THE ROLE OF GENE EXPRESSION IN TASK REGULATION OF THE WORKER CASTE OF THE RED IMPORTED FIRE ANT (*SOLENOPSIS INVICTA*, BUREN)

A Dissertation

by

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ABSTRACT

The reproductive ground plan hypothesis suggests that the evolution of social insects has resulted in genes being co-opted from reproductive functions into functions that enable the division of labor. To test this hypothesis in *Solenopsis Invicta,* I investigated expression of candidate genes important in colony organization. Transcriptome analysis of the worker brains showed no significant differences between carbohydrate and protein foragers, 194 differentially expressed genes were identified between workers in the presence or absence of brood. Among these, two vitellogenin (Vg) genes were identified. *S. invicta* genome harbors four distinct Vg genes, *Vg1*, *Vg2, Vg3* and *Vg4,* and both *Vg2* and *Vg3* were up-regulated in the brains of workers in the absence of brood. In the head, *Vg2* and *Vg3* were up-regulated in all morphological subcastes in the absence of brood, except for *Vg3* in minors and Vgs expression was higher in heads of nurses compared to foragers from colonies with brood. Further, *Vg2* was up-regulated in the head of workers in protein-starved colonies.

In the whole body, expression analysis of the four Vg genes in workers while in the presence or absence of brood revealed no difference but differences were identified among the morphological subcastes (major workers had higher levels of *Vg1* and *Vg2*). Expression of *Vg1* was also higher in carbohydrate foragers when compared to nurses and protein foragers.

A phylogenetic analysis of *S. invicta* hexamerins determined that each of the four predicted proteins clustered with one of the orthologous *Apis mellifera* hexamerins. Expression analyses revealed queens and nurses had significantly higher expression of

all genes compared to the foragers. *Hexamerin 1* was expressed at higher level in queens, while *hexamerin 2* and *arylphorin subunit beta-like* were expressed at significantly higher level in nurses. The relationship between the hexamerins and Vgs, and S-hydroprene, a juvenile hormone analog revealed *Hexamerin 1* and *arylphorin subunit alpha-like* expression were significantly lower after application in queens. *Hexamerin 2* and *arylphorin subunit beta-like* expression were significantly lower after application in foragers, and all hexamerins were significantly lower after application in nurses. No effect was seen in the expression of Vg in workers.

We have found several differences between *A. mellifera* and *S. invicta*, which show that some biological processes which regulate social behavior and colony structure are not conserved between social hymenopterans. These results, the structure of *S. invicta* colonies (polygyne versus monogyne colonies), and the complete sterility of workers enables future research into unique elements of eusociality which are not able to be observed in *A. mellifera* and may provide a foundation for the use of *S. invicta* as a model system to study eusociality in insects.

DEDICATION

For Grayson Tung

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INTRODUCTION: THE SIGNIFICANCE OF THE RED IMPORTED FIRE ANT, *SOLENOPSIS INVICTA* AND FACTORS AFFECTING SOCIAL ORGANIZATION AND GENE EXPRESSION IN SOCIAL INSECTS

THE RED IMPORTED FIRE ANT, *SOLENOPSIS INVICTA*

Red imported fire ants (*Solenopsis invicta* Buren; Hymenoptera: Formicidae) are native to South America, specifically to areas along the Parana river (Allen, Lutz, & Demarais, 1995). In 1930, *S. invicta* were introduced to the United States of America through the port of Mobile, Alabama (Vinson et al., 1986) from where they spread rapidly throughout the Southern regions of the USA. Most recently, they have become established in California (Gotelli & Arnett, 2000) while also expanding their range worldwide (Ascunce et al., 2011; Holway, Lach, Suarez, Tsutsui, & Case, 2002).

Infestation has resulted in over $90 million spent by Texas government agencies to control *S. invicta* between 1999 and 2000 (C. Lard, Willis, Salin, & Robison, 2002), which distinguishes them as a significant invasive pest. Even though they are not a major pest of any one crop or commodity, they have impacted sectors of public and medical health, agriculture, urban, wildlife, and environmental systems.

In a public health context, the workers of *S. invicta* are highly aggressive and attack any animal species that disturbs their nest or foraging activities. The workers deter potential threats by biting and administering a venom using their modified ovipositor (stinging) (MacConnell, Blum, & Fales, 1970). Secondary infections sometimes occur,

and occasionally hypersensitivity to the venom has been observed (Griffing, Kwan, Banks, & Dvorak, 1984). A higher incidence of stings have been reported in urban environments where *S. invicta* infest lawns, schoolyards, golf courses, and parks which require rigorous pest control strategies (Drees & Gold, 2003). Electrical and communication equipment can be severely damaged by *S. invicta* chewing on the insulation (C. Lard, Willis, Salin, & Robison, 2002). *S. invicta* also cause problems when they forage inside homes to seek food and moisture (Drees & Gold, 2003).

In an agricultural context, significant damage has been recorded through *S. invicta* feeding on the seeds of soybeans, corn, peanuts, beans, Irish potatoes, and cabbage. The greatest loss of plant production has been observed in soybean through disruption of arable land by the mounds (C. Lard, Willis, Salin, & Robison, 2002). Damage has also been found in citrus where *S. invicta* construct their nests near the tree bases and feed on the bark and cambium to obtain sap which kills new trees (Jetter, Hamilton, & Klotz, 2002). Vegetable production can also be affected by *S. invicta* feeding on developing fruit and seeds. Particularly high losses have been reported in soybeans in California (Apperson & Powell, 1983), and eggplants in Florida (Adams, 1983).

From a wildlife context, *S. invicta* impact populations of reptiles, birds, and mammals (Allen, Demarais, & Lutz, 1994). Allen, C. R., Epperson, D. M., & Garmestani, A. S. (2004) previously noted the impacts of *S. invicta* on birds and concluded they are a major threat to wood duck nestlings, cliff swallows, and act as a major predator of individual birds including prairie chicken, northern bobwhite, and the

scissor tailed flycatcher. They are also implicated in the deaths of newborn mammals through predation, especially rabbits and squirrels (Ferris, Killion, Ferris, Grant, & Vinson, 1998). In summation, the presence of *S. invicta* has major ecological and economic impacts in the areas which they inhabit.

SOLENOPSIS INVICTA COLONY STRUCTURE

Colonies of *S. invicta* are eusocial and composed of individuals that belong to different castes: the reproductive caste which consists of queens, and the worker caste which consists of sterile female workers. The males (drones) typically have a short lifespan, and their only intended purpose is to mate. The reproductive caste functions within a colony to produce offspring and contribute to colony growth (W.R. Tschinkel, 2006). Queens can live for up to eight years (W.R. Tschinkel, 2006).

Colonies develop into two distinct social forms (gynes), determined by a single allelic variation at the *Gp- 9* locus. Monogyne queens, which are homozygous dominant (BB) at the *GP-9* locus, produce colonies populated by BB workers exclusively. While polygyne queens, who are heterozygous (Bb), produce both BB and Bb workers (Dietrich Gotzek & Ross, 2009). Polygyne colonies differ substantially from monogyne colonies in their biology. Polygyne queens may be unrelated but cooperate by co-existing in the nest and sharing the reproductive function for colony growth, while monogyne colonies are more aggressive and kill Bb individuals upon introduction (W.R. Tschinkel, 2006; Valles & Porter, 2003). Differences in this behavior are crucial to understand the invasive success of polygyne colonies in the United States even if within

a polygyne colony, the individual queen fecundity and dispersal are reduced in comparison to monogyne queens (Holway et al., 2002). Large *S. invicta* colonies are exacerbated by their polygyny (the accumulation of multiple mated queens to cooperate) (W.R. Tschinkel, 2006; Tsutsui & Suarez, 2003).

Workers can be used to assess the genotype of the queens because if a monogyne queen (BB) returns to a polygyne (Bb) nest where there are Bb workers the BB queens will be executed.

Workers are the "labor central" of the colony and are responsible for carrying out the tasks that are associated with colony maintenance, survival, and acquisition of territory and resources (W.R. Tschinkel, 2006). Workers are allocated specific tasks that contribute to the growth and survival of the colony. These tasks include foraging for carbohydrate sources or protein sources, acting as guards and defending, nursing, or tending to the queen (Daugherty, Toth, & Robinson, 2011; Sudd & Franks, 2013; W. R. Tschinkel, 2011). In parallel to workers actively performing tasks, there is a category of ants referred to as "reserve". These ants can account for more than 50% of the total workforce and can perform various tasks as needed by the colony contributing to the overall health of a colony (Mirenda & Vinson, 1981). A major question in the study of insect task allocation is: Without a central control, what determines the task allocation of workers? In honey bee, *Apis mellifera,* and in several ant species this happens in an age-dependent manner. Younger workers tend to perform in-nest tasks related to brood care while older workers tend to act as foragers (Gro V Amdam, Csondes, Fondrk, & Page Jr, 2006; Elekonich & Roberts, 2005; Tofilski, 2006). However, differences exist among

species. For example, in *Formica selysi* gene expression profiles and body size are regulated independently, and body size tends to have a stronger influence on the task being performed. Foragers are typically significantly larger than in-nest workers (Schwander, Rosset, & Chapuisat, 2005). The preference tasks being performed by a given worker may also have a direct genetic basis independent of worker size in ant species (E. O. Wilson, 1978) and may occur as a response to a particular stimulus that can generate a specialization in their task in order to match the needs of the colony (G. E. Robinson, 1992). In fire ants, the workers show little variation in anatomical features, but show great range in their morphology, with their size ranging from 2 – 6 mm (E. O. Wilson, 1978). Thus, the workers are differentiated into morphological subcastes determined by head width and size. Majors are the largest workers with a head width between 0.92 and 1.26 mm. Medium-sized workers are the intermediate size workers, with a head width between 0.73 and 0.91 mm. Minors are the smallest workers with a head width below 0.72 mm. The size of the workers is largely subject to the age of the colony and the availability of food (Oster & Wilson, 1979). Changes in morphology between the subcastes of workers during development is a result of hormonal responses to environmental and social cues within the colony (Evans & Wheeler, 2001). This may allow the colony to modify the number of individuals belonging to each subcaste as a means of adaptation to changes both within and outside of the colony (R. E. Page Jr, Robinson, Britton, & Fondrk, 1992). Studies have highlighted a genetic component to the worker size and caste of ants, implying that the number of queens (polygyny) may broaden the phenotype of the colony to change or improve caste ratios (Fraser,

Kaufmann, Oldroyd, & Crozier, 2000; Hughes, Sumner, Van Borm, & Boomsma, 2003). Workers of *S. invicta* from polygyne colonies are smaller and less polymorphic than workers from monogyne populations (Goodisman & Ross, 1996). A correlation between the worker size and labor being performed exists. In general, younger and smaller workers concentrate on in-nest duties such as brood care, while larger and older workers tend to participate in out-of-nest duties such as foraging and defensive behaviors (E. O. Wilson, 1978). Workers of any size are able to switch tasks from source collection to larval care as needed, but smaller workers play a larger role in brood care in which larger workers play only a small role (Cassill & Tschinkel, 1999b). Medium workers are the most versatile, engaging in all colony tasks and displaying considerable variation in the frequency at which they switch from one task to another as the colony requires.

Casual links exist between gene regulation and worker task transition. The *foraging gene (for)* is associated with foraging behavior in numerous insects (Yehuda Ben-Shahar, 2005; C Lucas et al., 2010; Pereira & Sokolowski, 1993), and in social insects is associated with division of labor. In *A. mellifera,* workers express higher transcript levels of *for* compared with nurses (Y Ben-Shahar, Leung, Pak, Sokolowski, & Robinson, 2003). This pattern is not conserved across all eusocial Hymenoptera. In *Pogonomyrmex barbatus* and *S. invicta*, higher expression of *for* is observed in nurses when compared to foragers (Ingram, Oefner, & Gordon, 2005; Christophe Lucas, Nicolas, & Keller, 2015). A multitude of genes play a role in task transition in *A. mellifera*, such as *malvolio,* which is involved in sucrose responsiveness. High expression of *malvolio* is associated with pollen foraging compared with nurses (Yehuda

Ben-Shahar, Dudek, & Robinson, 2004). The insulin-signaling TOR pathway has also been implicated in foraging behavior, where foragers expressed higher levels of insulin/insulin like growth factor genes (*Ilp1* and *InR1*) in the brain and abdomen when compared to nurses (Ament, Corona, Pollock, & Robinson, 2008). In *Polistes metricus*, gene expression within the TOR pathway is also upregulated in foragers with lower nutrient reserves when compared to nurses (Toth et al., 2007). Of particular interest, in *S. invicta* the *foraging* gene has been assessed for its role in task allocation behavior (Bockoven, Wilder, & Eubanks, 2015).

Of particular interest is the role that storage proteins may play in task transition. Storage proteins such as vitellogenin(s) (Vg) and hexamerin(s)are essential in the development of oocytes in queens, yet these genes are also expressed in workers supporting a potentially coopted function of these genes in a caste specific manner.

In *A. mellifera*, Vg is associated with a multitude of physiological functions, such as immunity, aging, reproduction, and the insulin/insulin like growth factor pathway (Gro V Amdam, Norberg, Hagen, & Omholt, 2003; Corona et al., 2013; Corona et al., 2007; Huang & Robinson, 1996). Variation in the titer of Vg is related to worker task and its decrease can trigger the onset of foraging in workers (Guidugli et al., 2005). Silencing of Vg causes the workers to begin precocious foraging and activities related to those of older workers (Antonio, Guidugli-Lazzarini, Do Nascimento, Simões, & Hartfelder, 2008). In *Ectatomma tuberculatum* vitellogenin is associated with worker age polyethism in the colony (Azevedo, Zanuncio, Delabie, & Serrão, 2011). Workers begin their adult life by conducting tasks inside the nest associated with the brood, and

as they age vitellogenin production ends as workers begin to forage (Azevedo et al., 2011).

MOLECULAR BASIS OF LABOR DIVISION; THE REPRODUCTIVE GROUND PLAN HYPOTHESIS

The division of labor between reproductive and non-reproductive individuals in eusocial insects is considered a major event in the evolution of social insect species. Individuals within a colony can develop into the reproductive caste, or they can develop into the non-reproductive workers (Beshers & Fewell, 2001; G. E. Robinson, 1992; E. O. Wilson, 1978). The reproductive ground plan hypothesis (RGPH) suggests a mechanism for generating the division of labor among workers (West-Eberhard, 1987), stating that worker division of labor has evolved through the cooption of genetic and hormonal networks that normally control the reproductive cycle in solitary insects (Page & Amdam, 2007; West-Eberhard, 1987).

The RGPH predicts that reproductive traits can still be associated with workers performing brood care. In *A. mellifera*, nurses retain a higher titer of Vg compared to foragers (Gro V Amdam et al., 2006; Gro V Amdam, Norberg, Fondrk, & Page, 2004; Gro V Amdam, Norberg, Hagen, et al., 2003). In some species, including *S. invicta*, workers have completely lost ovaries. Ant species without ovaries, like *S. invicta*, still exhibit division of labor. In these species, differences in caste specifc phenotypes are likely affected by other components of the reproductive system, such as cooption of hormonal signals typically associated with reproduction in non-eusocial insects. In this

case, the presence or absence of ovaries would prove uninformative in some ant species. It is possible that ants without ovaries appear contrary to RGPH, but in this case their presence may not impact the overall neofunctionalization of other elements of the reproductive system (Tobias Pamminger & Hughes, 2017) and the physiological mechanisms of RGPH could vary between ant species. This difference may be indicative of different eusocial taxa evolving independently. This suggests that ancestral genetic and endocrinological networks which generate behavior and reproduction have been decoupled to generate non-maternal brood rearing behaviors in completely sterile individuals (Tobias Pamminger & Hughes, 2017).

It is unknown if the genetic mechanisms that regulate behavior are conserved across eusocial insects (Corona et al., 2013). One of the individual genes associated with social organization in eusocial insect colonies is Vg. Vg is a phospholipoglycoprotein that serves as the precursor for vitellin, a yolk protein, which is conventionally known for its role in female reproduction in insects being the source for embryonic nutrients (Tufail & Takeda, 2008). Vg is mainly synthesized in the fat body of females, but in social insects the production of Vg is not exclusive to the reproductive individuals (queens) and occurs in the sterile worker caste (Guidugli et al., 2005; Teresa Martinez & Wheeler, 1991; Claire Morandin et al., 2014; Seehuus, Norberg, Krekling, Fondrk, & Amdam, 2007), and in some social insects it has been found in the males (Piulachs et al., 2003). In *A. mellifera*, Vg expression in workers is related to (but not exclusive to) a specific task, and is expressed higher in nurses compared to foraging workers (Gro V Amdam, Norberg, Hagen, et al., 2003).

Different numbers of Vg genes are found among insect species. While one gene is present in the *A. mellifera* genome (Kent, Issa, Bunting, & Zayed, 2011), several copies are encoded in many ant species, including in *S. invicta* (Wurm, Wang, & Keller, 2010). Duplication and neofunctionalization of this gene could be involved in social organization. This can perhaps be explained by the greater morphological and physiological differences between workers and queens of *S. invicta* compared to *A. mellifera*, and the sterility of workers in many ant species (Corona et al., 2013; D. Gotzek, Robertson, Wurm, & Shoemaker, 2011; Hölldobler & Wilson, 1990).

THE INFLUENCE OF THE BROOD ON WORKER BEHAVIOR

The immature stages of *S. invicta*, the eggs, all larval instars, and the pupae, are collectively known as the brood. The foraging for proteinaceous sources is largely determined by the presence or absence of brood. Carbohydrate rich foods are mainly utilized among workers, proteinaceous foods are directed toward the larvae and the queen. This selective distribution of nutrients can be correlated with the nutritional needs of the brood and queen since they both require protein for structural growth as well as egg production in queens, and workers require higher amounts of carbohydrates for energy (Howard & Tschinkel, 1981). The brood, both type and amount, influence the task allocation of the workers. The fourth instar larvae are the only members of the colony that can metabolize solid protein sources for the rest of the colony to utilize (Petralia & Vinson, 1978). In contrast to other instars, the fourth instar larvae are morphologically adapted to feed on solid food, with a specialized hairless region located

anterior-ventrally on the body (O'Neal & Markin, 1973). In fact, the fourth instar larvae feed on anything placed in their anterior-ventral region. Solid protein metabolized by the fourth instar larvae is turned into a liquid source that can be utilized by the entire colony, therefore influencing the types of food being foraged by the workers. The nutrition of the colony is controlled through a chain of command where forager hunger determines the rate of food transported to the nest and larval hunger determines the rate that nutrients can be utilized by the colony (Cassill & Tschinkel, 1999b).

The process in which the workers respond to the brood has been investigated in *A. mellifera* and numerous ant species, which unlike *S. invicta* workers which are completely sterile, can produce fertile or trophic eggs. Thus, the expression of Vg in this caste suggests that in this species, Vg genes have been co-opted and acquired non-reproductive functions. For example, brood pheromone can affect the transition in task allocation, such as the onset of foraging as it can stimulate the foraging activity of bees competent to forage, and also the type of foraging they conduct (protein or carbohydrate biased) (Tanya Pankiw, 2004). Further, brain responses to brood pheromone were identified based on microarray studies (Alaux et al., 2009). These studies support the idea that the brood has an influence in the behavior of the workers and that this influence occur thorough changes in gene expression in the brain.

TASK ALLOCATION SIGNALING IN THE BRAIN OF WORKERS

Social insects require cognitive abilities that allow them to communicate extensively, navigate during foraging, and explore their environment. These social behaviors are important for survival of the colony. Insect brains are structurally different from mammalian brains, but the basic demands are the same. Social insects, particularly *A. mellifera*, have been a valuable model in studying neural pathways, learning, and memory in social behavior (Menzel, Leboulle, & Eisenhardt, 2006). Studies in *A. mellifera* have also related structural changes in the mushroom body, the area of the brain associated with learning and memory, with a change with task transition (Farris, Robinson, & Fahrbach, 2001). In social insects, even those which have evolved eusociality independently, common pattern of gene expression can be observed. For example, gene expression changes in response to food availability operates similarly in *A. mellifera* and *P. metricus* (Daugherty et al., 2011). Genes involved in insulin signaling, insulin-like peptide 2 (*ILP2)* and juvenile hormone signaling, ultraspiracle (*USP*) were found to be expressed at a lower level in starved individuals of *P. metricus*. In contrast to *A. mellifera*, no relationship was found between foraging behavior and vitellogenin at the whole body level, but higher levels of *PmVg* were found in response to a period of food deprivation (Daugherty et al., 2011).

In *A. mellifera* workers, Vg can be taken up by the hypopharyngeal glands in the head of nurses which functions to synthesize food for the brood (Gro V Amdam, Norberg, Hagen, et al., 2003; Seehuus et al., 2007). Although studies have suggested specific localization of Vg in the non-neuronal glial cells in *A. mellifera* nurses, no

synthesis of Vg has been reported in these cells (Münch, Ihle, Salmela, & Amdam, 2015). It is likely that glia-specific Vg accumulation relies on an uptake mechanism that is not shared with other cells in the brain. This is consistent with the hypothesis that Vg is synthesized elsewhere before being transported (Münch et al., 2015). It is therefore implied that Vg might affect behavior at the intersection of the head, fat body, and the brain. The *A. mellifera* worker transition in task (nest living vs foraging) is mediated by the inverse relationship between JH and Vg and this has been associated with transcriptional, physiological, and metabolic changes in the fat body and the brain (Nunes, Ihle, Mutti, Simões, & Amdam, 2013).

THE INTERPLAY BETWEEN VITELLOGENIN AND JUVENILE HORMONE

Juvenile hormone (JH) is a sesquiterpenoid that regulates insect development and reproduction in virtually all insect species (Hansen, Attardo, Rodriguez, & Drake, 2014). JH in adult insects also plays a role in un-modulating the response to environmental cues and maintaining queen longevity.

In *A. mellifera* workers, a behavioral switch in task from nursing to foraging for pollen and nectar is accompanied by an increase in JH titer and a decrease in Vg protein level (Gro V Amdam, Norberg, Hagen, et al., 2003; Bloch, Hefetz, & Hartfelder, 2000; Bloch, Wheeler, & Robinson, 2002; Guidugli et al., 2005). The unconventional pattern of JH and Vg in the workers of *A. mellifera* has suggested that both are involved in worker task transition and may be an important pacemaker of worker development (Fahrbach, 1997). In *A. mellifera*, the nurse bees produce brood food from their

hypopharyngeal glands, and the conversion of yolk protein for larval food would be compatible with the physiological state of nurse bees which have high Vg and low JH (Gro V Amdam & Page Jr, 2010; Seehuus et al., 2007). Earlier studies indicate that when JH is applied to newly eclosed adult *A. mellifera*, there is a precocious transition to foraging (Jaycox, 1976). The relationship between JH and Vg has fit into a mathematical model of the "double repressor hypothesis" which states that Vg and JH would mutually suppress each other (Gro V Amdam, Norberg, Hagen, et al., 2003). In the case of the workers, they would quickly shift to a new state in the event of an elevated concentration of either JH or Vg which results in rapid change of task fixation (nursing or foraging). Higher concentrations of Vg in the nurses would therefore repress the increase of the JH titer, and the behavior performed would be dependent on the amount of Vg in hemolymph (Antonio et al., 2008; Page & Amdam, 2007). Further studies have shown that Vg knockdown by RNAi results in a significant stable increase in the JH titer in worker *A. mellifera* independent of social setting (Antonio et al., 2008; Guidugli et al., 2005). The knockdown of Vg causes an increase in JH titer accompanied by the increase in *usp*, a candidate JH receptor (Guidugli et al., 2005).

Similarly in *E. tuberculatum*, JH application to the adult worker causes a decrease in the levels of Vg, showing that production of the Vg protein is downregulated by circulating JH (Azevedo, de Paula, Zanuncio, Martinez, & Serrão, 2016). Depletion of Vg in the hemolymph of *E. tuberculatum* is associated with a decrease in the protein synthesis in the fat body and a reduction in Vg gene expression, and this is coupled with the transition from nurses to foragers (Azevedo et al., 2011; de Azevedo & Hartfelder,

2008). In *Pogonomyrmex californicus,* JH is more abundant in foragers when compared to workers inside the nest, and while age may influence the endocrine state to indirectly alter behavior, in this species JH was the main component of foraging behavior suggesting foraging behavior is not strictly associated with age (G. E. Robinson, 1992). In these two ant species, workers make nutritive eggs, or trophic eggs which may function analogously to the hypopharyngeal glands of *A. mellifera* (Gro V Amdam, Norberg, Hagen, et al., 2003) where they are used to store and utilize Vg. Thus, it appears that similar to *A. mellifera*, these ant species have evolved mechanisms for exploiting Vg as a source of nourishment.

JH has been associated with division of labor in the workers of many Hymenoptera species including *A. mellifera, Streblognaths peetersi, Polistes sp.,* and *P. californicus* which indicates that these insects might share a similar physiological mechanism in the sterile workers. This mechanism may also be different based upon the reproductive capabilities of the workers. For example, Vg reduction in workers of *E. tuberculatum* that are producing trophic eggs was observed after JH application (Azevedo et al., 2016; Azevedo et al., 2011). In contrast, *S. invicta* workers are completely sterile. A gonadotropic role for JH has been identified in *S. invicta* queens which is related to the synthesis of Vg and uptake into developing oocytes (C. S. Brent & Vargo, 2003; Burns, Teal, Vander Meer, Nation, & Vogt, 2002; M. E. Chen, Lewis, Keeley, & Pietrantonio, 2004). The *S. invicta* queen displays a peak of JH in the hemolymph during the oviposition period (C. S. Brent & Vargo, 2003).

CHAPTER II

EXPRESSION ANALYSIS OF VITELLOGENINS IN THE WORKERS OF THE RED IMPORTED FIRE ANT, (*SOLENOPSIS INVICTA*)*

INTRODUCTION

Division of labor and foraging specialization are a key characteristic of the eusocial insect colony structure. Many factors can influence division of labor in insect societies, such as morphology, genetic variation, developmental and nutritional factors, experience, and age (Corona et al., 2013; Kohlmeier et al., 2017; Page & Robinson, 1991; G. E. Robinson, 1987; Toth & Robinson, 2007). In social insect colonies, vitellogenin (Vg) may control the division of labor, social behavior, and colony function (Beshers & Fewell, 2001; Guidugli et al., 2005; Nelson, Ihle, Fondrk, Page Jr, & Amdam, 2007; C. R. Smith, Toth, Suarez, & Robinson, 2008). Indeed, in the honey bee worker caste Vg is involved in royal jelly production (Gro V Amdam, Norberg, Hagen, et al., 2003) age polyethism regulation (Antonio et al., 2008), antioxidant and immunity regulation, and insulin/insulin-like signaling which controls growth, aging, and reproduction (Gro V Amdam et al., 2006; Keller & Jemielity, 2006). This relationship between Vg and task has also been recognized across species of ants (Corona et al., 2013; Graeff, Jemielity, Parker, Parker, & Keller, 2007; Hartmann & Heinze, 2003; Teresa Martinez & Wheeler, 1991).

Insects encode a variable number of Vg genes depending on the species. In social insects, difference in Vg expression among castes could play a key role in social organization as proposed by the ovarian ground plan and genetic "toolkit" hypotheses (Toth & Robinson, 2007; West-Eberhard, 1987). Similarly, Vg duplication and subsequent subfunctionalization could be linked with Vg's role in social organization. Differences in Vg and Vg-like gene expression between queens and workers were identified in several ant species (Feldmeyer, Elsner, & Foitzik, 2014; Claire Morandin et al., 2014; Oxley et al., 2014) and differences in Vg expression between workers performing different tasks also exist (Corona et al., 2013). Two Vg subfamilies were identified in the formicoid clade, subfamily A are Vg genes expressed at higher level in queens than in workers, and subfamily B are Vg genes preferentially expressed in workers (Corona et al., 2013). The Red imported fire ant (*Solenopsis invicta* Buren; Hymenoptera: Formicidae) genome harbors four adjacent Vg genes (*Vg1*, *Vg2*, *Vg3*, and *Vg4*) (Wurm et al., 2011), *Vg1* and *Vg4* clustering in the B subfamily and *Vg2* and *Vg3* clustering in the A subfamily (Corona et al., 2013). Previously, it was established that *Vg1* and *Vg4* transcripts are highly expressed in workers, while *Vg2* and *Vg3* are expressed at higher level in queens (Wurm et al., 2011), and SDS–PAGE analyses determined that Vg proteins are present in the hemolymph of reproductive queens, virgin alate queens, and workers (Lewis et al., 2001). However, the expression of these genes among task-allocated workers or different subcastes has not been studied.

