in hive bees and winter bees, making up about 30-50% of hemolymph (Chan, Howes et al. 2006). It is differentially expressed amongst the worker caste. The variable expression can be explained by the behavioral tasks. Younger in-hive workers (nurses) have upregulated Vg expression while older out-of-hive (foragers) workers express Vg at lower levels (Amdam, Norberg et al. 2003, Page and Amdam 2007). Vg is

produced at high levels in the queen, young workers, egg-laying workers, and long-lived winter workers (Amdam, Norberg et al. 2003). Conversely, JH is expressed highest in workers performing out-of-hive tasks such as foragers and lower workers performing in-hive tasks such as nurses (Robinson, Strambi et al. 1991).

However, hormone levels may be modulated by environmental and colony factors such as food availability and population structure. Pheromonal regulation of JH may be a mechanism underlying the ability of workers to respond to changing colony needs (Pankiw, Winston et al. 1998). Colony conditions are controlled by an assortment of chemical signals called pheromones. The queen is an important source of pheromones responsible for releasing and maintaining the chemical balance contributing to the social order within the colony. Pheromones are classified as either releaser or primer pheromones. Releaser pheromones cause a rapid but short-lived response. Primer pheromones are slower acting in influencing behavior via long-term physiologic effects. Primer effects are responsible for regulating colony organization, caste structure, and the division of labor (Slessor, Winston et al. 2005). Some pheromones such as Queen mandibular pheromone (QMP), released by the queen, and beta-ocimene, released by young worker larvae, have both types of effects. QMP is a key pheromone that contributes to maintaining queenright worker physiology (Kaminski, Slessor et al. 1990). However, there is little evidence where comparisons of workers developing in environments lacking the influence of these critical primer pheromones are challenged alongside normal, queenright, workers.

Queenright workers are exposed to the five-component QMP gland mixture (Pankiw, Winston et al. 1996) when the chemical is distributed throughout the colony by queen attendants. The queen is taken care of by a few workers through feeding, licking, and grooming. Subsequently, QMP is transferred through these workers who have contacted the queen (Winston, Higo et al. 1991, Pankiw, Winston et al. 1995). This gives the colony a sense of being "queenright". The queen pheromones stimulate pollen foraging (Higo, Colley et al. 1992), elicit short-term inhibition of queen rearing (Winston et al., 1989, 1990, 1991; Pettis et al., 1995), calms queenless workers (Naumann et al., 1990), and attracts foragers (Currie, Winston et al. 1992).

QMP also induces changes in worker endocrine physiology that affects reproduction and behavior. QMP is effective in repressing ovary activation in workers while stimulating hypopharyngeal glands. These glands produce the protein rich food fed to developing larvae (Knecht and Kaatz 1990). Larvae are restricted to their cells, unable to feed themselves. They rely on chemical signaling to alert nurse bees of their presence and needs. When they are young, the emission of the strong volatile pheromone beta-ocimene stimulates the nurses to provide them with appropriate nutrition (Maisonnasse, Lenoir et al. 2010). Brood requires a protein rich diet for development. Nurses mobilize their own protein stores to fortify the brood food (Amdam, Rueppell et al. 2009). So, workers become facultatively sterile to care for brood in the presence of a functional queen. If nurses are lacking in their own protein stores to provide adequate quality brood food, this could influence future foraging preferences for pollen (Pankiw, Page Jr et al. 1998). As the larvae age, the pheromone composition changes to less volatile

compounds, blends of fatty acid esters known as brood ester pheromones (BEPs) (Maisonnasse, Lenoir et al. 2010). Consequently, the behavioral response of nurse bees also changes. Nurses switch from feeding protein-rich royal jelly to a mixture of pollen and honey to meet the nutritional requirements of the growing larvae (Haydak 1970).

Nurses and foragers also release pheromones to indicate their presence in the population (Katzav-Gozansky, Soroker et al. 2000). The ability to adjust the division of labor between workers in response to changing colony dynamics is important for maintaining colony survival. For example, if there is an abundance of nurses present in the colony it will cause some of the workers to accelerate their development into foragers (Traynor, Le Conte et al. 2014). Similarly, if there is a wealth of foragers present in the colony, it will cause some of the foragers to revert to younger nurse bee behavior to ensure brood survival and general hive maintenance (Amdam, Aase et al. 2005).

The life progression of workers from nurses to foragers is associated with physiologic changes induced by QMP and BEPs. The transition from nurse to forager results in alterations to their nutrient storage (Toth and Robinson 2005), hormonal expression (Guidugli, Nascimento et al. 2005), oxidative stress resistance (Seehuus, Norberg et al. 2006), mortality dynamics (Rueppell, Bachelier et al. 2007), and immune pathways (Amdam, Simões et al. 2004). Queenless colonies lack the influential effects of QMP and brood pheromones. Therefore, a queenless colony develops its own hive make-up independent of the queen. Identifying a queenless colony is straightforward based on the egg laying pattern, number of eggs in cells, position of egg(s) in cells, and drone brood in worker cells (Chapman, Oldroyd et al. 2009).

In queenless colonies, the essential elements of social organization are broken down such as their ability to divide reproduction and behavior division between queen and workers (Page and Amdam 2007). Queenless workers maintain their colony much like communal bees and unlike their advanced eusocial queenright counterparts. Queenless workers have an investment in reproduction, brood care, and colony maintenance. Reproductive workers engage in foraging and colony defense. Foragers in queenless colonies have larger wax and hypopharyngeal glands than queenright nurses. These workers are generalists in their behavior, exhibiting some cooperative behaviors in colony defense and food storage (Robinson, Page et al. 1990). For example, some workers in queenless colonies can produce queen pheromones to suppress nest mates from reproducing. Reproductive workers compete in egg-laying and brood care of drones of genetic relatedness (Page and Robinson 1994). The kin selection theory suggests a basis for the behaviors of reproductive workers (Peters, Queller et al. 1999).

Although a queenless colony is not the ideal situation for beekeepers and some researchers because the colony will eventually die when only drones and no new workers are produced, queenless colony conditions may benefit workers by increasing individual longevity. The physiology of workers in a queenless colony is different than of queenright workers. One hallmark of queenless workers is their increased Vg expression levels (Peso et al. 2015). The level of reproductive hormones produced is correlated to ovary size (Amdam, Simões et al. 2004). There is a potential effect of hormonal differences among workers due to different ovary sizes and activation coupled with changes to the workers social role, presumably the onset of foraging, and hormone

dynamics (Page and Amdam 2007). Reproductive workers and queens do not exhibit the reproductive trade-off many other organisms experience. The intrinsic longevity advantage of reproductive workers can be attributed at least in part to their later onset of foraging (Dixon, Kuster et al. 2014), presumably due to their altered hormone titers. However, the physiological links of reproductive activation to Vg, immunity, and stress resistance in workers have not been studied so far.

Viral Infection

Honey bee colony declines, in the United States, correlate with the introduction of *Varroa* in 1980s (Wilfert et al. 2016). *Varroa* is an obligate ectoparasite relying on the developmental phase of the honey bee for reproduction. *Varroa* feeds on the hemolymph of honey bees weakening the host and transmitting virus. Once the adult honey bee emerges it is infested with multiple *varroa*, weakened, and diseased. *Varroa* originally was found to infest the males of *Apis cerana*. In *Apis mellifera*, *Varroa* can infest both the males and females. *Apis mellifera* is not as resistant to varroa infestation as *Apis cerana.* *Varroa* has been implicated in transmitting honey bee viruses. There are approximately 18 honey bee viruses, many of these are single stranded RNA viruses (Chen and Siede 2007).

Israeli Acute Paralysis Virus (IAPV) is the third most common viral infection in honey bee colonies after Black Queen Cell Virus (BQCV) and Deformed Wing Virus (DWV) (Cox-Foster, Conlan et al. 2007). IAPV is a single stranded RNA virus that was first discovered in 2004 (Maori, 2007). It has been associated with colony collapse disorder. The association between *Varroa* and virus transmission reduces honey bee

immunity and enables the increased replication of IAPV within the honey bee (Le Conte, Ellis et al. 2010). Honey bee symptoms to IAPV infection, and death, are related to the quantity of virus and transmission route (McMenamin and Genersch 2015).

Oxidative Stress

Honey bees come frequently into contacts with pesticides. For example, the plants that honey bees pollinate can be directly sprayed with pesticides, uptake or contain systemic pesticides, or honey bees can be exposed to pesticide drift (Krupke, Hunt et al. 2012). Consequently, foragers are exposed to pesticides while flying in areas where pesticides have been sprayed, volatization of these chemicals from the soil, ingesting contaminated pollen and/or nectar, and topical exposure by visiting flowers. The foragers can contaminate the colony with pesticides that are in the collected food or in their body. Additional, beekeepers use some pesticides to control *Varroa* and other bee diseases, further increasing the pesticide exposure (Traynor, Pettis et al. 2016).

