SHORT NEUROPEPTIDE F RECEPTOR IN THE WORKER BRAIN OF THE RED IMPORTED FIRE ANT (*SOLENOPSIS INVICTA* BUREN) AND METHODOLOGY FOR RNA INTERFERENCE

A Thesis

by

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ABSTRACT

The red imported fire ant (*Solenopsis invicta* Buren) is one of the worst invasive species in the United States. Investigating their physiology to understand its molecular basis could lead to the discovery of new gene targets for fire ant control. Neuropeptides are involved in the regulation of important physiological processes, and in insects the short neuropeptide F (sNPF) plays an important role as regulator of feeding, and involved in mechanisms of nutrient sensing, growth and reproduction. This study is focused into unveiling the physiological role of the sNPF and its receptor (sNPFR) in fire ants. In workers, we found a total of nine clusters of immunoreactive-sNPFR cells located near important neuropiles in the brain. These sNPFR-expressing cells are sensitive to the presence/absent of brood, perhaps in correlation with changes in the nutritional status of the colony. Also, these cell clusters are differentially expressed among worker subcastes, suggesting the sNPF/sNPFR pathway could be associated with mechanisms of division of labor. To discover where sNPF is synthesized, we attempted to localize the sNPF-expressing cells in the brain of queens and workers through *in situ* hybridization, unfortunately without success; thus, the site of synthesis of sNPF in the brain still remains unknown. Finally, we tried to elucidate the role of the sNPFR in social context by silencing its gene expression trough feeding of dsRNA using small laboratory colonies. We found differential effects when delivering the sNPFR-dsRNA through heat-killed dsRNA-producing bacteria or by delivering dsRNA purified from these bacterial cultures. Also, the type of food used as carrier and the concentration of

dsRNA were crucial for gene silencing success. Our results showed that feeding a large concentration of dsRNA in liquid and solid foods is required to induce RNAi in the queen midgut; and that the silencing of the sNPFR in this organ did not induce mortality in these queens, but instead it appears to increase larval mortality. In summary, our results are consistent with the sNPF/sNPFR signaling pathway in fire ants being involved in the regulation of division of labor and in sensing the nutritional status, and suggest its function is fundamental for larval development.

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NOMENCLATURE

JH	Juvenile hormone
ILPs	Insulin-like peptides
CA	Corpora allata
CC	Corpora cardiaca
IIS	Insulin/Insulin-like signaling pathway
IR	Insulin receptor
sNPF	short neuropeptide F
sNPFR	short neuropeptide F receptor
GPCR	G protein-coupled receptor
NPY	Neuropeptide Y
ISH	*In Situ* Hybridization
RNAi	RNA Interference
EGFP	Enhanced green fluorescent protein

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CHAPTER I

INTRODUCTION: FIRE ANTS SOCIAL ORGANIZATION AND DIVISION OF LABOR

The problem of fire ants as persistent invasive species

Ants, wasps, bees and termites are organisms that live in societies with a complex social structure, and they represent the best examples of "eusocial insects". The term "eusocial" refers to the three remarkable traits exhibited by these insect societies, as follows: first, the performance of cooperative brood care; second, the existence of reproductive castes (queens and drones) and sterile worker castes, and finally, the coexistence of overlapping generations of individuals. Usually the individuals in the reproductive caste are several times less numerous than in the worker caste, and they are specialized in producing offspring, while workers are specialized for tasks associated with brood care, foraging, nest construction and maintenance, and colony defense [1].

The red imported fire ants (*Solenopsis invicta* Buren; Hymenoptera: Formicidae) are eusocial insects native from South America; and they have become invasive in many temperate areas of the world due to the high reproductive capacity of the queens, and their extraordinary capacity of adaptation to different environments, which allowed them to survive in different habitats as well as to survive catastrophic weather events (i.e., flooding) [2]. In addition, these ants present two forms of social organization depending on the number of queens living in the colony. Monogyne colonies have only one mated

queen, while polygyne colonies have multiple mated queens; and it is believed that the origination of polygyne colonies is an evolutionary adaptation which allowed the proliferation of these ants and the perpetuation of the species [3]. These invasive ants were introduced in the United States in Alabama around the 1930s, and 35 years later they were present in nine states from the Carolinas to Texas [4]. Currently, populations of these ants are still in the mentioned southern states but they have been found in California and Oregon where they are being eradicated [5].

Fire ants build their nests in urban as well as wild areas and when their mounds are accidentally disturbed, they react aggressively and are able to sting people, pets, domestic and wild animals. Additionally, they are voracious predators representing a threat for several species of native animals and also causing millions of dollars of losses in agriculture every year [6–8]. Since 1957, considerable effort has been invested to control the spreading of fire ant populations through the application of pesticides in toxic baits [9,10], use of biological control agents [11,12], and pathogens [13,14]; but none of these techniques appear to be completely effective to eradicate this invasive species, thus the discovery of new molecular targets and the development of new strategies to efficiently control their populations are necessary.

Castes, division of labor and food flow inside the colony

As in other eusocial insects, fire ant colonies are composed by individuals belonging either to the reproductive (queens and drones) or to the worker caste, and both castes perform different functions. Fire ant adult reproductives (alate males and females), are specialized for flying, mating and reproduction while all the workers are sterile females in which the ovaries are not present. These workers exhibit large variations in body size and based in these size differences; this caste has been subdivided into three subcastes denominated as minors, media and majors [15]. Minor ants are the smallest workers, and major ants are the biggest; while media are of intermediate size between both. This size variation is known as worker polymorphism and constitutes one of the bases for division of labor. This division of labor refers to the specialization of the workers to perform a specific task, and includes adaptations of their body shape and size. In fire ants, a correlation between the worker's body size and the labor they perform had been described before, but there is considerable variability among subcastes [15]. In general, younger and smaller ants usually perform tasks related with brood care (nursing), while older and larger ants prefer to forage for food. However, the length of nursing and foraging "careers" and the age of transition between these activities vary considerably between and within size groups. However, it is certain that foragers never feed larvae and nurses never forage [16]. In addition to the main worker subcastes of nurses and foragers, there is a third subcaste of workers of heterogeneous age, size and behavior classified as reserves. This subcaste is found primarily in the nest periphery and

is inactive for much of the time; but ants of this caste could engage in different tasks such as nursing, foraging, storage of liquid food or in food relay from foragers to nurses [16].

The eggs laid by the fire ant queen go through three additional developmental stages: larva (first to fourth instar), pupa, and adult; and ants of all immature stages are collectively known as brood [17]. Eggs and pupae do not feed, but the larvae require feeding by the workers of the colony to complete their development. All the members of the colony feed via trophallaxis, and workers feed larvae of all instars by regurgitation of liquid foods such as oils, sugary and proteinaceous solutions; however, only 4^{th} instar larvae can be fed with solid protein-rich foods [18]. Larvae require protein for structural growth, and queens mainly consume protein-rich foods required for egg production. On the other hand, workers consume almost no proteins and they prefer to feed upon carbohydrates, which are quick sources of energy [19].

The interaction between the hungry larvae and the workers regulates colony nutrition through the regulation of the quality and quantity of food distributed and ingested between all the members of the colony [19]. It has been proven that the levels of hunger in individual workers promote foraging and determine the rate at which food is brought from the environment into the nest. Larval hunger as well as hunger of workers inside the nest determine the rate and direction in which food is moved among all members of the colony within the nest [20,21]. Additionally, 4^{th} instar larvae can act as donors of a liquid food known as “meconium” when they are about to pupate. The meconium is a product of their digested and undigested food accumulated in the midgut,

and is usually high in amino acids which may serve as food supplements for the workers as well as the queen and brood.

The molecular mechanisms of caste determination and division of labor

Until present, the molecular mechanisms involved in caste determination and division of labor in fire ants are not well understood. In eusocial hymenopterans including ants, any fertilized egg (diploid) has the potential to develop into a queen or a worker depending upon environmental and nutritional conditions [22]. Additionally, individuals of some characteristic genotypes are more likely to develop into queens than others, indicating the genetic background of the developing individual also plays a role influencing caste determination [23]. A previous study in fire ants showed that the gene expression profiles of queens, males and workers at the pupae stage of development are relatively similar, with less than 400 differentially expressed genes among them; but in the adult stage, there is a remarkable change of these profiles specially between queens and workers, in which more than 800 hundred genes are differentially expressed [24]. Even when it is known that differences in gene expression exist among all castes, how these transcripts' variations are achieved is still unknown. In a recent study, whole-genome microarrays were used to investigate differences in gene expression of foraging vs. non-foraging fire ant workers from colonies with and without queen. There were 1,387 transcripts differentially expressed between foraging and non-foraging workers, independently of the presence of the queen, indicating that these differences are most

probably due to the task performed by these workers [25]. Gene ontology analysis of these differentially expressed transcripts showed that 26 genes were involved in the generation of precursor metabolites and energy, and 17 genes were involved in oxidative phosphorylation; suggesting that nutritional signaling pathways could be involved in mechanisms of division of labor in these ants.

Gene expression patterns can vary due to different factors such as the developmental stage and age, the nutritional status, and even in response to environmental changes. One known way to permanently modify the expression of a gene is through its methylation. For insects in general, DNA methylation levels are considered lower in comparison with other invertebrates and mammals [26], and methylated genes tend to be ubiquitously expressed among cell types or phenotypes while unmethylated genes tend to show tissue or phenotype-specific expression patterns [27]. In holometabolous insects, such as hymenopterans (sawflies, wasps, bees and ants) the DNA methylation levels are higher in exons than introns, and is almost undetectable in the rest of the genome [28].

In bees and ants, recent studies demonstrated that epigenetic changes are involved in caste determination during larval development [29]. For example, in the honeybee *Apis mellifera*, it was demonstrated that when decreasing the methylation levels of one specific gene (*dynactin p62*) in the larvae, the proportion of adult queens obtained was significantly higher than the proportion of workers [30], indicating that differential methylation of this gene somehow determines the expression of queen-like traits [30]. Other examples are found in the carpenter ant *Camponotus floridanus* and the

jumping ant *Harpegnathos saltator*, in which DNA methylation patterns change during development and this is correlated with caste-specific gene expression and alternative splicing of genes [31]. Similarly, in the red harvester ant *Pogonomyrmex barbatus,* different DNA methylation patterns were correlated with division of labor [32].

Similarly to honeybees, fire ants possess a complete and functional set of DNA methyltransferases (DNMTs), including DNMT1, DNMT2 and DNMT3 which are responsible of maintenance of DNA methylation (DNMT1), tRNA methylation (DNMT2) and *de novo* methylation of the DNA (DNMT3), respectively [33]. However, both species differ in that honeybees exhibit a clear bimodal gene methylation profile (some genes are highly methylated while others are nonmethylated or weakly methylated) [34]; while fire ants lacks bimodality and present considerable lower levels of intragenic DNA methylation [28]. This is also in agreement with the fact that there are less methylated genes in fire ants (2,581) in comparison with honeybees (3,030) [28]. In addition, DNA analyses of methylomes of fire ant queens, workers, and males (both haploid and diploid [35]) indicate that differential DNA methylation is more closely linked to ploidy variation than differences in morphology, behavior or physiology associated with distinct queen and worker castes. More than one thousand differentially methylated genes were found between diploid queens, males and workers compared with haploid males; but only 248 differentially methylated genes were found between queens and workers. [36].

Role of neuropeptides as regulators of important physiological processes

Neuropeptides are short-chain polypeptides produced by the nervous system which can act as hormones, neurotransmitters or neuromodulators. The importance of these molecules is that they constitute the largest class of signal molecules, and are involved in the regulation of very important physiological processes such as development, growth, reproduction, metabolism and behavior. In fire ant queens as well as in other insects, nutrition and reproduction are processes closely related and finely regulated by the interaction of several hormones and neuropeptides. In order to reproduce, female insects must be well nourished to be able to transfer to their eggs essential nutrients which are indispensable for embryonic development [37].