As for other eusocial insects, *S. invicta* colonies are composed by individuals belonging either to the reproductive caste (queens) or the worker (sterile) caste. The

workers are central to colony maintenance and growth, their labor in turn is a central resource through which ants acquire a territory, defend it, and search for food sources to distribute to the entire colony. The workers show little variation in anatomical features, but are highly variable in body length ranging from 2 to 6 mm and are further categorized into subcastes according to their head width and size; these subcastes are known as major, medium, and minor workers (W.R. Tschinkel, 2006). The size of the workers depends upon the age of the colony, founding status of a colony, and it may also vary with food availability (W.R. Tschinkel, 2006). When colonies are newly established and small, workers are monomorphic and consist exclusively of minors, but as the colonies increase in size and age, approximately one month after colony establishment, the variation in size of the workers becomes increasingly noticeable as does the variation in tasks being performed. As colonies grow, the worker population shifts to an even larger fraction of large workers, suggesting that such shifts in the labor economy could be important to colony growth and reproduction (W.R. Tschinkel, 2006). While the molecular basis of task allocation in fire ants has not been elucidated, a correlation between the worker size and the labor performed was described, and in general younger and smaller ants concentrate more on brood care while older and larger ants tend toward foraging (E. O. Wilson, 1978). Medium sized workers are the generalists, performing nearly all colony tasks. They are the most versatile and can engage in recruitment to food sources, larval grooming, and larval feeding. They display considerable variation in the frequency in which they feed larvae before switching to other tasks, which suggests flexibility in tasks (Cassill & Tschinkel, 1999b). The major workers play a small role in

larval care, while the minor workers play a larger role. In addition to nurses and foragers, a third group of workers of heterogeneous age, size, and behavior exists, these ants are denominated as reserves, and are a transitional group from nurses to foragers (Mirenda & Vinson, 1981). This group does not conduct any specific task, but may nurse, forage, store liquid food, and/or relay food between nurses and foragers (Mirenda & Vinson, 1981; W.R. Tschinkel, 2006). Depending on the size of the colony, reserves might represent up to 30% of the workers (W.R. Tschinkel, 2006; E. O. Wilson, 1978). The amount of activity undertaken by each individual worker is different. On average, workers performing specific tasks were inactive 20 min of 30 min observation intervals (Mirenda & Vinson, 1981). Studies analyzing crop content of workers of a monogyne colony, a colony with only one mated queen, determined that workers of any size could switch tasks to food storage and source collection from larval care (Cassill & Tschinkel, 1999b). Differences in Vg expression between queens and workers exist in *S. invicta*, however, it is unknown whether there are there differences in Vg expression among the morphological worker subcastes, and/or among workers performing different tasks.

In many insects, juvenile hormone (JH) regulates Vg expression (Tufail, Nagaba, Elgendy, & Takeda, 2014). In social Hymenoptera, JH is involved in reproductive division of labor as well as in worker age-related division of labor, and differences in the role of JH between primitive and advanced eusocial species exist. For instance, in *Bombus terrestris*, a primitive eusocial insect in which JH retains its gonadotropic role, no changes in worker Vg expression were measured following JH application (Amsalem, Teal, Grozinger, & Hefetz, 2014). On the other hand, an inverse relationship

between JH and Vg exists in honey bee workers, and this relationship is involved in the behavioral switch as workers age: JH increases as a worker transitions from nursing to foraging while Vg protein level decreases. Furthermore, Vg is involved in the control of JH synthesis and the feedback loop between JH and Vg regulates the onset of the foraging behavior (Gro V Amdam et al., 2005; Gro V Amdam, Norberg, Hagen, et al., 2003; Gro Vang Amdam & Omholt, 2002; Antonio et al., 2008; Guidugli et al., 2005). Overall, few studies have assessed the role of JH in Vg regulation in workers. For example, topical application of JH to non-reproductive *Ectatomma tuberculatum* workers resulted in the downregulation of Vg protein synthesis and reduced Vg titers in the hemolymph (Azevedo et al., 2016). While JH has retained its gonadotropic role in the queen (M. E. Chen et al., 2004), it is widely unknown if the interplay of JH and Vg is important in regulation of tasks in workers of *S. invicta*, or whether Vg expression is regulated by JH in workers.

S. invicta is an ideal species to evaluate the role of Vg in task allocation because the workers are sterile, and therefore the role of these genes in reproduction and task allocation can be decoupled. The goals of this study were: (1) to investigate the expression of the four Vg transcripts in the three worker subcastes (minor, medium, and major) of *S. invicta*; (Kemp, deShazo, Moffitt, Williams, & Buhner) to investigate the expression of the four Vg transcripts in workers performing different tasks (nursing, foraging carbohydrates, and foraging proteins); and (3) to identify the potential role of JH in the expression of each of the four Vgs using topical applications of a JH analog.

MATERIALS AND METHODS

Insect colonies

Polygyne colonies of *S. invicta* were collected in Brazos County, TX from May to July 2015 and maintained in the laboratory in plastic trays (27 × 40 × 9 cm) with the walls of the containers covered with Fluon (Insect-a-slip; BioQuip products, Compton, CA, USA) in the Department of Entomology at Texas A&M University, College Station, TX, USA. The colonies were maintained at 27 ± 2 °C in a 12:12 h light–dark photoperiod. Ant colonies were provided with a 14 cm diameter petri dish half filled with damp Castone (Dentsply International Inc., York, PA, USA) as a nest area. Ant colonies were fed daily with a 20% honey water solution and crickets, *Acheta domestica*. Water was given ad libitum. Colonies contained mated queens, alate queens, males, brood (eggs, larvae, and pupae), and a polymorphic worker caste.

Classification and selection of worker ants

For subcaste analyses, ants were classified into majors, mediums or minors according to their head width, as previously described by (E. O. Wilson, 1978). Minor workers had a head width smaller than 0.72 mm; medium workers' head width was between 0.73 and 0.92 mm; and major workers had a head width larger than 0.93 mm (Fig. 1).

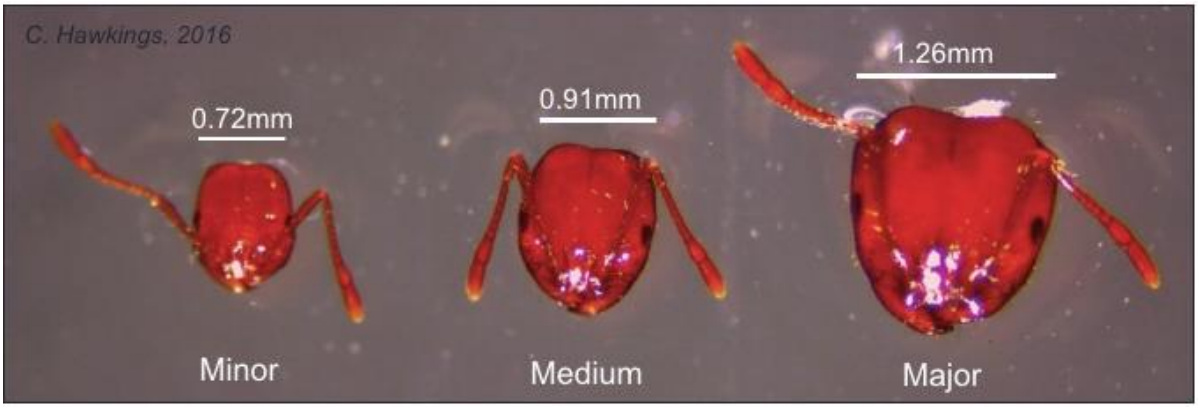

Figure 1: Comparison of the head width among individuals from the worker caste of a single colony (A) Minor, (B) Medium, (C) Major.

For task-allocated ant analyses, medium workers were collected while conducting a specific task: protein foraging, carbohydrate foraging, or nursing. Ants interacting with brood were considered as nurses. Foragers were determined as ants interacting directly with the specific food type. Ants were collected 30 min after the food source was renewed for the day. So, if a worker was found actively collecting food from the protein tray at this time it was classified as a protein forager, if a worker was found on the carbohydrate source it was classified as a carbohydrate forager, and if the worker was in the nest interacting with the brood at the time of food source introduction it was classified as a nurse. Food sources were replaced at approximately 9:00 AM, so ants for all experiments were collected between 9:30 and 10:00 AM.

Five different colonies were used for the morphological caste and task allocation Vg transcript expression assays (five biological replicates each). All replicates were collected within one month; this was done to prevent variations caused by natural circadian rhythms. For each experimental replicate, 10 ants were collected from the same colony. For the task allocation assay, medium workers were selected because of their versatility in the tasks they perform. Pools of 10 workers were flash-frozen in liquid nitrogen upon collection and kept at −80 °C until further use.

Selection of specific primers for each Vg

Primers for each Vg gene were designed using the UGENE software (Okonechnikov, Golosova, Fursov, & team, 2012). The four Vg transcript sequences were aligned using the UGENE alignment tool and specific regions of each Vg were identified. Primers were designed manually to amplify the specific regions. To further verify the accuracy of the qPCR primers (Table 1), DNA was extracted from whole body worker ants using the E.Z.N.A. Insect DNA kit (OMEGA Bio-Tek, Norcross, GA, USA). PCR was conducted using the following optimized temperature parameters: 94 °C for 2 min, then 35 cycles at 94 °C for 15 s, 60 °C for 15 s, and 68 °C for 30 s, followed by a final elongation step of 5 min at 68 °C. The reaction was conducted in a 50 μL volume containing 1× GoTaq Green Master Mix (Promega, Madison, WI, USA) and 0.4 nM of each Vg primer. The PCR products were separated in a 2% agarose gel, and then purified using the PureLink PCR purification kit (Invitrogen, Carlsbad, CA, USA), following the manufacturer's protocol. The amplicons were cloned into the pGEM®-T

easy vector (Promega, Madison, WI, USA). Plasmids were introduced into One Shot® TOP10 Chemically Competent *E. coli* (Invitrogen) and purification was conducted using the PureLink Quick Plasmid MiniPrep Kit (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. Five bacterial colonies were selected from each plate and sent to Eton Biosciences (San Diego, CA, USA) for sequencing. Sequences were analyzed using BLAST which confirmed the correct vitellogenin for each primer set.

Table 1: Primers used for gene expression analysis

Name	Sequence
SiVg1_F	5'-CTTACCATTCTGGCATCACC-3'
SiVg1_R	5'-GGGCAATAACGGACTCTCTG-3'
SiVg2_F	5'-CATGTGGTTCCCTGTCACC-3'
SiVg2_R	5'-GACTCGTCGCTAGGAACCTG-3'
SiVg3_F	5'-TATCGAACGGTCCGTATTCCA-3'
SiVg3_R	5'-TCGTGGATAATTCCGAAACA-3'
SiVg4_F	5'-AGTCGAGCCCCCAAAAGC-3'
SiVg4_R	5'-GATGAGAGCGGGTCCAGTT-3'

Gene expression analysis

Pools of 10 whole body insects were used for gene expression analyses. Pools were used to normalize the natural variation in gene expression in the colony, in addition they yielded a sufficient quantity of RNA for each sample. Insects in each biological replicate were ground in liquid nitrogen with a pestle and mortar into a fine powder. Total RNA extractions were performed using the Trizol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. RNA purification was completed using the Micro prep plus clean up kit (Zymo Research, Irvine, CA, USA) for sample clean-up. The RNA was resuspended in 20 µL of nuclease-free water. Genomic DNA was eliminated with the Turbo DNAse kit (Ambion, Waltham, MA, USA) following the manufacturer's instructions. Total RNA quantity and purity was assessed using an Infinite® 200 PRO NanoQuant (Tecan, Männedorf, Switzerland) and RNA integrity was visualized by electrophoresis using a 2% agarose gel stained with ethidium bromide. For expression analyses, RT-qPCR reactions were performed using the SensiFAST SYBR® Hi-rox one step kit (Bioline, Taunton, MA, USA) according to the manufacturer's instructions. Each reaction contained 50 ng of RNA, 250 nM of forward and reverse primer; and 1× of SYBR Green Master Mix; the volume was adjusted with nuclease-free water to 10 µL. The thermocycler program was 45 °C for 10 min followed by 95 °C for 2 min and 40 cycles at 95 °C for 5 s and 60 °C for 30 s. Real-time PCR assays were performed using an Applied Biosystems ABI 7300 real-time PCR Thermocycler (Applied Biosystems, Foster City, CA, USA) according to manufacturer's recommendations. Reactions for all samples were performed in duplicates with negative

controls for each reaction. The threshold cycle values and the efficiency of each primer set for RT-qPCR were determined using LinRegPCR software (Ramakers, Ruijter, Deprez, & Moorman, 2003) and primer specificity was monitored with the melt curve analysis using the Sequence detection system version 1.4.0.27 (Applied Biosystems, Foster City, CA, USA). The relative expression of each vitellogenin gene was estimated with the delta delta CT method (Schmittgen & Livak, 2008) by normalizing the levels of each Vg transcript to the internal control. Six putative housekeeping genes (ribosomal protein 9 (*RP9*), ribosomal protein L18 (*RP18*), translation elongation factor 1 (*ef1-beta*), actin, glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), and TATA box binding protein (*tbp*) (J. Chen & Zhang, 2013; Wurm et al., 2011) were tested among subcastes. Only *RP18* displayed low variation among groups and was thus used to normalize Vg expression values. For each Vg gene, the samples were calibrated using the minor subcaste or the carbohydrate forager relative expression in the Vg expression studies.

Juvenile hormone analog study

Major workers (1.26 mm head width) were collected and were treated topically on the abdomen with 1 μL of S-hydroprene (Sigma-Aldrich, St. Louis, MO, USA) (25 ng/μL dissolved in 80% acetone and 20% ethanol), a JH-mimic, or with 1 μL of a solvent (80% acetone and 20% ethanol; solvent control) once during the assay. This S-hydroprene dose was similar to that used previously to produce realistic changes in JH titers for other social insect workers (Cahan, Graves, & Brent, 2011; Tobias Pamminger

& Hughes, 2017; Shorter & Tibbetts, 2009). Fresh solutions of S-hydroprene were prepared for each biological replicate. Another group of ants were similarly manipulated but without any topical application (untreated control). After topical application of the S-hydroprene or the control solutions, treated and untreated ants were caged according to their treatment in a container within the original colony. Six different colonies were used as biological replicates for the JH assay (six replicates). After 12 h following treatment, 10 worker ants from each treatment were removed, pooled, flash frozen and kept at −80 °C until further analyses. RNA purification and expression analyses by RT-qPCR of *Vg1*, *Vg2*, *Vg3*, and *Vg4* were performed as previously described. All qPCR reactions were conducted in duplicates and assays were performed in six biological replicates. For the expression analysis, the untreated control was used to calibrate the Vg relative expression. As a positive control to validate the application of the JH analog to *S. invicta*, virgin alate queens were tested following the protocol by Vargo and Laurel (Vargo & Laurel, 1994) since JH application to alate virgin queens causes them to dealate. Twelve hours following the topical application virgin queens were checked for dealation.

Statistical analysis

All data are reported as means; error bars represent standard error of the mean. Statistical analyses were performed with the one-way ANOVA test and Tukey–Kramer post hoc using the JMP Version 13 (SAS Institute Inc., Cary, NC, USA, 1989–2017);

estimated *p*-values were considered significant below the 0.05 threshold.

Protein domain analysis of vitellogenin proteins

Vg protein sequences were downloaded from the NCBI database (ID numbers LOC105205865, LOC105205782, LOC105205783). LOC105205865 appears to encode both Vg1 and Vg4 proteins (see "results" section). Protein domains were identified by searching the NCBI Conserved Domain Database (CDD) (Marchler-Bauer et al., 2014). Signal peptides were identified using SignalP (Petersen, Brunak, von Heijne, & Nielsen, 2011). Protein structure was visualized in IBS (Liu et al., 2015).

RESULTS

Protein domain analysis of the vitellogenin proteins

Three sequences were identified in NCBI as Vg genes, LOC105205865, LOC105205782, and LOC105205783. These genes are annotated as Vitellogenin-1, Vitellogenin-2, and Vitellogenin-3, respectively, and are contiguous in *S. invicta* genome in the location NW_011804688.1. The encoded proteins were 3,312, 1,807, and 1,761 amino acids (AAs) in length, respectively. Further analyses of these proteins revealed that *Vg1* encoded a putative signal peptide predicted to be cleaved at position 29, two Vitellogenin N-terminus (lipoprotein amino terminal region) domains (AAs 34–736 and 1684–2,389), two domains of unknown function (DUF1943) (AAs 770–1,032 and 2,445–2,677), and two von Willebrand factor type D (VWD) domains (AAs 1,428–1,591 and 3,079–3,247) (Fig. 2). However, *Vg2* and *Vg3* had each a predicted signal peptide

cleaved after AA 16, one Vitellogenin N-terminus domain (AAs 26–751 and 24–730, respectively), one DUF1943 (AAs 784–1,043 and 764–1,016, respectively), and one VWD domain (AAs 1,444–1,615 and 1,397–1,570, respectively). Based on these results, we determined that both *Vg1* and *Vg4* genes were merged under the same ID number and that the *Vg1* and *Vg4* proteins have been merged (accession number XP_011173700.1). Indeed, inspection of the LOC105205865 sequence revealed a 350-base pair gap approximately at position 2,330,945 in the NW_011804688.1 location, therefore we concluded that *Vg1* corresponds to AA 1–1,662, and *Vg4* to AA 1,663–3,312 approximately.

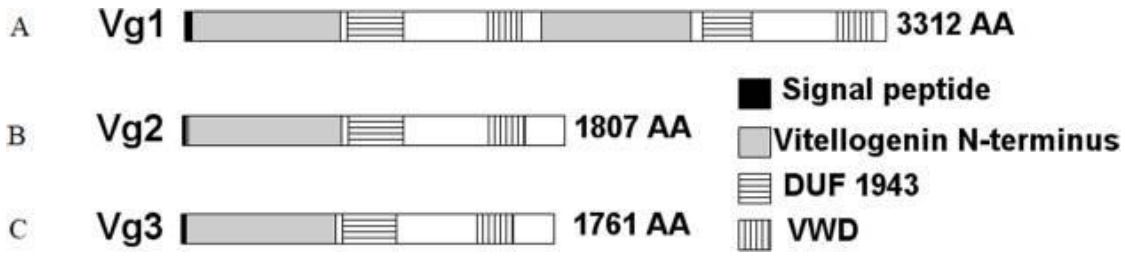

Figure 2: Structural domains identified in the *S. invicta* predicted Vg proteins, (A) Vg1, (B) Vg2, (C) Vg3.

Each protein encoded a putative cleaved signal peptide (black). These proteins possess the LPD-N (gray), DUF1943 (horizontal stripes), and VWD (vertical stripes) domains commonly found in Vg proteins. While *Vg2* and *Vg3* possessed one of each domain, *Vg1* possessed two. Therefore, *Vg1* might encode *Vg1* and *Vg4*.

Vitellogenin expression in morphological subcastes

The expression profile of each Vg transcript (*Vg1*, *Vg2*, *Vg3*, and *Vg4*) was evaluated in pools of 10 ants from each morphological subcaste of the worker caste conducting the task of carbohydrate foraging by RT-qPCR (Fig. 3). Differences of expression among the subcastes were identified for *Vg1* ($F = 20.93$, df = 2.00, $p = 0.0001$) and *Vg2* ($F = 10.03$, df = 2.00, $p = 0.0027$). On average, expression of *Vg1* was 4.6- and 2.9-fold higher in major workers relative to minor and medium workers, respectively. There were no significant differences ($p > 0.05$) in *Vg1* expression between minor and medium ants. On average, *Vg2* was 4.8-fold higher in major workers than in minor workers. No differences in the expression of *Vg2* were measured between medium and minor workers or between medium and major workers. No differences in the expression of *Vg3* and *Vg4* were measured among the morphological worker subcastes.

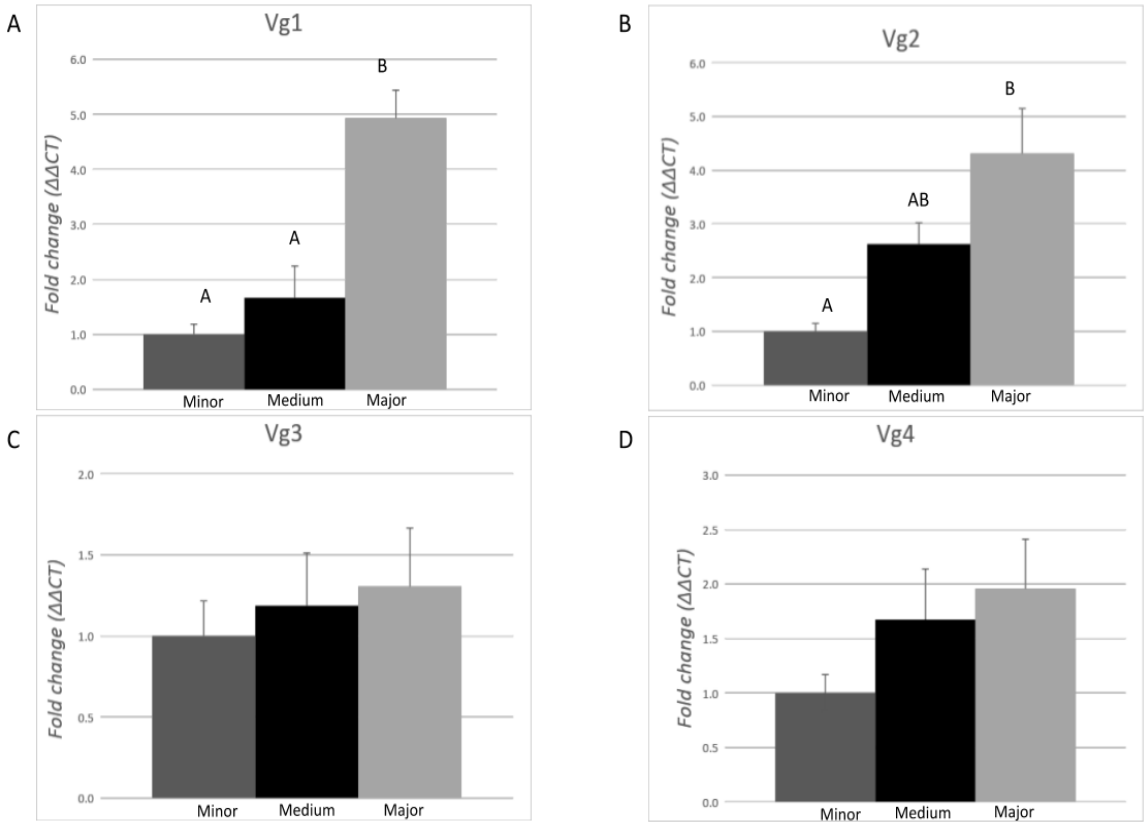

Figure 3: Expression analysis of the vitellogenin transcripts among the worker morphological subcastes.

Each bar represents the mean ± SEM (n = 5). All workers were carbohydrate foragers. (A) *Vg1* transcript expression. (B) *Vg2* transcript expression. (C) *Vg3* transcript expression. (D) *Vg4* transcript expression. Vg mRNA expression level was normalized relative to RP18 mRNA expression level. Statistical relationships between groups were assessed using one-way ANOVA with Tukey–Kramer post hoc test ($p < 0.05$), where different letters indicate statistical differences among the subcastes.

Vitellogenin expression in task allocated medium workers

The expression level of each Vg transcript was evaluated in task-allocated medium workers performing specific tasks: nurses (non-foraging), carbohydrate foragers and protein foragers (Fig. 4). Differences of expression among the ants performing specific tasks were identified for *Vg1* ($F = 14.90$, df = 2.00, $p = 0.0006$). On average, expression of *Vg1* was 2.4- and 1.5-fold higher in carbohydrate foragers relative to nurses and protein foragers, respectively, while no differences of *Vg1* expression were measured between protein foragers and nurses. There were no differences in *Vg2, Vg3*, or *Vg4* relative expression among the task-allocated medium workers.

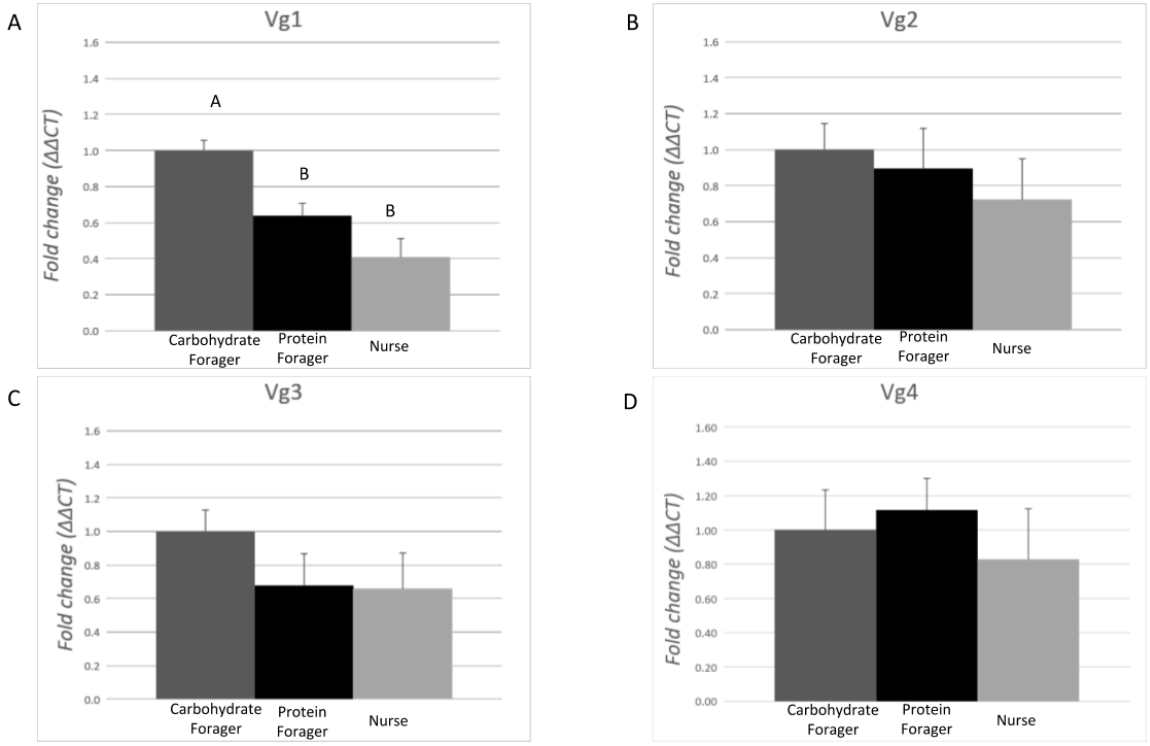

Figure 4: Expression analysis of the vitellogenin transcripts among medium workers performing different tasks.

Each bar represents the mean ± SEM (n = 5). (A) *Vg1* transcript expression. (B) *Vg2* transcript expression. (C) *Vg3* transcript expression. (D) *Vg4* transcript expression. Vg mRNA expression level was normalized relative to RP18 mRNA expression level. Statistical relationships between groups were assessed using one-way ANOVA with Tukey–Kramer post hoc test ($p < 0.05$), where different letters indicate statistical differences among the different task-allocated insects.