The effects of pesticides vary. Lethal responses result in the immediate death of the honey bee. This will reduce the number of foragers and subsequently food availability in the colony, trigger premature foraging, and potentially lead to the collapse of the colony (Perry et al. 2017). Sublethal effects are probably more widespread and have been a more recent focus of studies. For example, pesticide exposure has been shown to impair immunity and reproduction in honey bee queens. I will be using the herbicide paraquat, which was first produced for commercial use in 1961 primarily for weed and grass control. It is under restricted usage in the United States due to its toxic nature in inducing oxidative stress in a number of organisms. It has been shown to cause oxidative damage

through its redox cycling mechanism producing reactive oxygen species. Although the mechanism is not well established in the honey bee model, paraquat had been shown to induce oxidative stress in honey bees (Seehuus, Norberg et al. 2006, Corona, Velarde et al. 2007)

Vitellogenin as a Natural Defense

Honey bees have natural defenses against environmental stressors such as viral pathogens and pesticides. Honey bees, like many social organisms, exhibit a social immunity, a collection of behaviors that typically arise from the cooperation of the individual group members to combat the increased risk of disease transmission as a consequence of group living (Cremer, Armitage et al. 2007, Simone, Evans et al. 2009). Social immunity is particularly important for honey bees because they only have an innate immune response with relatively few immune genes (Evans, Aronstein et al. 2006). Individual immunity begins at the cellular level, increasing to the individual organism, and the total colony (Seeley 1989). Thus, the honey bee colony can be regarded as a complex super-organism with physical, chemical, and behavioral defenses in various levels of the colony hierarchy.

Insects do not exhibit the ability to create specific immune responses in the form of antibodies as in the case of adaptive immunity of most vertebrates. Innate immunity of honey bees is comprised of circulating immune cells, in the form of hemocytes, and antimicrobial peptides (Evans, Aronstein et al. 2006). Hemocytes clear pathogens in the hemolymph by phagocytosis, nodulation, or encapsulation (Wilson-Rich, Dres et al. 2008). Specific immune signaling pathways for the honey bee have been hypothesized

using information from the fruit fly *Drosophila melanogaster*. Yet, the honey bee has only one third of the immune-related genes of *Drosophila* (Evans, Aronstein et al. 2006). Even though honey bees presumably lack acquired immunity, an induced response is believed to occurs after pathogen exposure sometime after the fast-acting innate response. This response can last for weeks following exposure and can be transferred to nestmates and offspring (via immune priming), potentially increasing immunity in subsequent exposures. An induced response is characterized by the increased transcription of genes for antimicrobial peptides, which occurs primarily in the fat body (Evans and Pettis 2005).

The fat body of the honey bee also produces Vg, which is a potent Zn carrier in workers that is necessary for immune function (Amdam, Aase et al. 2005). Zn is an essential metal that is required to catalyze biochemical reactions. It also has a structural and regulatory role as well. A deficiency in this metal can induce oxidative stress and apoptosis in immune cells (Shankar and Prasad 1998). For honey bees, a lack of Zn causes the degeneration of their immune cells. Therefore, the onset of foraging not only reduces life span by exposing foragers to environmental risks but depletes their Vg levels and plasma Zn levels reducing their immune fitness (Amdam, Simões et al. 2004).

There is evidence to suggest that Vg also protects honey bees against pesticides because it is selectively oxidized by the herbicide paraquat (Seehuus, Norberg et al. 2006). Vg is found in a variety of tissues and cell types due to its ability to adhere to membrane structures. This feature enables the cell to be protected with Vg acting as an antioxidant and anti-inflammatory agent (Havukainen, Münch et al. 2013). The higher Vg

titers in queens have been associated with the queens' resistance to oxidative stress compared to workers (Corona, Velarde et al. 2007).

In queens, Vg titer is higher than in workers and drones, and the excess in reproductive hormone has been proven to protect the queen against various stressors, such as oxidative stress (Corona, Velarde et al. 2007) and could also make them more disease resistant. Under queenless conditions, workers start to become reproductively active and increase their Vg titers (Galbraith, Wang et al. 2015). These physiological changes are predicted to increase their stress resistance and immunity compared to queenright workers, but experimental data are lacking despite the fact that these comparisons could directly link social function and health without the many confounding factors that arise in worker-queen comparisons (Rueppell, Amdam et al. 2004)

Hypothesis and Predictions

Based on the information above, I hypothesized that increased levels of vitellogenin in reproductive workers protect against viral infection and oxidative stress. This study explored the relevance of non-reproductive functions of vitellogenin in worker bee defense against virus infection and oxidative stress by social manipulations. Specifically, I predicted that 1) altering the natural social structure of the hive will alter worker physiology, 2) workers in a queenless colony will undergo reproductive activation, 3) worker in queenless colonies will have an improved resistance to viral and pesticide stressors compared to workers in queenright colonies, and 4) workers from queenless colonies will have a pattern of gene expression that enables improved stress resistance as it relates to their reproductive status. These predictions were tested by

introducing workers to queenless colonies to induce reproductive activation, while controls were placed in queenright colonies. The two groups of workers were compared with regards to ovary activation, vitellogenin and other immune and oxidative defense gene expression, oxidative stress resistance, and disease resistance. Therefore, the experimental aims are 1) to compare ovary activation and the gene expression of the key regulator vitellogenin between workers in queenless and queenright conditions, 2) relate these social treatments and vitellogenin titers to the workers' resistance to Israeli Acute Paralysis Virus (IAPV) infection and oxidative stress induced by the pesticide paraquat, and 3) compare the expression of other candidate genes that may be involved in oxidative stress resistance and immune defense. Thus, my study has investigated how the physiology of honey bee workers is changed by a lack of reproductive suppression and how this might improve resistance to IAPV and pesticide-related oxidative stress.

CHAPTER II

MATERIALS AND METHODS

Field Design

Experimental colonies were established in May and maintained until August 2017. Four queenright and four queenless colonies were each made up of 600 random honey bees of mixed origins from the UNCG honey bee yard. Colonies were established by shaking workers, of unknown age, from frames into small three-frame mating nuc hives, with capacity of approximately 1,500 honey bees for both queenright and queenless colonies. The queenright colonies were headed by unrelated queens of unknown age. To compensate for worker mortality, the queenless colonies were provided with newly emerged workers to maintain population throughout the experiment. All colonies were monitored for parasites, pathogens, and adequate food stores. Routine powdered sugar treatments were applied to each colony to limit the effects of *varroa* mites on worker lifespan. Queen candy, a mixture of powdered sugar and water, was provided during poor weather conditions and times of low food stores.

At the onset of the experiment, frames with ready-to-emerge workers were collected from UNCG stock colonies. These frames were stored in an emergence incubator overnight. Newly emerged workers were carefully brushed from frames into containers. Individuals were marked with target colony-specific acrylic paint on their thorax, the date of introduction was recorded to keep track of age for future collection at

15- day old (non-foraging age) and 25-day old (foraging age). Approximately 1,000 workers were divided for introduction to the colonies. Multiple cohorts were introduced, starting in May. The collections for IAPV treatment and controls that were actually analyzed started in June and the beginning of July. Subsequently, the months of July and August were used for sample collections for paraquat treatment and controls.

Sample Collection

Of the four colonies established, only two maintained desired colony conditions, queenless and queenright, for the duration of experiments. Individuals were collected from these colony sources for all pre-treatment and treatment samples. Non-foraging and foraging workers from each social condition were collected at 15 (non-foraging age) and 25 days (foraging age), respectively. The two sampling time points were chosen because in-hive (non-foragers) and out-of-hive (foragers) exhibit significantly different rates of Vg expression and hemolymph levels (Amdam and Omholt 2003). To assess the effect of social condition on worker physiology, ten workers from each colony type and age group were collected, immediately stored in microcentrifuge tubes with 400 µL of RNAlater™, and transferred to the lab where they were stored in -80 °C. The remaining workers sampled from each social condition and age group were stored together in cages for transportation to the lab. Afterwards, the samples were divided into control and treatment groups and treatments were applied (see below). Each treatment and control group (n = 45) were kept in sterile plastic cups with three replicate cups per trial. A subsample of living workers was collected after 24h (n = 10 per age and treatment group) and 120h (n = 10) after treatment. Survival was monitored every 24 hours for five days while these

groups were maintained under optimal incubator conditions (33°C, and 50% humidity) with ad-lib water and food (Williams, Alaux et al. 2013).

Treatment Application

Individual workers were anesthetized on ice before treatment application. Workers inoculated with IAPV had the thorax shaved and 2 µL of diluted IAPV solution (10^{-2} stock solution as per Boncristiani et al. 2013) topically applied. Control groups were shaved with no virus applied to the thorax. Workers had oxidative stress induced by injecting 1 µL of a 150µg/g paraquat solution (Seehuus, Norberg et al. 2006). Controls were injected with 1 µL of PBS. Individuals that did not wake up from anesthetization were omitted from the study.