Oocyte formation in the ovaries involves the action of different hormones such as juvenile hormone (JH), ecdysteroids (i.e., 20-hydroxyecdysone), and insulin-like peptides (ILPs) [37]. JH is a sesquiterpenoid produced by the *corpora allata* (CA) which is required for oocyte maturation in fire ants [38], and stimulates vitellogenin production by the fat body as well as vitellogenin sequestration by the growing oocytes [39]. In fire ants, the interplay between the insulin/insulin-like signaling pathway (IIS), JH and ecdysteroids is not well understood, but previously was demonstrated that the incubation of ovaries from newly eclosed alate females with a JH analog (methoprene) *in vitro* significantly increases the levels of vitellogenin receptor (VgR) transcripts; while the incubation of these ovaries with 20-hydroxyecdysone does not change the VgR transcript levels [40]. In contrast with the fire ants, the interaction of these pathways in

the regulation of reproduction has been extensively studied in dipterans. In *Drosophila melanogaster*, it is known that nutritional signals influence the secretion of JH and ecdysteroids, and that adult flies with mutations in the insulin receptor (IR) have an impaired ovarian ecdysteroid synthesis [41] and reduced JH titers [42]. Also in *D. melanogaster*, it has been demonstrated that the transcriptional induction of ILPs 1 and 2 on insulin producing cells (IPCs) present in the brain is regulated by the upstream activity of another neuropeptide, the short neuropeptide F (sNPF) [43]. These results suggest that the sNPF could represent an important link between nutritional status and reproduction, making of this neuropeptide a very attractive candidate to study the hormonal signaling controlling these processes in other insects such as fire ants.

sNPF peptides have been identified in several insect species [44], and exert their action through their sNPF receptor (sNPFR), a G protein-coupled receptor (GPCR) related to the mammalian NPY receptor (most similar to the Y2 subtype). The main function of the sNPFs is the regulation of feeding behavior and growth, but additional functions in relation to the regulation of circadian clocks, stress responses, hormone release, etc. have been recently discovered [45]. The role of the sNPFs in relation to feeding behavior and nutritional sensing appear to vary among insect species, and sometimes contrasting functions have been reported for different insect species. For example, it is well known that this neuropeptide promotes food intake in *D. melanogaster* [46]; but the opposite effect was observed in the desert locust, *Schistocerca gregaria* [47]. Regardless, the sNPF signaling pathway is involved in the regulation of feeding behavior in both species. Similarly to *D. melanogaster*, in larvae of *B. mori,* the

injection of sNPF peptides stimulates feeding behavior; and interestingly, the levels of sNPF peptide in the brain are significantly reduced in response to starvation, similar to fire ant queens [48] (see next section). In addition, the gene expression levels of the sNPF receptor (sNPFR) in the silkworm are also significantly reduced in the central nervous system, foregut and midgut in starved larvae, suggesting the sNPF/sNPFR signaling pathway is sensitive to changes in nutrient availability [49].

The sNPF signaling pathway in fire ants

In fire ants, there is a paucity of information on the sNPF/sNPFR signaling pathway and most of the current knowledge is from previous studies from the Pietrantonio laboratory, performed only in queens. Similarly to *Drosophila*, in these queens only one transcript variant from the single sNPF receptor gene has been reported, which is expressed in the brain, midgut, hindgut, Malpighian tubules, fat body, and ovaries [48]. Additionally, the sNPFR has been immunolocalized in ovaries and brain of queens. In the ovary, this receptor was observed in mid- and early-oogenesis oocytes, but not in oocytes at the late-oogenesis stage; while in the brain, there are about 164 sNPFR-immunoreactive cells ubiquitously distributed in 12 clusters located in or near important sensory neuropils including the mushroom bodies, the antennal lobes, the central complex, and in different parts of the protocerebrum as well as in the subesophageal ganglion [50]. These results suggested this receptor could also be involved in processes associated with nutrient sensing, learning and memory, locomotion, olfaction, vision,

and feeding in the fire ant queen. Similarly to *B. mori*, it appears that in fire ant queens the sNPFR is also sensitive to changes in the nutritional status. In a previous study performed with mated queens it was demonstrated that the highest expression of the sNPF receptor transcripts occurs in the brain; but these transcripts levels could be significantly reduced after 5 days of starvation [48]. In addition, the decrease of the sNPFR transcript in starved queens was correlated with a decrease in oviposition, which completely stops after 72 h of starvation [48].

As mentioned above, the sNPF appears to modulate important behavioral and physiological functions in fire ants, but there is a paucity of information about how this signaling pathway works, especially in the workers. Based on the current knowledge available in fire ant queens and other insects, our hypothesis is that in the queen the ingestion of food promotes the activation of nutritional signals (probably from the gut, i.e. aminoacids or peptide hormones) which can act on the brain to induce the release of sNPF in the hemolymph. In the brain, sNPF could activate its receptor (sNPFR) in an autocrine or paracrine fashion to induce the transcription of ILPs, which could be released in the hemolymph to act as hormones in other tissues such as the CA, the ovaries and the fat body. In the CA, ILPs could stimulate the release of JH into the hemolymph triggering vitellogenesis in the fat body, and promoting the activation of the ovaries, as a consequence. For example, the vitellogenin receptor transcript increases in fire ant queen ovaries incubated with the JH analog methoprene [40]. Finally, the activity of JH and ILPs would lead to the induction of oogenesis and egg growth in the ovaries, promoting reproduction.

Thesis objectives

The overarching long-term goal is to elucidate the role of the sNPF/sNPFR signaling pathway in fire ants, specifically to investigate if and how this neuropeptide regulates feeding behavior and nutrient sensing at the colony level, and how its function could be affected by the social interactions among the queen, workers and brood. Toward accomplishing this goal, the specific objectives were:

Objective 1: *Determine the spatial distribution of cells expressing the sNPF receptor protein in the brain of all worker subcastes by immunohistochemistry*.

The spatial location of sNPFR-expressing cells is already known in the fire ant queen brain [50]. Thus, we want to investigate where the sNPFR-expressing cells are located in the brain of workers, to then, by comparing the patterns obtained from queens and workers, try to identify common and unique neural networks regulated by the sNPFR in each caste.

We hypothesize the role of the sNPFR in the queen brain is related to the regulation of reproduction while in workers is related to the regulation of feeding or foraging behaviors instead.

Objective 2: *Determine the spatial distribution of cells expressing snpf mRNA transcripts in the brain of queens and all worker subcastes by in situ hybridization.*

The identification of *snpf*-expressing cells in the brain of queens and workers is

important for the elucidation of neuronal networks involved in the synthesis and release of this neuropeptide. Our null hypothesis is that the spatial distribution of *snpf*-expressing cells should be similar across worker subcastes, but different when comparing workers and queens. This may be so especially because queens are able to reproduce, thus they have higher nutritional requirements than workers, which in this species are all sterile females [51]. Further, queens perform unique and more complex behaviors than each specific worker subcaste [52], and the distribution of *snpf* may reflect this higher complexity

Objective 3: *Silence the sNPF receptor through dsRNA feeding assays in small, single-queen laboratory colonies.*

The silencing of the sNPFR induced by feeding of dsRNA will allow us to simultaneously discover specific behavioral traits modulated by the function of the sNPFR in all castes, including immature ants at different developmental stages.

Our hypothesis is that disrupting the function of the sNPFR could be detrimental for the colony fitness due to a dysregulation of the colony nutritional status, which ultimately could lead to a reduced reproductive capacity of the queen, affecting colony growth. In addition, the implementation of this technology has the advantage that the ants can autonomously take up the dsRNA provided in food, opening the possibility to apply this strategy for population control.

In summary, this thesis focuses on elucidating the role of the sNPF/sNPFR signaling pathway in fire ants, which until now is unclear. It is expected that the results obtained from the proposed objectives will help us identifying specific neurons expressing the sNPF and/or sNPFR and "map" their spatial location in the brain with the aim to obtain some evidence on the specific physiological processes or behaviors regulated by this neuropeptide in the central nervous system of these ants. In addition, we also expect from objective three to observe alterations in foraging or feeding in all worker subcastes, which may lead to a reduction of the reproductive fitness of the queen, and perhaps to alterations in the development on the growing larvae.

CHAPTER II

IMMUNOLOCALIZATION OF THE sNPF RECEPTOR IN THE BRAIN OF ALL WORKER SUBCASTES*

Introduction

The red imported fire ants (*Solenopsis invicta* Buren; Hymenoptera: Formicidae) are eusocial insects native from South America with an extraordinary capacity for adaptation to different environments. They have invaded countries such as the United States, Australia, New Zealand, China and Taiwan [2]. They are considered a dangerous invasive species in the U.S., affecting the habitat of other native animals [53–55]. Fire ant colonies are composed by individuals from different castes, designated as the reproductives (males and females) and the workers. This reproductive division of labor refers to specialization of drones and queens for the generation of new individuals, while female worker ants co-operate and perform brood rearing, care for the queen, forage for food, defend the nest, dig soil for nest construction, etc.

*Reprinted with permission from "Differences in sNPF receptor-expressing neurons in brains of fire ant (*Solenopsis invicta* Buren) worker subcastes: indicators for division of labor and nutritional status?" by Castillo, P., Pietrantonio, P.V., 2013. PLOS one, 8.12: e83966, Copyright 2013 by PLOS one.

The worker caste is composed of a greater number of individuals compared to the reproductive caste, and its members present large variations in body size. This size variation is known as worker polymorphism and in fire ants is the basis for the division of the worker caste into three loosely defined subcastes, as follows. Minor ants are the smallest workers, while major ants are the biggest. The third subcaste corresponds to medium workers of intermediate size between the former [15]. Previous studies showed that there is a correlation between the worker body size and the labor they perform, and it is believed this specialization of workers is necessary to increase their efficiency. However, other factors such as worker age influence task performed, making the prediction of worker task based on size less accurate. In the fire ants there are two main categories of workers, “nurses” and “foragers”, each group composed by a wide age/size range of ants. A third category of workers called “reserves” is very heterogeneous in age, size and behavior; they may work as nurses or foragers, or they may store liquid food, to which the name “reserves” refers to. In general, the smallest and youngest ants are responsible of taking care of the brood while older ants prefer to forage. However, foragers never feed larvae and nurses never forage [16]. In other ants and bees the endocrine mechanism appears to be related to a higher juvenile hormone titer in older workers that promotes foraging in comparison to younger nurses that remain in the nest [56].

Importantly, fire ant female workers are completely sterile, without ovaries, and thus are devoid of reproductive plasticity. Only queens can produce eggs. This makes this species a good model to separate female gene networks related to reproduction

(queens) from those related to female worker tasks. Previous studies in other ant species have shown that the worker division of labor and the differences workers have when compared with the reproductive castes (drones, queens) are correlated with differences in the structure and organization of their brains [57–60]. For example, workers of several species of ants generally present small optic lobes and large antennal (olfactory) lobes compared with males and queens; and this correlates with the size of the mushroom bodies, especially in the latter input regions (lip and collar) [61,62]. In carpenter ants, *Camponotus ocreatus*, mushroom bodies are significantly larger in workers than in queens and males; perhaps related to the fact that worker ants need to memorize the location of food sources and develop a good sense of spatial orientation to remember the way back to the nest. Both processes could be integrated in the mushroom bodies, which are the most important center of learning and information processing [63]. Other differences have been observed among ant castes, such as those in the patterns of serotonergic immunoreactivity in the optic lobe of *Pheidole dentata*, where old major workers exhibit an increased number of serotonergic cell bodies than old minors [64]. Also in those ants, and in agreement with these findings, serotonin titer increases in the brain with age, and major workers showed significant differences in the branching of serotonin-immunoreactive calyx input neurons than minors [65]. Considering all of the above, it is clear that ant behavior correlates with some physical differences in body size and changes of signaling molecules in the brain. Other factors such as ovarian activity [66] (but not in fire ants, as indicated above), genetics [67,68], and even patterns of DNA methylation [32] could modify the division of labor among worker ants.

Neuropeptides could regulate behavior through the temporal and spatial coordination of several neuronal circuits that could involve the participation of sensory neurons, interneurons and motor neurons [69]. In most arthropods studied so far, short neuropeptides F (sNPFs) are 6-11 amino acid residues in length with the C-terminal consensus sequence xPxLRLRFamide. These neuropeptides are important because are involved in the regulation of several critical functions such as: feeding and growth, stress responses, locomotion, olfaction, hormone release, reproduction, learning and memory [44]. The short neuropeptide F (sNPF) exerts it action through the short neuropeptide F receptor (sNPFR), a G protein-coupled receptor (GPCR) related to the mammalian NPY receptor (Y2). The sNPFR was first identified and characterized in *Drosophila,* where just one variant of the receptor (sNPFR1) was found in brain and diverse tissues [70]. In animals, NPY signaling may play a role in the motivation towards foraging behavior [71]. In honey bees the NPY system is apparently represented only by the sNPF (sNPY) signaling system because the long NPF receptor is absent from the genome although the long NPF (NPY) peptide is present; it is yet unknown if both peptides activate the same receptor [72].