Vitellogenin expression in major workers after application with S-hydroprene

No changes in the expression of any of the Vg transcripts were measured 12 h after the topical application of S-hydroprene in task-allocated (carbohydrate foragers) major workers (Fig. 5). No significant differences were found between the non-treatment control and acetone, and no significant differences were found between S-hydroprene and acetone or S-hydroprene and the control treatment ($p > 0.05$).

Topical application of S-hydroprene resulted in 100% dealation of virgin queens, while no virgin treated with the acetone control solution or in the untreated control dealated (Fig. 6).

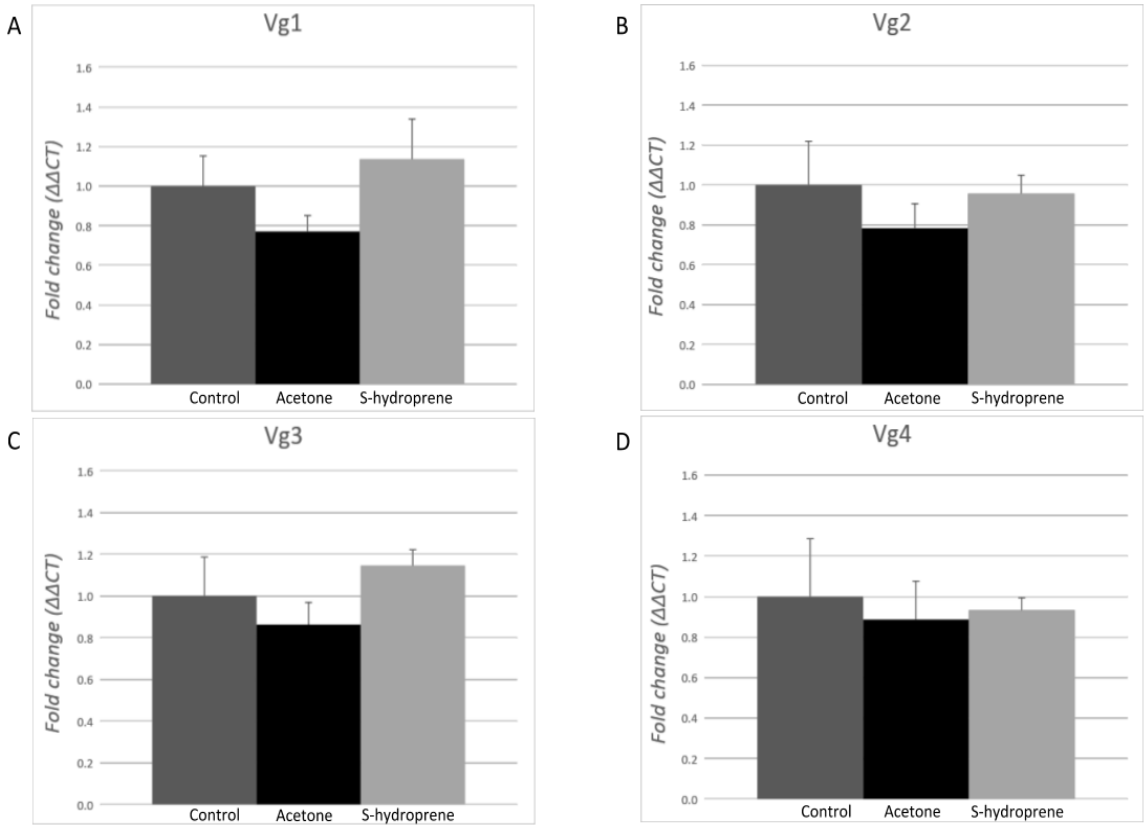

Figure 5: Expression analysis of the vitellogenin transcripts among major workers 12 h after topical application of S-hydroprene.

Each bar represents the mean ± SEM (n = 6). (A) *Vg1* transcript expression. (B) *Vg2* transcript expression. (C) *Vg3* transcript expression. (D) *Vg4* transcript expression. Vg mRNA expression level was normalized relative to RP18 mRNA expression level. Statistical relationships between groups were assessed using one-way ANOVA; no significant differences were found for any of the Vg transcripts ($p > 0.05$).

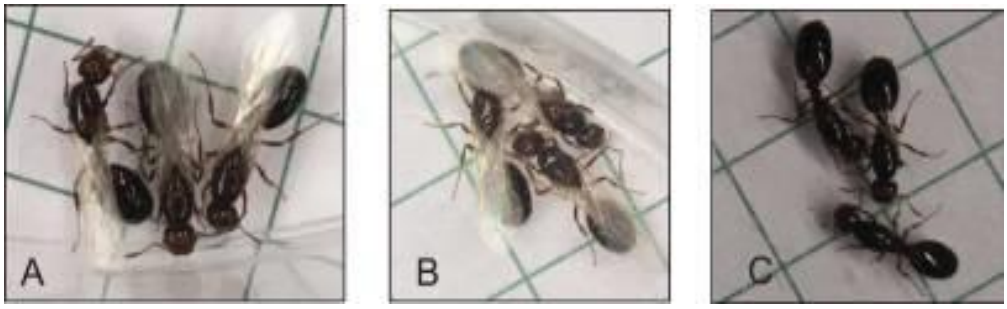

Figure 6: Evaluation of S-hydroprene application and effect in alate virgin queens 12 h after treatment.

(A) Untreated alate queens, 0% of the tested virgins dealated. (B) Alate queen treated with acetone, 0% of the tested virgin dealated. (C) Alate queens treated with S-hydroprene, 100% of the tested virgins dealated.

DISCUSSION

The present studies were undertaken to characterize the expression of the four Vg genes in *S. invicta* workers as a first step to investigating the functional role they may play in the worker caste. Changes in Vg sequence following duplication could be related to neofunctionalization in social insects. Because sequence evolution is faster in genes with a caste-biased expression in *S. invicta* than in genes with unbiased expression (B. G. Hunt et al., 2011), it was important to evaluate if the *S. invicta* Vg genes encoded the typical Vg domains. The four predicted *S. invicta* Vg genes encoded the typical Vg domains, lipoprotein N-terminal domain, DUF1943, and VWD. The roles of these

domains have not been identified so far, however, DUF1943 and VWD can recognize lipopolysaccharides and lipoteichoic acid from bacteria and may be involved in pattern recognition and Vg's role in immunity (Sun, Hu, Liu, Gao, & Zhang, 2013), while the lipoprotein N-terminal domain is involved in the interaction with the Vg receptor (Li, Sadasivam, & Ding, 2003; Roth et al., 2013). Since *S. invicta* workers do not have ovaries and lack the ability to lay either reproductive or trophic eggs (Khila & Abouheif, 2008), the role of Vg proteins in the worker caste remains unknown.

In the present study, differential expression of Vg genes among workers of different sizes (subcaste) and workers performing specific tasks were identified. *Vg1* was up-regulated in major workers compared to medium and minor workers, while *Vg2* was up-regulated in major workers compared to minor workers. *Vg1* was also up-regulated in carbohydrate foragers when compared with nurses and protein foragers. These results suggest that *Vg1* after gene duplication and subfunctionalization could have acquired a subcaste- and task-related expression in workers. Our studies also indicated that *Vg2* had a subcaste-related expression profile but no differential expression among task-allocated workers.

Two points need to be highlighted. First, Vg is expressed in the fat body (Arrese & Soulages, 2010; Corona et al., 2007), therefore the higher expression in major workers could be related to relative different amounts of fat body among the subcastes. However, in that scenario all Vgs should be expressed at a higher level in majors than in minors, which was not the case. Therefore, the up-regulation of *Vg1* and *Vg2* in major workers compared to smaller ants could be associated with functional differentiation in the fat

body as reported for *Monomorium pharaonic* (Jensen & Børgesen, 2000). Second, based on the phylogenetic relationship of *S. invicta* Vg proteins and on previous analyses of Vg expression in fire ant queens and workers (D. Gotzek et al., 2011), similar expression profiles might have been expected for *Vg1* and *Vg4* (subfamily Vg B) and for *Vg2* and *Vg3* (subfamily Vg A). However, the expression pattern in workers was different between the two most similar (most recent duplications) genes.

Differential expression of Vg genes between queens and workers were reported (D. Gotzek et al., 2011), with *Vg2* and *Vg3* being up-regulated in queens compared to workers, while *Vg1* and *Vg4* are up-regulated in workers compared to queens, which is consistent with the loss of reproductive constraints and evolution of new functions. *Vg3* is shown to be consistently similar throughout subcastes and tasks indicating that this gene could perhaps have a preferential functional role in ovarian activity. Previous studies in *Pogonomyrmex* sp. indicated that Vg expression was differential between foragers and nurses (Corona et al., 2013; Keller & Jemielity, 2006). In *Temnothorax longispinosus Vg2* and *Vg3* are up-regulated in queens compared to foragers, *Vg1* is up-regulated in foragers and infertile workers, while foragers have reduced expression of *Vg2*, *Vg3*, and *Vg6* (Feldmeyer et al., 2014). These results are inconsistent with our study as protein forager expression profiles were shown to be more closely related to nurses than to carbohydrate foragers. A potential explanation for this would be that *Pogonomyrmex* sp. workers can produce trophic eggs which are thought to be the main method of nutrient distribution because trophallaxis has not been observed in this species (Corona et al., 2013). Another recent study in *E. tuberculatum* showed that

nurses have significantly increased ovarian activity compared with foragers, suggesting that trophic eggs are produced by the nurses which could ultimately result in the differential expression of Vg in that species (Azevedo et al., 2016). This pattern of nutrient sharing differs from *S. invicta* where the solid protein food sources must be provided to the fourth instar larvae for digestion before redistribution to the remainder of the colony.

Juvenile hormone appears to have maintained its gonadotropic role in primitive eusocial wasps and fire ants but not in other advanced eusocial insects and some other ant species (Bloch et al., 2000; G. E. Robinson & Vargo, 1997). In adult *A. mellifera* workers, an increase in JH titer in the hemolymph is related to lower Vg titers (Edwards, 1975; Excels, 1974) and topical application of JH inhibits Vg expression (Corona et al., 2007). Our data indicate that *S. invicta* does not follow this pattern of an inverse relationship observed in the honey bee and other ant species (Azevedo et al., 2016; C. Brent, Peeters, Dietmann, Crewe, & Vargo, 2006). The JH analog, S-hydroprene, had no effect on the expression of the four Vgs 12 h after topical application on the abdomen of workers. Previously, it was reported that topical application of a JH analog to virgin queens resulted in queen dealation coupled with some degree of ovary development 8 and 12 h after JH analog application to queens (Vargo & Laurel, 1994). It was suggested that in *S. invicta* queens, Vg is constitutive, while yolk formation is regulated through the level of Vg uptake into the oocyte, rather than at the level of Vg synthesis by the fat body (M. E. Chen et al., 2004). Also, topical application of S-hydroprene to queens resulted in dealation and down-regulation of a hexamerin-like

gene 12 h after treatment (Calkins et al., 2018). Similar dealation results were obtained when we performed the applications of S-hydroprene to queens, which validated our method of JH analog application for the workers. However, visible physiological changes are undetectable in workers because of their lack of wings and ovaries. Our study could also suggest that increased JH may be involved with uptake of the protein rather than increased transcript, consistent with previous studies on the queen in *S. invicta* (C. S. Brent & Vargo, 2003; Lewis et al., 2001), which would result in no change at the transcript level. This result could be indicative of the functional component of the duplicated Vg genes. *Solenopsis invicta* have four Vg genes while *A. mellifera* have only one, and the inverse relationship of Vg and JH may be correlated with reproductive capabilities. Our findings open a new avenue to test whether JH has a different regulatory pathway, and its potential influence on age polyethism in *S. invicta.* This study was aimed at evaluating the expression of Vg genes in the whole body of *S. invicta* task-allocated and different subcaste workers. Future studies should analyze the expression of each Vg in specific body regions (Seehuus et al., 2007) of queens and workers and should also explore the potential relationship of JH and Vg in specific body regions or tissues.

CONCLUSION

In conclusion, the results of this study suggest that *Vg1* is correlated to both subcaste size and task allocation, suggesting that it could have been co-opted to regulate behavior. *Vg2* is correlated with subcaste size, potentially suggesting a size-biased

expression in the workers, in particular in the major workers, which have a higher expression than smaller workers. *Vg3* and *Vg4* showed no significant differences in expression among subcaste sizes or task allocation. *Vg1* and *Vg2* expression pattern among subcastes could be consistent with the relative amount of fat body present in the ants. However, this was not the case for *Vg3* and *Vg4*. This result likely reflects the existence of specific regulation of these genes. Furthermore, it is possible that future analyses performed using age-specific workers or increasing the number of replicates analyzed might uncover more subtle differences in Vg gene expression among subcaste or task-allocated *S. invicta* workers. While expression at a whole body level may not be significantly different, further exploration of Vg expression in specific tissues may reveal subcaste- or task-associated changes in workers. Application of a JH analog had no significant effect on the expression of any of the four Vg genes in workers 12 h after topical application. Overall, these results might support the co-option of reproductive pathways to regulate the behavior of the sterile worker caste, however the role of Vg in the *S. invicta* workers still needs to be elucidated.

CHAPTER III

CASTE DIFFERENTIAL HEXAMERINS EXPRESSION IN RESPONSE TO A JUVENILE HORMONE ANALOG IN THE FIRE ANT (*SOLENOPSIS INVICTA*)

INTRODUCTION

Eusocial insects utilize caste-based systems of division of labor to ensure colony success. The reproductive ground plan hypothesis proposes that the gene networks that regulate foraging behavior and reproductive female physiology in social insects emerged from ancestral networks of genes and endocrine factors (Gro V Amdam et al., 2004; Barden, Held, & Graham, 2011; Wang et al., 2010). Examples of this co-option of gene networks to regulate division of labor have been shown in several social hymenopteran species (Dolezal, Brent, Gadau, Hölldobler, & Amdam, 2009; Nelson et al., 2007; Rueppell, Hunggims, & Tingek, 2008; A. A. Smith, Hölldober, & Liebig, 2009). One of the genes recognized for its co-optive role is vitellogenin (Vg). Vg encodes a soluble, yolk protein precursor involved in the production of eggs in oviparous species. In general, Vg protein is synthesized and secreted by the fat body, and processed into vitellin, which accumulates in the developing oocytes *via* receptor-mediated endocytosis (Hansen et al., 2014; Lu, Vinson, & Pietrantonio, 2009). These proteins provide the sustenance and nutrients for the developing embryo (Hansen et al., 2014). This conventional role of Vg has been documented in a wide array of insect orders (Tufail & Takeda, 2008). In social insects, Vg expression in the queen is linked with its conventional role in reproduction, while in the workers is linked to social organization

and task allocation (Gro V Amdam, Norberg, Hagen, et al., 2003; Guidugli et al., 2005; Hawkings & Tamborindeguy, 2018; Wurm et al., 2011). In *Apis mellifera*, Vg plays a role in the social organization and age polyethism of workers. Nurses exhibit high Vg protein titers, and as the workers ages, Vg levels decrease and the individuals transition into foraging tasks (Gro V Amdam, Norberg, Hagen, et al., 2003). Furthermore, silencing of the Vg gene in nurses causes precocious foraging (Antonio et al., 2008). Similar studies conducted in several ant species have also identified a potential role of Vg in task allocation and colony organization based on the patterns of expression in task allocated insects or in response to changes in social context. For instance, *Pogonomyrmex barbatus* nurses express higher levels of Vg transcript which decrease as the individual transitions into foraging tasks (Corona et al., 2013).

The behavioral transition of workers, and the dominance position and reproductive status of hymenopteran queens have also been linked to juvenile hormone (JH) titers (Amsalem et al., 2014; Azevedo et al., 2016; Bloch et al., 2000; Norman & Hughes, 2016; T Pamminger et al., 2016; Rembold, Czoppelt, & Rao, 1974), suggesting that JH may play a role in the expression of proteins that will change the behavior or task of an individual (Bloch et al., 2000). JH is a developmental hormone in insects that plays a role in switching the expression of genes at critical periods, such as larval development, reproduction, and task transition in social insect colonies (Nijhout & Wheeler, 1982). The role of JH in social insect age polyethism has been extensively studied in *A. mellifera* (Amsalem et al., 2014; Jaycox, 1976; Rembold et al., 1974; G. E. Robinson & Vargo, 1997). In this species, JH level is typically low in nurses, and

increases as the workers transition into foraging tasks.

In addition to Vg, hexamerins, a second family of storage proteins have been also associated with social organization. Hexamerins, also known as larval storage proteins, are synthesized in the fat body, and are secreted and accumulate in the hemolymph of the larvae. Later in development, they are reabsorbed into the fat body, broken down, and incorporated into adult tissues during the pupal molt (Levenbook & Bauer, 1984). In termites, silencing of hexamerins results in a higher proportion of soldiers. In these insects, hexamerins are involved in the regulation of caste determination (Xuguo Zhou, Oi, & Scharf, 2006; X Zhou, Tarver, Bennett, Oi, & Scharf, 2006). This latter function may be through an interaction with JH signaling (Xuguo Zhou, Tarver, & Scharf, 2007). In *Polistes metricus*, a social wasp, insects that will emerge to become gynes have higher levels of Hexamerin 1 protein compared to those that will become workers (Toth et al., 2007). Furthermore, several studies reported differences in hexamerin expression between castes of social Hymenoptera (Colgan et al., 2011; Evans & Wheeler, 1999; Hoffman & Goodisman, 2007; J. H. Hunt, Buck, & Wheeler, 2003). Specifically, in *A. mellifera* two hexamerin genes are expressed in the adult fat body in a caste- and sex-specific manner, with higher expression in workers than in queens (Martins, Nunes, Cristino, Simões, & Bitondi, 2010). In ants, these proteins accumulate during the alate stage of the adult queen life and may serve as amino acid storage (D. E. Wheeler & Buck, 1995). In some species, this storage is critical to the claustral period of colony formation; hexamerins allow queens to produce the first generation of workers without having to leave the nest to forage (Teresa Martinez & Wheeler, 1994; D. Wheeler &

Buck, 1996; D. E. Wheeler & Buck, 1995).

Investigating the expression and function of storage proteins in *S. invicta* is of particular interest because this species displays extreme reproductive division of labor. *S. invicta* colonies consist of two basic caste forms. The worker caste is composed of polymorphic sterile females (Khila & Abouheif, 2008) responsible for the tasks that support the growth and maintenance of the colony, while the reproductive adults are queen(s) and drones which are responsible for producing the offspring. The *S. invicta* genome harbors four annotated hexamerins. Previously, we have shown that when comparing brain transcriptomes of virgin alates and dealate mated queens, the expression of two hexamerins identified as *hexamerin-like* (LOC105192919, hereafter called *hexamerin 1*) and *arylphorin subunit alpha-like* (LOC105192898) is significantly reduced in brains of mated queens (Calkins et al., 2018). In the same transcriptome analysis, no differences in expression were observed for the other two hexamerin genes, *hexamerin-like* (LOC105204474, hereafter called *hexamerin 2*) and *arylphorin subunit beta-like* (LOC105192897). Moreover, the application of the JH analog S-hydroprene decreased the expression of *hexamerin 1* in whole body samples of virgin queens (Calkins et al., 2018). However, the effect of S-hydroprene on the expression of the other hexamerin genes in queens and on the expression of all hexamerins in adult workers were not determined.

Despite the knowledge about the involvement of storage proteins in caste differentiation in social insects, the expression and roles of these genes in *S. invicta* has not yet been investigated. In particular, knowledge gaps exist regarding the relationship

between hexamerin expression and JH, a key regulator of social organization. In this study we evaluated the expression of the four hexamerin genes in *S. invicta* workers and queens and investigated whether those genes were regulated by a JH analog in a caste- or task-specific manner.

MATERIALS AND METHODS

Insect colonies

Polygyne colonies of *S. invicta* were collected in College Station, Brazos county, Texas, from July to September of 2017 and maintained as laboratory colonies in plastic containers (27 x 40 x 9 cm). The inside of these containers was coated with Fluon to prevent ant escape (Insect-a-slip, Bioquip products, CA). The colonies were maintained in the Department of Entomology at Texas A&M University, College Station, Texas at 27± 2 °C in a 12:12 hour dark-light photoperiod. Colonies were provided with half-filled water tubes, blocked with cotton, as a damp nesting area and fed daily with both 20 % honey solution and crickets, *Acheta domestica*. Water was provided *ad libitum*. Colonies contained mated queens (dealate), virgin queens (alate), drones, polymorphic workers and brood (eggs, larvae and pupae).

Phylogenetic analysis

Apis mellifera, Nasonia vitripennis, Camponotus floridanus, Harpegnathos saltator, Acromyrmex echinatior, Atta colombica, and Lasius niger hexamerin proteins were identified using Blastp searches or mining the respective genomes. The sequences

were downloaded in FASTA format and aligned using MAFFT v.7 (Katoh, Standley, & evolution, 2013) using the amino acid alignment default parameters. The alignments were visually assessed using Mesquite v. 3.03 (Maddison & Maddison, 2016) and the sequences were manually trimmed. The phylogenetic tree was reconstructed by Bayesian inference (Mr. Bayes v 3.2.3) (Ronquist & Huelsenbeck, 2003) on the CIPRES supercomputer (Miller, Pfeiffer, & Schwartz, 2010) with four runs of a mixed amino acid model for 1,000,000 generations, and a 10 % burnin. Convergence of the runs were assessed in Tracer 1.6. The consensus tree was visualized in FigTree version 1.4.2 (http://tree.bio.ed.ac.uk/software/figtree/) and rooted *Reticulitermes flavipes* hexamerin proteins as the outgroup.

Classification and selection of ants

Virgin queens (alate) were selected based on the presence of wings. The workers were classified based upon their head width (W.R. Tschinkel, 2006) and for this study only medium-size workers were used (head width between 0.73 and 0.92mm), which are the intermediate size workers within the colony. Foraging workers were individuals in the foraging arena who were actively interacting with food resources outside of the nest. Nurses were individuals inside the nest and actively interacting with the brood (any immature developmental stage).

Juvenile hormone analog study

Alate queens, medium-size workers were collected and treated topically on the

dorsal surface of the abdomen using a pipette with 1 µl of the JH analog, S-hydroprene (analytical standard catalogue number 46426, Sigma-Aldrich, St. Louis, MO) (25 ng/µl dissolved in acetone) or with 1 µl of acetone on the abdomen once during the assay. The S-hydroprene dose was used in previous experiments on ants (Cahan et al., 2011; Hawkings & Tamborindeguy, 2018; Penick, Prager, & Liebig, 2012; Vargo & Laurel, 1994). Fresh solutions of S-hydroprene were prepared before each replicative application. Also included in this experiment was a non-treatment control group, which consisted of ants that were handled similarly to the treatment group but to which no solution was topically applied. Following treatment, ants from each control or treatment group were caged together in separate containers within the original colony. Cages were enclosed with very fine nylon mesh, which separated each group from the rest of the colony. Six replicates were conducted using six independent colonies. Pools of 10 medium-size foragers, medium-size nurses, or virgin queens from each treatment were collected twelve hours after S-hydroprene treatment, flash-frozen with liquid nitrogen upon collection and stored at -80 °C until gene expression analysis. For the queens, only the S-hydroprene treatment group induced dealation, and 100 % of the queens used in this group had completely dealated.

Gene expression analysis

Gene expression analyses from whole ant bodies were conducted using pools of ten individuals from the same colony to account for individual variation and obtain sufficient RNA. Workers were pooled according to the task performed (nursing or

foraging). Pooled virgin queens were of unknown age. Pools were flash-frozen in liquid nitrogen upon collection and stored at -80 °C until gene expression analysis. Insects in each biological replicate were ground in liquid nitrogen to a fine powder using a mortar and pestle. Total RNA extractions were conducted using Trizol reagent (Invitrogen, Carlsbad, CA) following the manufacturer's protocol. Purification of RNA was performed using the Direct-zol microprep kit (Zymo Research, Irvine, CA) for clean-up of the samples. RNA was resuspended in 20 µl of nuclease-free water. Genomic DNA was eliminated using the Turbo DNAse kit (Ambion, Waltham, MA) following the manufacturer's protocol. Total RNA was quantified, and the purity evaluated using the Infinite® 200 PRO NanoQuant (Tecan, Männedorf, Switzerland) and the integrity of total RNA was assessed using gel electrophoresis, visualized on a 2 % agarose gel stained with ethidium bromide.

For gene expression analyses, RT-qPCR reactions were performed using the SensiFAST SYBR® Hi-rox one step kit (Bioline, Taunton, MA) following the manufacturer's protocol. All reactions contained 50 ng of RNA, 250 nM of forward and 250 nM of reverse primers (Calkins et al., 2018) and 1X SYBR Green Master Mix; the volume was adjusted with nuclease-free water to 10 µl. The thermocycler parameters were set to 45 °C for 10 min, then 95 °C for 2 min and 40 cycles of 95 °C for 5 sec and 60 °C for 30 sec. RT-qPCR was performed using the Applied Biosystems QuantStudio™ 6 Flex Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA) according to manufacturer's instructions. Each reaction was performed in duplicate, and negative controls for each reaction were included. Primer specificity was

monitored with a melting curve analysis using the QuantStudio™ software V1.3 (Thermo Fisher Scientific). Relative expression of each hexamerin gene was determined using the ΔΔCt method (Schmittgen & Livak, 2008) by normalizing the level of each hexamerin transcript to the internal control gene, *rp18* which is stable among different *S. invicta* castes (Cheng, Zhang, He, & Liang, 2013). Six independent biological replicates were analyzed; each replicate consisted of ten pooled individuals from the same colony; six different colonies were used in total.

Statistical analysis

The data in this study are reported as means and error bars represent the standard error of the mean. Statistical analyses on data with a normal distribution were conducted using the one-way ANOVA test and Tukey-Kramer Post Hoc with JMP Version 13 (SAS Institute Inc., Cary, NC, 1989-2017) and the estimated *p*-values were considered significant when below the 0.05 threshold. For data with non-normal distribution a log-transformation was conducted before the ANOVA and Tukey-Kramer Post Hoc tests were conducted.

RESULTS

Phylogenetic analysis

A Bayesian analysis was conducted to evaluate the phylogenetic association of *S. invicta* hexamerins with other hymenopteran hexamerins (Fig 1). The tree was reconstructed using Bayesian methods in MrBayes. The potential scale reduction factor values reached 1.0 which indicates convergence had been reached. Strong support for nodes was found on the phylogeny. Most nodes had 100 % posterior probability value; three nodes did not but achieved high support (84 %, 89 % and 97 %). The obtained phylogenetic tree has two major clades as in Cristino et al. (2010). Alignment of hexamerin proteins can be found in supplementary information in appendix (A1 Fig).