Ovary Dissections

The ovaries of the pre-treatment, 24 hours post-treated, and 120h post-treated workers were assessed by dissecting abdomens that had been thawed overnight in RNAlater ICE™. Left and right ovaries were scored on a four-point scale according to previous studies (Graham, Munday et al. 2011). Reproductive activation was quantified by yolk presences in ovaries. Ovary score was recorded in SPSS and separated by social condition: queenless and queenright, and age: 15-day old and 25-day old. For analysis, the maximum ovary development between the left and right ovaries was used. Ovaries were classified as inactive if there was no yolk present (stages 0-1), and active if yolk was present (stages 2-4). Results (see below) indicated that ovary activation was only significantly different between social conditions in the older 25-day old but not the

younger 15-day old workers. Therefore, molecular analysis was only conducted on older 25-day old workers from each social condition and treatment group.

RNA Extraction

RNA was extracted from abdominal tissue using Trizol™ following the manufacturer's recommendations. Briefly, the abdomens were placed on dry ice and then crushed to powder with a disposable pestle. The pulverized tissues were mixed with 1mL of Trizol™. 200 µL of chloroform was added to separate the desired RNA and other soluble extracts after centrifugation for 15 minutes at 12,000 rcf. Undesired extracts were further separated by precipitating the RNA with 0.5 mL isopropanol on ice for 15 minutes, followed by centrifugation for 10 minutes at 12,000 rcf. The resulting RNA pellet was washed with 1 mL of 75% ethanol, to ensure purity, and air dried. The dried RNA was resuspended in 100 microliters of molecular-grade water and stored at -80°C for future processing.

cDNA Synthesis

Once the RNA was quantified using a Nanodrop™ spectrophotometer, synthesis of the cDNA was performed with 20 ng RNA using the SensiFAST™ cDNA Synthesis Kit according to the manufacturer's recommendations. All required calculations for dilutions and reagents were completed using an excel template.

Quantitative Real Time PCR

The Sensifast SYBR Green™ Kit was used to complete qPCR analysis to quantify levels of the following target genes: Vitellogenin, the immune genes Dicer-like, Defensin, Apidaecin, and Argonaute-2, and the oxidative stress defense genes Glutathione-s-transferase, CuZn Superoxide dismutase, and Catalase (Table 1). The average values of two technical duplicate runs were used for all reactions. Ct value determination was standardized using a single gene-specific fluorescence threshold value across all reactions. Gene expression was expressed relative to the mean of two reference genes, actin and RPS5, using the ΔC_T method (Schmittgen and Livak 2008). Gene expression of 25-day old (foraging age) was determined by subtracting average cycle time (C_T) values of reference genes from target genes C_T values. Average ΔC_T values were compared between experimental (treatment vs. control) and social (queenright vs. queenless) groups for the following samples: no treatment, 24h post PBS injection, 120h post PBS injection, 24h post paraquat injections, 120h post paraquat injections, 24h post shaving without virus application, and 24h post IAPV inoculation. Note that there were no 120h post virus inoculation or non-virus application samples as there were too few individuals that survived for molecular analysis.

Statistical Analysis

All statistics were run using the software IBM SPSS version 25. Ovary activation was analyzed by age (15day old and 25-day) and social (queenless and queenright) using a 2-way ANOVA. Survival comparisons were performed using Kaplan-Meier survival function. The effect of social treatment on survival rate and the difference between

survival rates of treatment groups was determined using log-rank test. The gene expression was analyzed for social, treatment, and social x treatment interaction using a 2-way ANOVA with additional bootstrapping to correct p-values because the data did not conform to parametric assumptions. Post-Hoc analysis using Bonferroni test was used for comparison of treatment effect.

CHAPTER III

RESULTS

Social Manipulation and Age Effect On Reproductive Activation

Histograms of the frequency of ovary activation scores (Figures 5 and 6) indicated differences between social and age groups. Overall, queenless workers had a significantly higher ovary activation than queenright workers ($F_{(3,199)} = 58.5$, $p < 0.001$). A significant effect of sampling age (15d vs. 25d) was also observed ($F_{(3,199)} = 36.5$, $p < 0.001$). However, a significant interaction between social condition and sampling age existed ($F_{(3,199)} = 39.0$, $p < 0.001$) and therefore the effect of social condition was evaluated separately for both age groups. Foraging age (25d) queenless workers had significantly higher ovary activation than queenright workers ($F_{(1,99)} = 49.3$, $p < 0.001$), while the differences at 15d were not significant ($F_{(1,99)} = 0.042$, $p = 0.84$).

Social Manipulation Effect On Stress Resistance

Stress resistance was measured as a decrease in mortality after exposure to two acute stressors. Survival analysis combined data from younger 15-day old and older 25-day old workers from both social conditions. There was a significant difference between treatment groups and within each treatment group according to social condition. Across all social groups, paraquat injection decreased survival significantly compared to the PBS-injected control group (Figures 1 and 3). Queenless workers overall had an

increased resistance to paraquat stress compared to queenright workers ($X^2 = 82.5$, $p < 0.001$). Across all social groups, IAPV application decreased survival significantly compared to the control shaved group (Figures 2 and 4). Queenless workers overall had an increased resistance to viral stress compared to queenright workers ($X^2 = 26.5$, $p < 0.001$).

Social Manipulation and Treatment Effect On Gene Expression

Gene expression was only analysed among foraging age workers due to the effect of social condition on ovary activation in that age group but not the young workers. Treatment and/or social condition had a significant effect on the expression of Vg, Toll-6, Apidaecin, Argonaute-2, and Catalase expression (Table 2). Treatment alone had a significant effect on Apidaecin gene expression. Social condition and treatment individually had a significant effect on Argonaute-2 gene expression. There was a significant interaction between social condition and treatment for Vg, Toll-6, and Catalase. Splitting the data for target genes that had a significant interaction effect, determined that Vg expression was higher in queenless workers in pre-treated ($F_{(1,18)} = 7.2$, $p = 0.015$) and 24h post-PBS, shaving, paraquat, and IAPV treated 25-day old workers ($F_{(1,19)} = 14.1$, $p = 0.001$; $F_{(1,17)} = 82.3$, $p < 0.001$; $F_{(1,18)} = 12.1$, $p = 0.003$; $F_{(1,19)} = 11.5$, $p = 0.003$ respectively). However, social condition was not significant in 120h post-paraquat and PBS treated 25-day old workers ($F_{(1,19)} = 1.1$, $p = 0.032$; $F_{(1,19)} = 0.04$, $p = 0.83$ respectively) (Figure 7). Similarly, social condition had a significant effect on Toll-6 gene expression for 24h post PBS, IAPV, and shaving treatmets for 25-day old workers ($F_{(1,18)} = 17.1$, $p = 0.001$; $F_{(1,19)} = 4.9$, $p = 0.40$; $F_{(1,18)} = 11.1$, p 0.004

respectively) (Figure 8). There was not a significant effect of social condition for 25-day old workers 120h post paraquat treatment. There was significant effect of treatment for gene expression of Catalase for 24h post paraquat and shaving treatments, and 120h post PBS treatment groups. There was an efffect of social condition on Catalase gene expression for 24h post paraquat and shaving treatments ($F_{(1,18)} = 5.8$, $p = 0.02$; $F_{(1,17)} = 4.4$, $p = 0.05$ respectively) and 120h post PBS treatment ($F_{(1,16)} = 4.1$, p 0.05) (Figure 13). All other genes (Dicer-like and CuZn Superoxide Distmutase (Figures 10 and 12)) were not significantly different among social or treatment groups or could not successfully be amplified (Table 2).

CHAPTER IV

DISCUSSION

There are a variety of stress factors that likely contribute to honey bee decline. Studies have alluded to the effect of viruses and demonstrated the effect of pesticide exposure on honey bees in a caste-dependent manner. Honey bee physiology varies among caste and social conditions but there is a lack of information for reproductive workers.This study demonstrates that honey bee worker response to stress can be improved by manipulating the social environment. Specifically, reproductive activation in workers is beneficial in improving stress resistance as evidenced by a decrease in mortality and increase in immune activity and oxidative stress defense.

Honey bees exhibit caste-dependent differences in longevity, where queens live an order of magnitude longer (a few years) than workers (5-7 weeks in the summer and a few months in the winter). Studies have elucidated the mechanisms of queen longevity to their reproductive activation and Vg levels. Few studies have demonstrated the consequence of reproductive activation in the worker caste. Majority of the literature focuses on the parallels of queenless workers in regards to hormonal regulation (ecdysone, JH, and Vg) and ovary composition. Queenless workers are able to undergo reproductive activation, display similar patterns of gene expression, and exhibit increased stress resistance as demonstrated in queens.

The consequence of queen absence on overall colony health is not ideal, however individuals in the colony benefit from their physiological changes. Queenless workers reveal the impact of social conditions on both colony and individual regulation. For instance, social condition was not previously found to have an effect on the age of worker reproductive activation in queenless colonies (Peso, Elgar et al. 2015). However, this study shows social condition had a significant effect on age of reproductive activation. Hive size may alter the efffects of social conditions, where smaller hives could intensify the effects of queenlessness on worker reproductive activation. Some evidence suggests reproductive activation is dependent on ovary composition, and can take time for phenotypic changes to occur. Workers could undergo physiological changes as consequence of reproductive activation before phenotypic changes occur, indicating there might be effect of social condition on younger (15-day old) worker physiology. Reproductive activation in honey bees does not follow the same trend as almost all other organisms, where in honey bees reproductive activation increases lifespan of workers (Rueppell, Aumer et al. 2016), as paralleled to honey bee queens (Kuszewska, Miler et al. 2017). The results associated with reproductive activation in queenless workers indicates that this longevity advantage under atypical conditions may be due to increased stress resistance as established in fruit flies (Morrow, Samson et al. 2004, Rose, Vu et al. 2004).