In honey bees the sNPF signaling system is involved in the regulation of foraging behavior and its receptor transcript expression is higher in foragers than nurses, and lower in workers well fed with sugar and pollen (where pollen is a protein source), than in food deprived workers (two days with honey followed by two days starvation) [72]. Foragers exhibit higher levels of sNPFR transcripts when given a poor diet of sugar in comparison with younger bees and nurses feeding the same diet [72]. The higher

sensitivity of foragers to nutritional cues could be mediated through sNPF and sNPFR signaling [72]. In agreement, in bees the sNPF peptide level varied in workers collecting either nectar or pollen and between foragers arriving or departing from a feeder, and these latter changes in peptide expression were highly dynamic, within minutes, supporting the sensitivity of the sNPF peptide expression to nutrition and foraging [73]. In fire ant queens we previously cloned the sNPFR cDNA; the receptor transcripts were highly expressed in brain and other tissues [48]. We were the first to show that the expression of the sNPFR transcript was significantly reduced in the brain of queens in response to starvation when they were provided only water, suggesting this receptor could be involved in the regulation of feeding behavior because its transcript level was affected by variation in the nutritional status [48]. We later published a detailed description of the localization of 164 sNPFR-immunoreactive cells in the queen brain [50].

Here we focused on the immunolocalization of the short neuropeptide F receptor (sNPFR) in the brain of fire ant workers, investigating if there is a relationship between the sNPF/sNPFR signaling pathway and worker division of labor (subcastes), and their sensing/or responding to colony nutritional requirements, which could be affected by the presence or absence of brood. Protein digested by larvae provides amino acids that are used for further brood growth which results in colony growth during the summer, while carbohydrates are the preferred energy source for the colony [74]. In summary, in fire ants presence of larvae reflects a demand for amino acids [20]. Based on the dynamic changes found in the sNPF peptide in honey bee worker brains in response to nutritional

cues (pollen vs. nectar) and foraging behavior, herein we investigated the distribution of the sNPFR in the brain of fire ants workers in colonies with and without brood. Our hypothesis states that changes in protein requirements and/or availability as consequence of the presence of hungry larvae would be reflected centrally in the sNPF signaling system in workers, specifically in sNPFR expression. These experiments were planned to reveal functions of sNPF signaling in sterile workers, and thus unrelated to reproduction at the individual level, but relevant to worker tasks and colony growth status and nutritional requirements.

Materials and Methods

Insects

Fire ants are invasive in Texas and ubiquitous. Polygyne colonies of *S. invicta* were a gift of Dr. R. Puckett at Texas A&M University. All colonies were collected at the "5-Eagle Ranch" (30°37'49.92"N; 96°40'19.37"W) in Burleson County, Texas, from May to July 2012. The Ranch owner authorized Dr. Puckett to collect fire ant colonies which are unwanted in the property. The field collections were limited to fire ants and did not involve endangered or protective species. Colonies with and without brood (egg and larvae) were used, and all of them had mated queens. Colonies without brood were those which queens have had the capacity to lay eggs, as observed for months previously, but had stopped producing brood. All the colonies were maintained in the laboratory on plastic trays, whose walls were covered with Fluon (Insect-a-slip©, BioQuip products, CA, USA) at 27± 2°C in a 12:12 h light-dark photoperiod. Each plastic tray contained at least one nest (10 cm diameter Petri dishes half-filled with Castone® (Dental Supply International Inc., York, PA, USA). The ants were fed daily with 15% honey-water and frozen crickets (Fluker's Cricket Farm, Port Allen, LA, USA). Water was provided *ad libitum.*

Classification and selection of worker ant subcastes

Worker ants were collected from colonies with and without brood, and classified into majors, media and minors according to the head width (H.W.) as described previously by Wilson [15]. In Wilson's study, the distribution of sizes of the head width (H. W.) was determined in the range from 0.48 to 1.46 mm. Minor workers are considered the smallest members of the worker caste, with H. W $\leq$ 0.72 mm; media workers have a H.W = 0.73 - 0.92 mm, and in major workers the H.W is $\geq$ 0.93 mm [15]. The differences in H.W. we observed in our colonies are shown in Figure 2.1. Major workers were collected on the tray open areas, far from the queen nest, or collected on the small food dishes were the crickets were provided. Medium workers were collected on the way in/out the nest, or on the surrounding open areas outside the nest. Minor workers were collected from inside the nest, around the mated queen or carrying brood. Depending on the different tasks workers were performing when collected for dissection, we considered the major workers as "foragers" and minor workers as "nurses". The media workers selected, however, were not performing any specific task, sometimes they were found inside the nest carrying brood, outside the nest foraging, or just standing outside the nest.

Dissection of the worker ant brains and subesophageal ganglion (SEG)

All worker ants were dissected using the same procedure, as described below. Selected ants were anesthetized on ice for about 10 min; the head was cut off and placed on a dissection dish with silicone on the bottom (Sylgard®, Dow Corning Corporation, Midland, MI, USA). Then, using thin forceps, antennae were removed by pulling and the head was held through the mouth. Several small punctures were made on the sides of the head above the mandibles, using a fine dissection pin (#2). After breaking the cuticle, PBS was added to the dissection dish, until the head was completely submerged. Using thin forceps, the cuticle was removed very carefully, starting from the previously made punctures. With a spooning movement of the closed forceps all tissues inside the head capsule were removed carefully including esophagus, tracheae and glands, with the brain/SEG being enclosed in these tissues and rarely easily observed. The brain and SEG were finally exposed by removing the surrounding tissues under buffer. All the brains/SEG were collected individually in Eppendorf tubes for fixation that was performed using 4% paraformaldehyde/PBS solution at 4°C for 2 h.

Antibodies

To immunolocalize the cells expressing the sNPF receptor in the fire ant worker brain, we used the same specific anti-peptide antibody developed against the *S. invicta* sNPFR described previously by Lu *et al.* and used for receptor expression analyses in brain and ovaries of fire ant queens [50]. The hydrophilicity and antigenicity profiles of sNPFR amino acid sequence (GenBank: DQ026281) had been analyzed using

DNASTAR and ExPAsy software to determine and select antigenic regions for anti-peptide antibody production, and the sequence "CRGDKIDNGNNTMQETL" was selected for antibody production. This amino acid sequence is located toward the receptor C-terminus encompassing residues 331 to 347. The polyclonal and affinity purified antibody was developed by Pacific Immunology (CA, USA) in New Zealand female rabbits, using the synthetic peptide conjugated with keyhole limpet hemocyanin (KLH). After purification, the specificity of the antibodies was verified by ELISA (tested by Pacific Immunology, CA), and additional characterization of this antibody was performed by western blot as described by Lu *et al* [50].

Immunolocalization of the sNPFR on brains/SEG of worker ants

From colonies with brood, 40 brains from majors, 52 each from media and minors were dissected, processed and analyzed for sNPFR immunoreactivity. Brains for negative control treatments were additionally dissected. From colonies with brood, a minimum of 6-10 brains were used per subcaste for either preimmune or antigen-preabsorbed antibodies (12-20 total per subcaste). Initially, additional negative controls were run with secondary antibodies only (about 4 brains per subcaste, not shown). From colonies without brood, 26 brains from majors, 26 from media and 16 minors were processed and analyzed for sNPFR immunoreactivity. For negative controls for all subcastes four brains each for preimmune, preabsorbed and secondary antibody only were dissected and processed (n=12 per subcaste). In summary, a total of at least 292 brains were used in this study. In all cases half of the brains for all treatments from

colonies with or without brood were mounted for analysis of either anterior or posterior brain view. Due to the time consuming task of selecting workers performing specific behaviors followed by brain dissection, the results were obtained through ten independent experiments, each with negative controls.

The expression of the sNPFR in brains/SEG of workers in all subcastes (minors, media and majors) was determined by whole mount immunofluorescence as described previously [50]. Briefly, after fixation, the brains/SEG was washed with 70% ethanol on ice. Then, the tissues were rinsed with PBST (PBS with 0.1% Tween) and incubated with 12 µg/ml protease K (Sigma-Aldrich, St. Louis, MO, USA) in PBS for 10 min. Both steps were performed at room temperature. The protease was removed by washing with PBST and then, the tissues were blocked for 24 h at 4°C using PBST with 10% normal goat serum (NGS) (Jackson ImmunoResearch, West Grove, PA, USA). After blocking, tissues were incubated with a 2 µg/ml primary antibody solution containing 2% NGS in PBST for 48 h at 4°C. A goat anti-rabbit IgG conjugated with Alexa Fluor® 546 dye (Invitrogen™) was used as secondary antibody (10 ng/ml) in the same solution as above. Finally, the brains/SEG were mounted on glass slides using Vectashield™-DAPI (Vector, Burlingame, CA, USA) for nuclear staining. Cover slips for tissues examined under microscopy were 0.16-0.19 mm thick (No. 1.5; Fisher Scientific).

To ensure the specificity of the primary antibody, negative control tissues were included in this study as follows: antigen pre-absorbed anti-sNPFR antibody (500 µg of peptide antigen was incubated with 4 µg in one ml solution and diluted 1:2 for final use); pre-immune rabbit serum (1:1000 dilution) instead of primary antibody; and tissues

incubated with secondary antibody only (10 ng/ml; 1:200 dilution of commercial product).

Data collection and analysis

Worker ant brains were analyzed for immunofluorescence from the anterior and posterior views. The anterior view corresponds to the frontal side of the brain, where the antennal lobes are clearly seen and oriented forward; while the posterior view corresponds to the back side of the brain/SEG were the subesophageal ganglion is more prominent. The tissues were analyzed using a Carl Zeiss Axioimager A1 fluorescent microscope, coupled with an AxioCam MRc color camera (Carl Zeiss). These images were captured and processed using the Axiovision software (version 4.8.2) provided with the microscope. All pictures were taken at a resolution of 1388 x 1040 pixels and were saved as TIFF files. No colored images were used, and in some images brightness and contrast were adjusted to clearly show the fluorescent signal found on the tissues by using the shading correction tool provided in the Axiovision software. For this, the whole image was corrected to improve image quality with no partial sections of images modified. Schematics were manually drawn and colored using PowerPoint software (Microsoft™).

Results

Characteristics of the immunostaining pattern of sNPF receptor (sNPFR) in brain and subesophageal ganglion (SEG)

The number of individuals analyzed is comparable to previous studies of insect NPY-signaling system [71]. Majors had an average brain width (B.W.) of 668.46 µm, medium workers 602.39 µm and minors have a B.W. of 573.30 µm in average. A total maximum of 9 clusters of cells expressing the sNPFR is present across worker subcastes in the fire ant. Figures shown are representative of the staining that was very consistent across subcastes within colonies with or without brood. When any small variation was observed, this is specifically mentioned.

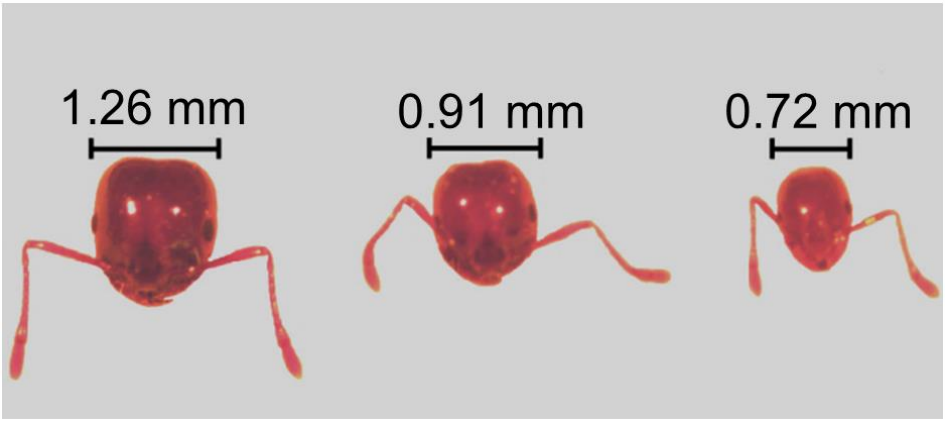


Figure 2.1 Comparison of the head widths among worker subcastes in fire ants. Majors are considered the biggest workers, minors are considered the smallest, and media are intermediate in size between majors and minors. The heads of a major worker (left; 0.93-1.46 mm H.W.), a medium worker (center; 0.73-0.92 mm H.W.) and a minor worker (right; ≤ 0.72 mm H.W.) are shown.