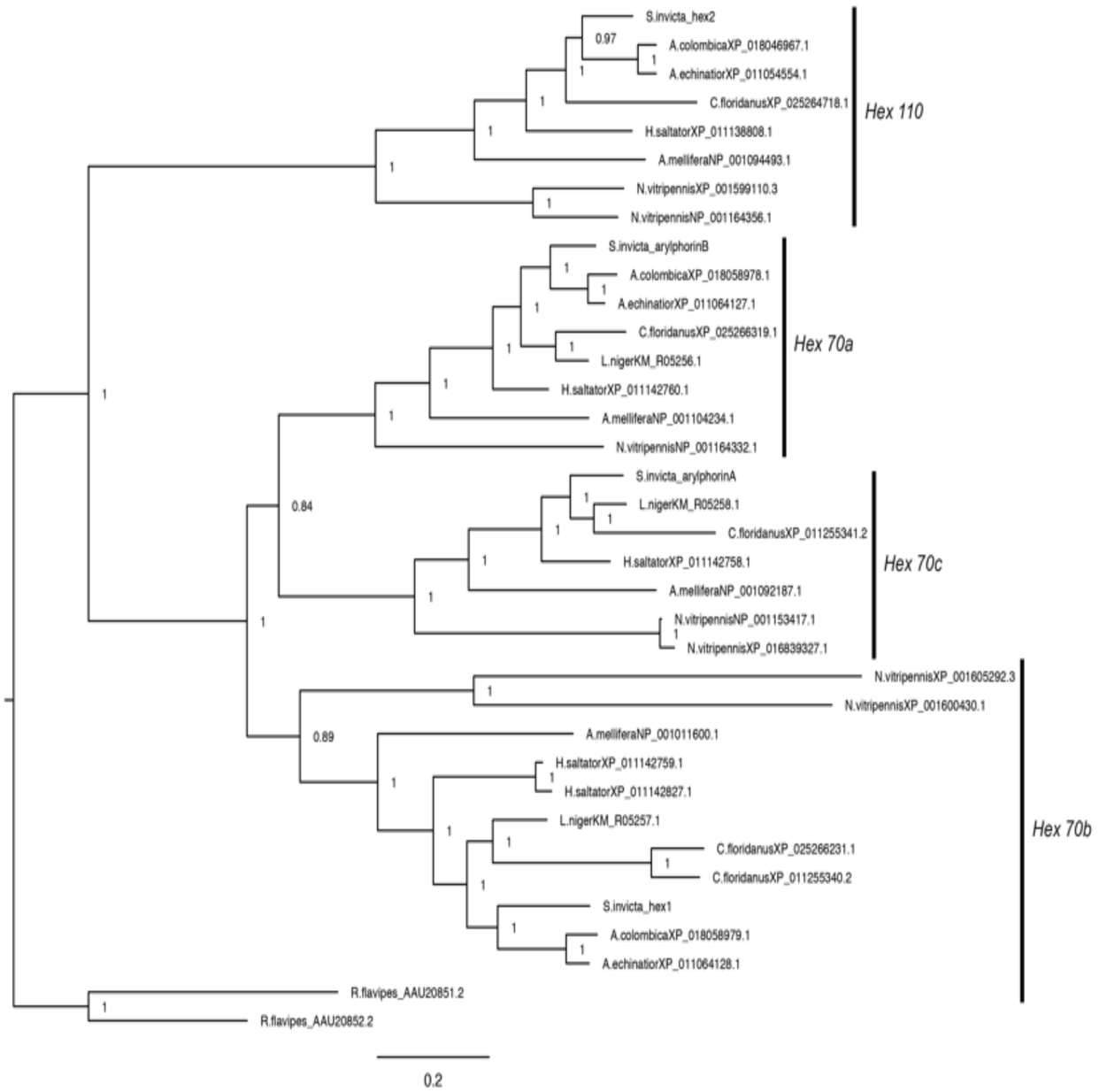

Fig 7: Phylogenetic analysis of S. invicta hexamerins and other hymenopteran hexamerin candidate proteins under Bayesian inference.

Node values posterior probability was rounded to two significant figures. Scale bar indicates branch length. The four clade names represent the four hexamerin proteins that have been described in A. mellifera. The two hexamerins of R. flavipes were used as the out group. The hexamerins of S. invicta clustered as follows: Hexamerin 1 (XP_025995150.1) with Hex70b, Hexamerin 2 (XP_011171862.1) with Hex110, Arylphorin alpha subunit-like (XP_011155485.1) with Hex70c, and Arylphorin beta subunit-like (XP_011155484.1) with Hex70a.

The smaller hexamerin clade included the largest *S. invicta* hexamerin, Hexamerin 2 (*S. invicta_hex2* in Figure 7) (1027 predicted amino acids) which clustered with *A. mellifera* NP_001094493.1 (Hex 110), *N. vitripennis* NP_001164356.1 and *N. vitripennis* XP_001599110.3. Similar to these proteins, Hexamerin 2 is characterized by a high glutamic acid/glutamine (Glx) content (25.4 %) (Table 1). *S. invicta* Hexamerin 2 also clustered with hexamerins from four ant species, *H. saltator* XP_011138808.1, *A. colombica* XP_018046967.1, *A. echinatior* XP_011054554.1, and *C. floridanus* XP_025264718.1.

Table 2: Composition of *S. invicta* hexamerins. The number of residues typically enriched or characteristic in insect hexamerins in each full predicted sequence and their corresponding percentage (in parenthesis) [Glx are glutamic acid and glutamine].

Hexamerin ID	N° of Aromatic amino acids (%)	Glx (%)	Methioinine (%)	Leucine (%)
Hexamerin 1 XP_025995150.1 (686 amino acids)	98 (14.29 %)	60 (8.75 %)	21 (3.06 %)	48 (7.00 %)
Hexamerin 2 XP_011171862.1 (1027 amino acids)	102 (9.93 %)	261 (25.41 %)	2 (0.19 %)	79 (7.69 %)
Arylphorin subunit alpha-like XP_011155485.1 (696 amino acids)	145 (20.83 %)	55 (7.90 %)	27 (3.88 %)	58 (8.33 %)
Arylphorin subunit beta-like XP_011155484.1 (696 amino acids)	129 (18.53 %)	58 (8.33 %)	19 (2.73 %)	56 (8.05 %)

The other three *S. invicta* hexamerins, which are adjacent in the *S. invicta* genome on the same contig (NW_020521759.1), show a higher degree of similarity (Table 2) as shown in the phylogenetic analysis. *S. invicta* Hexamerin 1 (686 predicted AA) clustered with *A. mellifera* NP_001011600.1 (Hex 70B), *N. vitripennis* XP_001605292.3, and *N. vitripennis* XP_001600430.1. *S. invicta* Arylphorin subunit alpha-like (696 predicted AA) clustered with *A. mellifera* NP_001092187.1 (Hex 70C), and *N. vitripennis* NP_001153417.1 and XP_016839327.1. Finally, *S. invicta* Arylphorin subunit beta-like (696 predicted AA) clustered *N. vitripennis* NP_001164332.1 and *A. mellifera* NP_001104234.1 (Hex 70A). Both *S. invicta* arylphorin proteins have high aromatic amino acid content (Table 3). Hexamerin 1 fails to meet the 15% aromatic amino acid criterion to be considered an arylphorin (Table 3). None of the *S. invicta* hexamerins are leucine-rich (>10% leucine) or methionine-rich (> 4%methionine) proteins (Telfer & Kunkel, 1991).

Table 3: Sequence similarities among the *S. invicta* hexamerins.

Percentage sequence identities and similarities were determined by BlastP analyses. Identities are shown below, similarities above the diagonal line.

	Arylphorin subunit alpha-like	Hexamerin 1	Arylphorin subunit beta-like	Hexamerin 2
Arylphorin subunit alpha-like	-	44	50	31
Hexamerin 1	62	-	46	33
Arylphorin subunit beta-like	67	66	-	31
Hexamerin 2	53	54	51	-

Expression of hexamerins in workers and queens

The patterns of expression of *hexamerin 1*, *hexamerin 2*, *arylphorin subunit alpha-like* and *arylphorin subunit beta-like* transcripts were obtained for alate queens, medium-size foragers and medium-size nurses. Significant differences were found in the expression of all genes. Foragers had the lowest expression of all tested genes (Figure 8). For *hexamerin 1* (Figure 8A), significant differences were found among the three groups ($F= 3656.829$, $df = 2$, $p <0.0001$). Queens had significantly higher levels of expression compared to both nurses and the foragers, and nurses had significantly higher levels than foragers. For *hexamerin 2* (Figure 8B), significant differences were found among the three groups ($F = 698.8919$, $df = 2$, $p < 0.0001$) where nurses had significantly higher levels than both queens and foragers, and queens had significantly higher levels than foragers. For *arylphorin subunit alpha-like* (Figure 8C), significant differences were found among the groups ($F = 893.4681$, $df = 2$, $p < 0.0001$). No significant difference of expression was found between nurses and queens, while foragers had significantly lower expression than the nurses and queens. For *arylphorin subunit beta-like* (Figure 8D) significant differences were also found among all groups ($F = 2730.836$, $df = 2$, $p < 0.0001$) where nurses had significantly higher levels than foragers and queens, and queens had higher levels than foragers.

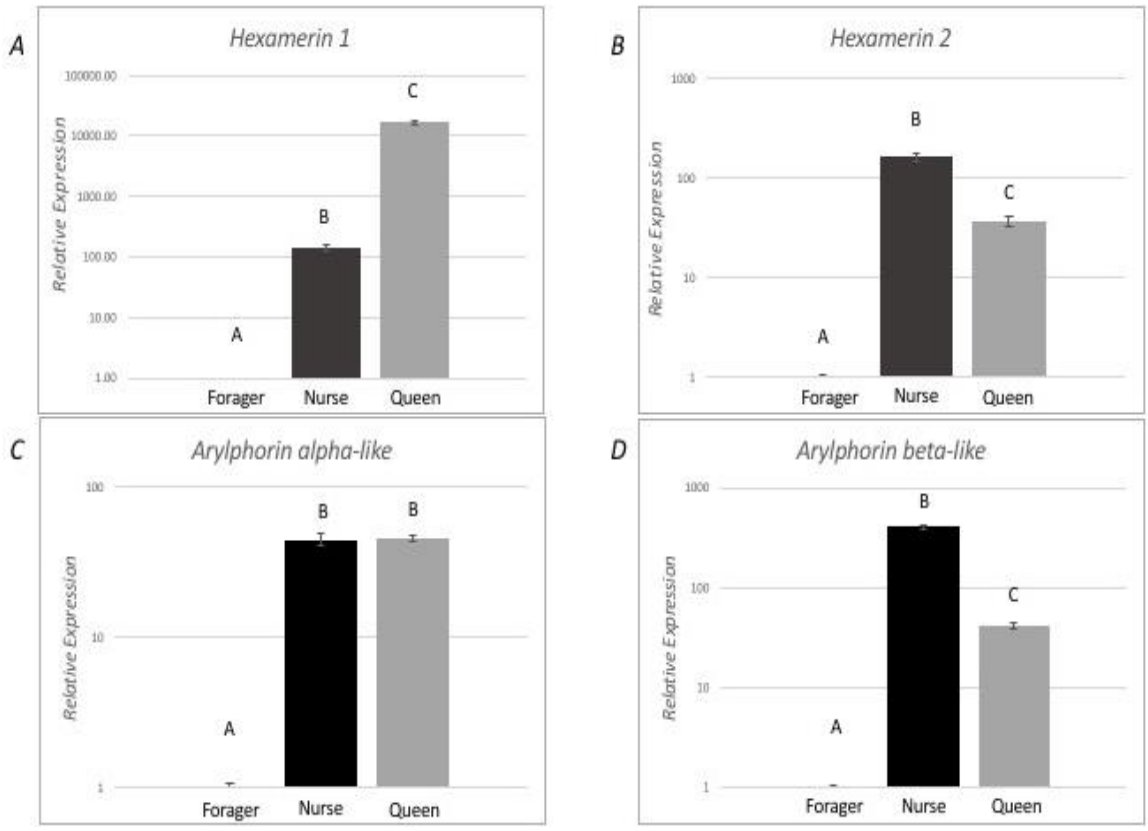

Figure 8: Expression analysis of hexamerin transcripts among alate virgin queens, medium-sized foragers and medium-sized nurses.

(A) *Hexamerin 1* transcript relative expression. (B) *Hexamerin 2* transcript relative expression. (C) *Arylphorin alpha subunit-like* transcript relative expression. (D) *Arylphorin beta subunit-like* transcript relative expression. Gene expression was quantified using RT-qPCR and analyzed using the ΔΔCt method. Each gene mRNA expression level was normalized relative to *rp18*. Bars represents mean ± SEM fold change relative to foragers ($n = 6$). Statistical relationships between groups were assessed using one-way ANOVA with Tukey post hoc test ($p < 0.05$), different letters indicate statistical differences among the groups.

Expression of hexamerins in medium-size foragers following topical application of S-hydroprene

The expression of the four hexamerin genes was evaluated from pools of ten individual medium-size foragers 12 hours after S-hydroprene application (Figure 9). No significant differences in expression were found for *hexamerin 1* (Figure 9A) and for *arylphorin subunit alpha-like* (Figure 9C) among the treatment groups ($p > 0.05$). However, significant differences in expression were identified for *hexamerin 2* ($F = 17.48$, $df = 2$, $p = 0.003$, Figure 9B) and for *arylphorin subunit beta-like* ($F = 77.96$, $df = 2$, $p < 0.0001$, Figure 9D). In both cases, the expression was lower in the S-hydroprene treatment when compared to both control treatments (non-treatment and acetone) indicating a decrease in expression of *hexamerin 2* and *arylphorin beta subunit-like* in response to the JH analog.

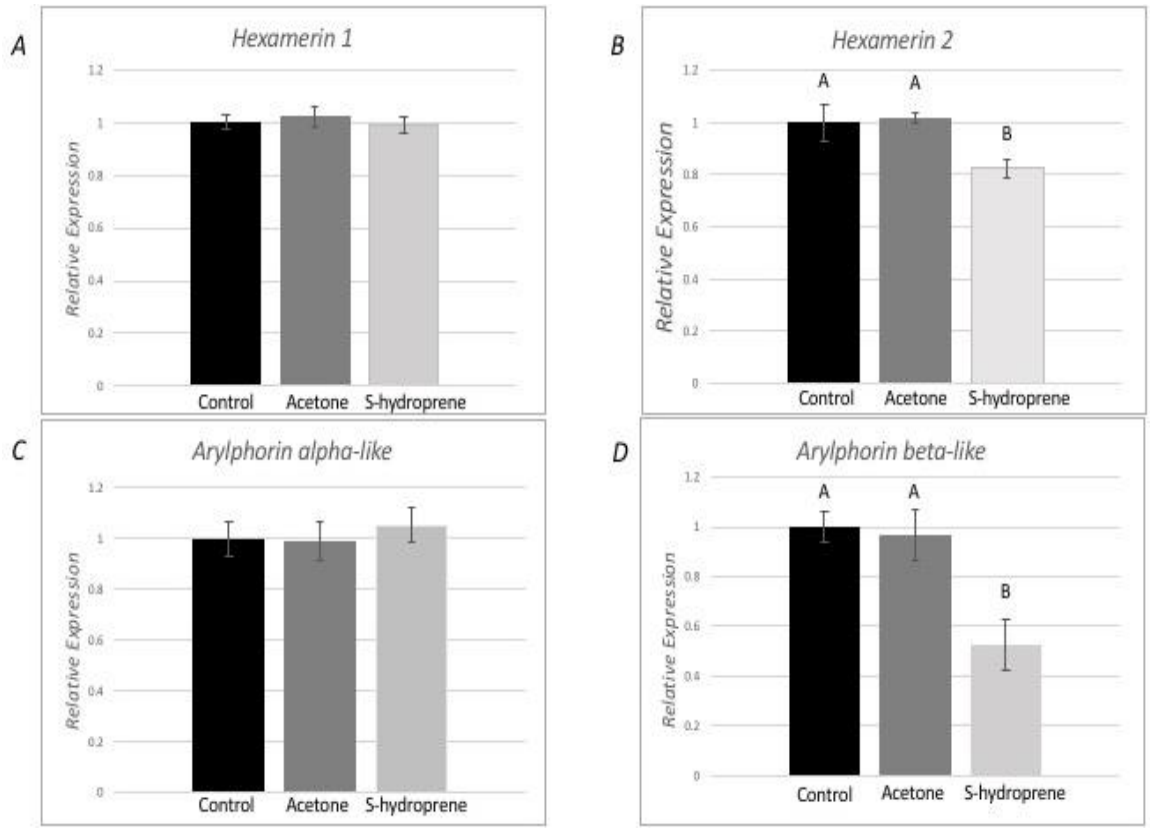

Figure 9: Expression analysis of hexamerin transcripts in medium-sized foragers 12 h following topical application of S-hydroprene.

(A) *Hexamerin 1* transcript relative expression. (B) *Hexamerin 2* transcript relative expression. (C) *Arylphorin alpha subunit-like* transcript relative expression. (D) *Arylphorin beta subunit-like* transcript relative expression. Gene expression was quantified using RT-qPCR and analyzed using the ΔΔCt method. Each gene mRNA expression level was normalized relative to *rp18*. Bars represents mean ± SEM fold change relative to the untreated control (n = 6). Statistical relationships between groups were assessed using one-way ANOVA with Tukey post hoc test ($p < 0.05$), different letters indicate statistical differences among the groups.

Expression of hexamerins in medium-sized nurses following topical application of S-hydroprene

The pattern of expression of the four hexamerin genes was evaluated from pools of ten individual medium-size nurses 12 hours after S-hydroprene application (Figure 10). In this case, the expression of all genes was significantly reduced in the S-hydroprene treatment group compared to the control treatments: *hexamerin 1* ($F = 4.79$, $df = 2$, $p = 0.025$, Figure 10A); *hexamerin 2* ($F = 17.48$, $df = 2$, $p = 0.0001$, Figure 10B); *arylphorin subunit alpha-like* ($F = 36.88$, $df = 2$, $p < 0.0001$, Figure 10C), and *arylphorin subunit beta-like* ($F = 77.96$, $df = 2$, $p < 0.0001$, Figure 10D). These differences indicate that the JH analog regulated the expression of these genes in nurses.

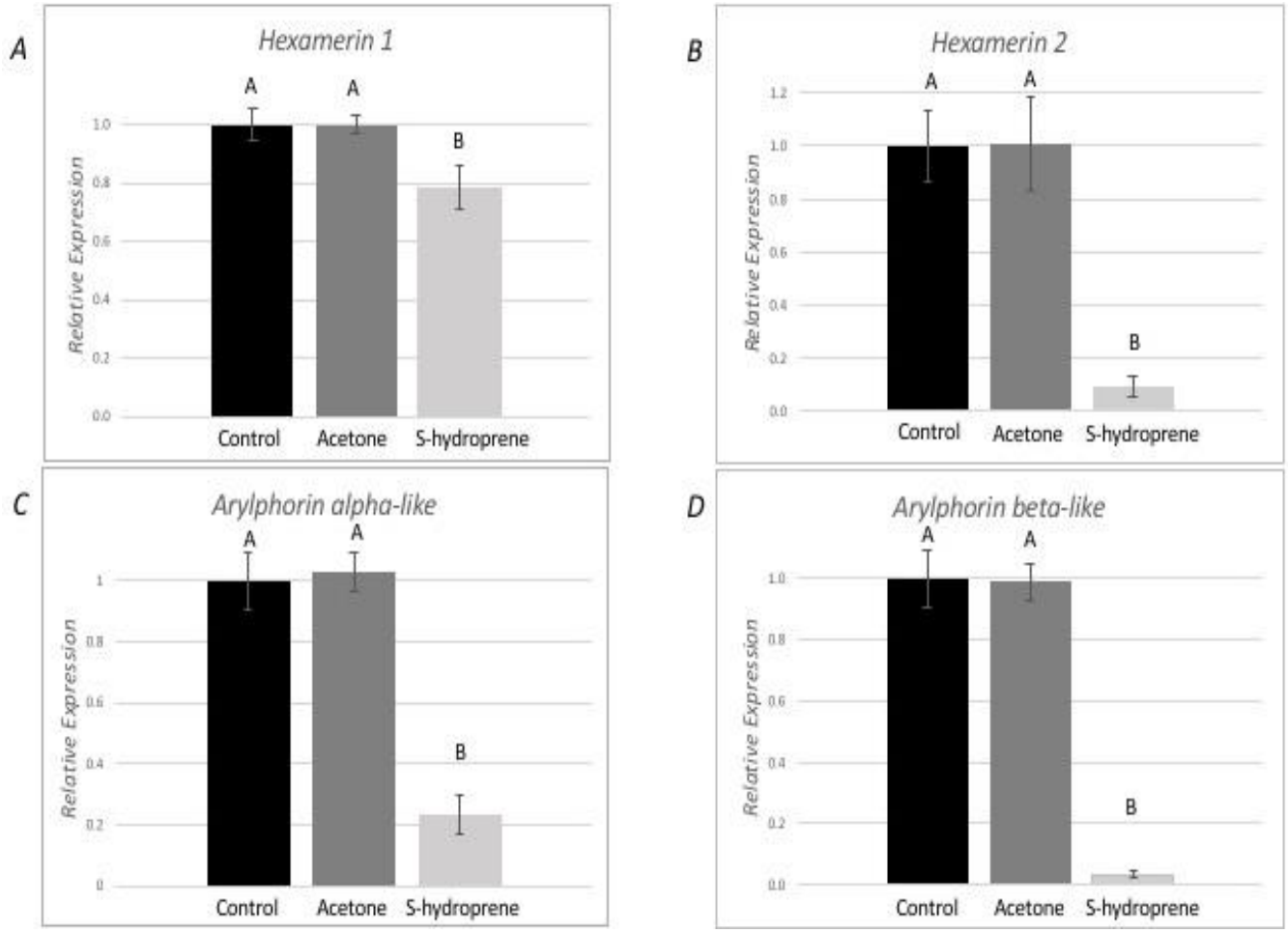

Figure 10: Expression analysis of hexamerin transcripts in medium-sized nurses 12 h after topical application of S-hydroprene.

(A) *Hexamerin 1* transcript relative expression. (B) *Hexamerin 2* transcript relative expression. (C) *Arylphorin alpha subunit-like* transcript relative expression. (D) *Arylphorin beta subunit-like* transcript expression. Gene expression was quantified using RT-qPCR and analyzed using the ΔΔCt method. Each gene mRNA expression level was normalized relative to *rp18*. Bars represents mean ± SEM fold change relative to untreated control ($n = 6$). Statistical relationships between groups were assessed using one-way ANOVA with Tukey post hoc test ($p < 0.05$), different letters indicate statistical differences among the groups.

Expression of hexamerins in alate virgin queens following topical application of S-hydroprene

The pattern of expression of the four hexamerin genes was evaluated from pools of ten virgin queens 12 hours after S-hydroprene application. No differences in the expression of *hexamerin 2* (Figure 11B) and of *arylphorin subunit beta-like* (Figure 11D) were found among the treatments ($p > 0.05$), while differences were measured for *hexamerin 1* ($F = 40.94$, $df = 2$, $p = < 0.0001$, Figure 11A), and for *arylphorin subunit alpha-like* ($F = 33.27$, $df = 2$, $p = <0.0001$, Figure 11C). In both cases, the expression of was lower in the S- hydroprene treatment group than in the control treatment groups.

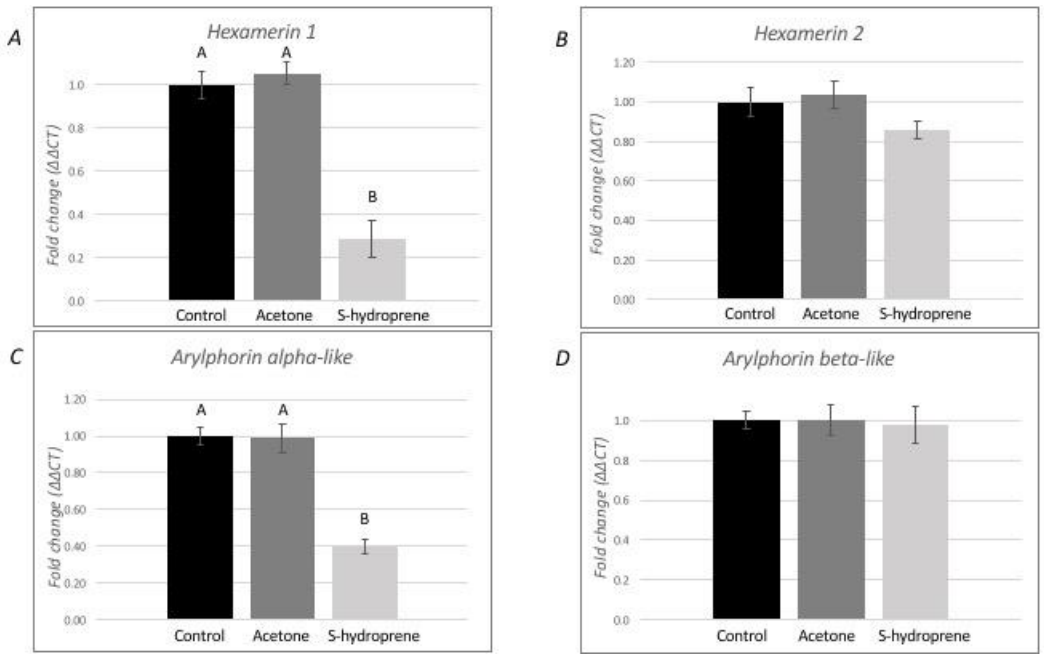

Figure 11: Expression analysis of hexamerin transcripts in virgin alate queens 12 h following topical application of S-hydroprene.

(A) *Hexamerin 1* transcript relative expression. (B) *Hexamerin 2* transcript relative expression. (C) *Arylphorin alpha subunit-like* transcript relative expression. (D) *Arylphorin beta subunit-like* transcript relative expression. Gene expression was quantified using RT-qPCR and analyzed using the ΔΔCt method. Each gene mRNA expression level was normalized relative to *rp18*. Bars represents mean ± SEM fold change relative to untreated control (n = 6). Statistical relationships between groups were assessed using one-way ANOVA with Tukey post hoc test ($p < 0.05$), different letters indicate statistical differences among the groups.

DISCUSSION

Hexamerins have been characterized in numerous insect species. In holometabolous insects, hexamerin proteins accumulate during larval development (Telfer & Kunkel, 1991). These proteins serve as an amino acid source during the pupal and pharate adult stages. Insects

encode several hexamerins, and the copy number of hexamerin genes varies within social insects. Previously, it was determined that hymenopteran genomes harbor at least one copy of each of the four hexamerins commonly identified in *A. mellifera* (*hex 70a, hex 70b, hex 70c, hex 110*) (Suen et al., 2011). Four hexamerin genes are predicted in the *S. invicta* genome (LOC105192919, LOC105192898, LOC105204474, and LOC105192897). Based on the phylogenetic analysis, they correspond to each of the four hexamerin types. Interestingly, our search of hexamerins among the chosen ant species failed to identify the homolog of *hex 70c* in *A. colombica* and in *A. echinatior*, two leaf-cutting ant species. The absence of *hex 70c* in *Atta cephalotes* had been reported, and the absence of this gene was linked with the mutualism established between this species and fungi (Suen et al., 2011). Our finding that two other leaf-cutting ant species do not encode *hex 70c* is consistent with the hypothesis linking the absence of this gene with the fungal mutualism. In addition to Hex70c in the aforementioned ants, a homolog of *hex 110* was not found in the current annotation of *L. niger* genome.

While hexamerins are commonly known for their role as storage proteins used during molting, hexamerins can act also as storage proteins for reproductive purposes in adults, in particular for gamete formation (Teresa Martinez & Wheeler, 1994). Further, other functions have been linked to these proteins in different insect species. However, the function of hexamerins in hymenopteran sociality needs to be further investigated, in particular, for their potential co-option for different functions across castes. Indeed, hexamerins are expressed in sterile hymenopteran workers which indicates the potential adaptation of these proteins for the colony function (D. E. Wheeler & Martinez, 1995). *S. invicta* workers are completely sterile, their ovaries do not develop (Khila & Abouheif, 2008). Therefore, analysis of hexamerin expression in this species could help understand the role of these genes in sociality.

Potential for involvement of hexamerin genes in sociality in hymenopterans has been hypothesized based on expression analyses (Evans & Wheeler, 1999; Hoffman & Goodisman, 2007; Pereboom, Jordan, Sumner, Hammond, & Bourke, 2005). In *A. mellifera, hex 70a* and *hex 110* are the only hexamerin genes with detectable expression in the worker fat body (M. M. Bitondi et al., 2006). The expression of both genes in queens is relatively low (when compared to workers) and occurs in the ovaries of egg-laying queens (Martins, Anhezini, Dallacqua, Simoes, & Bitondi, 2011; Martins & Bitondi, 2016; Martins et al., 2010). *Hex 70a* and *hex 110* are also expressed in adult workers, in which they are regulated by nutrition and by the activation of reproduction (M. M. Bitondi et al., 2006; Martins, Nunes, Simões, & Bitondi, 2008). Further, a decrease in the expression of *hex 70a* in workers coincides approximately with the transition to foraging. In ants, expression of hexamerin genes in adults, and differences of expression between the worker and queen castes were recorded in *Formica exsecta* (C Morandin et al., 2015). Moreover, the accumulation of hexamerin proteins in virgin queens of several ant species has been associated with claustral colony founding (Teresa Martinez & Wheeler, 1994; D. Wheeler & Buck, 1996; D. E. Wheeler & Buck, 1995).