As predicted, queenless workers were able to survive abiotic and biotic stressors significantly better than queenright workers. The difference in survival for control treatments (PBS injection and thorax shaving without virus application) was not

predicted (Figures 1 and 2). However, it was not too suprising, given that reproductive workers also have a survival advantage under natural field conditions (Dixon, Kuster et al. 2014). Furthermore, the control groups could have also experienced some stress due to treatmet application. It has been shown that wounding and injection are forms of stress for honey bees (Evans, Aronstein et al. 2006), shaving and the cage environment might have been an additional form of stress. Treatment application stress could have been coupled with the introduction of paraquat and IAPV, but the differences between treatment and control groups demonstrated that the treatment stress itself was the more significant stressor. Results from the survival studies (Figures 1 through 4) indicate that queenlessness can afford workers resistance to a variety of stressors.

The differences in survival between social conditions could be attributed to differences in gene expression. The observed differences in gene expression were in general as predicted: Reproductlively active, queenless workers had an increased expression of Vg compared to queenright workers. Results from comparisions of Vg expression between pre-treated and 24h post PBS, paraquat, IAPV, and shaving treatment, all indicate that social conditions had a significant effect on Vg expression, with queenless workers expressing Vg consistently more than queenright workers. The change in Vg expression is an indication that social condition alters the relative expression, and 24h is not sufficent time to revert the gene expression differences. In contrast, the 120h post treatment worker comparision showed no difference in Vg expression between social groups. Although queenless workers may have had increased circulating levels of Vg, after prolonged presence in a different social envrionment, the

normal pattern of Vg gene expression can be recovered. This finding suggests that the effect of social condition on worker physiology is reversable, and also suggests Vg as a defense mechanism is not specifically induced by exposure to paraquat, viral, or control stressors. While the physicological responses to stress are not well understood, it has been determined in queenright workers that acute sublethal exposures to pesticides do not cause changes in hormones associated with behavior (JH and Vg) (Bordier, 2017). Normally, JH suppresses Vg synthesis to promote the transition from in-hive nurses to out-of-hive forgarers (Amdam, 2003). Immune challenges, such as viral infection, alter the expression of JH and Vg where there is a respective increase and decrease in hormone levels (Bordier, 2017). However, such effect was not observed in the IAPV inoculation experiments. This study demonstrates queenless workers appear to also maintain the ability to reduce disease transmission by having decreased Vg expression in response to treatment, possibly as a means to induce foraging.

Queenless workers also had a pattern of immune gene expression that was expected as a consequence of reproductive activation, where there was a down-regulation in expression of the Toll-6 gene. In response to IAPV inoculation, queenright workers had an increase in Toll-6 expression compared to queenless workers. The toll pathway is activated by certain combinations of virus-host interecations (Brutscher et al. 2015) that may not have been triggered in queenless workers due to increased immune activity provided by Vg that queenright workers did not have. Similar to Vg, the effect of social condition on Toll-6 expression appeared to be reversed 120h post-treatment. However, queenright workers expressed Toll-6 significantly higher compared to queenless workers

(Figure 8). Toll-6 gene expression appeared to be regulated by IAPV treatment as expected, where evidence suggests different responses to IAPV infection: IAPV-inoculated workers in laboratory exhibited increased expression of Toll-6, as supported in this study. In contrast, workers that were naturally infected with IAPV in the field did not exhibit increased antiviral defense using the toll pathway (Brutscher, 2015).

Correspondingly, queenless workers followed a pattern of gene expression for the RNAi pathway as expected, where queenless workers expressed Argonaute-2 significantly higher than queenright workers at least in one of the comparisons. The RNAi pathway is one of the major antiviral mechanisms in honey bees (Evans, 2006). However, this study demonstrates 24h post treatment there is a greater paraquat treatment effect association with increased expression of Argonaute-2 rather than IAPV. The significant difference in argonaute-2 expression 120h post PBS treatment suggests that the stress of injection may initiate an immune response for pre-exisiting infections (Figure 11). The mechanism of paraquat induced oxidative damage in honey bees is not well understood, and could be associated with pathways outside of oxidative defense, such as the RNAi pathway. All together, the unusual activation of the RNAi pathway by paraquat and PBS treatment could unmask viruses already present in the system.

The expression of Apidaecin, an antimicrobial peptide end product of both the RNAi and toll pathways, was not significantly affected be social condition overall. Apidaecin was differentially expressed between social groups in 24h post paraquat treated workers only. This finding supports previous results of differential regulation of both the toll and and RNAi pathways which intiate antimicrobial peptide (AMP)

synthesis. However, AMP synthesis is not the primary role for the RNAi pathway unlike the toll pathway that primiraly utilizes AMP synthesis for immune activity (Brutscher et al. 2015). Apidacein, along with other antimicrobial peptides, act as immune effector proteins (Evans et al. 2006) and although expression was not significantly different in IAPV treated workers it was still differentially expressed. Queenright workers expressed more Apidaecin in almost all treatment groups although the difference was only significant for the 24-hour post paraquat injection comparison (Figure 9).

Workers in the absence of a queen are able to undergo reprductive activation that induces physiological changes that enable an upregulation of immune activity that otherwise declines in the transition from younger (15-day old) to older (25-day old) workers (Amdam, Aase et al. 2005). Traditionally, the transition from in-hive tasks to the more risky out-of-hive foraging tasks is linked to a significant decline in immune function. This immunosenescence in honey bee workers is reversable with a reversal in task (forager to nurse) (Amdam, Aase et al. 2005). This study demonstrates that the immune system is also responsive to other social manipulations and the resulting plasticity of worker physiology.

Oxidative stress resistance is also an important defense affored to queens, possibly as a consequence of reproduction and increased Vg titers (Corona, Velarde et al. 2007). Catalase, Glutathione *S*-transferase (GST), and Superoxide Dismutase have been shown to be increased in queens, however in a tissue specific manner (Corona, 2005; Weirich, Collins et al. 2002). In this study, catalase was the only differentially expressed oxidative defense gene between social groups which could be attributed to catalase being

not being extracellular (Corona and Robinson 2006). For example, catalase is free in the cytosol of cells and requires no peptide for release (Corona and Robinson 2006), indicating that its activity may be limited to oxidative defense inside the cell. In contrast, SOD was not differentially expressed between social conditions or treatment groups (Figure 12). GST was removed from the analysis because it did not amplify in any sample, which may be a consequence of primer defects.

Unexpectedly, reference gene expression was also significatnly affected by social condition and treatment even when averaged together. Therefore, patterns of gene expression in treatment groups with high ΔC_T values, corresponding to less gene expression, could be due to lower averages in reference genes. Additionally, IAPV infection in pupae has been shown to effect ribosomal RNAs where there was increased expression of RPS5 (Boncristiani, 2013). The transcriptional profiles from the fat bodies of young IAPV infected workers had 753 genes differentially expressed, including genes in the RNAi and toll pathways (Galbraith et al. 2015) as oberved in this study. In more general terms, IAPV infected adult workers exhibited differentially expressed genes that were associated with developmental growth (down-regulated) and hormone synthesis (Galbraith et al. 2015). Understanding that viral infection can alter reference gene expression, the relative gene expression profiles reported in this study can only be interpreted cautionarily as evidence for stress resistance observed in queenless honey bee workers.

Honey bees serve as model organisms to understand fundamental biological processes and the evolution/adaptations for sociality. A queenless colony can reveal

characteristics of the honey bee otherwise masked in the queenright colony dynamic. Queenlessness is not the desired colony make-up for researchers and beekeepers alike because of its practical limitations: a colony populated mainly by drones will eventually die out (without the addiditon of workers) without much honey produced or pollination performed. Even if queenless colonies are not a remedy to honey bee health decline, the exploitation of colony dynamics exposes queenless workers to have advantages to certain stressors such as pesticides and viruses. Insights provided through this study on the benefits of reproductive activation and associated gene expression patterns could help our fundamental understanding of how colony dynamics regulate honey bee physiology, how reproductive activation in honey bees is a benefit to the individual, and how differences between and within the honey bee female caste affect stress resistance and longevity. Specifically, the lack of the conventional trade-off between reproduction and survival functions in honey bees is not restricted to the honey bee queen but also occurs within the worker caste. This finding implies that fundamental physiological regulatory circuits have been reshaped by social evolution and are enabled through the social structure of colonies that liberates individuals from constraints that mediate the conventional tradeoff. Future research should focus on further characterizing the activity of queenless worker genomes compared to the established honey bee queen and worker transcriptomes. Common patterns of gene expression could indicate predispositions for stress resistance in combination to applying stressors. More definitive correlations between reproductive activaiton, Vg expression, and longevity would be required to better and more

realistically understand underlying biological processes workers can activate in response to environmental challenges.