We observed consistent differences among subcastes as defined (Figure 2.1), in the number of immunolabeled clusters present, with certain clusters being present only in one subcaste. A schematic representation summarizing the location of the cell clusters expressing the sNPFR in majors, medium and minor workers, from the anterior and posterior views of the brain is showed in figure 2.2. Different cell clusters could be observed only from either the anterior or posterior view of the brain and others from both, providing subjective but relevant information about their depth relative to the surface of the brain observed. The anterior view of the brain shows the antennal lobes toward the front; and the posterior view shows the SEG towards the front (Figure 2.2). In the queen brain twelve clusters designated C1- C12 were previously reported [50]. Some of the cell clusters expressing this receptor in fire ant worker brains were highly reminiscent of those observed in the queen brain. This similarity determined that the number assigned to these clusters was retained for the worker brain but utilizing the small letter c (for cluster) followed by the same cluster number observed in the queen. We identified in workers as a group five of these apparently common clusters with the queen: c2, c5, c7, c9 and c12. However, except c5 which is present in all subcastes, the rest are differentially present among worker subcastes. Novel clusters found exclusively in workers were numbered c13-c16 (Figure 2.2; Table 1).

An important observation is that the total number of cells expressing this receptor decreases from minor to major workers (Figure 2.2). The higher number of cells in minors is due both to a larger number of cell clusters and to the presence of more cells in the specific cluster. Major worker ants exhibited a total of five cell clusters (19-26

sNPFR immunolabeled cells), while minor workers showed eight clusters (47-59 sNPFR immunolabeled cells). Medium workers were intermediate in immunolabeled cell number, having seven clusters of cells (29-39 sNPFR immunolabeled cells) (Figure 2.2; Table 1). While the location and the characteristic cell number of certain clusters (c5, c13, c16) was constant in all worker subcastes (perhaps except for c5 in majors with some individuals showing increased number from 4-6 cells vs. 4 in others) in colonies with or without brood (Table 1), others showed an apparent increasing gradient in the number of cells from majors to minors such as cluster c7, absent in majors, with 2-4 cells in each side of the brain in media and four in each side in minors (see Figure 2.2, D-F, Table 1).

Distribution of the sNPFR in the brain superior protocerebrum in worker subcastes

The most remarkable differences among subcastes in the pattern of cells expressing the sNPFR were observed in this region of the brain (Figure 2.3). In Figure 2.3-A, a summary of immunoreactive cells that could be collectively found in minors and media, c2 (lateral) and c7 (center), are shown. In major workers these were not found (Figure 2.3, B-D). Cluster c7 is located above the fan-shaped body (FB) of the central complex in the superior medial protocerebrum (smP), under the medial calyces (mCa) of the mushroom bodies (Figure 2.3, E, F, H, K).

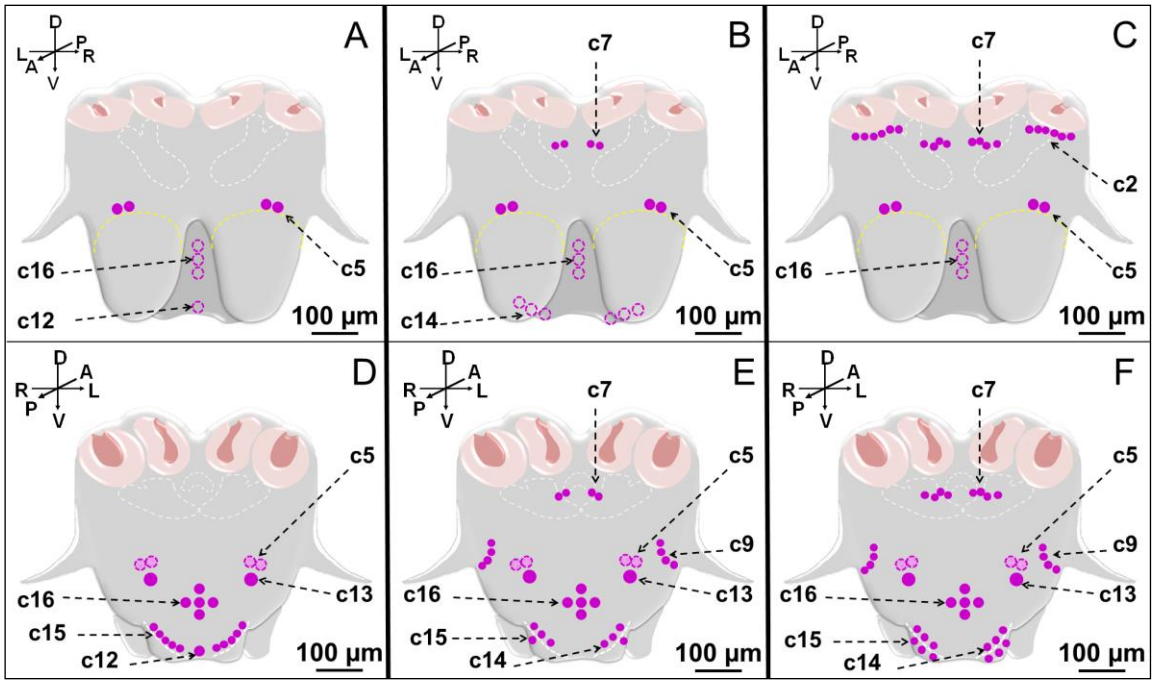


Figure 2.2 Schematic representation of the short neuropeptide F receptor immunolocalization in the brain and subesophageal ganglion of all worker subcastes. Anterior (top panel) and posterior (bottom panel) views of the brain show different cell clusters expressing the sNPFR. (A, D) represent the localization in majors; (B, E) in media and (C, F) in minors. Dashed-empty circles indicate the cells can be observed faintly from the anterior view but are located deeper in the brain; purple checkered-filled circles indicate the same, but when the brain is seen from the posterior side. Within clusters the depth of certain cells may vary. The brain orientation is indicated by the direction of the arrows shown in the top left corner of each subpanel: A= anterior, P= posterior, D= dorsal, V= ventral L= left, R= right.

In medium workers c7 was observed from both brain views (anterior, Figure 2.2, B, and Figure 2.3, E, F, and posterior, not show, but see Figure 2.2, E) and the number of cells in this cluster was variable, from two to four cells. Also, not always both groups were identical in the same individual, as shown in Figure 2.3, F, where three cells are clearly labeled on the right side and two on the left side of the brain. In media, similarly to majors, c2 is completely absent (Figure 2.3, G, I).

In minor workers, clusters c2 and c7 were present, and c2 could be observed only from the anterior view of the brain. Cluster c2 is located right below the lateral calyces (lCa) of the mushroom bodies, symmetrically on both brain hemispheres and the number of cells in each lateral cluster varies from 4 (Figure 2.3, J) to 10 cells (Figure 2.3, L). Also, the shape and size of the cells in this cluster is variable, and some of them seem to be located deeper into the brain. Cluster c7 could be observed from both views, in the same location observed in medium workers (Figure 2.3, H) but this cluster was always observed as two groups of four cells each, similar in size and shape (Figure 2.3, H, K). No labeled cells were observed in negative controls of all subcastes, as expected (not all shown) (Figure 2.3, M-O).

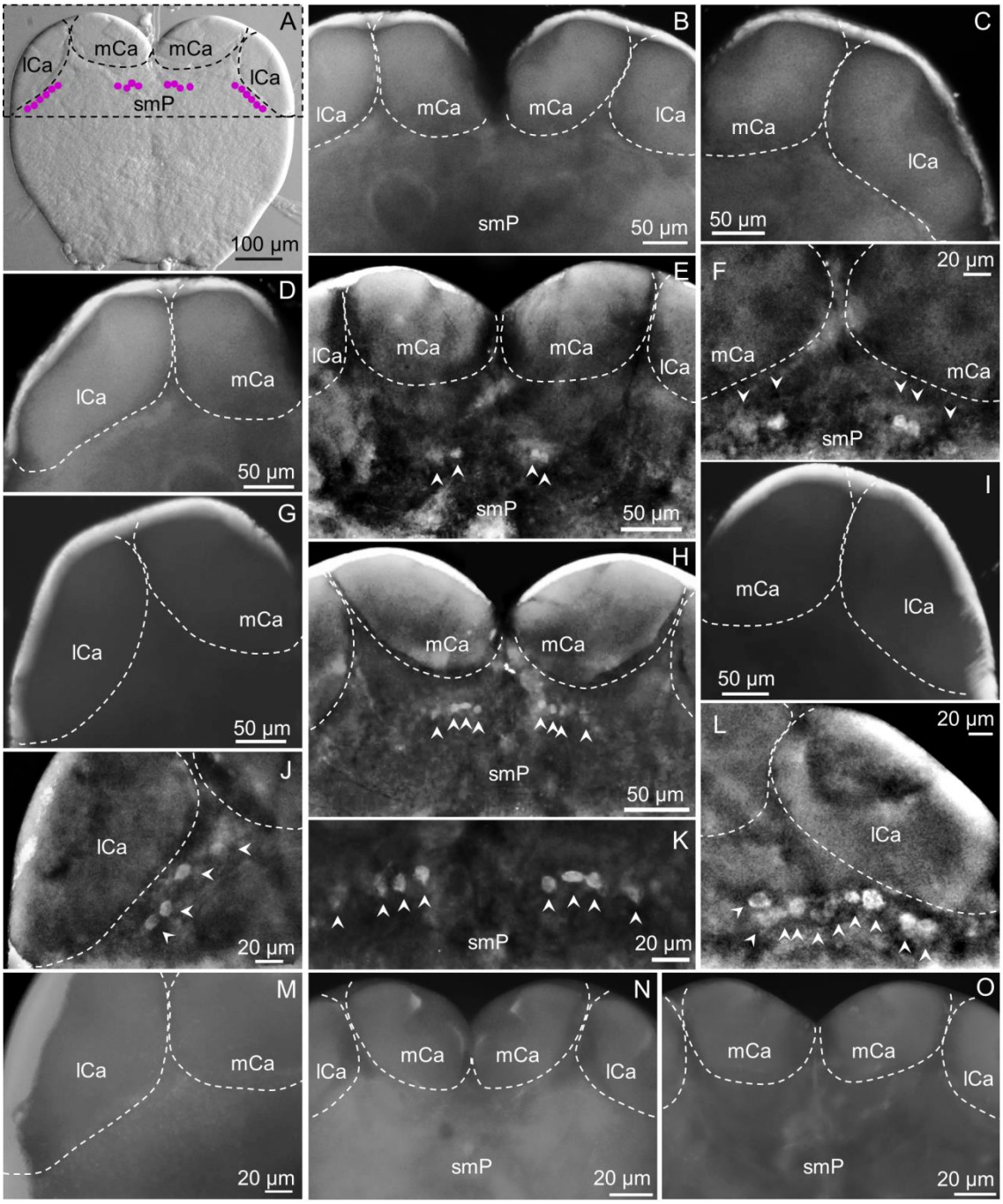
Distribution of the sNPFR in the brain central region in worker subcastes

This region includes the inferior protocerebrum and the area corresponding to the superior edge of the antennal lobe above the deutocerebrum (if observed from the anterior view where cluster c5 is detected (Figure 2.4)), or corresponds to the superior commissure of the SEG (if observed from the posterior view where cluster c9 is detected

(Figure 2.5)). From the anterior view, cluster c5 can be seen in the brain of major (Figure 2.4, A), medium (Figure 2.4, B) and minor workers (Figure 2.4, C). Cluster c5 is composed of two strongly labeled cells, located symmetrically and horizontally aligned on the superior edge of each antennal lobe as shown in the schematic (Figure 2.4, D. Purple dots); in some majors the two cells appear to touch each other while in other individuals they are ~10 microns apart (Fig. 2.4, A). Viewed from the posterior side, these cells appear to be located deeper in the brain than when viewed from the anterior side (Figure 2.4, E. checkered-filled dots). Sometimes a third cell could be observed in c5 in majors (Figure 2.4, F, G), but only two cells were observed in media (Figure 2.4, H, I) and minors (Figure 2.4, J, K). Representative negative controls (Figure 2.4, L, M) did not show any immunoreactivity, as expected.

From the posterior view of the brain, cluster c9 is observed in the inferior lateral protocerebrum, near the lobula of the optic lobe (Figure 2.5, A). This cluster is present in minor (Figure 2.5 B, G) and medium workers (Figure 2.5 C, H), but is not detected in majors (Figure 2.5 D, I). Usually this cluster is observed as four cells similar in size and shape, with cells distributed vertically forming a curved line (Figure 2.5 B, G, H). Nevertheless, sometimes immunoreactive cells in this brain region appear to be closely grouped in a circular fashion, just as in the brain of media shown in Figure 2.5 C.