In *S. invicta* the four hexamerin genes were expressed in virgin queens and in workers (Figure 8). Interestingly, *hexamerin 1* (that clustered in the hex 70b group) was expressed at higher level in virgin queens than in workers (Figure 8A). We previously showed that this gene was expressed in the heads and in the thorax/abdomens of virgin queens, and that its expression was reduced in response to a JH analog (Calkins et al., 2018). The expression of all hexamerins also higher in virgin queens than in foragers. In *F. exsecta* all hexamerin transcripts are expressed at higher level in emerging queens than in emerging workers, while the opposite occurs when comparing old workers and old queens (C Morandin et al., 2015).

It is noteworthy that differences in hexamerin expression between *S. invicta* nurses and foragers were also measured for the four genes; all hexamerins were down-regulated in foragers when compared to nurses. This finding is in accordance with transcriptomic results comparing gene expression between *S. invicta* nurses and foragers (Qiu, Zhao, & He, 2017). Similarly, in *A. mellifera*, the expression of *hex 70a* and of *hex 110* decreased as the workers aged, and this decrease corresponded with the transition to foraging (Martins et al., 2010). This change in gene expression coincides with the increase in the JH titer in the *A. mellifera* workers age. Therefore, the expression of these genes could be associated with the specific task being performed by the worker or with the age of the worker. Comparison of the *hex 110* and *hex 70a* expression between workers of similar age but under different social contexts, queenless (in this case the worker ovaries developed and became active) and queenright (the worker ovaries remain inactive) showed activation of the expression of both genes in *A. mellifera* workers with active ovaries (Martins et al., 2010). Contrary to *A. mellifera* nurses, *S. invicta* nurses do not have the potential for reproduction; therefore, the expression of hexamerins in this ant species is likely linked to other functions. As such, nurses could be using hexamerins as reserves to nourish brood (Teresa Martinez & Wheeler, 1994), and/or in nutritional signaling (Calkins et al., 2018; Xuguo Zhou et al., 2006).

Because in *S. invicta, hexamerin 1* is down-regulated by topical application of a JH analog to virgin queens (Calkins et al., 2018), whereas in *A. mellifera* larvae the four hexamerin genes are up-regulated by JH (Martins et al., 2010), the expression of the four *S. invicta* hexamerin genes was evaluated following topical application of the JH analog in virgin queens, nurses, and foragers. Opposite to JH-induced expression in *A. mellifera*, all four genes were down-regulated in *S. invicta* nurses in response to the JH analog. While only *hexamerin 2* and

arylphorin subunit beta-like were down-regulated in foragers, *hexamerin 1* and *arylphorin subunit alpha-like* were down-regulated in virgin queens by the JH analog. This unexpected result may suggest the existence of different regulatory modules that regulate gene expression in a caste-specific manner in *S. invicta*. In workers, JH regulates the expression of the four hexamerin genes. Due to low hexamerin expression in foragers, it is possible that RT-qPCR was not sensitive enough to detect a reduction in the expression of *hexamerin 1* and of *arylphorin subunit alpha-like* following the application of the JH analog, however, the four genes were down-regulated in nurses following the JH analog application. In virgin queens, only *hexamerin 1* and *arylphorin subunit alpha-like* appear to be regulated by JH. Despite *hexamerin 2* and *arylphorin subunit beta-like* being expressed at higher levels in virgin queens than in forages, these genes were not regulated after the treatment with the JH analog. Thus, it appears that *hexamerin 2* and *arylphorin subunit beta-like* are regulated by JH in workers, that is, in a caste-specific manner.

Alate virgin queens are repressed by the queen primer pheromone, and as a result they have low levels of JH, which prevent their ovaries from developing (D. Fletcher & Blum, 1981). When virgin queens are released from the influence of the queen primer pheromone, their JH titers increase, and as a consequence they shed their wings and their ovaries develop. The same results (wing shedding and ovary development) are obtained by topical application of JH or its analogs (Calkins et al., 2018; D. Fletcher & Blum, 1981; D. J. Fletcher & Blum, 1983). Thus, based on the effect of the JH analog on the expression level of *hexamerin 1* and *arylphorin subunit alpha-like* in virgin queens observed here, we hypothesize that newly mated queens have lower expression of these two genes than the virgin queens. In support of this hypothesis, we have previously shown that *hexamerin 1* expression was indeed lower in the brains of newly

mated queens than in virgin queens (Calkins et al., 2018). The changes in *hexamerin 1* and *arylphorin subunit alpha-like* expression could be linked to the mobilization of reserves for ovary development. Additionally, these changes could also be linked to the potential involvement of hexamerins in signaling nutritional status; the down-regulation of these hexamerins could signal the end of the period in which virgin queens actively accumulate reserves. Similarly, these genes are probably expressed at low levels in mature queens which have a higher JH titer than virgin queens (C. S. Brent & Vargo, 2003).

Some hexamerins bind JH (Braun & Wyatt, 1996; Xuguo Zhou et al., 2007), thus it is hypothesized that these proteins might bind JH in social insects to modulate JH titers (X Zhou et al., 2006). Among the *S. invicta* hexamerins, *arylphorin subunit alpha-like* contains a region that is the most similar to the sequence of the locust hexamerin, which if deleted eliminates the JH binding activity (Braun & Wyatt, 1996; Calkins et al., 2018). Therefore, this protein could be involved in modulating JH titers in different *S. invicta* castes: in queens, it might be involved in maintaining a low JH titer in virgins to prevent precocious reproductive development; while in workers, it might be involved in maintaining a low JH titer in nurses preventing transition to foragers. Indeed, JH titer is low in the *A. mellifera* nurse and it increases as the insect ages (Amsalem et al., 2014; Jaycox, 1976; Rembold et al., 1974; G. E. Robinson & Vargo, 1997). The down-regulation of all hexamerins in *S. invicta* nurses following JH analog application, and their down-regulation in foragers compared to nurses supports the existence of changes in JH titer as the *S. invicta* worker ages, and a similar role of JH in *S. invicta* age polyethism such as that found in other social insect species.

CONCLUSION

In conclusion, in *S. invicta* a caste- and task-biased expression of hexamerins was established. Further, the regulation of the four genes by the JH-analog, S-hydroprene, in a caste- and task-specific manner was also established. While expression of these genes in *A. mellifera* workers might be associated with their potential for reproduction, *S. invicta* workers cannot reproduce. Thus, the expression of hexamerins might indicate the potential role of these genes in other aspects of *S. invicta* biology. In queens, *hexamerin 1* and *arylphorin subunit alpha-like* may be associated with signaling nutritional status, dominance status and/or reproductive activity. In workers, hexamerin expression might be associated with nutritional signaling, task-allocation and/or age polyethism. Co-option of these genes may have given *S. invicta* colony the ability to respond to cues involved in social organization and queen dominance, and the expression of these storage proteins in adults may be based on colony demands. The gene expression analysis presented here illustrates the potential for hexamerins to play important roles in fire ant social physiology, warranting protein level analysis and reverse genetic manipulation to further elucidate the evolutionarily coopted roles of these genes/proteins in relation to the reproductive ground plan hypothesis (Tobias Pamminger & Hughes, 2017).

CHAPTER IV

BRAIN GENE EXPRESSION ANALYSES IN *S. INVICTA* FIRE ANT FORAGERS IDENTIFY GENES REGULATED BY CHANGES IN SOCIAL CONTEXT: VITELLOGENIN AS A CANDIDATE MARKER

INTRODUCTION

The existence of 'behavioral genetic toolkits' that regulate behavior across different species has been proposed. These toolkits are sets of genes repeatedly used during evolution to generate novel forms of behavior (Rittschof & Robinson, 2016). *Vitellogenin* (Vg), a gene encoding for an egg yolk protein is a very well-known example of a gene candidate in the genetic toolkit because of its involvement in division of labor and social behavior in social insects. In the honeybee (*Apis mellifera*), Vg is involved in the queen reproduction, while in the workers a decrease in the expression of Vg is associated with the onset of the foraging behavior in an age-specific manner (Gro V Amdam, Norberg, Guidugli, Omholt, & Simões, 2003; Guidugli et al., 2005). Ant species harbor multiple copies of Vg; duplication and subsequent sub functionalization of each copy has been associated with social organization. For example, the harvester ant *Pogonomyrmex barbatus* encodes two Vg genes; *Pb_Vg1* is up-regulated in nurses compared to foragers, while *Pb_Vg2* is up-regulated in foragers compared to nurses (Corona et al., 2013). Furthermore, differences in Vg and Vg-like gene expression between queens and workers have been identified in several ant species (Feldmeyer et al., 2014; Claire Morandin et al., 2014; Oxley et al., 2014).

Interestingly, foragers from eusocial colonies forage to sustain all members of the colony, which may or may not be kin, therefore, communication among nestmates to ascertain the colony

nutritional needs is necessary (Toth & Robinson, 2007). The red imported fire ant *Solenopsis invicta* polygyne colony contains several egg-laying mated queens, these queens and their worker progeny cooperate to grow and maintain the colony. Foragers collect both proteinaceous and carbohydrate food sources.

Carbohydrates can be immediately utilized by the workers or can be fed to the larvae, while the proteinaceous food are mainly utilized by the queen and brood. Food is passed among *S. invicta* colony members following a chain-of-demand flow in which recipients solicit food from donors (Cassill & Tschinkel, 1995, 1999a). It has been established that *S. invicta* colonies regulate their protein intake (Cook, Eubanks, Gold, & Behmer, 2011), and larvae play a strong role in protein intake regulation behavior of the workers; ant colonies that lack brood prefer carbohydrate-biased diets, while those with brood prefer a more balanced protein-carbohydrate intake (Dussutour & Simpson, 2009). Further, solid proteins can only be metabolized by the fourth instar larvae (Petralia & Vinson, 1978). Therefore, it can be expected that the amount of protein collected is directly related to the amount of brood in the colony and the mechanisms of communication could be a "chain of demand" from this larval stage (Cassill & Tschinkel, 1999a). *Apis mellifera* brood produces brood pheromone which impacts foraging by workers and results in more pollen (protein) being foraged (Dreller, Page Jr, & Fondrk, 1999; T. Pankiw, 2004). Brood pheromones in ants have been less studied. In *S. invicta,* triolein was identified as a brood-tending pheromone isolated from the sexual brood (Bigley & Vinson, 1975). Whether *S. invicta* brood pheromone exists or if it stimulates protein foraging by workers remains unknown. In *S. invicta* colonies, foragers do not interact directly with brood so a global communication system within the colony could exist to regulate protein intake. In this context, it is possible that the presence/absence of brood has an effect on the behavior of all workers including foragers that

are not in direct contact with the brood.

The brain centralizes the perception and integration of information and coordinates behavioral responses. Importantly, the brain also communicates with peripheral tissues. Division of labor in ants is mediated by the brain to cause upstream changes in physiology and behavior when an individual perceives a social signal or cue leading to actions or changes in behaviors (Muscedere & Traniello, 2012). Therefore, the brain is an obvious target to evaluate the causal relationships or associations between gene regulation and behavior (Rittschof & Robinson, 2016).

The main goal of this study was to identify changes in the brain transcriptome of foragers in response to presence/absence of brood and to evaluate the role of key candidate genes in signaling and communication. For this purpose, brain transcriptomes from carbohydrate foragers from colonies in the presence or absence of brood were compared. This worker category was chosen because it is present in both types of social context (as opposed to nurses, which are only present in colonies where brood are present). Because in *S. invicta* presence/absence of brood can be used as a proxy for protein availability, this work can lead to the identification of molecules involved in signaling social context and colony nutritional requirements, as well as genes that could be candidates for the genetic toolkit model.

MATERIALS AND METHODS

Insect colonies

Polygyne *S. invicta* colonies used in this study were collected in Brazos County, TX from May to July 2015. They were maintained in the laboratory at Texas A&M University in plastic trays (27 x 40 x 9 cm) with the walls of the containers covered with Fluon (Insect-a-slip,

BioQuip products, CA, USA). Each colony was provided a 14 cm-diameter petri dish half-filled with damp Castone (Dentsply International Inc., York, PA, USA) as a nest area. The colonies were maintained at 27 ± 2 °C in a 12:12 hour light - dark photoperiod. The colonies were fed daily with a 20% honey water solution and crickets, *Acheta domestica*. Water was given *ad libitum.*

Colonies which contained workers with a reproductive (dealate) queen, several alate virgin queens and brood were distinguished as brood colonies (B). Colonies which contained workers with a reproductive (dealate) queen, several alate virgin queens, but no brood were distinguished as no brood colonies (NB). NB colonies were queenright and were fed daily similar amounts and types of food as the B colonies. The queen in those colonies had previously laid eggs but had stopped producing brood for some weeks.

Classification and selection of worker ants

For subcaste analyses, ants were classified as major, medium, or minor workers according to their head width as previously described (E. O. Wilson, 1978). Specifically, minor workers had a head width smaller than 0.72 mm, medium workers' head width was between 0.73 and 0.92 mm, and major workers had a head width bigger than 0.93 mm.

For transcriptomic analysis, foragers used were determined as ants interacting directly with the specific food type. So, if a worker was found actively collecting food from the carbohydrate tray (honey) at the time of sampling it was classified as a carbohydrate forager and were used for transcriptomics (see below). For further qRT-PCR analysis on task performed, workers actively collecting food from the protein tray (cricket) were classified as protein foragers. Nurses which were inside of the nest area, directly interacting with brood were classified as nurses. Food

sources were replaced between 9:30 AM and 10:00 AM. Ants were collected at approximately at 11:00 AM, 1 hour after the food source was renewed for the day.

RNA extraction and purification

RNA was extracted using the Trizol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. The Direct-zol™ micro prep plus clean up kit (Zymo Research, Irvine, CA, USA) was used for sample clean up and RNA purification to remove any trace of TRIzol from the solubilized pellet. RNA was then resuspended in 20 µl of nuclease free water. The Turbo DNAse kit (Ambion Waltham, MA, USA) was used to eliminate genomic DNA using the manufacturer's protocol. Quantity and purity of the total RNA was assessed using an Infinite® 200 PRO NanoQuant (Tecan, Männedorf, Switzerland) and the integrity of RNA was visualized on a 2% agarose electrophoresis gel stained with ethidium bromide. RNA was then stored at -80 °C until further use for RT-qPCR analyses.

Transcriptomic analyses

For transcriptomic analyses, brains of carbohydrate- foraging workers were dissected on ice-cold PBS buffer and placed immediately in 100 µL of TRIzol Reagent (Thermo Fisher Scientific, Carlsbad, CA) maintained on dry ice and pooled. Once approximately 30 brains had been collected in a tube, they were kept at -80°C until further needed. For the final sample, brains were combined into one tube (approximately 150 to 200 brains), and RNA purification was performed as described in (Calkins et al., 2018) and as mentioned above. Three total RNA samples of workers (150 – 200 brains in each) from a colony containing brood and three total RNA samples of workers from a colony without brood were obtained. Approximately 1 µg of

total RNA from each sample were submitted to the Texas A&M AgriLife Genomic and Bioinformatic Center for transcriptome analysis and library preparation.

The libraries for RNAseq analyses were prepared with Illumina TruSeq Stranded Total RNA library preparation kit and sequenced with the HiSeq 2500 System (Illumina) in four lanes of 125SE (single end). The sequence raw reads were cleaned using cutadapt 1.0 to remove barcode tags and adaptors. Individual samples were processed with FastQC, and the QC reports were checked as final confirmation of sequence quality and trimmed as needed up to the first 15 base pairs.

All bioinformatic analyses were performed in the Discovery Environment web interface and platform at CyVerse (S. A. Goff et al., 2011). The RNA-seq reads that passed the quality filters (FASTQC tools) were mapped to the *S. invicta* genome (Ensembl Metazoa, GCA_000188075.1.34) using TopHat 2.0.9 (Trapnell, Pachter, & Salzberg, 2009). Cufflinks 2.1.1 was used to estimate the transcript abundance (L. A. Goff, Trapnell, & Kelley, 2012). Differentially expressed genes (DEGs) were identified using Cuffdiff 2.2.1 with the JS (Jensen-Shannon) option (Trapnell et al., 2013). The identification of DEGs was performed with the following criteria: false discovery rate (FDR < 0.01) and a fold change and a fold change > 2 based on Reads Per Kb per Million reads (RPKM) values. DEGs were researched and annotated using UniProt Knowledgebase (Consortium, 2016). GO term enrichment analyses were performed using the g:Profiler web server with Benjamini-Hochberg False Discovery rate correction (Reimand, Kull, Peterson, Hansen, & Vilo, 2007).

Transcriptome validation by qRT-PCR of selected genes

The transcriptome analysis was verified using three replicates of worker brains from three

independent colonies different than those used in the sequencing analysis. Brains were dissected as previously described and RNA was purified as before. cDNAs were synthesized from 140 ng of total brain RNA using the Verso cDNA synthesis kit (Thermo Fisher Scientific) following manufacturer's protocol and 50 ng of cDNA was used for q-RTPCR.

The genes chosen for verification were Notch 4, Ejaculatory bulb protein, Vitellogenin 2 and Vitellogenin 3. The reference gene *RPL18*(Cheng et al., 2013) was used as a control gene. Primer sequences are presented in appendix (Table A2), primers for vitellogenin genes were as in (Hawkings & Tamborindeguy, 2018).

Reactions for qPCR were performed using the SensiFast Hi Rox kit (Bioline, Taunton, MA, USA) following the manufacturer's protocol. Reactions contained 1 x SYBR Green Master mix (Applied Biosystems), primers (250 nM forward and 250 nM reverse) and 50 ng cDNA. The volume was adjusted to 10 µl with nuclease-free water. Reactions were run with two technical replicates for each gene and cDNA sample, and three biological replicates were performed. The following thermocycling program in a QuantStudio Flex 6 qPCR thermocycler (ThermoFisher) was used: 2 min at 95°C, then 40 cycles of 95°C for 5 s and 60°C for 30 s. Primer specificity was monitored with "melt curve" analysis in the QuantStudio 6 program. Data were analyzed via the ΔΔCt method utilizing the reference gene as an internal control (Hawkings & Tamborindeguy, 2018; Schmittgen & Livak, 2008). Statistical differences were determined by comparing ΔCts of each gene between samples with unpaired *t*-test or ANOVA using JMP software (Ye, Liu, Ren, & Okafo, 2000).

qRT-PCR analyses of vitellogenin expression in worker whole body and head

Expression of *Vg1*, *Vg2*, Vg3 and *Vg4* was analyzed in the whole body and head of

carbohydrate foraging workers for all three subcastes (minor, medium and major) from NB and B colonies. Workers were selected based on size as above. In the task performance experiments heads were taken from protein foragers, carbohydrate foragers, or nurses from B colonies. All ant specimens were collected one hour after the daily food source was renewed, at 11:00am. For head region analyses, ants were decapitated under a light microscope and placed directly in an Eppendorf tube on ice. For each replicate, 10 ants were pooled to normalize the natural variation within the colony and flash-frozen using liquid nitrogen. Bodies of the samples were pooled together separately. 10 worker heads generated approximately 200 ng of RNA.

For the RT-qPCR, Vg primers were selected as previously described above (Hawkings & Tamborindeguy, 2018) and the reference gene, *RPL18* (Cheng et al., 2013) was used (Table A2). Reactions were performed using the SensiFAST SYBR® Hi-rox one step kit (Bioline, Taunton, MA, USA) according to manufacturer's protocol. Reactions contained 1 x SYBR Green Master mix (Applied Biosystems), primers (250 nM forward and 250 nM reverse) and 50 ng RNA. The volume was adjusted to 10 µl with nuclease free water. Reactions were run with two technical replicates for each gene and RNA sample, and three biological replicates were performed. The following thermocycling program in a QuantStudio Flex 6 qPCR thermocycler (ThermoFisher) was used: 10 min at 45°C, followed by 2 min at 95°C, then 40 cycles of 95°C for 5 s and 60°C for 30 s. Primer specificity was monitored with "melt curve" analysis in the QuantStudio 6 program. Data were analyzed via the ΔΔCt method as previously described.

Starvation assay

For the starvation assay, small colonies were created using a larger stock colony collected in

Brazos County during July 2018 (Figure 12). The small colonies were maintained in plastic trays (16 x 10 x 5 cm) and contained an approximately equal weight of brood and equal distribution of brood stages, and a mated queen. All colonies had workers of similar sizes and similar worker age (the age distribution was presumed to be even considering workers were randomly taken from one stock colony). Three individual small colonies were used as biological replicates for each treatment group (3 non-starved colonies and 3 protein-starved colonies). Prior to the experiment period, colonies were allowed to acclimate for three days and were fed daily with 10% honey water and a single cricket.

On day 0, all colonies contained proteinaceous food source. Ten medium carbohydrate foraging workers were taken from each colony and decapitated under the microscope, the 10 head and 10 body regions were pooled in separate tubes and flash-frozen in liquid nitrogen. After day 0, protein was eliminated from the experimentally-starved colonies but replacement of carbohydrate sources continued throughout the duration of the experiment. Medium carbohydrate foragers were collected every ten days one hour after the colonies had been fed (carbohydrate only in starved colonies) at 11:00 AM, they were decapitated as previously described, and the head and body regions were flash-frozen and stored in 80 °C until further needed. On day 21, the amount of brood appeared to be reduced in the protein-starved colonies. Protein food sources were reintroduced on day 21: one cricket was added daily for the remainder of the experiment. Collections continued every ten days until day 40. Control colonies were continuously fed both crickets and honey/water during the duration of the experiment. RNA extraction from the head regions and RT-qPCR analysis of Vg genes was conducted as mentioned above.

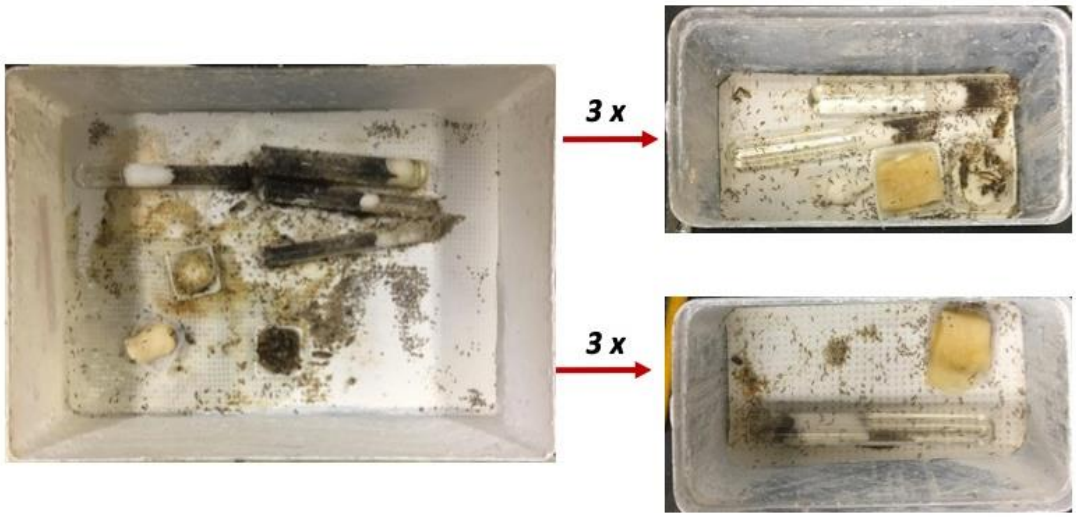

Figure 12: Experimental set up of starvation assay. Small colonies for the starvation assay were created from a large stock colony. Control colonies contained both protein and carbohydrate food sources. Starved colonies contained no protein source during the starved portion of the experiment.

RESULTS

Transcriptome description

Six purified RNA samples from carbohydrate foraging worker heads (three biological replicates per condition of B and NB) were sequenced, and a total of 299.89 million reads were obtained. On average, 82.1% of the reads mapped to the *S. invicta* genome (Supplementary Table 2). A total of 194 genes were differentially expressed (q value ≤ 0.01; with a fold change ≥ 2) between worker brains from the B and NB colonies (Table S3), 97 DEGs (50 %) were upregulated on the brains of NB workers, and 97 DEGs (50 %) were upregulated on the brains of B workers. In total, 72 DEGs were annotated as uncharacterized in the genome (Ensembl Metazoa).

g:Profiler identified enrichment of Biological Process and Molecular Function GO terms among the DEGs (Table 1). Of interest, there was enrichment of GO terms associated with the Biological Process GO:0035176 Social behavior. The two genes associated with this GO term were *pheromone-binding protein Gp-9 (LOC105194487)* and *pheromone-binding protein Gp-9-like (LOC105194495),* and were both up-regulated in the brain of B workers. A second *pheromone-binding protein Gp-9-like (LOC10519450),* without any associated GO term was also up-regulated in this condition. Also, there was enrichment of the GO term associated with the Molecular Function GO:0045735 Nutrient reservoir activity. This GO term was associated with two Vg genes, *vitellogenin 2* and *vitellogenin 3* which were both up-regulated in the brain of NB workers.

Nine genes involved in lipid metabolism were regulated. Among those, *lipase 1-like,* three *lipase 3-like* genes and *fatty-acid amide hydrolase 2-like* were up-regulated in the brain of B workers and *venom carboxylesterase-6,* and three *sterol O-acyltransferase 1-like* were up-regulated in the brain of NB workers. An *apolipoprotein D-like,* a potential carrier of lipophilic molecules in neurons and glial cells, was up-regulated in the brain of B workers. Two alpha-amylases, *alpha-amylase* and *alpha-amylase A-like,* involved in carbohydrate metabolism and the sugar transporter *SWEET1* were also up-regulated in the brain of B workers.

Several genes potentially involved in oxidoreductase reactions were regulated; *cytochrome P450 4C1-like, cytochrome P450 6d3-like, cytochrome P450 6k1-like, probable cytochrome P450 6a14, probable cytochrome P450 6a13,* and *laccase-5-like* were up-regulated in the brain of B workers, while *cytochrome P450 9e2-like* and three *prenylcysteine oxidase-like* were up-regulated in the brain of NB workers. Interestingly, *sestrin-3,* a metabolic regulator that may be involved in protection against oxidative and genotoxic stresses was also down-regulated

in the brain of NB workers.

Eight genes potentially involved in brain remodeling were up-regulated in the brain of NB workers. These genes were *brain tumor protein, homeobox protein slou-like, homeotic protein female sterile-like, microtubule-associated protein futsch, ras-related protein Rap-2a, mothers against decapentaplegic homolog 6, myoblast determination protein 1 homolog, semaphorin-1A-like*, and *transcription factor AP-1*. Further, *headcase, forkhead box protein O* (also known as FOXO), and the *protein tramtrack, beta isoform* were also up-regulated in the brain of NB workers. *Headcase* is a downstream regulator of the insulin receptor / mechanistic target of rapamycin (mTOR) pathway controlling the timing of neurogenesis, while *FOXO* and *tramtack* regulate the post-embryonic glial proliferation in *D. melanogaster*. FOXO is a transcription factor involved in the regulation of the insulin signaling pathway and integrating insulin responses to glucose and lipid homeostasis. *Tramtrack* is required for the proliferation of glia and to promote photoreceptor development in eye development during *Drosophila* melanogaster pupal stage. On the other hand, the *transcription factor Adf-1-like* that regulates dendrite growth was up-regulated in brain of B workers.

Other DEGs potentially involved in nervous system physiology included *ataxin-2 homolog*, required for *D. melanogaster* normal eye development and bristle morphology, *espin-like*, required for the formation and maintenance of inner ear hair cell stereocilia and staircase formation and normal hearing in humans, both genes were up-regulated in brain NB workers. The *ejaculatory bulb-specific protein 3-like,* a putative chemosensory protein CSP9, was up regulated in the brain of NB workers.