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APPENDIX A

LIST OF TABLES

Table 1. Primers for Target Genes used for Real-Time Quantitative PCR

Target gene	Primer Sequences	Reference
Actin	F: CCTAGCACCATCCACCATGAA R: GAAGCAAGAATTGACCCACCAA	Galbraith et al. 2015
RPS5	F: AATTATTTGGTCGCTGGAATTG R: TAACGTCCAGCAGAATGTGGTA	Brito, McHale, and Oldroyd 2010
Vitellogenin	F: GGAACCTGGAACGAACAAGA R: GCTCGTAGTCACCGACGATT	Brito, McHale, and Oldroyd 2010
Toll-6	F: TCCGAGGCGTCAACAGGAATCGACC R: GACAGGTCGAACGTCTCCAG	Galbraith et al. 2015
Apidaecin	F: TAGTCGCGGTATTTGGGAAT R: TTTCACGTGCTTCATATTCTTCA	Evans et al. 2006
Dicer-like	F: CCAACAGGAGCTGGAAAAAC R: TCTCCACTAAGTGCTGCACAA	Galbraith et al. 2015
Argonaute-2	F: TCAACAGCAGCAATCGGATA R: TTGCGGTGAACTTTGTTGTT	Galbraith et al. 2015
Glutathione-S-Transferase	F: GCCGCTTCAAAAGAAGTACG R: GTGGCGAAAACAAGGATGAT	Schmehl et al. 2014
CuZn Superoxide Dismutase	F: GTCGTTCCGTGTAGTCGAGAA R: TCCTTTGACTTCACCCTGAAGA	Corona et al. 2005
Catalase	F: TGGAGCAAGTCCTGATAAAATGC R: TGGGCCAAGACGATGTCTATG	Corona et al. 2005

Table 2. Factors and Interactions Effecting Gene Expression from a Full Factorial General Linear Model

Gene	Social	Treatment	Social x Treatment
Average Reference	F = 10.4, p = 0.002	F = 6.6, p < 0.001	F = 0.7, p = 0.58
Actin	F = 6.3, p = 0.01	F = 8.1, p < 0.001	F = 0.27, p = 0.95
RPS5	F = 19.2, p < 0.001	F = 5.6, p < 0.001	F = 1.7, p = 0.124
Vitellogenin	**F = 71.7, p < 0.001**	**F = 6.2, p < 0.001**	**F = 8.1, p < 0.001**
Toll-6	**F = 14.7, p < 0.001**	**F = 22.4, p < 0.001**	**F = 3.5, p < 0.003**
Apidaecin	F = 0.43, p = 0.51	**F = 10.2, p < 0.001**	F = 1.1, p = 0.37
Dicer-like	F = 0.78, p = 0.38	F = 0.39, p = 0.88	F = 0.19, p = 0.97
Argonaute-2	**F = 5.3, p = 0.023**	**F = 5.6, p < 0.001**	F = 1.1, p = 0.38
CuZn Superoxide Dismutase	F = 0.03, p = 0.85	F = 0.85, p = 0.53	F = 0.14, p = 0.99
Catalase	F = 1.98, p = 0.16	**F = 4.9, p < 0.001**	**F = 3.5, p = 0.003**

APPENDIX B

LIST OF FIGURES

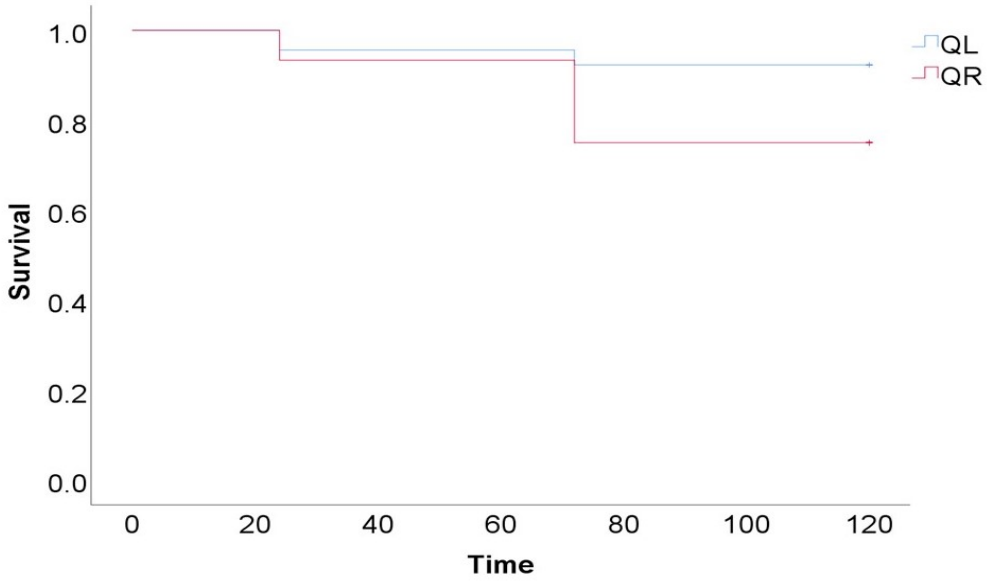

Figure 1. Overall Survival of PBS Injected Workers. Social groups (QL = queenless and QR = queenright) differed significantly (**$X^2 = 8.3$, $p = 0.004$)** in survival. Time was measured in hours. Log-rank analysis of social groups included survival data from both 15-day old and 25-day old workers injected with 1 μL of PBS

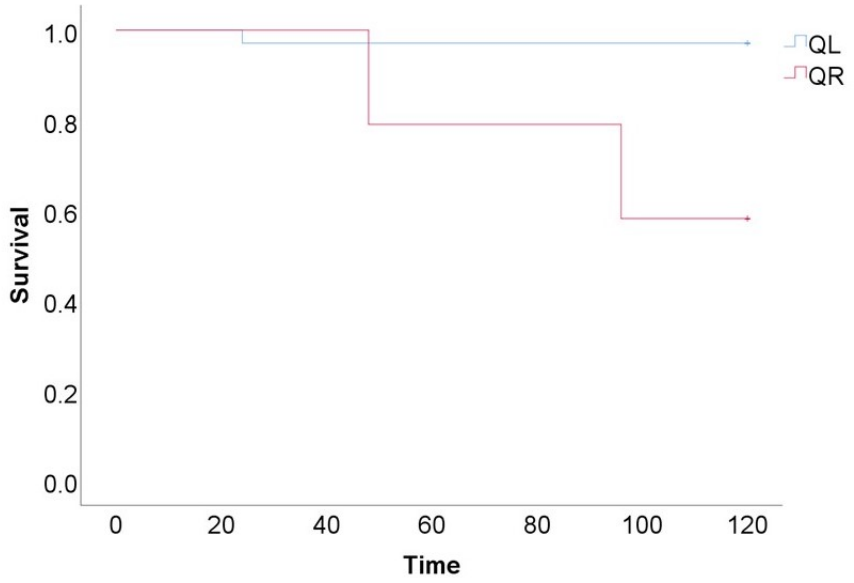

Figure 2. Overall Survival of Non-IAPV Inoculated Workers. Social groups (QL = queenless and QR = queenright) differed significantly (**$X^2 = 26.5$, $p < 0.001$).** Time was measured in hours. Log-rank analysis of social groups included survival data from both 15-day old and 25-day old workers with thorax shaved and no virus application.

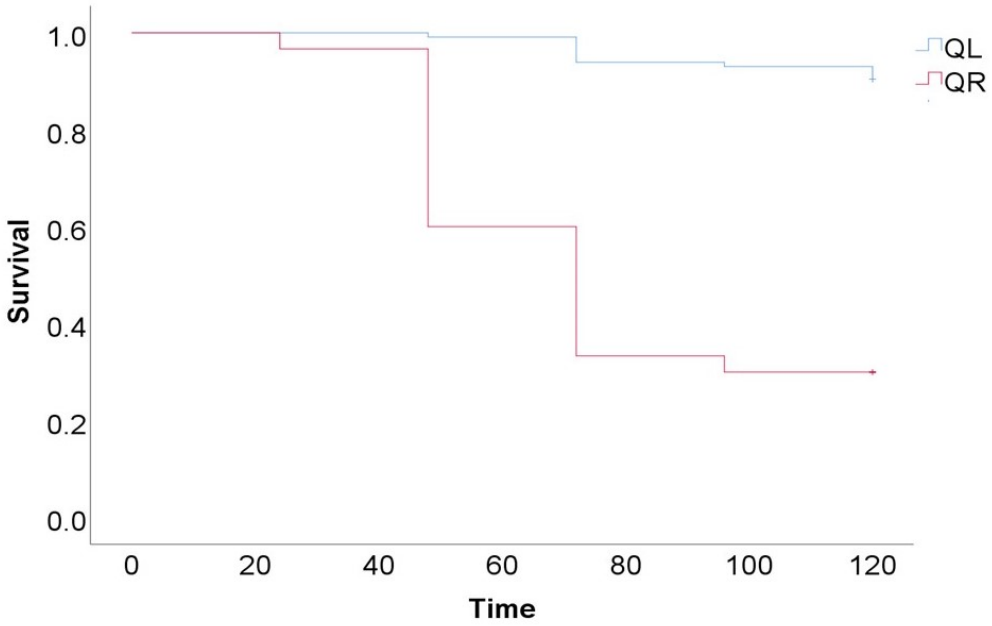

Figure 3. Overall Survival of Paraquat Injected Workers. Social groups (QL = queenless and QR = queenright) differed significantly (**$X^2 = 82.5$, $p < 0.001$).** Time was measured in hours. Log-rank analysis included survival data from both 15-day old and 25-day old workers injected with 1 μL of 0.150 ug/g of paraquat.