Figure 2.3 The superior protocerebrum exhibits differential short neuropeptide F receptor immunoreactive neurons among subcastes (anterior brain view). A: Nomarsky image of a minor worker brain showing the location of clusters c7 (center) and c2 (lateral). In majors, clusters c7 (B, center), and c2 (C, right; D, left) were not detected. E: shows c7 in the brain of a medium worker (detail in F); however, cluster c2 normally under the lCa is not observed (G, left; I, right). In minors, c7 is clearly visible close to the mCa (H, detail in K) and c2 is located under the right (L) and left lCa (J). The lack of fluorescent signal on the left lCa of a minor worker brain treated with pre-absorbed antibody as negative control is shown in (M), and both brain hemispheres are shown in (N). No fluorescent signal was observed for the pre-immune negative control (O). mCa: median calyces, lCa: lateral calyces, smP: superior medial protocerebrum. Left or right refers to the brain hemisphere shown.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Distribution of the sNPFR on the posterior-inferior brain and SEG in worker subcastes

This region of the brain contains the largest number of cells expressing the sNPFR in worker ants of all subcastes (Figure 2.6, A). Across all subcastes, clusters 12-16 are present (Figure 2.2, D-F); however, there are differences among subcastes. In majors, clusters c12, c13, c15 and c16 are observed, while c14 is not detected (Figure 2.2, D; Figure 2.6, B-D). In media and minors c13-c16 are present while c12 is not detected (Figure 2.2 E, F and Figure 2.6, E-O). Cluster c12 is formed by a group of two or three cells located at the center and bottom edge of the SEG (Figure 2.2, D and Figure 2.6, B, see arrow and dashed inset). Cluster c13 is present in workers of all sizes, and is composed of two very large cells (average 13.9 μm diameter each) located symmetrically, apparently at the upper commissure of the SEG, and at an intermediate, but yet variable distance between the foramen and the lateral edge of the brain/SEG (Figure 2.6, B in majors; E, F in media and J, K in minors). At the bottom of the SEG, clusters c14 and c15 are located somewhat parallel to one another on a seemingly curve trajectory and symmetrically on both sides of the SEG (Figure 2.6, A). Cluster c15, is present as a row of cells closest to the inferior lateral edges of the SEG; while c14 is internal to c15. In minors, c14 and c15 are usually formed by a group of three cells each (Figure 2.6, M); however, sometimes a fourth cell could be observed in c14 (Figure 2.6, O). In medium workers both clusters (c14, c15) are more irregular in their bilateral spatial arrangement compared with minor workers, and cell numbers may vary from 1 to 3 in each cluster even within the same individual. For example, in a medium worker only one cell is seen in c15, and three are detected in c14 in left hemisphere (Figure 2.6, H)

while in the right hemisphere there are 3 cells in c15 with c14 showing only two cells (Figure 2.6, I). In majors cluster c14 is absent, but c15 is clearly visible very close to the lateral edge (Figure 2.6, B-D) and could be composed from 3 to 5 cells.

In all worker subcastes c16 is located at the center of the SEG. It is composed of a group of five cells, most commonly distributed in a cross-like fashion (Figure 2.6, A, E, G) sometimes showing some distortion in the cross-like distribution as seen in Figure 2.6 B, L and N. These cells are very similar in shape and size, but usually two of them (center, bottom two cells in c16, Figure 2.6, G, L) appear with a higher fluorescence intensity compared with the other three. It is not known yet if this is due to a higher expression of the sNPF receptor, or as a consequence of the variable distance of these cells to the surface of the SEG. Negative controls did not show immunoreactivity (Figure 2.6 P, Q). In some instances these c16 cells appeared to be attached or in close proximity to trachea (Figure 2.7).

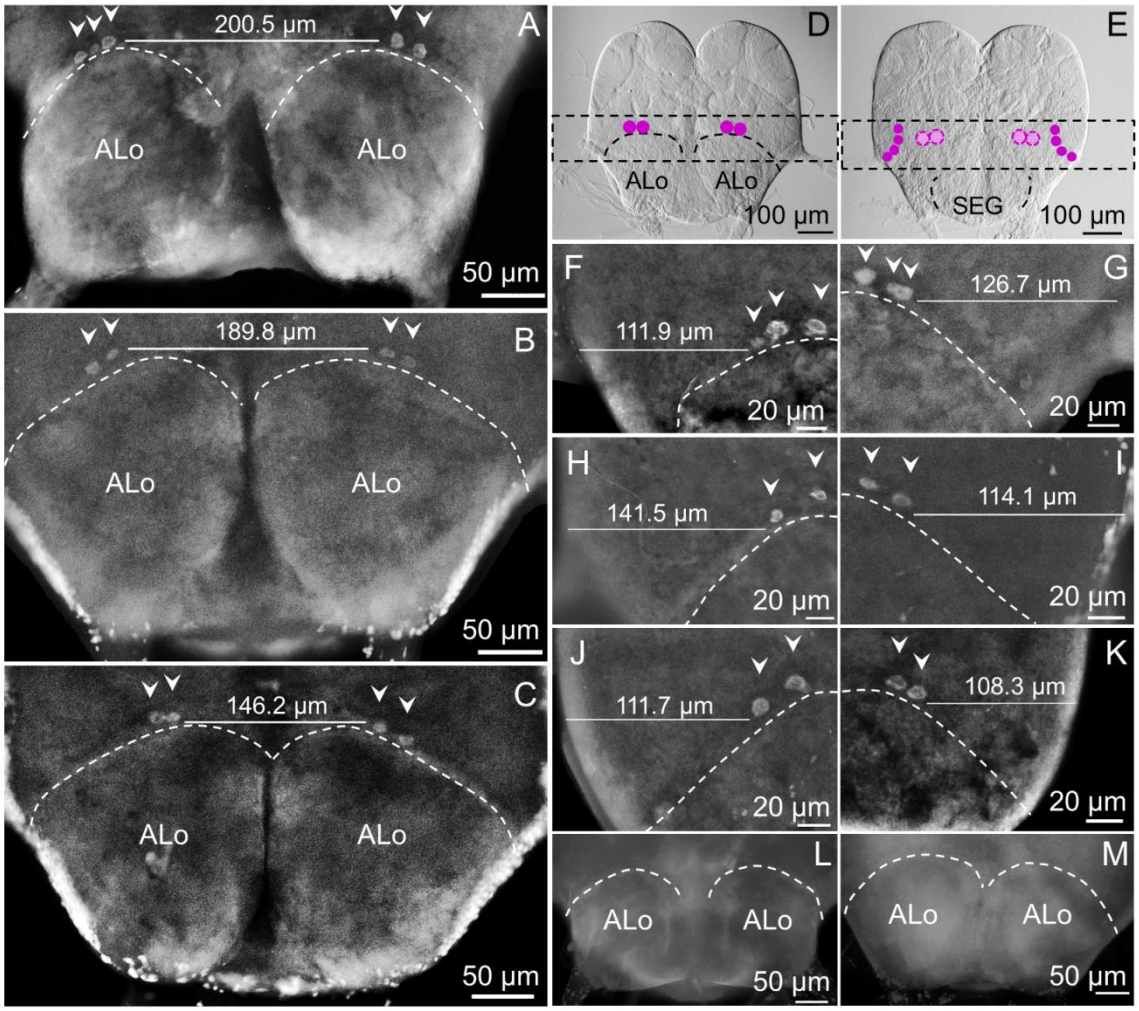


Figure 2.4 Cluster c5 is immunolocalized at the superior edge of the antennal lobe in all subcastes. Cluster c5 could be observed in the brain of majors (A), medium (B) and minor (C) workers more clearly from the anterior view but also from the posterior view, slightly deeper in the brain. A schematic representation is shown over a Nomarsky image of a medium worker brain from the anterior view in D. Cluster c5 is most often represented by two cells, which are symmetrically located at the superior edge of the ALo. In the posterior view of the brain in E, c5 is depicted using checkered dots; purple solid dots correspond to cluster c9. Cluster c5 in majors is shown in detail in F (left), G (right); notice that in those images c5 is composed of three cells. H (left), I (right) show a detail of c5 in media, and the same is shown in J (left), K (right) for minors. No fluorescent signal was observed in negative controls with pre-immune serum (L) or with the antigen-preabsorbed antibody (M). Left or right refers to the brain hemisphere shown.

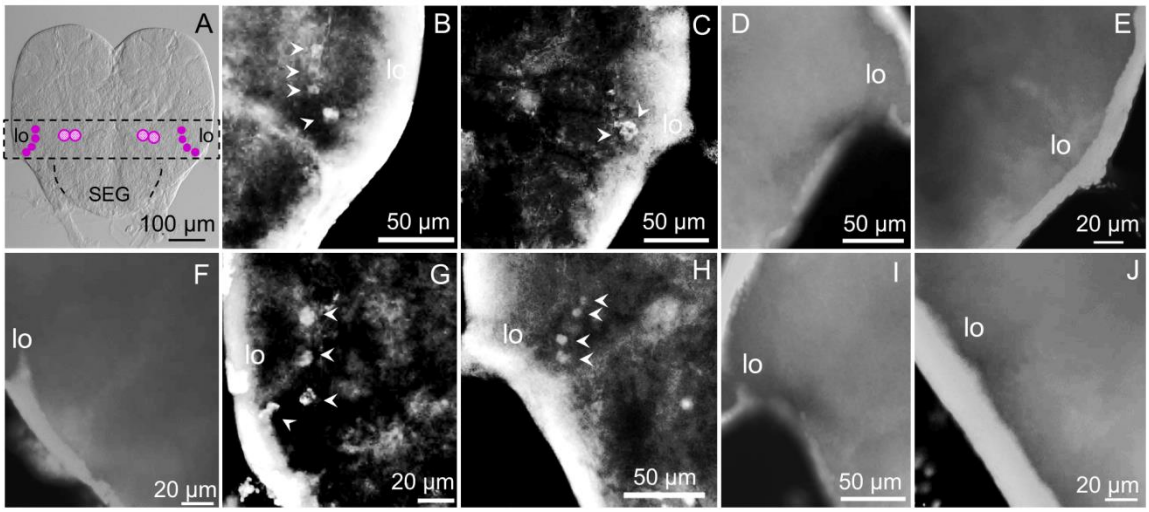


Figure 2.5 Short neuropeptide F receptor immunoreactive cluster c9 is only detected in the posterior lateral protocerebrum of media and minors (posterior view). (A) Schematic representation of the location of c5 (central) and c9 (lateral). Images in the top panel show the right hemisphere and the bottom images, the left one. In minor worker brains cluster c9 is detected near the optic lobe, symmetrically on both sides of the brain (B, G). Also, c9 is present in medium worker brains (C, H). In majors, cluster c9 is undetectable (D, I). No fluorescent signal was observed in negative controls, with pre-immune serum (E, F) or antigen-preabsorbed antibodies (J). Lo: lobula.