Table 4: GO term enrichment analysis performed with g:Profiler.

Type	Term ID	Term name	p-value	Gene list
Biological Process	GO:0051703	intraspecies interaction between organisms	0.0276	LOC105194487 LOC105194495
Biological Process	GO:0051705	multi-organism behavior	0.0276	LOC105194487 LOC105194495
Biological Process	GO:0035176	social behavior	0.0276	LOC105194487 LOC105194495
Biological Process	GO:0042219	cellular modified amino acid catabolic process	0.0141	LOC105198266 LOC105206458 LOC105206662
Biological Process	GO:0000096	sulfur amino acid metabolic process	0.0141	LOC105198266 LOC105206458 LOC105206662
Biological Process	GO:0006575	cellular modified amino acid metabolic process	0.045	LOC105198266 LOC105206458 LOC105206662
Biological Process	GO:0030329	prenylcysteine metabolic process	0.000818	LOC105198266 LOC105206458 LOC105206662
Biological Process	GO:0044282	small molecule catabolic process	0.0447	LOC105195261 LOC105198266 LOC105206458 LOC105206662
Biological Process	GO:0044273	sulfur compound catabolic process	0.0133	LOC105198266 LOC105206458 LOC105206662
Biological Process	GO:0016054	organic acid catabolic process	0.0223	LOC105195261 LOC105198266 LOC105206458 LOC105206662
Biological Process	GO:0046395	carboxylic acid catabolic process	0.0223	LOC105195261 LOC105198266 LOC105206458 LOC105206662
Biological Process	GO:0009063	cellular amino acid catabolic process	0.00821	LOC105195261 LOC105198266 LOC105206458 LOC105206662
Biological Process	GO:0000098	sulfur amino acid catabolic process	0.000818	LOC105198266 LOC105206458 LOC105206662
Biological Process	GO:0030328	prenylcysteine catabolic process	0.000818	LOC105198266 LOC105206458 LOC105206662
Molecular Function	GO:0016670	oxidoreductase activity, acting on a sulfur group of donors, oxygen as acceptor	0.00321	LOC105198266 LOC105206458 LOC105206662
Molecular Function	GO:0001735	prenylcysteine oxidase activity	0.000818	LOC105198266 LOC105206458 LOC105206662
Molecular Function	GO:0008374	O-acyltransferase activity	0.031	LOC105197438 LOC105197475 LOC105206796
Molecular Function	GO:0045735	nutrient reservoir activity	0.0208	LOC105205782 LOC105205783

Validation of genes

The expression of *Vg2, Ejaculatory bulb protein, Notch 4* and *Vg3* genes were evaluated in brains from ants from three independent colonies from those used in the bioinformatic analysis. Among these genes four candidate genes were chosen for validation of the transcript analysis. *Vg2* and *Vg3* were the two most highly differentially expressed genes in the transcriptome and the functional role and expression of these genes in the brain and in the heads of social insects has been previously explored (Münch et al., 2015; Nunes et al., 2013). Ejaculatory bulb protein was chosen because of its associated role as an odorant binding protein in other insect species (Bohbot, Sobrio, Lucas, & Nagnan-Le Meillour, 1998; Pelosi, Zhou, Ban, & Calvello, 2006), and Notch 4 was chosen because of its role in development and immunity (Artavanis-Tsakonas, Rand, & Lake, 1999; Tu et al., 2005). For each of these genes, higher expression was found in the brains of NB workers compared to that of the B worker brain ($p < 0.005$) (Figure 13). *Vg2* displayed the highest fold change (150 times) between the brains in the absence of brood when compared to in the presence ($p = 0.0003$).

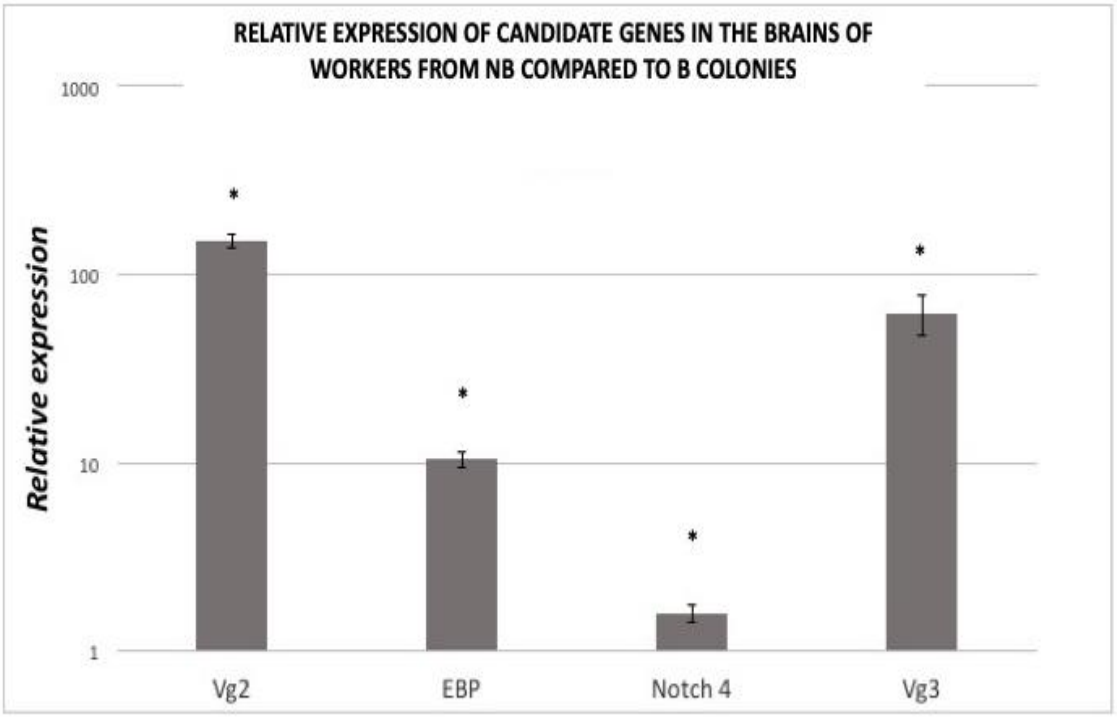

Figure 13: Expression analysis of *Vg2, Ejaculatory bulb protein, Notch 4*, and *Vg3* transcripts in brains of B and NB major workers.

Gene expression was quantified using qRT-PCR and analyzed using the ΔΔCt method. For each gene, the mRNA expression level was normalized to *RP18*. Bars represents mean ± STDERR fold change relative to B (n = 3). For simplicity, only relative expression in NB brains is shown (for all genes relative expression in B brains was 1). Statistical relationships between groups were assessed using student t test. * represent significant expression difference between B and NB workers.

Vg expression in whole bodies of carbohydrate foragers from each subcaste from B or NB colonies

To asses if the differences in Vg expression between B and NB workers were also detectable at the whole body level, the expression profile of each Vg (*Vg1*, *Vg2*, *Vg3* and *Vg4*) transcript was evaluated by qRT-PCR in pools of 10 whole bodies of ants from each worker subcaste, minor (Figure 14), medium (Figure 15), and major (Figure 16) performing carbohydrate foraging in either B or NB colonies. No significant differences in expression were identified for minor, medium or major worker whole body workers between B and NB ($p > 0.05$) in any of the Vg genes.

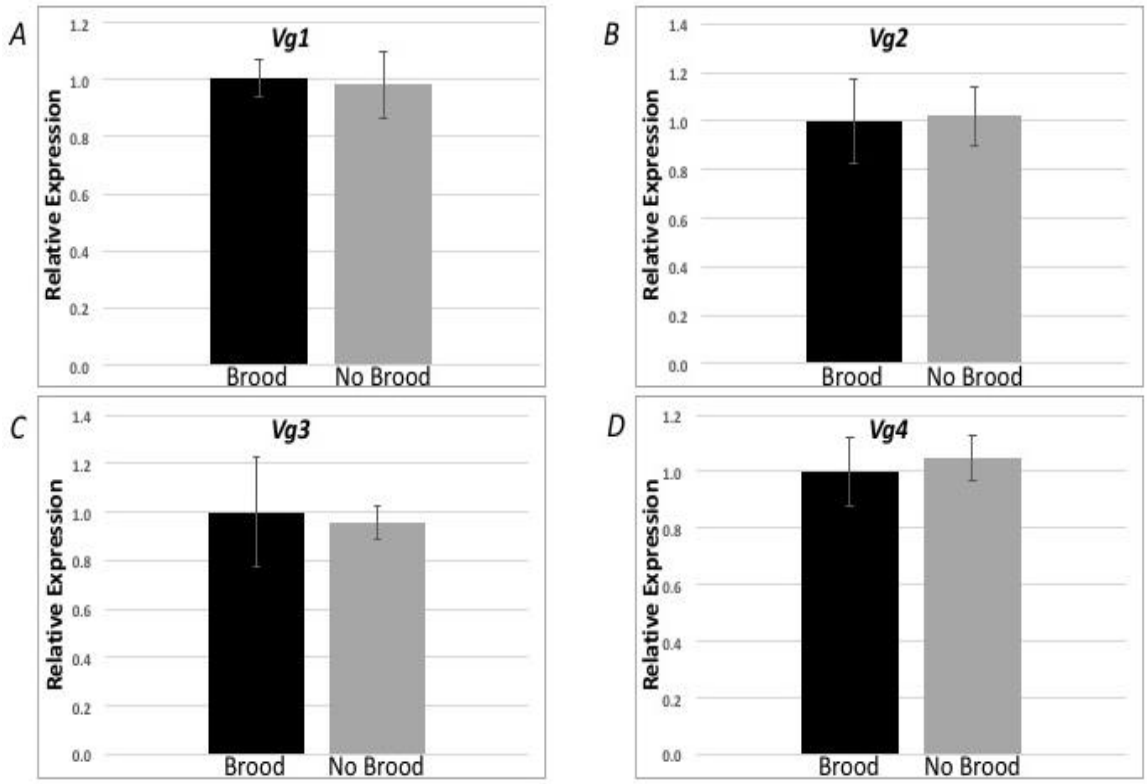

Figure 14: Expression analysis of Vg transcripts in whole body of B minor and NB minor workers.

(A) Vg1 transcript relative expression. (B) *Vg2* transcript relative expression. (C) *Vg3* transcript relative expression. (D) *Vg4* transcript relative expression. Gene expression was quantified using RT-qPCR and analyzed using the ΔΔCt method. Each gene mRNA level of expression was normalized to *RP18*. Bars represents mean ± STDERR fold change relative to B (n = 3). Statistical relationships between groups were assessed using t-test.

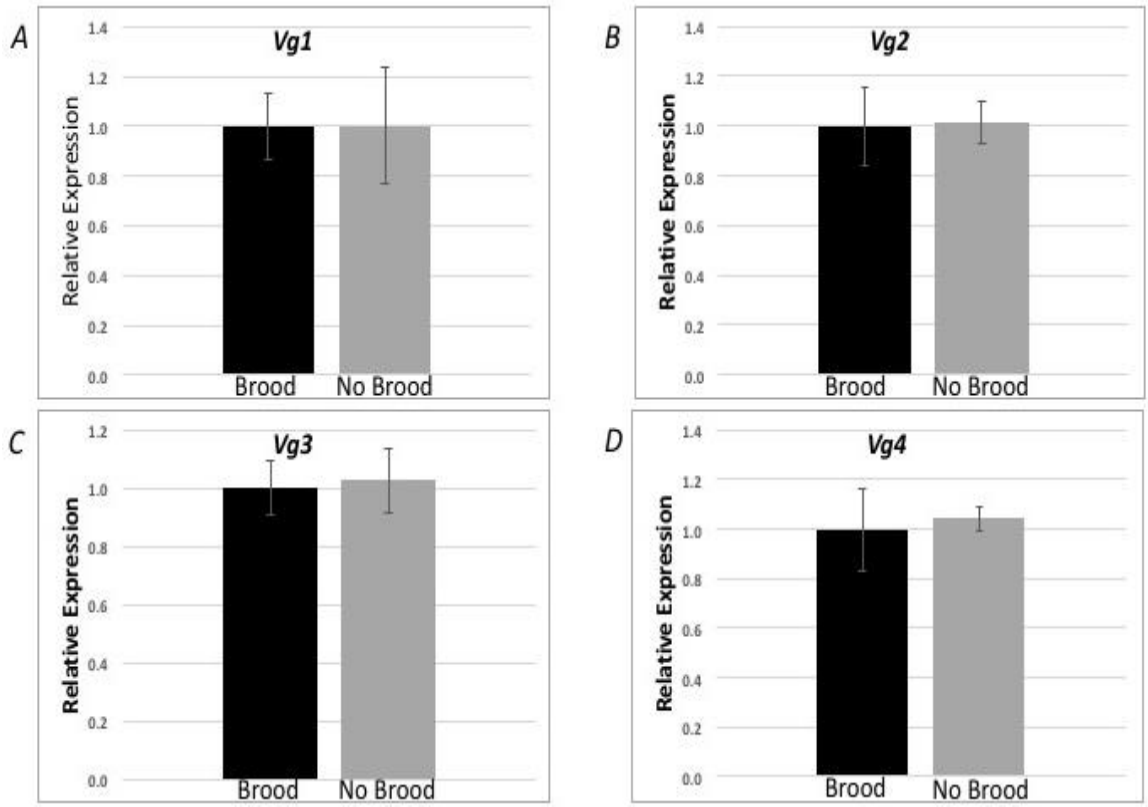

Figure 15: Expression analysis of Vg transcripts in whole body of B medium and NB medium workers.

(A) *Vg1* transcript relative expression. (B) *Vg2* transcript relative expression. (C) *Vg3* transcript relative expression. (D) *Vg4* transcript relative expression. Gene expression was quantified using RT-qPCR and analyzed using the ΔΔCt method. Each gene mRNA level of expression was normalized to *RP18*. Bars represents mean ± STDERR fold change relative to B (n = 3). Statistical relationships between groups were assessed using t-test.

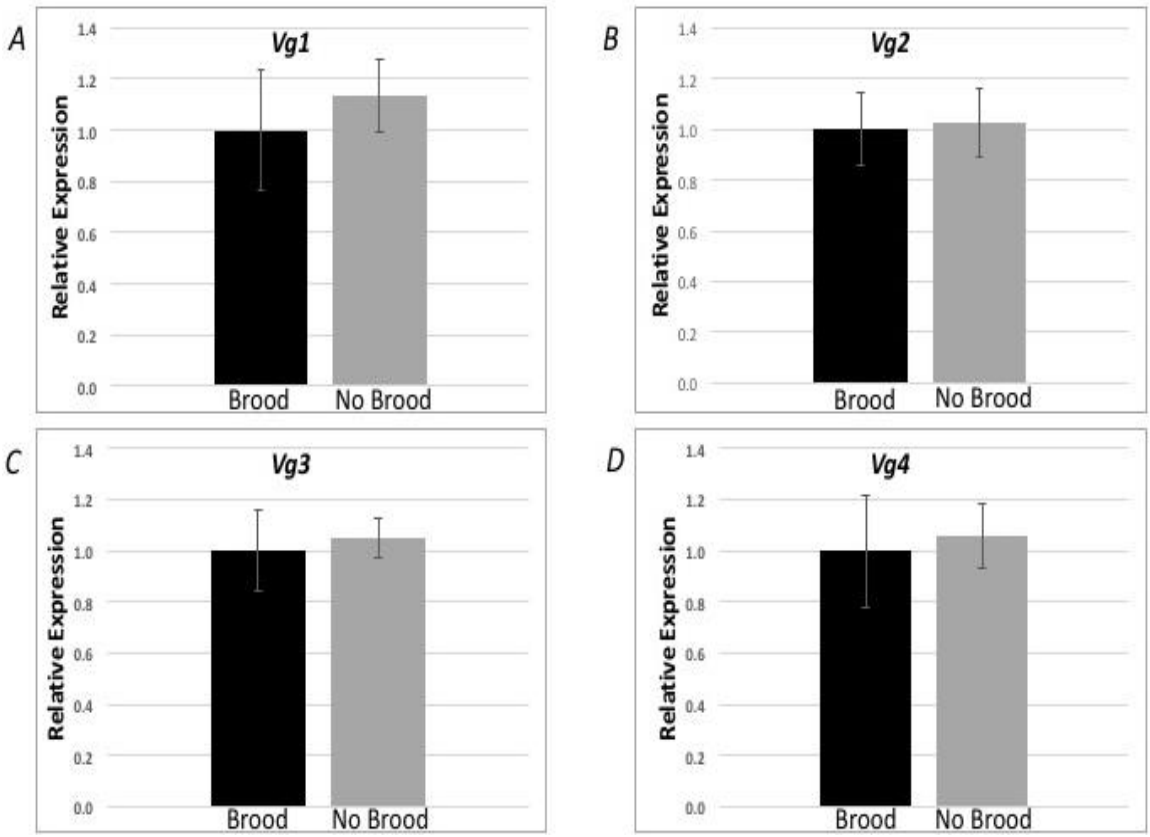

Figure 16: Expression analysis of Vg transcripts in whole body of B major and NB major workers.

(A) *Vg1* transcript relative expression. (B) *Vg2* transcript relative expression. (C) *Vg3* transcript relative expression. (D) *Vg4* transcript relative expression. Gene expression was quantified using RT-qPCR and analyzed using the ΔΔCt method. Each gene mRNA level of expression was normalized to *RP18*. Bars represents mean ± STDERR fold change relative to B (n = 3). Statistical relationships between groups were assessed using one-way ANOVA with Tukey post hoc test ($p < 0.05$), different letters indicate statistical differences among the groups.

Vg expression in heads and decapitated bodies (without head) of carbohydrate foragers

To asses if the differences in Vg expression between B and NB workers were detectable in the head region, the expression profile of each Vg transcript was evaluated in the heads of B and NB decapitated carbohydrate foraging workers from each subcaste (minor, medium and major) by qRT-PCR. Further, the expression of these genes was compared between the head and rest of the body. Thus, for each subcaste, 10 worker heads and the remainder of the 10 worker bodies were pooled in separate tubes. In minor workers (Figure 17), significantly higher expression of *Vg2* ($F = 2.9185$, $df = 3$, $p = 0.10$) was measured in the heads of NB compared to the heads of B workers (Figure 17B). No significant difference was found between the head and body samples for any of the Vg genes.

In medium size workers, differences in *Vg2* ($F = 22.5869$, $df = 3$, $p = 0.0003$) (Figure 18B) and *Vg3* ($F = 4.9161$, $df = 3$, $p = 0.0319$) (Figure 18C) expression were identified. In both cases, expression in NB heads was higher when compared with B heads, and with B and NB bodies. No significant differences of *Vg1* and *Vg4* expression were identified.

In major workers, differences in the expression of all Vg genes were identified. For *Vg2* ($F = 107.7666$, $df = 3$, $p < 0.0001$) (Figure 19) and *Vg3* ($F = 75.3416$, $df = 3$, $p < 0.0001$) (Figure 19C) higher expression was measured in the NB heads compared to the B heads. Expressions in B and NB bodies were also significantly higher than in B heads. For *Vg1* ($F = 48.0329$, $df = 3$, $p < 0.0001$) and *Vg4* ($F = 7.4465$, $df = 3$, $p = 0.0106$) higher expression was measured in the bodies compared to the heads (Figure 19A). However, no differences of expression between B and NB samples were observed.

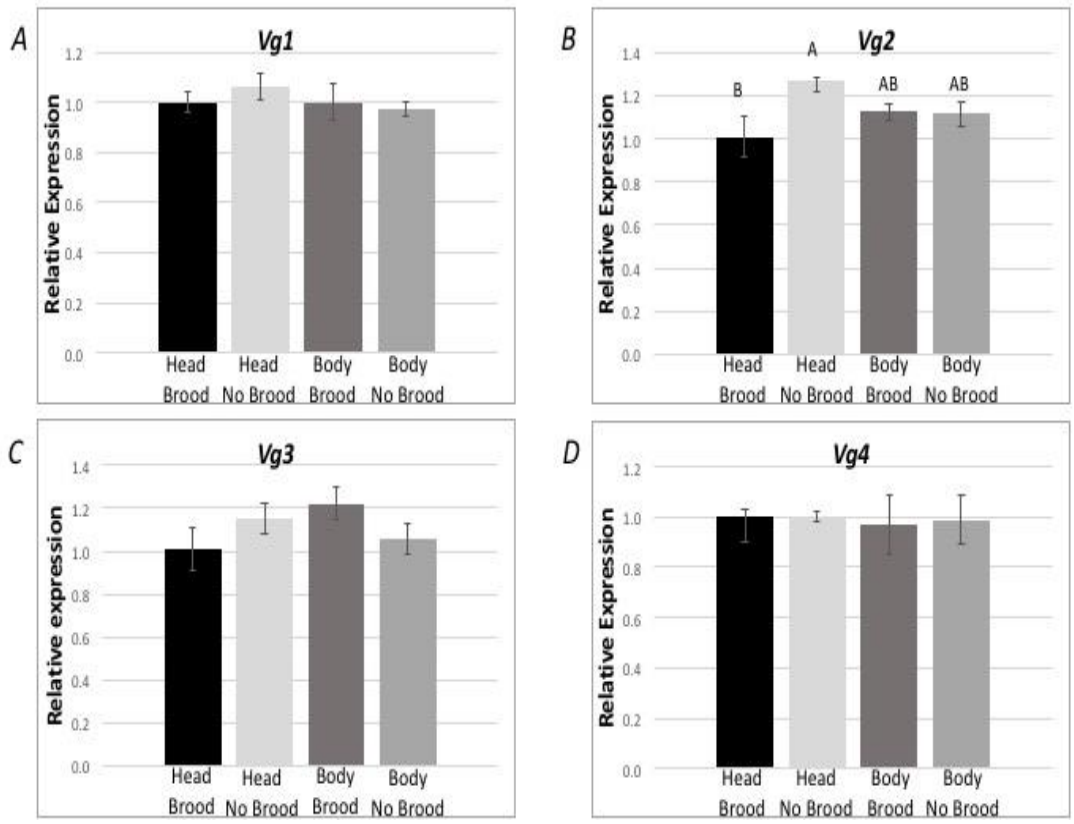

Figure 17: Expression analysis of Vg transcripts in heads and decapitated bodies (without head) of B minor and NB minor carbohydrate foraging workers.

(A) *Vg1* transcript relative expression. (B) *Vg2* transcript relative expression. (C) *Vg3* transcript relative expression. (D) *Vg4* transcript relative expression. Gene expression was quantified using RT-qPCR and analyzed using the ΔΔCt method. Each gene mRNA level of expression was normalized to *RP18*. Bars represents mean ± STDERR fold change relative to B heads (n = 3). Statistical relationships between groups were assessed using one-way ANOVA with Tukey post hoc test ($p < 0.05$), different letters indicate statistical differences among the groups

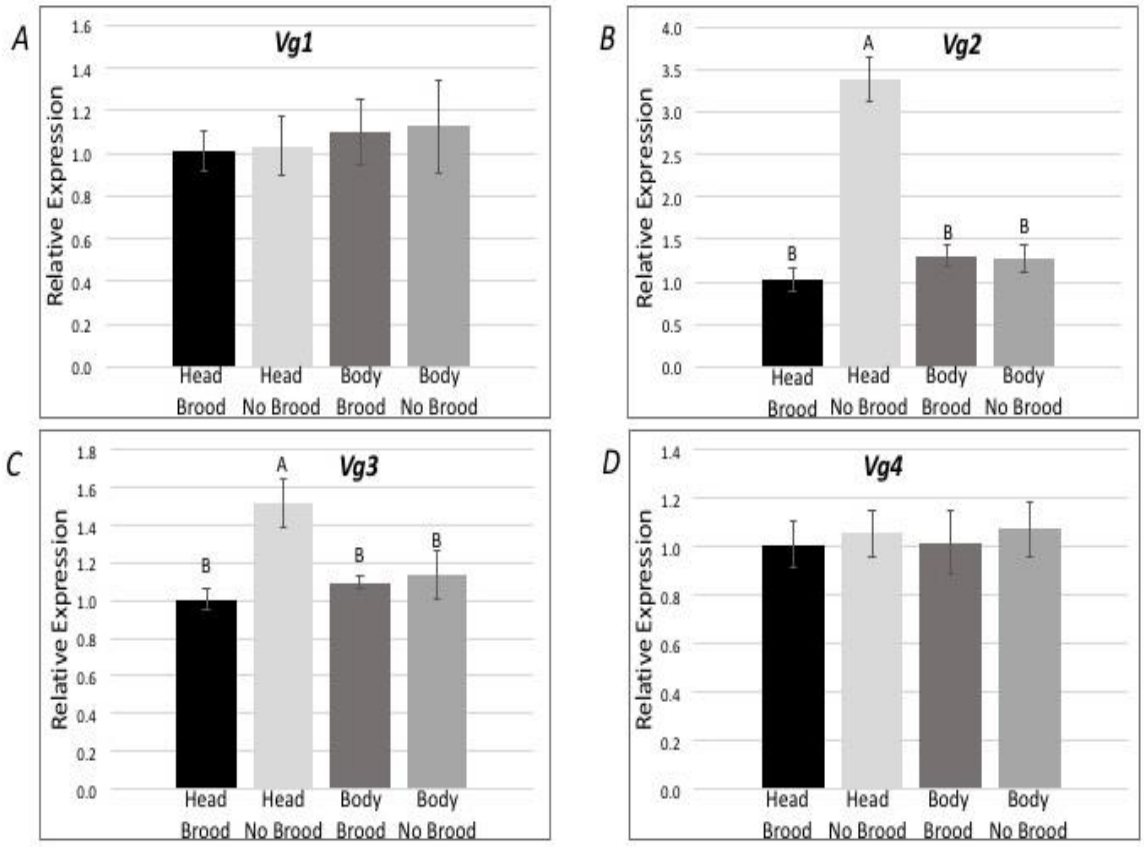

Figure 18: Expression analysis of Vg transcripts in heads and decapitated bodies (without head) of B medium and NB medium carbohydrate foraging workers.

(A) *Vg1* transcript relative expression. (B) *Vg2* transcript relative expression. (C) *Vg3* transcript relative expression. (D) *Vg4* transcript relative expression. Gene expression was quantified using RT-qPCR and analyzed using the ΔΔCt method. Each gene mRNA level of expression was normalized to *RP18*. Bars represents mean ± STDERR fold change relative to B (n = 3). Statistical relationships between groups were assessed using one-way ANOVA with Tukey post hoc test ($p < 0.05$), different letters indicate statistical differences among the groups.