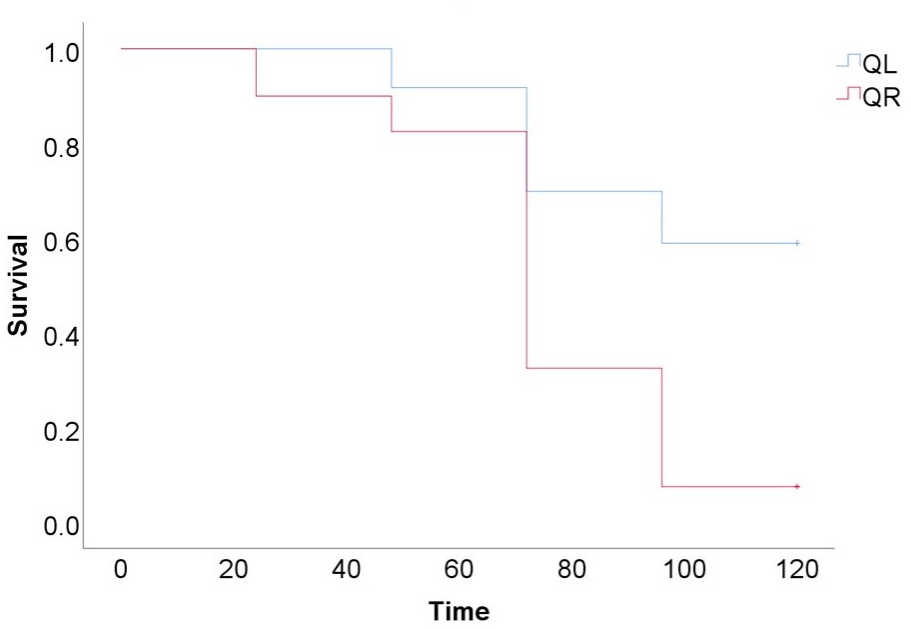

Figure 4. Overall Survival of IAPV Inoculated Workers. Social groups (QL = queenless and QR = queenright) differed significantly (**$X^2 = 29.1$, $p < 0.001$**). Time was measured in hours. Log-rank analysis included survival data from both 15-day old and 25-day old workers with thorax shaved and 2 µL of 10^{-2} IAPV applied.

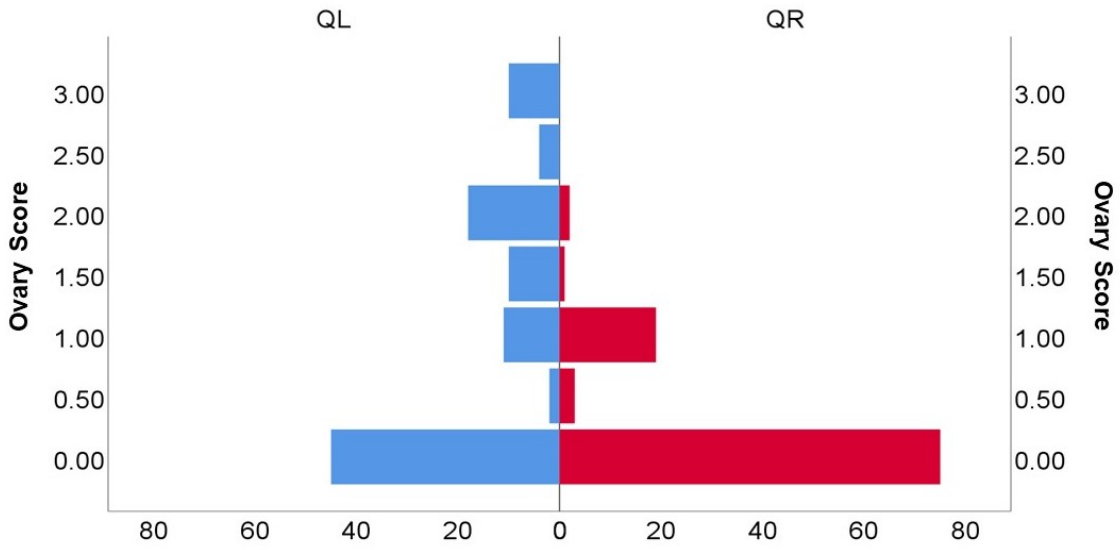

Figure 5. Effect of Social Condition on Reproductive Activation. Social groups (QL = queenless and QR = queenright) differed significantly in ovary activation ($\mathbf{F_{(1,199)} = , p < 0.001}$). ANOVA analysis of social groups included ovary score of both 15-day old and 25-day old workers.

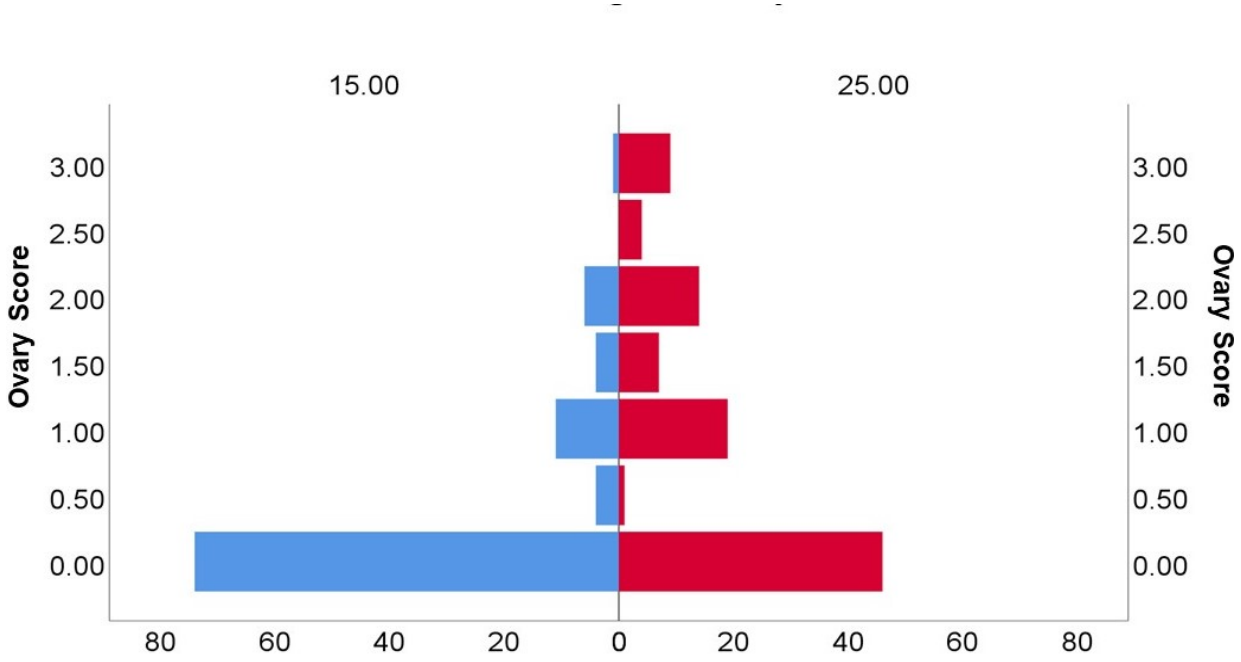

Figure 6. Effect of Age on Reproductive Activation. Age groups (15-days and 25-days) differed significantly in ovary activation (**$F_{(1,199)}$ =, p < 0.001**). ANOVA analysis of age groups included ovary scores from both social conditions (queenless and queenright).