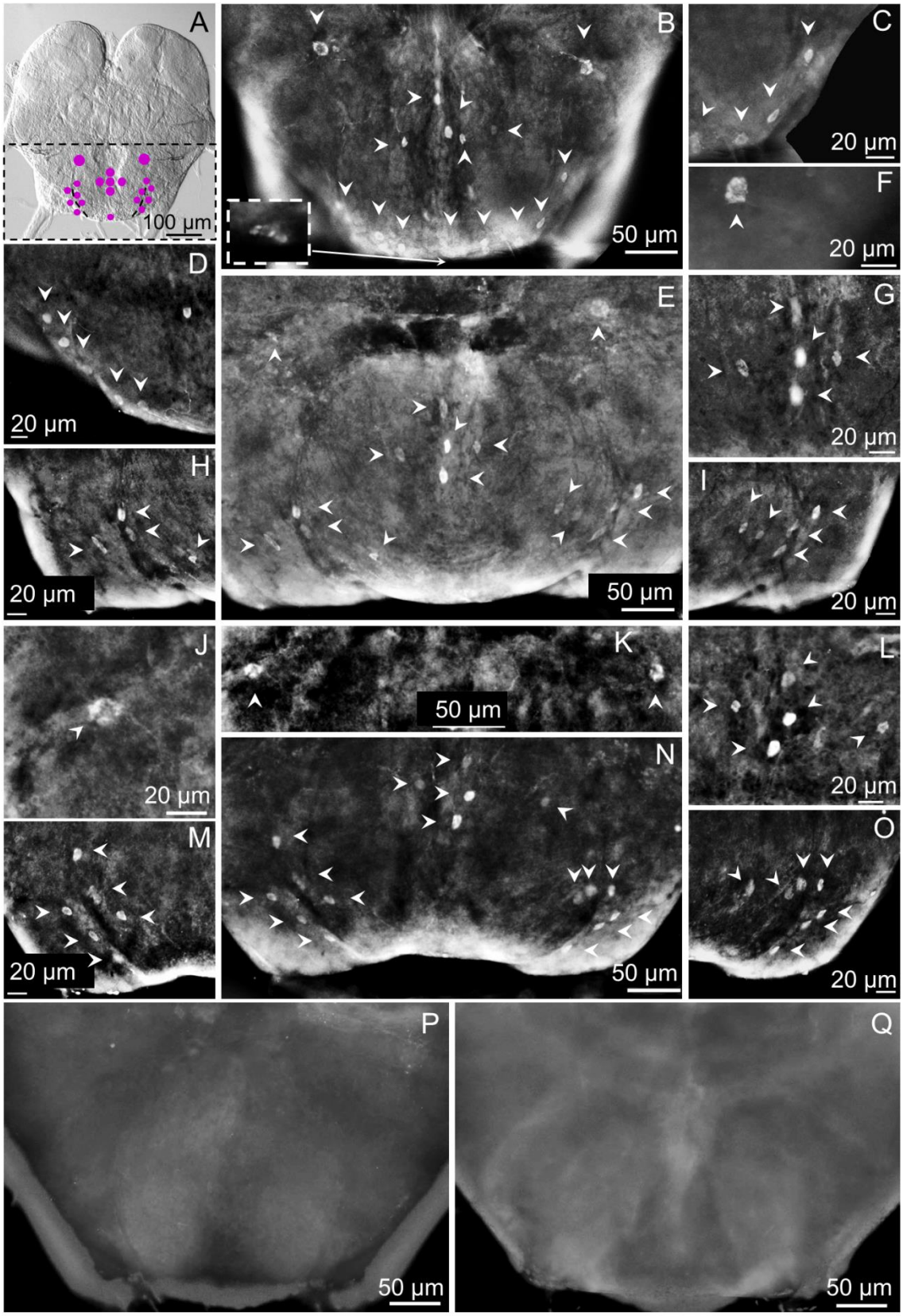
Expression of the sNPFR on the brain/SEG of worker ants from colonies without brood

We investigated differences in the number of clusters and cells within clusters expressing the sNPFR that correlated with the presence/absence of brood in the colony (Figure 2.8, A-H). Some worker brain clusters remain unchanged with respect to colonies with brood. For example, in workers from colonies without brood clusters c5, c13 and c16 were detected in all worker subcastes in the same location and with the same characteristics mentioned above (data not shown and Figure 2.8 B, D, F, H), and c14 was also present in minor (Figure 2.8 H, O (left), P (right) and medium (Figure 2.8 D, Q (left), R (right) workers as before; c14 is never present in majors (Table 1). For

other clusters, in workers from colonies without brood the number of immunoreactive cells for the sNPFR was considerably reduced in comparison to workers of colonies with brood. This was most strikingly seen in the major-exclusive cluster c12 that became undetectable (Figure 2.8 B, S (right)) and in the worker caste ubiquitous c15 (Figure 2.8 B, D, H; O, P, minors; Q, R, media and S, T, majors) that remained only observable in majors (Figure 2.8 B, S, T) while being undetectable in minor (Figure 2.8 H, O, P) and media (Figure 2.8, D, Q, R) in colonies without brood. In majors, the number of cells in c15 was drastically reduced from 3-5 in colonies with brood to 1-2 cells in those without brood (Figure 2.8, compare A to B; and compare majors without brood S and T with c15 in Figure 2.6, B-D).

Two clusters, c7 and c9, were undetectable in medium and minor worker brains from colonies without brood. Cluster c7, normally composed of 2-3 cells in media (Figure 2.8, C) was not detectable (Figure 2.8 D, K) while in minors, normally composed of 4 cells (Figure 2.8 E, G), was also absent in colonies without brood (Figure 2.8 H, I). Cluster c9, was undetectable in both media (Figure 2.8 D, L; compare with Figure 2.5, C, H) and minors (Figure 2.8 H, M, N; compare with Figure 2.5 B, G) in colonies without brood. Cluster c2 normally only present in minors of colonies with brood (Figure 2.8 E, and Figure 2.3 J, L) was undetectable in those from colonies without brood (Figure 2.8 F, J).

Figure 2.6 Common and subcaste-differential short neuropeptide F receptor immunoreactive clusters are present in the posterior brain and subesophageal ganglion. A schematic of all possible clusters detected in the posterior SEG across subcastes are shown in (A) over a Nomarsky image of a major. In all subcastes clusters c13 (top arrows in B), c15 (B, bottom arrowheads) and c16 (B, center) are present. Clusters c12 and c14 are differentially detected among subcastes. (B) In majors, c12 (dashed inset, arrow) is present but c14, that should be located internal to c15, is not detected. A detail of c15 in the right hemisphere of majors is shown in (C) and left, in (D). Medium and minors exhibit all clusters except c12, but although c14 and c15 are present, they have reduced cell number with respect to those in majors (compare D with H and M). In these two subcastes c14 and c15 could be observed from both sides of the brain. (E) Medium worker, clusters c13 to c16; details in F (c13), G (c16), H (c14, c15, left) and I (c14, c15, right). In minors, c13 is shown in (J), and in both brain hemispheres, in (K). (L) Brain of minor; sometimes cells in c16 are not perfectly distributed in a cross-like pattern (compare with G). (N) Distribution of clusters c13 to c16 in minors. Clusters c14 and c15 are show in M (left) and O (right). No fluorescent signal was observed in negative controls with pre-immune serum (P) or antigen-preabsorbed antibodies (Q).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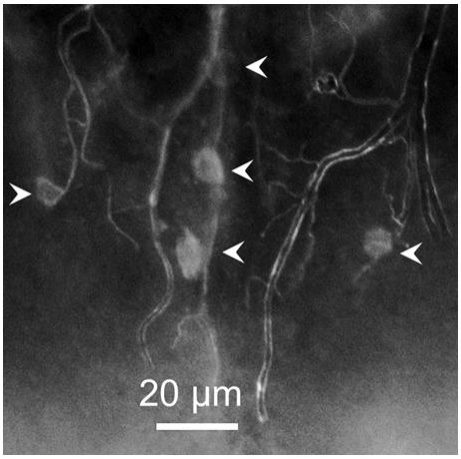


Figure 2.7 Cluster c16 is located near trachea in the posterior brain. This cluster is observed in all subcastes, generally located at the center of the SEG, with 5 cells distributed in a cross-like fashion. Sometimes, the location of each individual cell can vary, and they could be observed near, or perhaps in association with trachea, as shown here in a minor brain.

Differences among castes and subcastes in sNPFR cell clusters

In summary, sNPFR immunoreactive clusters detected in workers that are apparently in similar location in the queen brain are: c5 (also in all worker subcastes); c2, c7, c9 and, c12. Clusters c13-c16 are present exclusively in workers, of which c13, c15 and c16 are detected in all subcastes, the latter likely representing worker specific functions. About clusters and characteristics that discriminate subcastes: majors are the only subcaste that always lack c14 immunostaining but in which c12 is present in colonies with brood, while c12 is always absent in media or minors (Table 1). Minors can be distinguished because are the only subcaste that exhibit c2 in colonies with brood and this is a critical difference with media with which they share others clusters. In colonies with brood, media can be distinguished from majors in that media have c7, c9

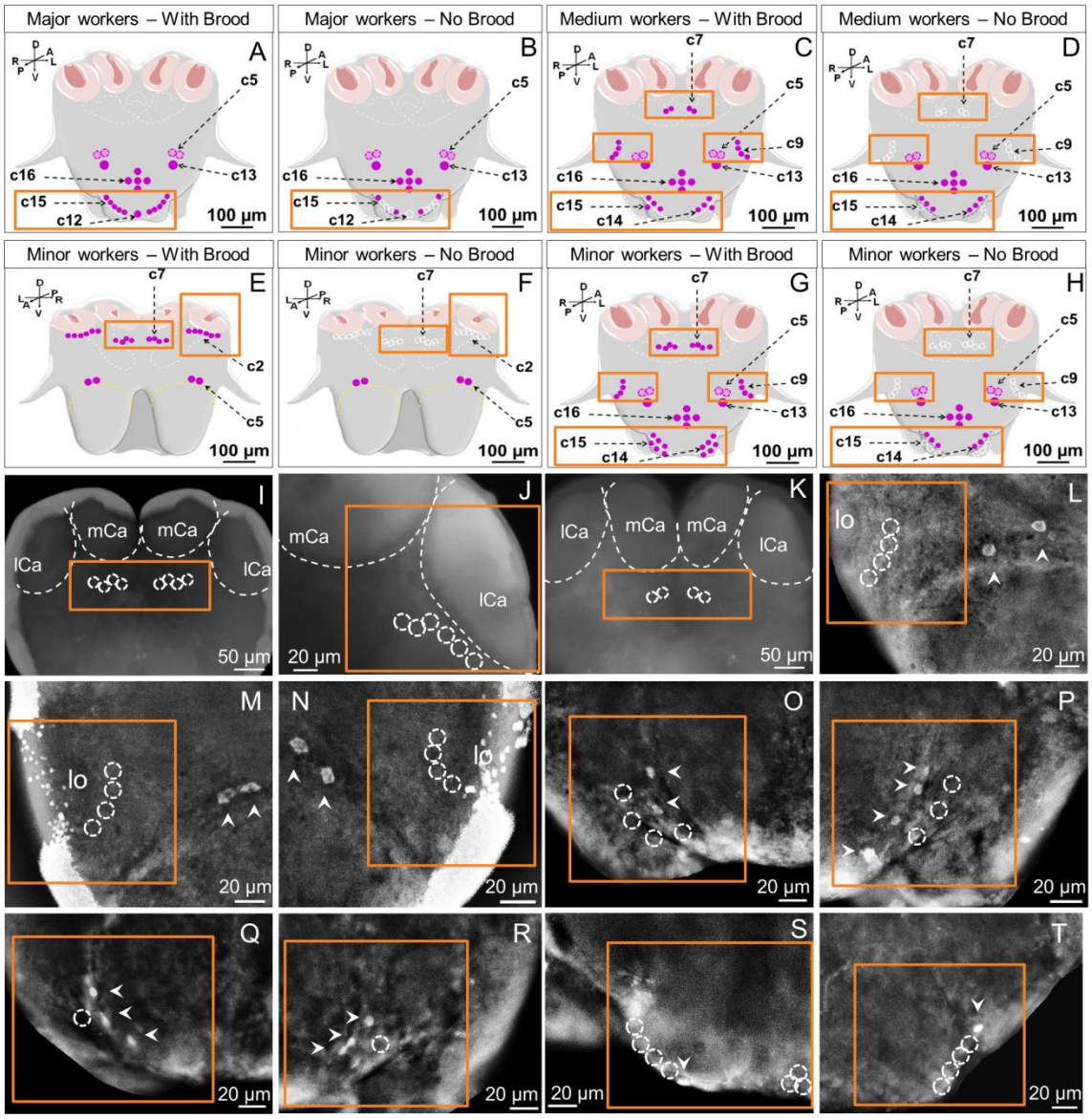
and c14 immunoreactivity; and from minors, in that media lack c2 (Table 1). In colonies without brood majors can be distinguished from both media and minors in that majors retain c15 immunostaining, even when the number of cells is reduced, while media and minors cannot be discriminated by sNPFR staining in these colonies.

Discussion

The spatial expression pattern of the sNPFR in the fire ant worker brain was analyzed in all subcastes, the first time this is reported for workers of a social insect. The sNPF and sNPFR have been characterized in *Drosophila* [75–78] where brain sNPF expression is broad; in contrast, in honey bee workers brain, only a few neurosecretory cells are labeled by sNPF *in situ* hybridization [72], pointing to striking differences between solitary and social insects. There is a paucity of information on this signaling system in other social insects other than honey bees. Therefore, our previous study on fire ant queen brains was now followed by the analyses on workers.

Our results are particularly important for fire ants because worker subcastes are loosely defined by age and size, with observed performed tasks varying with the individual's age creating temporal castes. Therefore, currently there are not known clear morphological or neurobiological markers for determination of worker subcaste as to precisely define the number of individuals most likely performing a particular task at a particular time in a colony. Behavioral plasticity adds to the impossibility of defining the division of labor precisely [52].