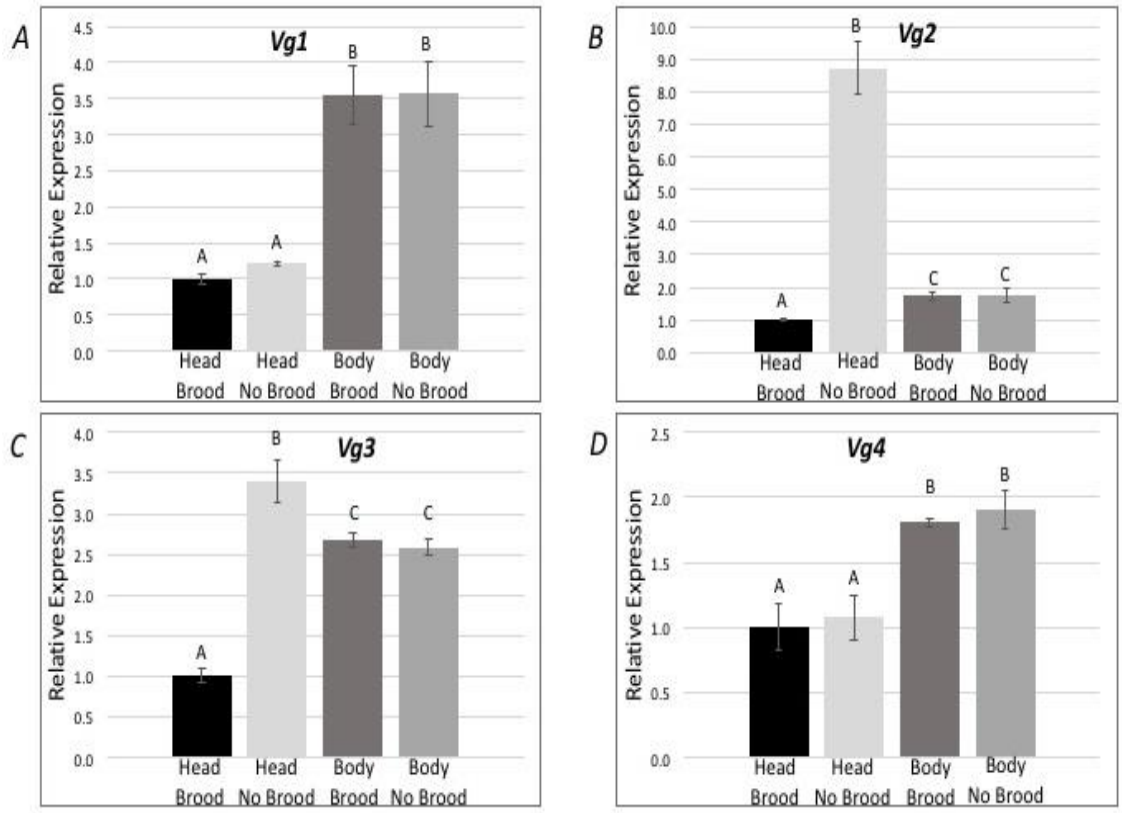

Figure 19: Expression analysis of Vg transcripts in heads and decapitated bodies (without head) of B major and NB major carbohydrate foraging workers.

(A) *Vg1* transcript relative expression. (B) *Vg2* transcript relative expression. (C) *Vg3* transcript relative expression. (D) *Vg4* transcript relative expression. Gene expression was quantified using RT-qPCR and analyzed using the ΔΔCt method. Each gene mRNA level of expression was normalized to *RP18*. Bars represents mean ± STDERR fold change relative to B (n = 3). Statistical relationships between groups were assessed using one-way ANOVA with Tukey post hoc test ($p < 0.05$), different letters indicate statistical differences among the groups.

Vg expression in the head of task performing medium-size workers foraging carbohydrate or protein and nurses in a brood-containing colony.

The expression profile of each Vg transcript was evaluated by qRT-PCR in the heads of medium protein foragers, carbohydrate foragers, and nurses from colonies containing brood. For each sample, 10 workers were decapitated, and their heads were pooled for each task.

Higher expression was measured in the nurses for all genes, *Vg1*, *Vg2*, *Vg3* and *Vg4* (Figure 20). In *Vg1* ($F = 23.0251$, $df = 2$, $p = 0.0015$) (Figure 20A), *Vg3* ($F = 7.6594$, $df = 2$, $p = 0.0223$) (Figure 20C), and *Vg4* ($F = 5.3196$, $df = 2$, $p = 0.0469$) (Figure 20D), no significant differences were found between the carbohydrate and protein foragers. In *Vg2*, protein foragers had also significantly higher expression compared to the carbohydrate foragers ($F = 84.3662$, $df = 2$, $p < 0.0001$).

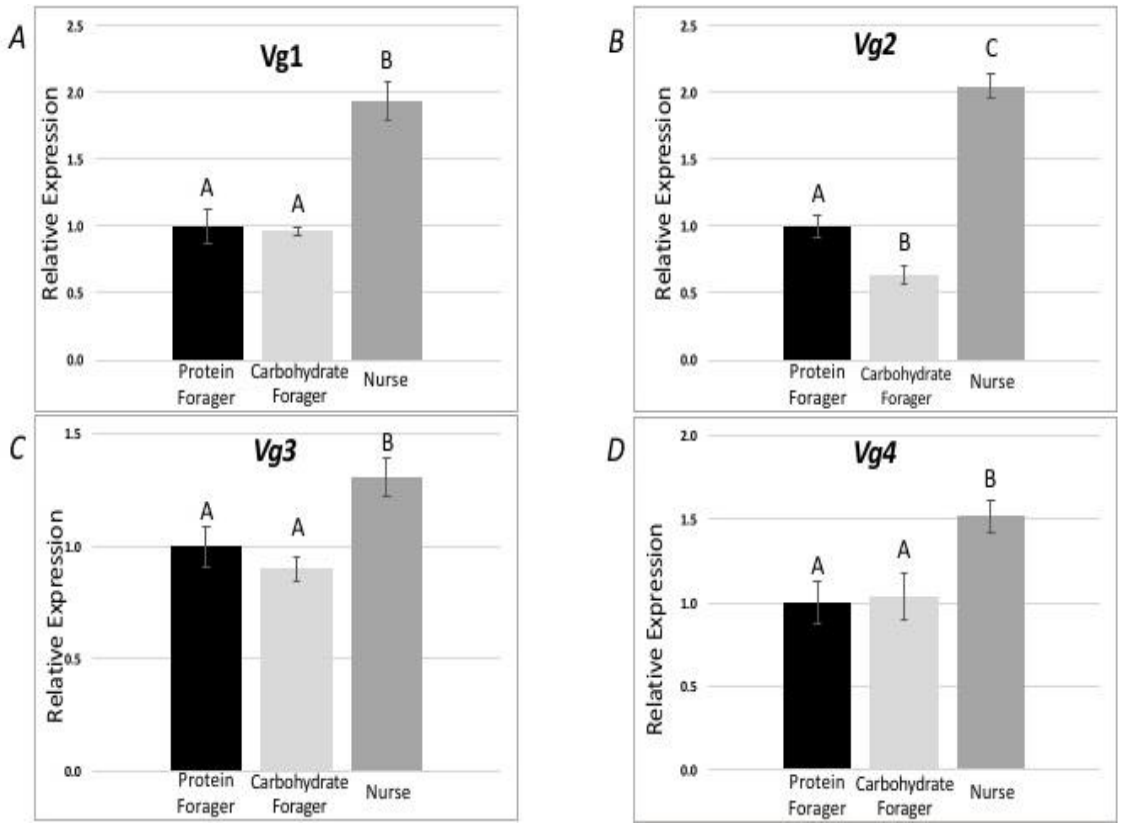

Figure 20: Expression analysis of Vg transcripts in heads of medium nurses, protein foragers and carbohydrate foragers in a colony with brood.

(A) *Vg1* transcript relative expression. (B) *Vg2* transcript relative expression. (C) *Vg3* transcript relative expression. (D) *Vg4* transcript relative expression. Gene expression was quantified using RT-qPCR and analyzed using the ΔΔCt method. Each gene mRNA level of expression was normalized to *RP18*. Bars represents mean ± STDERR fold change relative to protein foragers (n = 3). Statistical relationships between groups were assessed using one-way ANOVA with Tukey post hoc test ($p < 0.05$), different letters indicate statistical differences among the groups.

Vg expression in the heads of medium-size workers starved of protein food source

The expression profile of each Vg transcript was evaluated in the heads of medium carbohydrate foragers in protein-starved (manipulated) and control colonies (Figure 21). No differences in *Vg1* (Figure 21A) and *Vg4* (Figure 21D) expression were observed in the heads of workers from the control and manipulated colonies. For *Vg2*, higher expression was identified in the heads of the manipulated colonies compared to the control colony 20 days after initial starvation ($F = 29.7931$, $df = 1$, $p = 0.0055$) (Figure 21B). For *Vg3*, significantly lower expression was identified in the heads of the manipulated colonies 30 days after initial starvation, coinciding with the 9th day after the reintroduction of protein, which occurred at day 21 ($F = 9.1583$, $df = 1$, $p = 0.0390$) (Figure 21C).

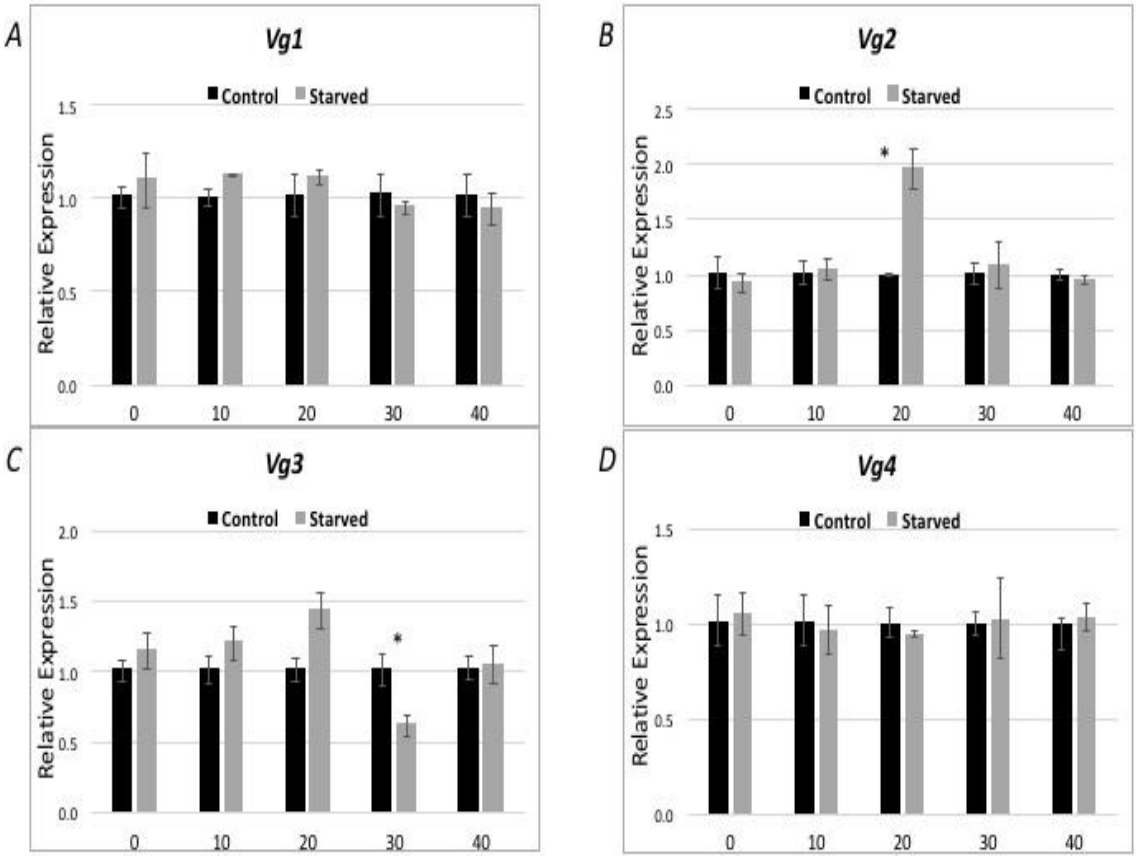

Figure 21: Expression analyses of Vg transcripts in heads of medium foragers starved of protein food source.

(A) *Vg1* transcript relative expression over time points. (B) *Vg2* transcript relative expression over time points. (C) *Vg3* transcript relative expression over time points. (D) *Vg4* transcript relative expression over time points. Gene expression was quantified using RT-qPCR and analyzed using the ΔΔCt method. Each gene mRNA level of expression was normalized to *RP18*. For each time point, bars represent mean ± STDERR fold change relative to the control colonies (n = 3). Statistical relationships between groups were assessed using one-way ANOVA with Tukey post hoc test ($p < 0.05$), different letters indicate statistical differences among the groups ($p < 0.05$). * represent significant differences between control and manipulated colonies.

DISCUSSION

Here, we asked if brood can influence the expression of genes involved in social organization we found and if this expression is regionalized. We found that Vg expression responds to changes to the brood or nutritional context of a colony.

For this study, we characterized the brain transcriptome of workers in colonies with and without brood and validated the transcriptome using four genes of interest. Transcriptome results pointed to two vitellogenins as differentially regulated in the presence or absence of brood. Therefore, we then we analyzed changes in gene expression by qRT-PCR of four vitellogenin genes in whole body and heads of foragers feeding either carbohydrate or protein (major, media and minors), and of nurses under those two conditions of brood present or absent. Finally, we also proceeded to subject colonies to protein starvation and similarly determine changes in Vgs expression in heads.

Of the genes upregulated in the transcriptome in NB, those related to metabolism (14 genes), transcription factors (10 genes), and cell growth (7 genes) were of particular interest. These processes have been shown to be potentially important in social organization and caste structure of social insects.

The genome of eusocial insects has been characterized as having a high number of gene regulatory elements, which are thought to contribute to the evolution and phenotypic plasticity shown in insect societies (Simola et al., 2013). Within the NB group, transcription factors comprised 10 of the upregulated genes, suggesting that the absence of brood has an effect on global gene expression. Studies have observed the effects of transcription factors in shaping behaviors and social structure (Ament et al., 2012; Shpigler et al., 2010), and our results may provide some insight into the social context in which these regulatory elements are active.

Although several transcription factors were observed as being overexpressed in NB, it is unknown at this time which genes or gene networks are being targeted and further studies are needed to understand how these regulatory elements directly influence worker behavior. Genes involved in nutrient metabolism were also observed as being upregulated in NB workers. This may be reflective of the interdependence of the members of the colony: a change in social context affecting gene expression. In this case, presence or absence of brood affects gene expression in the brain of foragers. Interestingly, this shift in metabolic gene expression could highlight the concept of brood serving as the "colony midgut" as well as potential physiological adaptations to the changing of the colony composition which affects nutrient availability for the colony. Genes related to cell growth and biosynthesis may be indicative of changes in the neuroanatomy of ants as they transition between tasks, as NB ants are incapable of performing nursing tasks. Two axon guidance genes were also regulated suggesting neural development taking place. Changes in the morphology of the mushroom bodies of *A. mellifera* post-metamorphosis have been investigated in nurses as they transition to foragers (Kiya, Kunieda, & Kubo, 2007). Our results may reflect a similar system in *S. invicta* where changes in task and social context could affect the neuroanatomy of the insect as the workers adapt to increase colony fitness.

The present studies were untaken to investigate how the gene expression patterns in the brain are changed by the absence or presence of brood, and the potential importance of the regulated genes on social regulation and neofunctionalization of genes in social insect systems. The results of this study provide evidence that the absence or presence of brood influence the gene expression patterns in the brain of foraging workers. These changes in gene expression may cause neural and molecular changes in the brain of these workers. This supports studies that have

been conducted in the brain of *S. invicta* where the precedent of the sNPF receptor changes in protein expression and in the number of clusters and number of cells in clusters (Castillo & Pietrantonio, 2013). Additionally, in the brain of *A. mellifera* workers in which brood pheromone regulates their behavior by modifying the expression of behaviorally relevant genes in the brain of workers (Alaux et al., 2009). Our results emphasize the importance of gene expression in the brain as a regulator of behavior and may operate in a similar fashion to Africanized honey bee reactions to alarm pheromone. Alarm pheromone has been shown to create distinct transcript patterns within the brains of *A. mellifera* in addition to changes in behavior (Alaux et al., 2009). Additionally, this reaction not only produces immediate behavioral changes, but also induces an extended change in behavior in which the bees show higher activity for several hours after exposure to the pheromone (Alaux et al., 2009). This supports the idea that exposure to brood could affect the foraging behaviors in *S. invicta*. This is further supported by the changes in type of food foraged for while brood are present. In social insects, the presence of brood causes an increase in the number of protein foragers, suggesting that the protein foraging state is directly tied to exposure to brood (Dussutour & Simpson, 2009; Tanya Pankiw, 2004; Pankiw, Page Jr, & Fondrk, 1998). The observed differences in the transcriptome between B and NB workers may be reflective of distinct neurogenetic states that may be shared across multiple species of ants (specifically those that require brood to metabolize proteinaceous food).

In this study, we identified two Vg genes as differentially expressed in the brains of B or NB workers. After confirmation by RT-qPCR of the differential expression of *Vg2* and *Vg3* in the brain transcriptomes, we investigated whether those differences also existed when analyzing whole bodies. Vg proteins are produced in the fat body, and while the transcriptomes were produced using dissected brains, fat body contaminations could have occurred. No differences in

the expression of any of the Vg genes were identified when comparing B and NB whole bodies of workers (Figures 14, 15 and 16). Previously, we analyzed the expression of Vg genes in the *S. invicta* worker subcastes and while performing specific tasks (Hawkings & Tamborindeguy, 2018), and found differences in *Vg1* expression depending on the task performed by the worker, and differences in *Vg1* and *Vg2* expression associated with the worker subcaste. However, the previous analysis did not compare the expression between workers from colonies with and without brood.

Further analysis of the expression of the Vg genes in the head of worker ants of each subcaste confirmed the differential expression of Vg genes between NB and B workers. The expression of *Vg2* and *Vg3* was significantly higher in the heads of NB medium and major workers compared to the heads of B forager workers (Figures 18 and 19), while only *Vg2* was up-regulated in the head of the NB minor forager workers (Figure 17).These results support the idea of neofunctionalization of the Vg genes in *S. invicta* after duplication: the up-regulation of *Vg2* and/or *Vg3*, but not *Vg1* and *Vg4* evidence the existence of different regulatory mechanisms for these genes. Thus, this analysis completed our previous work, and suggested that the changes in Vg expression in workers from B and NB colonies might be restricted to the worker head region.

S. invicta workers are completely sterile. Thus, the expression of Vg in this caste suggests that in this species, Vg genes have been co-opted and acquired non-reproductive functions. Our analyses also identified difference of expression between the heads and rest of the body for the four Vg genes in major workers. The selective up-regulation of *Vg 2* and/or *Vg3* in the heads but not whole bodies of the workers support the idea of fat body regionalization (Münch et al., 2015). Therefore, *Vg2* and *Vg3* might be playing specific and maybe similar roles in the heads of

workers. *A. mellifera* nurses use Vg as a proteinaceous source for royal jelly production by the hypopharyngeal glands (Hrassnigg & Crailsheim, 1998; Nelson et al., 2007). Vg expression in the head region of *S. invicta* could be linked to a similar mechanism of Vg utilization to feed brood, however this mechanism is unknown in this species.

Vgs could also play a signaling role in the heads of *S. invicta*. Because Vgs are phosphoglycolipoproteins that can bind many compounds, their signaling role could be direct, or through the compounds they carry. Indeed, in *A. mellifera* workers Vg and JH regulate caste-specific behaviors through their mutual repressor relationship (Azevedo et al., 2016; Sheng, Xu, Bai, Zhu, & Palli, 2011). Based on our results, Vg could be involved in nutritional-status signaling at the individual or colony level as well as signaling the presence, quantity, and/or developmental status of the brood. Therefore, Vg could be an important player in integrating nutritional and reproductive signals at the colony level.

In the formicoid clade, Vgs can be classified in two groups, subfamily A, which includes *S. invicta Vg1* and *Vg4* is preferentially expressed in workers, while subfamily B, which includes *S. invicta Vg2* and *Vg3* is preferentially expressed in queens (Corona et al., 2013). Remarkably, the differential expression of Vg genes in the absence of brood was only observed for *Vg2* and *Vg3*, which are believed to have maintained the reproductive role in *S. invicta*. Therefore, it cannot be excluded that this regulation is a remnant from a time when workers could reproduce.

Previously, differences in the expression profile between the Vgs from a same subfamily were identified, specifically, *Vg1* was regulated in a task-specific manner, but not *Vg4*. In the present analyses, fewer differences in the expression profiles were identified for *Vg2* and *Vg3*, and *Vg1* and *Vg4* when analyzing gene expression in head and body regions.

In our previous study we showed the higher *Vg1* expression in whole bodies of medium

carbohydrate foragers compared to medium protein foragers and nurses. Here, expression analysis of the Vg genes in the head of task-allocated workers revealed that all Vg genes were expressed at higher level in the head of the nurses compared to carbohydrate foragers and protein foragers. We can hypothesize that the higher expression in the head of the nurses may imply that Vg could be used for brood food. This expression also could also be linked to the Vg localization in the glial cells of *A. mellifera* worker brains (Münch et al., 2015). Localization of Vg in these cells could be associated to a role maintaining the functional integrity of the brain. The reduction of Vg expression in the head of the foragers compared to nurses could be linked to age polyethism. Indeed, in *A. mellifera, Vg* expression decreases as the workers transition from nursing to foraging tasks. Further in that species, the decrease in Vg titer in the hemolymph of workers coincides with the switch from a carbohydrate/protein to a pure carbohydrate diet (M. Bitondi & Simoes, 1996). Similarly, in *Pogonomyrmex barbatus* the *Pb_Vg1* expression is higher in nurses than in foragers (Corona et al., 2013). *Pb_Vg1* is the Vg gene that clusters in the *Vg* subfamily B (associated to the queen). Intriguingly, the expression of *Pb_Vg2*¸ which clusters in the *Vg* subfamily A (worker associated), is higher in foragers than in nurses when evaluated at the while body level. It would be interesting to evaluate the expression of these genes in the heads of these ants. *P. barbatus* nurses produce trophic eggs to feed the brood, thus expression analyses of Vgs in the head of these insects could help elucidate whether Vg expression in the head of *S. invicta* nurses is linked to brood feeding or other roles such as signaling.

Finally, *Vg2* was also differentially expressed in the head of carbohydrate and protein foragers. We could speculate differences in *Vg2* expression between foragers is linked to "chain signaling" as the protein foragers transfer proteinaceous food to nurses, which then interact with the brood. Importantly, no difference of expression was identified for *Vg3* between the two

foraging types. Thus, these results also confirm that these two genes have specific regulation mechanisms, and that while they might have similar functions in the head of the workers, they also have some distinct functions.

The existence of distinct functions for *Vg2* and *Vg3* was supported by the starvation assay. In that assay differential expression of *Vg2* and *Vg3* was identified. In the starved workers, *Vg2* expression in the head increased 20 days after starvation began compared with the unstarved (control) colony. We can assume that *Vg2* responded to the reduced protein availability in the colony or the reduced amount of brood. Further, this response was detected in the head region. At that time point, an increased expression of *Vg3* (fold change of 1.4) also was observed but it did not reach significance. After 21 days, the protein sources were reintroduced to the colony, and 30 days after the initial starvation the level of *Vg2* expression was indistinguishable in the manipulated and control ants. However, at day 30, *Vg3* expression was reduced in workers in the manipulated colonies compared to the control colony. This indicates that *Vg3* and *Vg2* may respond differently to nutritional cues as they did not show similar expression patterns. It could also mean that *Vg3* was responsive to the reintroduction of food and/or that transcript levels are reduced as more protein is being made in response to a surge of protein resources. *Vg3* may also be acting as a nutritional reserve within workers who are at a protein deficit. However, at this time it is unclear if *Vg3* transcripts are translated into their protein counterpart. Alternatively, these transcripts could be regulated in response to changes on the amount or type of brood. Indeed, by day 21, brood was reduced in the manipulated colonies.

In this study we did not evaluate the effect of age in gene expression. Further studies should be aimed at identifying if the total protein content of the food could cause differential expression changes. In the starvation assay, crickets had previously been the protein source, but

reserves, a category of workers, could have stored proteins within the manipulated colonies. Additionally, workers might obtain proteins from the honey-water in the colony. Alternatively, cannibalism could have supplied proteins. Manipulation of the amount and developmental stage of the brood in the colonies might reveal the importance of these factors in Vg regulation and its potential role in signaling.

CONCLUSION

In conclusion the results of this study confirm the hypothesis that differential expression of genes occurs in the brain of workers is regulated by the brood. Our study demonstrated the region-specific regulation of Vg expression in the workers of *S. invicta*. Differential expression in the head and brain occurred in foragers in response to the absence of the brood and the task being performed in colonies with brood. Greater differences in gene expression occurred in major ants. Medium workers conducting different tasks within a colony with brood showed differential Vg expression, of which the nurses showed the highest expression of every Vg, hinting that this may also be impacted by the interaction with the brood. When workers are deficient of proteins, *Vg2* levels are increased after 20 days, while *Vg3* is decreased 30 days after initial starvation but 9 days after the reintroduction of proteinaceous food sources. It is possible that this regional expression could also be influenced by the age of the workers, and future experiments conducted to evaluate the effect of age may uncover more differential expression in the head region.

CHAPTER V

COMPARATIVE TRANSCRIPT ANALYSIS OF THE BRAIN OF *SOLENOPSIS INVICTA* WORKERS FORAGING FOR CARBOHYDRATE OR PROTEIN

INTRODUCTION

It is widely accepted that eusocial insect colonies are characterized by division of labor in which workers conduct the majority of tasks associated with colony maintenance and survival (W. M. Wheeler, Wheeler, & Bouvier, 1928). The regulatory mechanisms of division of labor in social insects have been a major focus of research and a genetic-basis for the division of labor has been established in several hymenopteran species. This genetic variation affects the division of labor between the reproductive and the worker castes, as well as the behavioral division of labor among workers. This latter question has also been much studied and several components have been identified (C. R. Smith et al., 2008). There can be a genetic component to the task performed by the worker and the genetic diversity among workers can increase the colony fitness by generating a superior task allocation system (Libbrecht & Keller, 2013; Oldroyd & Fewell, 2007). This genetic diversity among workers can be the result of polyandry (a queen mates with multiple males), or polygyny (cooperation among queens) (Oldroyd & Fewell, 2007). Age-dependent polyethism also affects the task performed by a specific worker. In this case, usually younger insects perform in-nest tasks, while older workers perform out-of-nest tasks (Mersch, Crespi, & Keller, 2013; E. O. Wilson, 1978). Finally, the insect size and morphology, which can be linked to the genetic component, can also determine task in species in which workers display polyphenism (E. J. Robinson, Feinerman, & Franks, 2009; C. R. Smith et al., 2008). Phenotypic plasticity can be influenced by nutritional stimuli, and the behavioral states of nurses and

foragers are accompanied by physiological changes and changes in gene expression (Libbrecht & Keller, 2013).

One of the most studied species to elucidate the mechanisms of division of labor is the honey bee *Apis mellifera.* In this species, workers display age-dependent polyethism but not morphological polyphenism. Division of labor among workers is regulated by nutritional signals associated with lipid reserves where workers loose half their lipid stores during the transition from nursing to foraging (Fox et al., 2010). The insulin pathway has also been correlated with task transition in A. *mellifera* (Ament et al., 2008). Several genes have been identified as involved in division of labor. For example, greater reserves of the protein vitellogenin are found in nurses than in foragers, and a reduction of the amount of the vitellogenin protein in nurses stimulates foraging (Nelson et al., 2007). The *foraging (for)* gene has also been associated with division of labor in *A. mellifera* (Yehuda Ben-Shahar, 2005; Y Ben-Shahar et al., 2003; Daugherty et al., 2011; Ingram et al., 2005). Workers performing out-of-nest tasks have significantly higher transcript levels of this gene when compared to in-hive workers. The gene *malvolio* encodes a manganese transporter and is thus potentially involved in sucrose responsiveness (Yehuda Ben-Shahar et al., 2004; Rodrigues, Cheah, Ray, & Chia, 1995). *Malvolio* is upregulated in the brain of foragers compared to nurses, and this upregulation is mostly related to behavior rather than chronological age. Microarray and RNA-seq analyses have been used to compare changes in gene expression in the brains of insects associated with specific behaviors (Daugherty et al., 2011; Toma, White, Hirsch, & Greenspan, 2002). Nurses and foragers of social insets differ in expression of genes within the brain (Whitfield, Cziko, & Robinson, 2003) as well as in brain structure and neurochemistry (Kucharski & Maleszka, 2002; G. E. J. T. A. N. Robinson, 2002). Further, the brain gene expression profile of worker bees can

distinguish them as either a forager or a nurse regardless of age, colony type, or prior experience. Therefore, gene expression in the brain can be used as an indicator of the types of behavior of the worker (Whitfield et al., 2003). Further, evidence for a strong genetic influence on behavior exist (Zayed & Robinson, 2012). Artificial selection studies produced strains of bees which show strong preference toward either protein foraging or carbohydrate foraging, and preferentially collect high amounts of pollen or nectar respectively (Wang et al., 2010). This suggests there is a plastic genetic component to the type of foraging being performed by worker bees. Similarly, differences in brain neuropeptide profiles between bees which forage for pollen and those which forage for nectar were identified (Brockmann et al., 2009), and differences in peptide abundance are also observed prior to arrival at the preferred food source, suggesting that they are associated with foraging choices.