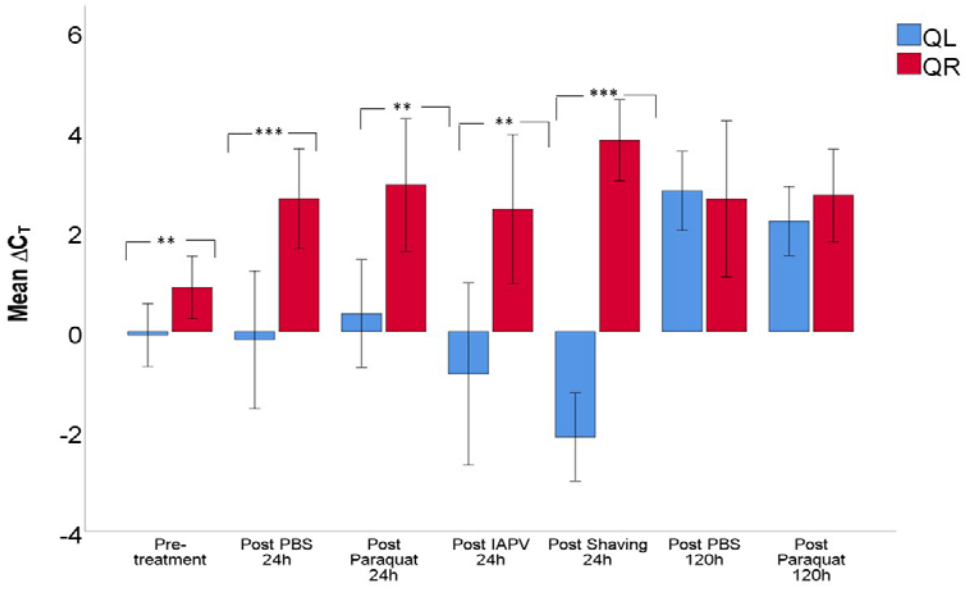

Figure 7. Differences in Gene Expression of Vitellogenin Between Social Groups in Response to Treatment. Asterisk indicate significant difference as follows: (* corresponds to $p \leq 0.05$) (** corresponds to $p \leq 0.01$) (*** corresponds to $p \leq 0.001$). ΔC_T values were calculated as the difference between the target gene and mean of the two reference genes, so a smaller ΔC_T value indicates an increase in expression. Significant difference between social groups (QL = queenless and QR = queenright) was calculated using ANOVA tests.

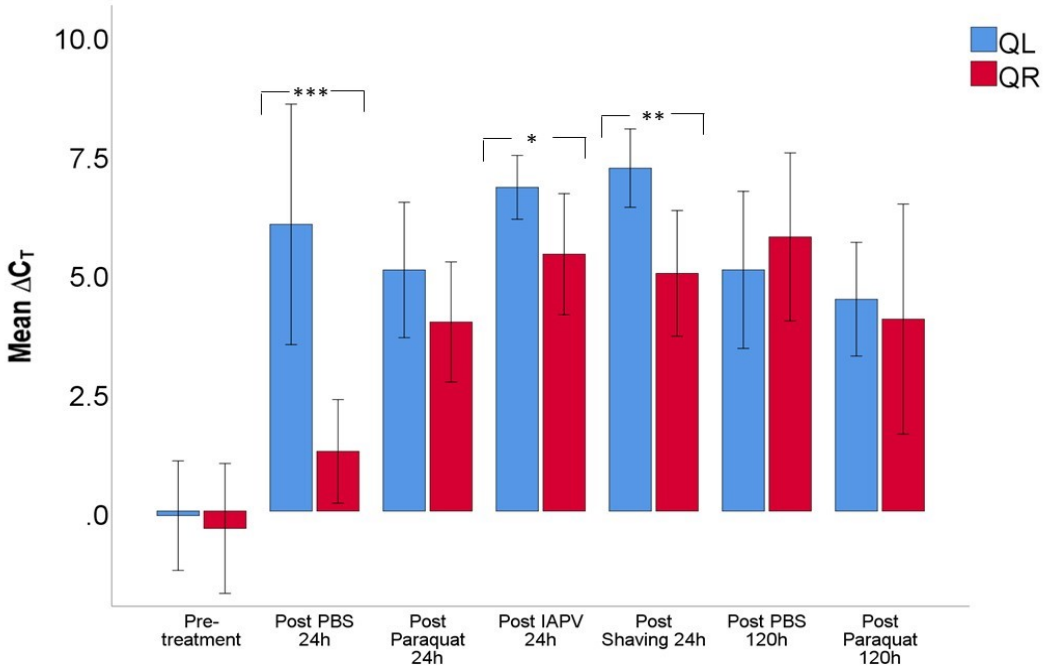

Figure 8. Differences in Gene Expression of Toll-6 Between Social Groups in Response to Treatment. Asterisk indicate significant difference as follows: (* corresponds to $p \leq 0.05$) (** corresponds to $p \leq 0.01$) (*** corresponds to $p \leq 0{,}001$). ΔC_T values were calculated as the difference between the target gene and mean of the two reference genes, so a smaller ΔC_T value indicates an increase in expression. Significant difference between social groups (QL = queenless and QR = queenright) was calculated using ANOVA tests.

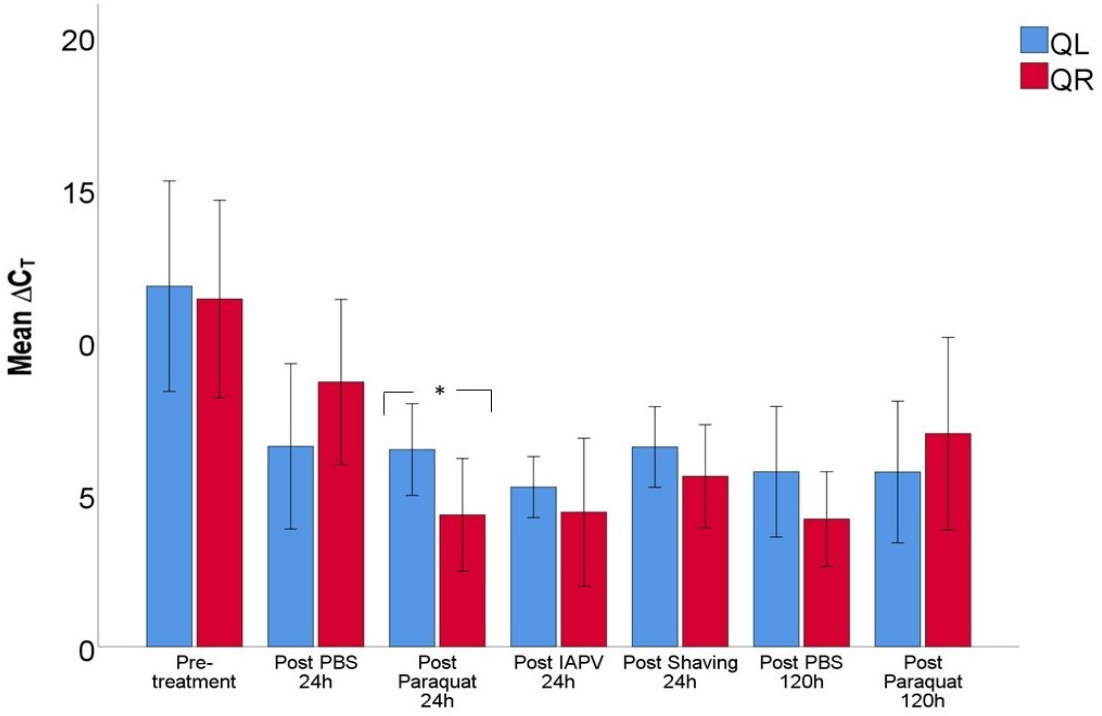

Figure 9. Differences in Gene Expression of Apidaecin Between Social Groups in Response to Treatment. Asterisk indicate significant difference as follows: (* corresponds to $p \leq 0.05$) (** corresponds to $p \leq 0.01$) (*** corresponds to $p \leq 0{,}001$). ΔC_T values were calculated as the difference between the target gene and mean of the two reference genes, so a smaller ΔC_T value indicates an increase in expression. Significant difference between social groups (QL = queenless and QR = queenright) was calculated using ANOVA tests.