Figure 2.8 Brain immunolocalization of the short neuropeptide F receptor in worker subcastes from colonies without brood and comparisons with those with brood. (A-H) Schematic of the location of cell clusters expressing the sNPFR in all subcastes, comparing colonies with and without brood. Areas in orange boxes enclose specific brain areas for comparison. Dashed white circles correspond to the expected location of immunoreactive cells in colonies with brood. A-D, G and H show the receptor signal in the posterior brain; E and F show the anterior brain view. In colonies without brood cluster c7 became undetectable both in minor (H, I) and medium workers (D, K); compare with C, G. Additionally, cluster c2 became undetectable in minors from these colonies (F, J). In the posterior view, cluster c9 also became undetectable in minors (H, and insets in M, N) and media (D, L, inset). Cluster c14 remained present in minor (O, P) and medium workers (Q, R), but cluster c15 became undetectable in these workers, and only observed in majors, however, composed by lower number of immunoreactive cells (S, T) in comparison to majors in colonies with brood. Cluster c12 also is undetectable in majors (B, and in S, lower right corner) when brood is absent.


Major workers – With Brood
Major workers – No Brood
Medium workers – With Brood
Medium workers – No Brood
Minor workers – With Brood
Minor workers – No Brood
Minor workers – With Brood
Minor workers – No Brood
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
P
Q
R
S
T
c5
c7
c9
c13
c16
c15
c12
c14
c2
100 µm
mCa
lCa
lo
50 µm
20 µm

Although workers were not discriminated by age in this study, it appears that age variation may have been coincidentally minimized perhaps because ants were chosen of a particular size and performing a specific task; as a consequence, the cellular expression pattern of sNPFR remained quite constant within subcastes. We did not use ants of different sizes performing the same task (perhaps of different ages; i.e. an older small nurse now foraging); this is especially true for minors and majors. The overall decrease in sNPFR immunoreactivity in colonies without brood (Table 1) supports a relationship between sNPFR expression and colony higher nutritional status or requirements for protein when brood is present because of the known ability of larvae to digest protein [79].

In contrast to the paucity of information in fire ants, there is more knowledge about mechanisms regulating division of labor and behavioral plasticity in worker honey bees. In these bees, about 5,500 brain genes are differentially expressed between nurses and foragers [80], and it is also established that division of labor is related to feeding behavior, nutritional status and age of each individual [81–83]. Additionally, there is evidence that the sNPF/sNPFR signaling pathway regulates the foraging behavior in honey bees, and that the sNPFR transcript appears to be upregulated in the brain of foragers compared to nurses [72], and strikingly, the sNPF peptide level changes in association with nectar or pollen foraging, and between foragers arriving or departing from a feeder [73]. Pollen-collecting bees departing from feeders with pollen on their legs had higher sNPF peptide in their brains [73]. These findings are in agreement with our results with nurses in colonies with brood, in which we found a significant higher

number of cells exhibiting sNPFR immunoreactivity in the brain/SEG. Similar to pollen-collecting bees, fire ant nurses handle protein, and feed proteins to larvae and queens preferentially; this is more evident when protein supplies are limited [84]. Although we have not yet investigated sNPF expression in fire ants, it seems that in bees and ants, protein sensing correlates with increased sNPF signaling effector proteins, sNPF or sNPFR, respectively.

Globally, the worker caste exhibited a total of 9 cell clusters expressing the sNPFR, with different number of clusters observed in the different subcastes (Figure 2.2). Clusters 13, 15 and c16 are present in all worker subcastes but not the queen, indicating that represent worker exclusive functions. There is an inverse relationship between worker size and number of clusters expressing sNPFR, with majors having the lowest cluster number of five. Interestingly, the range in the total number of sNPFR cells did not overlap among subcastes in colonies with brood (Table 1) indicating that the sNPFR clusters indeed correlate with functional subcastes (size and task performed). The lesser total number of sNPFR immunoreactive neurons found in majors (19-26) correlates with their known simpler behavioral suite: only 15 behaviors were registered for majors compared to 20 for minors, in which we observed higher sNPFR immunoreactive cells [15]. Further, 72% of majors lifespan is spent as reserves and about 27% as foragers, with only a minimal (1% of life span) time caring for brood [16]. We observed that in majors cluster c15 decreased in the number of sNPFR stained cells in the absence of brood, perhaps also reflecting a "protein-starved" phenotype (Table 1), while immunoreactivity fully disappeared in media and minors.

Worker fire ants are completely sterile, offering a model in which queen reproductive functions could be differentiated from other functions in workers, contrary to the honey bee in which workers may retain reproductive ability [85]. With respect to this, some of the cell clusters found in the worker brain are also present in the fire ant queen brain, as described by Lu *et al* [50]; thus, it appears there are indeed common circuits and functions modulated by the sNPFR in workers and queens, such as exemplified by C5 (Figure 2.4, Table 1). Cluster c5 located above the antennal lobes (Figure 2.4), was present in all subcastes from colonies with and without brood. In the queen brain C5 was identified as possible local and/or projection interneurons, which would transmit the information generated on the olfactory receptor neurons (ORNs) to higher processing centers in the brain [50]. We also observed C5 in males (data not shown). This suggests that its function is independent of size, age and specific labor of the workers and it appears it has constitutive expression in all castes (Table 1).

As expected, others clusters were exclusive to one caste (see Table 1; in pink, queen exclusive clusters, and c13-16, worker caste exclusive), indicating there are pathways differentially regulated by this receptor in queens and workers. Further, the number of cells expressing the sNPFR in queens (~164) is much higher compared to worker ants. This caste difference in sNPFR expression supports the notion that sNPF signaling is involved in regulation of additional complex functions in queens, such as reproduction, nutrient storage functions which queens display or others [50]. For example, cluster C1 present in the queen anterior protocerebrum which was postulated to correspond to insulin producing cells is not present in fire ant workers [50].

The most striking differences in the pattern of expression of the sNPFR among worker subcastes were found in the superior protocerebrum, below the mushroom bodies, and recent studies show the mushroom bodies seem to be involved in mechanisms related to the division of labor in ants [57,58,63,65], bees [86,87] and a wasp [88]. In minor fire ants, cluster 2 (c2) located below the mushroom bodies is in similar location as in the queen brain (C2), and it was hypothesized that they could be neurosecretory, due to their similar location to the lateral neurosecretory cells (FMRFamide-like immunoreactive) in the honey bee brain [50]. Because C1 cells are not present in workers it would be interesting to know if C1 and C2 in queens and c2 in workers represent two separate functions of the sNPF signaling pathway, related and unrelated to insulin production in queens and workers, respectively. Similar to the central location of C1 in fire ant queens, in the *Drosophila* brain a cluster of centrally located cells, the medium neurosecretory cells, are insulin producing and express sNPFR, however, in the same cluster, a few sNPFR expressing cells do not appear to produce insulin peptides, setting a precedent for the separation of expression of sNPFR and insulin like peptides in the insect brain [89].

Cluster c2 immunoreactivity was only observed in minor workers extracted from colonies with brood (Figure 2.3 J, L); while immunoreactivity was absent in minors from colonies without brood (Figure 2.8, F, J). This suggests that c2 could control common functions in the brain of queens and the minor workers studied here (likely “nurses”), and this hypothetic function could be related to protein sensing associated with brood handling and care. This subcaste appears to be more sensitive to the presence/absence of

brood than media and majors, with a 63-71% reduction in the number of sNPFR cells in minors from colonies without brood (Table 1).

In colonies with brood, with respect to cluster changes in the superior medial protocerebrum, cluster c7 was detected only in medium (Figure 2.3 E, F) and minor (Figure 2.3 H, K) workers; being undetectable in media (Figure 2.8 D, K) and minors (Figure 2.8 E, I) from colonies without brood (Table 1). This cluster was never observed in major workers. sNPFR immunoreactive cells in approximately the same location were named C7 in the fire ant queen brain [50], and both clusters are located in a similar area as the cluster of octopaminergic neurons G4d, in the honey bee brain [90]. Due to the location of c7 near the central complex, we hypothesized the sNPF/sNPFR signaling pathway could also be regulating the function of this neuropil in the ant brain. In *Drosophila*, cells expressing the sNPF and the sNPFR are in the central complex [91,92], and a reduction of sNPF expression in the fan-shaped body of female flies increases their walking distance and their mean walking speed [93]. The presence of c7 in minors and media (see Table 1) is also consistent with perhaps higher protein transfer near the nest when brood is present [74]. If c7 is involved in locomotion, it would be interesting to test if the immunoreactivity of these c7 cells changes with age in smaller workers as an age related, temporal polyethism, associated with foraging and the leaving of the nest in older workers. Similar to the cluster c7, cluster c9 appears to be immunoreactive only in minors and medium workers from colonies with brood. In the queen brain C9 could correspond to optical projection neurons, similar to the ones observed in the ant *Cataglyphis albicans* [50,94]. We speculate that in fire ants these cells are involved with

the regulation of visual input and brood care.

Most clearly seen from the posterior view of the brain, clusters c12 to c16 are located in the inferior protocerebrum and in the subesophageal ganglion (SEG) (Figure 2.6). We hypothesized they are involved in the regulation of functions that are processed by the SEG, such as regulation of movement of the mouthparts [95,96], gustation [97] and feeding behavior [98]. In different insect species including social insects several neuropeptides had been detected in the SEG, such as FMRFamide-like peptides, FXPLRamide related peptides (e.g. PBAN), neuropeptide F (NPF) and short neuropeptide F (sNPF) [75,99,100]. The expression pattern of the sNPFR in the SEG varies slightly among worker subcastes as shown under results (Figure 2.6), however, dramatic differences were observed in the SEG when comparing workers from colonies with and without brood (Figure 2.8). In the latter workers the number of sNPFR immunoreactive cells is considerably reduced specifically in clusters c15 (Figure 2.8, O-T) and c12 (Figure 2.8, B, S), which apparently could be due to a diminished nutritional status, maybe representing a mechanism of protein sensing. Clusters C12 is reminiscent of the octopaminergic ventral unpaired medium (VUM) neurons described in honey bee forager (worker) brains [90]; perhaps these cells integrate foraging behavior for protein in majors. Octopamine is broadly expressed in the SEG of honey bees and the fire ant sNPFR signal overlapping with known patterns of octopaminergic neurons in worker bees points perhaps to the integration of olfactory (VUM, c12) and nutritional signals (sNPF/sNPFR) and olfactory learning in ants [90].

Based on the fact that a) majors are not associated behaviorally with brood care but exhibit c12, c13, c15 and c16, and that b) some clusters were present in all castes (C5), it is reasonable to assume that the mentioned clusters are not particularly associated with the presence or absence of brood as it pertains to brood presence per se. Among these, clusters that appear to be protein sensing are c15 and C12. It appears that different subcastes may perceive the lack of protein differentially through these clusters (depending on their worker subtask priority, i.e. nurses being more sensitive through cluster c2). The queen exhibits sNPFR immunoreactivity in 12 clusters, higher than any other worker caste, giving her perhaps a higher sensitivity to either protein availability/requirements or presence of brood. Further, queens and larvae are always preferentially fed protein even when protein is limiting [19,101,102]. Therefore, it follows that the queen may always have sNPFR immunoreactivity in these clusters.

Conclusions

Dynamic changes in sNPFR appear to occur in worker brains in association to the presence/absence of brood, specifically in the protocerebrum and the SEG. Our study supports our previous work with starved fire ant queens in that the sNPFR signaling may change in response to nutritional requirements, not only in queens but workers. To our knowledge this is the first if not one of the few studies with social insects that correlate body size, behavioral state and colony brood presence (proxy for colony nutritional status/requirements) with the actual protein expression of a brain neuropeptide receptor, sNPFR, and not a transcript. In the absence of brood, the overall decrease in the sNPFR signal observed in worker brains supports the idea that workers may be less motivated to search for food. It is apparent that in fire ant workers, similar to bees, changes in sNPFR expression depend at least partially on foraging needs. However, it is not known if the cells that express the receptor in colonies with brood stop receptor expression in colonies without brood and could regain it if brood were present. Alternatively it is possible that these cells simply die. Therefore, to answer these questions it would be useful to identify a second marker for the cells expressing the sNPF receptor and manipulate the same colonies with or without brood present and investigate receptor expression after manipulations.

Table 1 Number of short neuropeptide F immunoreactive cells observed in the brain of worker subcastes from colonies with and without brood and comparison to those reported previously for queen brains.