In ants, the existence of age polyethism and physical polymorphism influencing division of labor among workers is well documented (Schwander et al., 2005). Regulation of task performed involves changes in neural organization that can be regulated by age and biogenic amines (R. E. Page Jr et al., 1992; Toth & Robinson, 2007), by hormones (Antonio et al., 2008), and/or linked to age-based polyethism (Beshers & Fewell, 2001). In fire ants, *Solenopsis invicta*, younger workers conduct tasks inside the colony such as nursing and queen tending while older workers perform their tasks extranidally, such as foraging (Mirenda & Vinson, 1981; E. O. Wilson, 1971). Studies based on *S. invicta* whole-body gene expression analyses have been used to identify genes and pathways associated with division of labor between nurses and foragers (Manfredini et al., 2014; Qiu et al., 2017).

In fire ants, foragers search for one of two broad categories of food resources, protein or carbohydrate sources. Once brought to the colony, protein food sources are first fed to the fourth

instar larvae, which are the only members of the colony able to digest solid proteinaceous food before they can be utilized by the rest of the colony (Cassill & Tschinkel, 1995; Petralia & Vinson, 1978). Carbohydrate sources, however, can be utilized by the individual worker directly or distributed throughout the colony (W.R. Tschinkel, 2006; W. R. Tschinkel, Mikheyev, & Storz, 2003). Studies using the whole body of workers suggest differential gene expression based on task. For instance, *vitellogenin 1* is differentially expressed in carbohydrate foraging workers compared to protein foragers and nurses at the whole-body level (Hawkings & Tamborindeguy, 2018). Similarly, while all vitellogenins (*vitellogenin 1*, *2*, *3*, and *4)* are expressed at higher level in the head of nurses than foragers, *vitellogenin 2* is also differentially expressed between the heads of workers foraging for carbohydrates and proteins (Hawkings and Tamborindeguy, unpublished Chapter IV). In our study, we used RNA-seq through the Illumina platform to compare brain gene expression profiles between protein forager and carbohydrate foragers of *S. invicta*.

MATERIALS AND METHODS

Insect colonies

Polygyne *S. invicta* colonies were collected in Brazos County, TX from May to July 2015 for this study. After collection, they were maintained in the laboratory at Texas A&M University in plastic trays (27 x 40 x 9 cm). The walls of the containers were coated with Fluon (Insect-a-slip, BioQuip products, CA, USA). Each colony was provided glass tubes, half-filled with water and blocked with cotton, and 14 cm-diameter petri dishes half-filled with damp Castone (Dentsply International Inc., York, PA, USA) as a nest area. Colonies were maintained at 27 ± 2

°C in a 12:12 hour light - dark photoperiod. The colonies were fed daily with crickets, *Acheta domestica,* and a 20% honey water solution. Water was given *ad libitum.* All colonies contained mated and virgin queens, brood and workers.

Classification and selection of worker ants

Firstly, ants were classified into subcaste according to their head width as previously described (E. O. Wilson, 1978). For this experiment, medium workers were selected because they are the most versatile subcaste in terms of the tasks they perform (W.R. Tschinkel, 2006). Workers were considered as medium workers if their head width was between 0.73 and 0.92 mm.

Foragers were classified as ants that were directly interacting with a specific food type. If a worker was actively collecting food from the carbohydrate tray during the collection it was determined to be a carbohydrate forager, if a worker was actively collecting food from the protein tray during the collection it was determined to be a protein forager. Food sources were replenished daily between 9:30 AM and 10:00 AM and ants were collected and dissected between 11:00 AM and 3:00 PM, a minimum of 1 hour after the food source was reintroduced that day.

Dissection of worker brains

For transcriptomic analyses, forager worker brains were dissected on ice-cold PBS buffer and placed immediately in 100 μL of TRIzol Reagent (Thermo Fisher Scientific, Carlsbad, CA) maintained on dry ice. Once approximately 30 brains had been collected in a tube, they were kept at -80°C until further needed. For the final sample, brain pools were combined into one tube

(approximately 150 to 200 brains). There were three colonies used and each replicate represented pools of brains from a same colony.

RNA extraction and purification

RNA from brains of carbohydrate and protein foragers were purified. There were three replicates per treatment (150 – 200 brains constituted a replicate, which yielded approximately 1 µg of total RNA). RNA purification was performed as described in (Calkins et al., 2018). In short, total RNA was extracted using the Trizol Reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. The Direct-zol™ micro prep plus clean up kit (Zymo Research, Irvine, CA, USA) was used for sample clean up and RNA purification to remove any trace of TRIzol from the solubilized pellet. RNA was then resuspended in 20 µl of nuclease free water. The Turbo DNAse kit (Ambion, Waltham, MA, USA) was used to eliminate genomic DNA using the manufacturer's protocol. Quantity and purity of the total RNA was assessed using an Infinite® 200 PRO NanoQuant (Tecan, Männedorf, Switzerland) and the integrity of RNA was visualized on a 2% agarose electrophoresis gel stained with ethidium bromide. RNA was then stored at -80 °C.

Transcriptomic analyses

RNA samples were submitted to the AgriLife Genomic and Bioinformatic Center (TAMU) for transcriptomic sequencing and libraries were prepared with Illumina TruSeq Stranded Total RNA library preparation kit and sequenced with the HiSeq 2500 System (Illumina) platform over four lanes of 125PE (paired end). The sequence raw reads were cleaned to remove barcode tags and adaptors using cutadapt 1.0. Individual samples were processed with

FastQC, and the QC reports were checked as final confirmation of sequence quality and trimmed as needed up to the first 15 base pairs.

All bioinformatic analyses were performed in the Discovery Environment web interface and platform at CyVerse (S. A. Goff et al., 2011). The RNA-seq reads that passed the quality parameters (FASTQC tools) were mapped to the *S. invicta* genome (Ensembl Metazoa, GCA_000188075.1.34) using TopHat 2.0.9 (Trapnell et al., 2009). Cufflinks 2.1.1 was used to estimate the transcript abundance (L. A. Goff et al., 2012). Differentially expressed genes (DEGs) were identified using Cuffdiff 2.2.1 with the JS (Jensen-Shannon) option (Trapnell et al., 2013). The bioinformatic analyses were visualized using the CummeRbund package in R (L. A. Goff et al., 2012).

RESULTS

Transcriptomic analyses

Six purified RNA samples (three biological replicates per condition) were sequenced, and a total of 718.05 million reads were obtained. On average, 92.6% of the reads mapped to the *S. invicta* genome (Table 5)

Table 5: Sequenced libraries summary. Where "L" are left reads and "R" are right reads

LIBRARY	NUMBER OF TOTAL READS	NUMBER OF MAPPED READS (%)
PROTEIN 1 L	74,883,943	70,777,225 (94.5% of input)
PROTEIN 1 R	51,045,643	46,249,609 (90.6% of input)
PROTEIN 2 L	51,045,643	47,857,122 (93.8% of input)
PROTEIN 2 R	51,073,657	46,559,907 (91.2% of input)
PROTEIN 3 L	51,073,657	47,997,530 (94.0% of input)
PROTEIN 3 R	60,372,135	55,662,612 (92.2% of input)
CARBOHYDRATE 1 L	60,372,135	56,891,585 (94.2% of input)
CARBOHYDRATE 1 R	74,883,943	68,626,510 (91.6% of input)
CARBOHYDRATE 2 L	67,909,629	63,945,009 (94.2% of input)
CARBOHYDRATE 2 R	53,740,296	48,626,534 (90.5% of input)
CARBOHYDRATE 3 L	53,740,296	49,864,624 (92.8% of input)
CARBOHYDRATE 3 R	67,909,629	62,024,399 (91.3% of input)

No significance difference of gene expression was identified between the brains of protein foragers and carbohydrate foragers ($q > 0.05$). A cluster dendrogram showing the similarity among the sequencing replicates is shown in Figure 22. This representation shows that the replicates did not cluster according to the type of foraging conducted by the workers: one of the protein forager replicates (Protein 2) was more similar to two of the carbohydrate forager replicates (Carbohydrate 2 and Carbohydrate 3) than to the other protein forager replicates. Similarly, the carbohydrate replicate, Carbohydrate 1 clustered with two protein forager replicates.

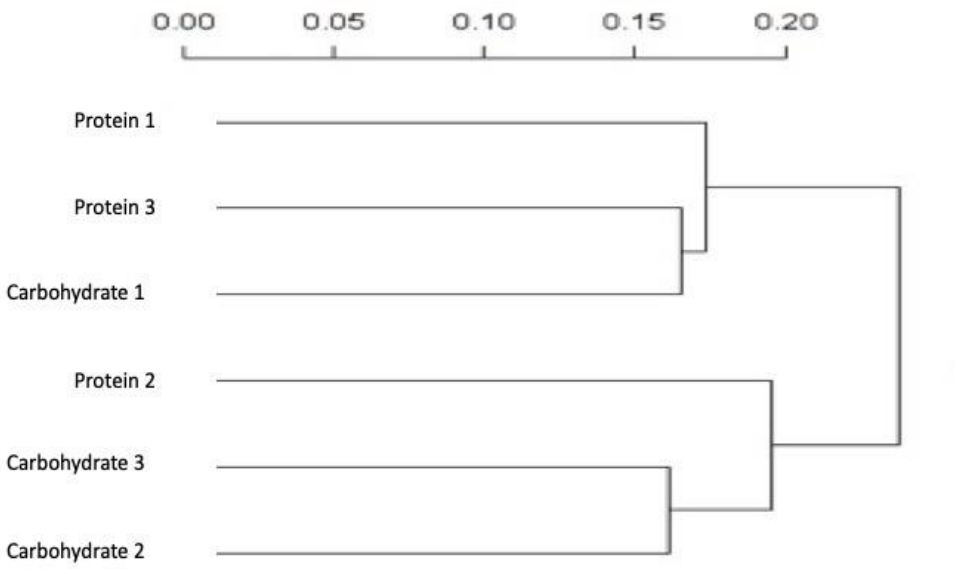

Figure 22: Transcriptome analysis overview

Cluster dendrogram of the replicates sequenced for the transcriptomic analyses. The three brain replicates obtained from the workers foraging carbohydrates are labeled Carbohydrate 1, Carbohydrate 2, and Carbohydrate 3. The three brain replicates obtained from the workers foraging proteins are labeled Protein 1, Protein 2, and Protein 3. The branch height indicates distances between replicates.

Using a less stringent q-value for the analyses, ten genes were identified as differentially expressed between the samples ($q < 0.08$) (Table 5). All differentially expressed genes were expressed at higher levels in the brain of carbohydrate foragers.

Table 6: Differentially expressed genes between carbohydrate foragers and protein forgers with a q < 0.08. Fold change [log ($FPKM_{carbohydrate}$ - $FPKM_{protein}$)]. Inf indicates infinite.

Gene ID	Gene name	Protein (FPKM)	Carbohydrate (FPKM)	Fold change	q-value
LOC105196674	branched-chain-amino-acid aminotransferase, cytosolic	18.2511	66.8898	1.8738	0.07345
LOC105196694	probable salivary secreted peptide	0	1.33374	inf	0.07345
LOC105196696	probable salivary secreted peptide	0	1.24068	inf	0.07345
LOC105196835	branched-chain-amino-acid aminotransferase, mitochondrial-like	35.2514	112.429	1.67326	0.07345
LOC105197985	serine protease 48	0	1.30034	inf	0.07345
LOC105199000	probable cytochrome P450 6a13	1.59363	6.84285	2.10229	0.07345
LOC105199597	purine nucleoside phosphorylase-like	34.4812	103.024	1.57909	0.07345
LOC105199862	uncharacterized	0	3.13177	inf	0.07345
LOC105205188	cytochrome P450 9e2-like	10.0592	26.8397	1.41585	0.07345
LOC105207006	alanine/arginine aminopeptidase-like	0	3.36471	inf	0.07345

DISCUSSION

In this study, we found no significantly differentially expressed genes between the brains of carbohydrate and protein foragers at $q < 0.05$. However, when we relaxed the q-value threshold, ten genes were identified as differentially expressed ($q < 0.08$). All the genes identified were expressed at higher level in the brains of the carbohydrate foragers. Among these genes were two branched-chain-amino-acid aminotransferase (BCAT) genes, one aminopeptidase gene, one serine protease gene, two probably secreted salivary peptide, two probable cytochrome p450 (6a13 and 9e2-like), one purine nucleoside phosphorylase-like, and one uncharacterized gene.

BCAT genes are interesting candidates because they are involved in the transamination of branched-amino acids. These amino acids play key roles in the brain and in particular they are linked to food ingestion and appetite (Fernstrom & Wurtman, 1972). Importantly, branched-chain amino acids can pass the brain-blood-barrier (Cooper & Plum, 1987) and they may influence the brain function and affect appetite, as well as other physical conditions (Fernstrom, 1985). For instance, branched-chain amino acids could compete for entry into the brain with aromatic amino acids (Fernstrom & Wurtman, 1972), and decreased levels of aromatic amino acids, and tryptophan in particular, could affect the synthesis of serotonin and other neurotransmitters. In humans, the branched-chain amino acids play an important role in the synthesis of brain glutamate (Yudkoff, 1997), a major neurotransmitter. Further, the levels of branched-chain amino acids can increase in response to food ingestion (Fernstrom, 2005). Finally, leucine, one of the branched-amino acids, activates the mechanistic target of rapamycin, mTOR, a serine/threonine protein kinase, which is involved in the control of cell growth and proliferation (Arquier, Delanoue, & Léopold, 2010).

Two probably secreted salivary peptide were also identified as potentially differentially expressed. These genes could be expressed in glands associated with the brain. While genes in this category could be related to neofunctionalization of gene function between workers and queens of ant species (Claire Morandin et al., 2016), they could also be related to the types of food being ingested. In this latter case, the expression of these genes could change after the insect starts foraging, and not before.

Interestingly, the two-probable cytochrome P450s identified in this study (6a13 and 9e2-like), were also identified as differentially expressed when comparing the brain transcriptomes of carbohydrate foragers from colonies with and without brood (Hawkings et al., unpublished; Chapter IV).

Differences in the gene expression profiles of forager and nurse brains are probably greater. Already, studies at a whole body level revealed differences between these two worker categories and highlighted the potential role of lipid storage and fatty acid biosynthesis in task allocation (Qiu et al., 2017). Further, the expression of hexamerins, storage proteins, is caste- and task-specific (Hawkings et al. unpublished; Chapter IV). Finally, while we identified differences in *vitellogenin 2* expression in the head of carbohydrate and protein medium foragers, this gene was not differentially expressed when comparing the brain transcriptomes of these types of foragers ($q = 1$).

Because of the similarity of the libraries sequenced, it is difficult at this time to determine if the behavior of the fire ant forager (carbohydrate or protein forager) can be identified based on the transcriptional profile of their brains as it was determined for *A. mellifera* (G. J. Hunt, Page, Fondrk, & Dullum, 1995; R. Page Jr et al., 2000; Rueppell et al., 2005), *S. invicta* workers prefer foods with equal or moderately protein-biased p:c ratios (Cook, Eubanks, Gold, & Behmer,

2010). Therefore, the macronutrient bias of the food resources in a natural setting for *S. invicta* may not be as clearly defined as that for *A. mellifera* where pollen and nectar can be selectively foraged.

Although the workers analyzed in the current study were of unknown age, previous studies in *A. mellifera* have indicated that most of the changes in physiology and gene expression in workers are more closely associated with their behavior and not chronological age (G. E. J. T. A. N. Robinson, 2002). Future experiments should be aimed at increasing the number of replicates sequenced to determine whether the changes in gene expression identified by relaxing the q-value threshold is biologically relevant. Further, future experiments should differentiate from foragers arriving to the food source from those departing. Indeed, we can speculate that major changes in the brain transcriptome could occur as the insect ingests the food (Brockmann et al., 2009).

It is important to note a few observations of this study. The analysis revealed that two protein replicates and two carbohydrate replicates clustered together, but the analysis has shown an outlier replicate (Protein 2) sample. To ensure the accuracy of the conclusions future studies would need to sequence more RNA replicates or re-sequencing of the samples again to validate the results (while there is no reason to suspect that a carbohydrate and a protein sample replicate were switched, human errors are always a possibility). Another issue in our experimental set-up could be linked to the foraging arena which is not equivalent with a natural setting. In a natural context, the ants would have a differing range of experience. Scouting ants may represent a clear intermediate in the foraging behavior, in our colonies because of the limit in colony size and space we do not see scouting ants. The gene expression profiles may not be distinct within task allocated workers because the food resources are close in proximity and distance traveled to

forage for them is short, so there is a limited need for a high degree of task specialization. Furthermore, qRT-PCR validation could confirm the differential expression of the genes identified with a relaxed q-value.

CONCLUSION

No significant differences in brain gene expression were found between workers foraging carbohydrates or proteins. These data help distinguishing the molecular mechanisms regulating foraging in ants and bees: *S. invicta* display an opportunistic mode of foraging. These results will therefore enhance our knowledge of social organization and foraging, and how these differ between social insect species.

CHAPTER VI

CONCLUSIONS

In this dissertation I focused on the molecular mechanisms that underlie the basis of *S. invicta* social behavior. In other social insect species, candidate genes have been implicated in the task allocation of workers through expression analysis studies (Choi, Kohler, Vander Meer, Neupert, & Predel, 2014; Corona et al., 2013; D. Gotzek, Shoemaker, & Ross, 2007; Christophe Lucas et al., 2015; Whitfield et al., 2003; Xuguo Zhou et al., 2007). *S. invicta* workers are completely sterile, which allows one to differentiate candidate genes involved in reproductive pathways as these phenotypes are separate. I hypothesized that there has been subfunctionalization of genes networks between workers and queens, specifically hexamerins and vitellogenins (Vg) which have been characterized as caste-biased in other eusocial insects (Gro V Amdam, Norberg, Hagen, et al., 2003; Antonio et al., 2008; Azevedo et al., 2011; Corona et al., 2013; Nelson et al., 2007). Additionally, I hypothesized that these genes have differing functions among different groups of social insects as social clades have evolved independently. While the Vg transcript is expressed in the workers and is important in task allocation in *A. mellifera* (Corona et al., 2007), only *S. invicta vitellogenin 1* was differentially expressed among workers performing different tasks at a whole body level, and contrary to what is known in *A. mellifera* and other ant species, the expression of this gene was higher in carbohydrate foragers than in nurses. There is a correlation of Vg titer with the onset of foraging in *A. mellifera* and other ants (Antonio et al., 2008; Azevedo et al., 2016; Corona et al., 2007), however this correlation may be unique to a few social insects. Further, Vg expression in *S. invicta* workers was not affected by the topical application of a JH analog, S-hydroprene, as it is in other ant

species (Azevedo et al., 2016; Rembold et al., 1974), supporting the hypothesis that in *S. invicta* Vgs may not be involved in age polyethism. I did however identify differential Vg expression among the morphological subcastes (major workers had higher levels of *Vg1* expression relative to both medium and minor workers, and major workers had higher levels of *Vg2* compared to minor workers) (Hawkings & Tamborindeguy, 2018). This could indicate possible signaling mechanisms of larger workers who may pose nutritional reserves within the colony. Differences in the relationship between Vg and JH in *S. invicta* when compared to *A. mellifera* may be attributed to their separate evolutionary path to eusociality. These results suggest that while reproductive ground plan hypothesis provides an explanation for how reproductive genes and physiological processes can be co-opted for social functions, the exact mechanism in which specific genes affect individual behaviors and colony structure may differ between taxa.

Interestingly, differences in Vg gene expression in the head and brains of *S. invicta* workers were observed when comparing colonies in the presence or absence of brood indicating the importance of social context and the brood on gene expression patterns in the brains of workers. We also see higher gene expression of Vg in the heads of nurses compared with protein and carbohydrate foragers. This correlates with *A. mellifera* where higher Vg has been found in the head region of nurses (Gro V Amdam, Norberg, Hagen, et al., 2003). This may indicate that Vg functions similarly in the interaction of brood, a mechanism that may be conserved among all social insect species. The Vg protein has been localized in the head of *A. mellifera* where it is suspected to be used to produce food for the brood (Münch et al., 2015).

Our transcriptome analysis results identified significantly differentially expressed genes potentially important in communication and signaling pathways, among which were *Vg2* and *Vg3*. These two Vg genes were more highly expressed in the brains of carbohydrate foragers in

the absence of brood. Since the fourth instar larvae are essential to the digestion of solid protein (Petralia & Vinson, 1978), the absence of the larvae (specifically fourth instar) may result in increased expression of these genes either as a storage protein in anticipation of a period of protein deficiency, or for signaling functions to signal the absence of protein flow. Surprisingly, the difference in *Vg2* and/or *Vg3* expression was also detected when assaying heads from decapitated carbohydrate foragers from colonies with and without brood (for each subcaste), but not when assessing their expression in the rest of their body (carcass minus head), or at the whole-body level. From this, we can assume that changes in Vg gene expression is region-specific, and in this case within the brain and/or head region. Vg is also differentially expressed among the workers in the head and brain regions of *A. mellifera* depending on the task being conducted (Münch et al., 2015; Whitfield et al., 2003).

I also showed a link between the reduction in the amount of brood in a colony and the increased expression of *Vg2* in the head region of carbohydrate foragers. After 20 days of a colony being deprived of a protein food source, the amount of brood in colonies was reduced. It is important to note that this could not be attributed to whether the queen had stopped laying eggs or if the workers were feeding on the larvae, both could be a response to protein deficiency. These changes in the expression of *Vg2* in the head were not reflective of the mechanisms occurring within the whole body, which may indicate a specialized function of this gene in the head at the intersection between behavior, brain, and communication. The differential expression of *Vg2* and *Vg3* may be dependent on the brood being able to digest the proteins for overall colony nutrition.

I also compared the brain transcriptomes of workers foraging for protein and for carbohydrate but did not identify any differences in their gene expression (at a q-value of <

0.05). This is unlike *A. mellifera*, which display preferential foraging behavior (protein-bias or carbohydrate-bias) based on gene expression (Hunt 1995) where candidate genes have been identified and manipulated to induce protein foraging (R. Page Jr et al., 2000). Perhaps in *S. invicta*, carbohydrate and protein foragers are interchangeable based on opportunity which may explain why no differential expression was found between the brains of carbohydrate foragers or protein foragers as opposed to having a genetic predisposition for a type of forager like *A. mellifera* workers. Differences in the natural history between *S. invicta* and *A. mellifera* may also provide insight into the lack of differences in gene expression between foragers. Foragers of *A. mellifera* will either search for nectar or pollen, which are heavily carbohydrate or protein biased respectively, and are able to fly great distances in search of food. Because of this, maintaining a balance of workers who are genetically predisposed to collecting either carbohydrates or protein is necessary to maintaining the proper macronutrient ratios for the whole colony as opposed to more opportunistic foraging strategies which may cause a skew in colony nutrition. In contrast, *S. invicta* derive their nutrition from interchangeable parts of the heterogeneous landscape of available resources. *S. invicta* workers incapability to fly also limits the distance in which foragers can search the environment for resources, therefore limiting how selective a single forager can be. Our results may reflect these differences between workers of *S. invicta* and *A. mellifera*. A previous study has also shown that *S. invicta* foragers exhibit seasonal changes in foraging behaviors, where foragers collected greater amounts of food during the Summer compared to Fall but regulated total macronutrient consumption to similar levels through selectively consuming and discarding food (Simpson 2011). This suggests that environmental influences, such as seasonal changes and colony status (number of brood), may be more influential in directing foraging decisions in workers than genetic elements in *S. invicta*. Overall,

this plasticity in workers could contribute to competitive advantages in exploiting food resources and in turn their ability to adapt in a wide range of environments as an invasive species.

In addition to Vgs, other genes have been implicated in the reproductive ground plan hypothesis. Hexamerins have been investigated in social insect task allocation (T Martinez, Burmester, Veenstra, & Wheeler, 2000; Xuguo Zhou et al., 2006; X Zhou et al., 2006; Xuguo Zhou et al., 2007), and here I identified a caste-specific bias of hexamerin expression between workers and queens. Differences in expression between nurses and foragers were identified which suggests possible functions of these genes in task allocation in workers. In our studies, *S. invicta* hexamerin genes were regulated by the topical application of a JH analog suggesting that perhaps some caste-specific functions might be dependent on the titer of this hormone to control the level of hexamerin expression. It could be possible that the expression of these genes involved in task allocation are unique to *S. invicta* and not shared amongst all social insect species. This is supported by the pattern of expression of hexamerins and their relationship with JH in *S. invicta* which correlates with the interaction of Vg and JH in *A. mellifera*. However, we see no correlation between Vg and JH in *S. invicta* as would be expected in *A. mellifera*. Future investigations would seek to identify additional differentially expressed genes from the transcriptome and a more thorough investigation of potentially key genes in task allocation and social organization. RNAi experiments may give us an insight to the potential behavioral determinants of hexamerin and Vg in *S. invicta* much like has been shown in *A. mellifera* and *Reticulitermes flavipes* (Antonio et al., 2008; Xuguo Zhou et al., 2006). The investigation of these genes might outline genes that are used in a unique in *S. invicta* social organization and those which may be general and used across all social insects. *A. mellifera* are a model used to study the molecular basis of behavior in a social insect context. While a lot is known about *A.*

mellifera, there is still a gap of knowledge for *S. invicta*, which could have a unique system for social organization as workers are completely sterile. Further, these studies can be used to outline the genetic differences between monogyne and polygyne colonies. It is known that Gp-9 expression is higher in monogynes than polygynes, and that this difference correlates with differences in the expression of the foraging gene (Christophe Lucas et al., 2015). Thus, we may hypothesize that additional candidate genes may be differentially expressed based on social form and therefore may be specifically important in the development of the more invasive polygyne form.

The elucidation of gene expression patterns is an important step in understanding of the molecular framework of social insects, especially pest species and targeting pest control through colony disruption. The abundance of *S. invicta*, its impact as a pest, and its continued spread across continents has emphasized the need for research into this species. We have found several differences between *A. mellifera* and *S. invicta*, which show that some biological processes which regulate social behavior and colony structure are not conserved between social hymenopterans. These results, the structure of *S. invicta* colonies (polygyne versus monogyne colonies), and the complete sterility of workers enables future research into unique elements of eusociality which are not able to be observed in *A. mellifera* and may provide a foundation for the use of *S. invicta* as a model system to study eusociality in insects.

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APPENDIX

Appendix 1: Alignment of the four S. invicta proteins using MAFT. ( ) indicates non conservative mutations, (*) indicates conserved regions, (:) indicates conservative replacement mutations and (.) indicates semi conservative mutations. Chapter III

Table A2: Primers used for gene expression analysis. Chapter IV

Name	**Sequence**
Si_Ejaculatorybulb_F	*GTGGCAGGCCATGGTAGAAA*
Si_Ejaculatorybulb_R	*CAGTTGGCAACATCATGCGG*
Si_Notch_F	*CCGCGGTGATGAATTGGGTA*
Si_Notch_R	*ACTGGTGGCACTTCGCAAAT*

Table A3: Summary information of the sequenced libraries Chapter IV

Library	**Total number of reads obtained**	**Mapped reads (percent of reads)**
Colony with brood 1	66,924,062	53,392,962 (79.8%)
Colony with brood 2	38,075,897	35,278,031 (92.7%)
Colony with brood 3	38,007,390	35,053,017 (92.2%)
Colony without brood 1	50,126,933	39,974,516 (79.7%)
Colony without brood 2	50,411,828	39,385,612 (78.1%)
Colony without brood 3	56,344,731	43,159,031 (76.6%)