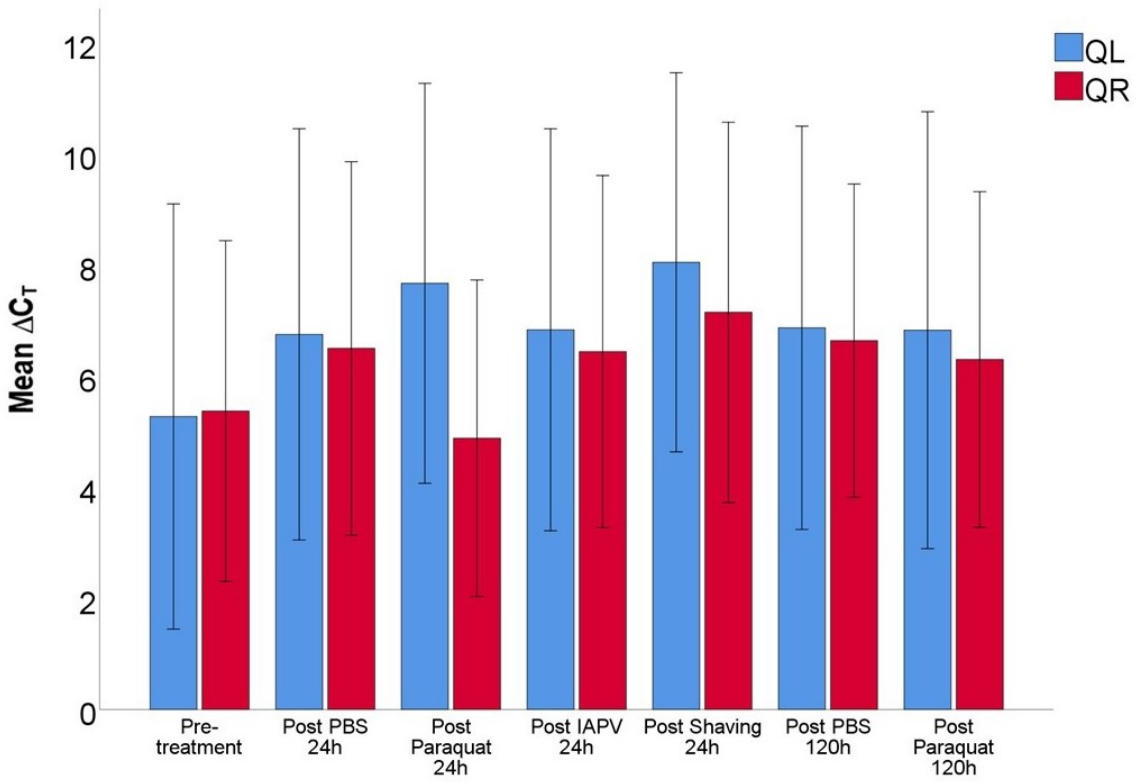

Figure 10. Differences in Gene Expression of Dicer-like Between Social Groups in Response to Treatment. ΔC_T values were calculated as the difference between the target gene and mean of the two reference genes, so a smaller ΔC_T value indicates an increase in expression. There was no differential expression of Dicer-like between social groups (QL = queenless and QR = queenright) of any treatment.

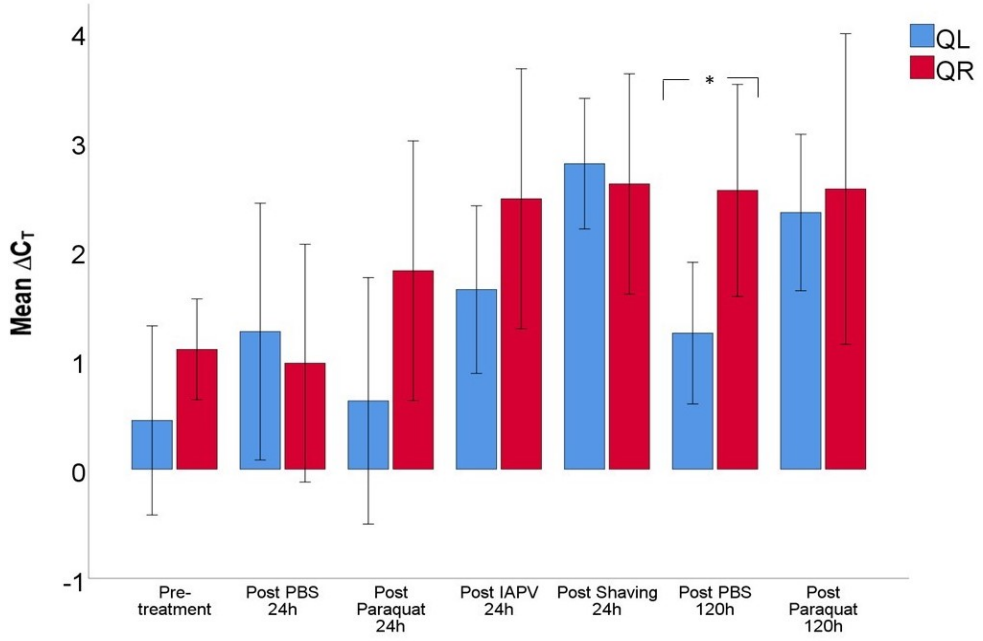

Figure 11. Differences in Gene Expression of Argonaute-2 Between Social Groups in Response to Treatment. Asterisk indicate significant difference as follows: (* corresponds to $p \leq 0.05$) (** corresponds to $p \leq 0.01$) (*** corresponds to $p \leq 0{,}001$). ΔC_T values were calculated as the difference between the target gene and mean of the two reference genes, so a smaller ΔC_T value indicates an increase in expression. Significant difference between social groups (QL = queenless and QR = queenright) was calculated using ANOVA tests.

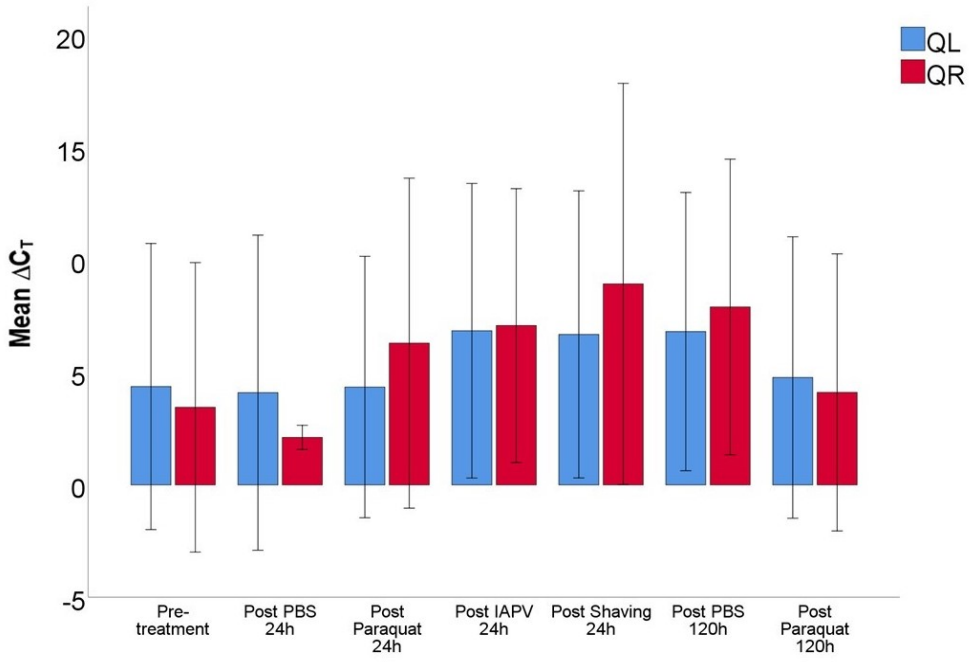

Figure 12. Differences in Gene Expression of CuZn Superoxide Dismutase Between Social Groups in Response to Treatment. ΔC_T values were calculated as the difference between the target gene and mean of the two reference genes, so a smaller ΔC_T value indicates an increase in expression. There was no differential expression of CuZn Superoxide dismutase between social groups (QL = queenless and QR = queenright) of any treatment.

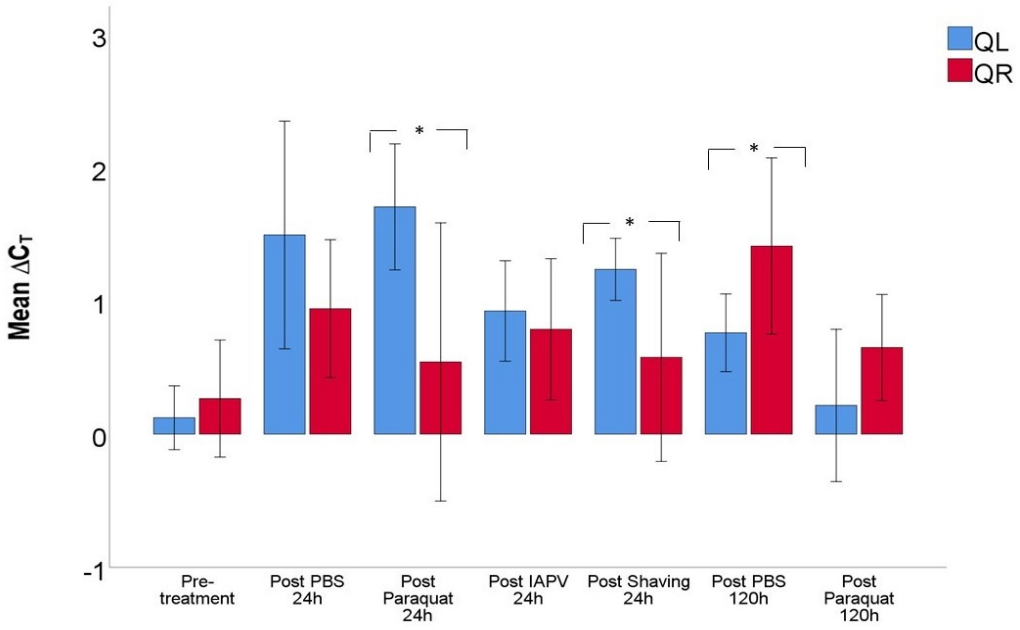

Figure 13. Differences in Gene Expression of Catalase Between Social Groups in Response to Treatment. Asterisk indicate significant difference as follows: (* corresponds to $p \leq 0.05$) (** corresponds to $p \leq 0.01$) (*** corresponds to $p \leq 0{,}001$). ΔC_T values were calculated as the difference between the target gene and mean of the two reference genes, so a smaller ΔC_T value indicates an increase in expression. Significant difference between social groups (QL = queenless and QR = queenright) was calculated using ANOVA tests.