	Workers from colonies with brood						Workers from colonies without brood						Queens from colonies [2]	
	Majors		Media		Minors		Majors		Media		Minors			
Cluster	Cell Nº per brain/SEG hemisphere[1]	Total Nº of cells per brain	Cell Nº per brain/SEG hemisphere[1]	Total Nº of cells per brain	Cell Nº per brain/SEG hemisphere[1]	Total Nº of cells per brain	Cell Nº per brain/SEG hemisphere[1]	Total Nº of cells per brain	Cell Nº per brain/SEG hemisphere[1]	Total Nº of cells per brain	Cell Nº per brain/SEG hemisphere[1]	Total Nº of cells per brain	Cell Nº per brain/SEG hemisphere[1]	Total Nº of cells per brain
C1	-	-	-	-	-	-	-	-	-	-	-	-	N/A	3
C2*	-	-	-	-	4- 10	8- 20	-	-	-	-	-	-	25	50
C3	-	-	-	-	-	-	-	-	-	-	-	-	8	16
C4	-	-	-	-	-	-	-	-	-	-	-	-	6	12
C5	2- 3	4- 6	2	4	2	4	2	4	2	4	2	4	3	6
C6	-	-	-	-	-	-	-	-	-	-	-	-	N/A	30
C7*	-	-	2- 4	4- 8	4	8	-	-	-	-	-	-	4	8
C8	-	-	-	-	-	-	-	-	-	-	-	-	11	22
C9*	-	-	4	8	4	8	-	-	-	-	-	-	4	8
C10	-	-	-	-	-	-	-	-	-	-	-	-	1	2
C11	-	-	-	-	-	-	-	-	-	-	-	-	1	2
C12*	N/A	2- 3	-	-	-	-	-	-	-	-	-	-	N/A	5
c13	1	2	1	2	1	2	1	2	1	2	1	2	-	-
c14	-	-	2 - 3	4- 6	3	6	-	-	2- 3	4- 6	3	6	-	-
c15*	3- 5	6- 10	1 - 3	2- 6	3	6	1- 2	2- 4	-	-	-	-	-	-
c16	N/A	5	N/A	5	N/A	5	N/A	5	N/A	5	N/A	5	-	-
Total cell number (range)		19-26		29-39		47-59		13-15		15-17		17		164
Percent change in cell Nº [3]								32-42		48-56		63-71		

Footnote: Names of clusters in capital letters correspond to those in the queen, but cell numbers under minor, media and majors correspond to worker clusters in similar position to those clusters found in queens and that we identified with small letter c throughout the manuscript. Note that clusters only present in the midline of the brain, and therefore not symmetrically distributed are: C1, C6, C12 and c16; clusters c13 through c16 are exclusively found in workers, but c14 is absent in majors.
(1)The number of cells per cluster in one brain/SEG hemisphere is indicated only for clusters that show a symmetrical distribution; in workers, numbers separated by a hyphen indicate the range in the number of cells observed in different individuals. N/A refers to clusters in the midline of the brain which cell number is only indicated in the total number per brain column. (2) From Lu *et al* [50].(3) The percentage decrease in the cell number range per subcastes in colonies without brood was calculated with respect to the respective range in cell numbers in colonies with brood. Clusters in pink are queen exclusive; in green, queen and majors exclusive; in light blue common to media, minors and queens; in white, minors and queen exclusive. In yellow (C5 and c13, c14, c16) are worker clusters that are present regardless of presence or absence of brood; in light yellow are clusters common to all females (all worker subcastes and queen). In workers, clusters with asterisks change in cell number depending on the presence or absence of brood; c15 is the only worker exclusive cluster that responds to the absence of brood (in orange).

CHAPTER III

TOWARD ANALYSIS OF sNPF GENE EXPRESION IN THE BRAIN OF QUEENS AND WORKERS

Introduction

In most arthropods studied so far, short neuropeptides F are 6–11 amino acid residues in length with the C-terminal consensus sequence xPxLRLRFamide, and several studies in different insect species showed that these neuropeptides are involved in the regulation of molting and development, feeding, stress responses, locomotion, olfaction, circadian rhythms (sleep cycles), reproduction, learning and memory [37,44,103–105]. In fire ants, two mRNA transcripts of a sNPF peptide precursor were cloned from brain tissue of newly mated queens. These mRNAs share the same ORF and they only differ on the length of the 3' UTR region, both being polyadenylated [106]. Interestingly, the prepropeptide gives origin to one of two possible active sNPFs peptides by means of further processing at an additional potential cleavage site towards the N-terminus. Most interesting is the mature neuropeptide ends in an amidated tyrosine (Y) instead of the amidated amino acid phenylalanine (F). This feature appears to be exclusive of fire ants, because in other insect species, sNPF peptides end in the characteristic amidated F, or W (mosquitoes and *Drosophila* [44]) [106].

In larval and adult *Drosophila melanogaster*, many neurons expressing *snpf* mRNA transcripts had been identified by *in situ* hybridization (ISH), and they are

ubiquitously distributed in the central nervous system (CNS), especially in Kenyon cells of the mushroom bodies in the brain, and in chemosensory neurons of antennae and maxillary palps. A total of at least 280 neuronal cell bodies had been detected in the adult brain, including the subesophageal ganglion [78]. Some of these *snpf*-expressing cells also synthesize other important neurotransmitters and neuropeptides such as acetylcholine, GABA, dopamine, corazonin, and pigment dispersing factor (PDF) [78,107]. In contrast to the solitary *D. melanogaster* where there is a large population of sNPF expressing neurons, in the eusocial honeybee *Apis mellifera,* there are few neurons in the brain near the mushroom body calyces which express *snpf* mRNA transcripts [72]. A recent study showed that the sNPF peptide is found in large amounts only in the *corpora cardiaca/allata* and the frontal ganglion in the CNS [108].

In ants, until now there is only one published neuropeptidomic analysis showing the spatial location of the sNPF peptide in specific regions of the brain and other areas of the nervous system. This pioneer study showed that in workers of the carpenter ant *Camponotus floridanus*, the sNPF peptide is present in the central brain, antennal lobes, optic lobes, gnathal ganglia, retrocebreral complex (*corpora cardiaca/corpora allata*), and the thoracic and abdominal ganglia [109]. These results indicate that the sNPF peptide is quite abundant and widely distributed throughout the nervous system of these ants, but still the site of synthesis and release remains unclear; that is, it is unknown if this neuropeptide is synthetized in all these regions or if it is produced in other more limited zones and then transported and released as a neurohormone elsewhere. In fire ants, the detection of the endogenous sNPF peptide(s) from intact brain of newly mated

queens was previously attempted by MALDI-TOF MS with no success. However, a partial sequence of the predicted sNPF peptide was detected from an extract of eggs and brood [106]. As an alternative to the identification of the sNPF peptide through peptidomic analysis or specific MALDI-TOF detection, in this study we proposed to identify the spatial distribution of *snpf*-expressing cells in the brain of fire ant queens and workers through *in situ* hybridization (ISH). This technique will allow us to detect the mRNA transcripts of the sNPF, without the need to develop a specific antibody against this neuropeptide, as required when performing immunohistochemistry. We previously attempted the immunohistochemistry of this neuropeptide with an antibody targeting the prepropeptide without success (western blot analysis showed that this antibody was not specific).

The identification of *snpf*-expressing cells is important for the construction of a complete map of neurons involved in the sNPF/sNPFR signaling pathway in the fire ant brain, as it was done in *Drosophila* [78,110]. The localization of sNPFR-immunoreactive cells in the queen and worker brain is already known [50,111], and investigating which cell clusters are responsible of the synthesis of sNPF will complement previous work. This knowledge as well as the identification of other neuropeptides/neurotransmitters co-expressed in neurons expressing the sNPF or its receptor will be fundamental to start unveiling the physiological role of the sNPF/sNPFR signaling pathway in these ants. We hypothesize the *snpf*-expressing cells in the brain of queens and workers are located near the mushroom bodies, maybe close to neurosecretory cells.

Materials and methods

Insects

Fire ants are invasive in Texas and abundant in the College Station city area. Polygyne colonies of *S. invicta* were collected on the west campus of Texas A&M University, in College Station, Texas. The field collections were limited to fire ants and did not involve endangered or protected species. All the colonies were maintained in the laboratory at 27± 2°C in a 12:12 h light-dark photoperiod on plastic trays, which walls were covered with Fluon (Insect-a-slip©, BioQuip products, CA, USA). Each plastic tray contained at least one nest, which is a 10 cm diameter Petri dish half-filled with Castone® (Dental Supply International Inc., York, PA, USA) prepared as per manufacturer's instructions. The ants were fed daily with 15% honey-water, frozen crickets (Premium crickets, Atlanta, GA, USA), and an artificial solid diet specially formulated for ants, which last up to a month under refrigeration at 4°C [112]. Detailed instructions to prepare this solid diet are shown in Appendix C at the end of this manuscript. Water was provided inside test tubes with a cotton plug. All foods and water were provided *ad libitum*.

Primer design for cloning regions of the sNPF peptide and its receptor (sNPFR) for ISH probe synthesis

Primers were designed to obtain an amplicon of 377 bp (53.6% GC content) corresponding to the whole ORF of the *snpf* gene (primers encompass from -18 to 359 bp corresponding to figure 1 in Bajracharya, *et. al.*, 2014 [106]), and a fragment of 535 bp long (46% GC content), located between the first and fifth predicted transmembrane regions of the *snpf* receptor gene (Table 2). This sequence encompasses the amino acid residues 42 to 218 of the sNPFR. For each gene, separate PCR reactions were performed using the Advantage® 2 PCR Enzyme System (Clontech, Mountain View, CA, USA) following the manufacturer's protocol. In both PCR reactions, a cDNA from mated queen brains was used as the template. This cDNA was synthesized from 1 μg of total RNA using oligo dT and the SuperScript® III First-Strand Synthesis System (Life Technologies, Grand Island, NY, USA) following the standard protocol. These PCR products were loaded in a 1.2% agarose gel and ran at 100 V for 30 min using 1X TAE buffer. The bands corresponding to the expected size were purified from the gel using the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA, USA) and ligated in the pGEM®-T Easy Vector using T4 ligase (Promega, Madison, WI, USA) at 4°C overnight. Then, One Shot® TOP10 chemically competent *E. coli* cells (Life Technologies, Grand Island, NY, USA) were transformed with the recombinant vectors following the standard protocol, allowing them to grow overnight at 37°C in LB agar plates supplemented with 100 μg/mL of ampicillin and X-gal. Randomly selected white colonies were re-plated to ensure the clones correspond just to one bacterial colony. Finally, each selected clone

was individually grown in a liquid culture for isolation of plasmid DNA using the QIAprep Spin Miniprep kit (Qiagen, Valencia, CA, USA). To confirm the presence of the correct inserts, about 200 ng of this plasmid DNA was digested with *Eco*RI for 1h at 37°C, and ran in a 1.2% agarose gel as described above. Clones showing an insert of the expected size were selected and sent for sequencing to Eton Biosciences (San Diego, CA, USA). The sequencing reactions were performed by Eton Biosciences using their T7 and SP6 universal primers. The sequence of the inserts and their orientation were analyzed using the BioEdit software (Ibis Biosciences, Carlsbad, CA, USA), and clones with the correct sequences for sNPF and sNPFR were selected for further synthesis of DIG-labelled RNA probes.

Table 2 DNA primers used for amplification of short fragments of the short neuropeptide F and short neuropeptide F receptor cDNAs for cloning in pGEM-T Easy vector

Primer name	**Sequence**	**Amplicon size (bp)**
Primers for cloning the short neuropeptide F fragment in the pGEM®-T Easy Vector (primers encompass from -18 to 359 bp corresponding to figure 1 in Bajracharya, et. al., 2014 [106])		
sisNPF F2	5' CCGAGGACAGAGGAGGCCATGTATGC 3'	377
sisNPF R2	5' CCCTGGAACAGCCTCACCGATTAATTG 3'	
Primers for cloning the short neuropeptide F receptor fragment in the pGEM®-T Easy Vector (region encompassing the amino acid residues 42 to 218)		
sNPFR_probe_F2	5' ATGTGTTGGTGATCTTCGTCGTCGGG 3'	535
sNPFR_probe_R2	5' TTAGCGCGCACTCGATCGTTCAGTTT 3'	

In vitro DIG-labelled RNA probes synthesis

For each gene sNPF and sNPFR (Table 2), recombinant plasmid DNA was digested in separate reactions with the restriction enzymes *Spe*I and *Sph*I for 16 hours (overnight) at 37°C. This reaction is necessary to *in vitro* transcribe only the cloned insert. After digestion, the enzymes were heat-deactivated and the linearization of the plasmid was verified on a 1% agarose gel. Linearized plasmids were precipitated with 0.3 M sodium acetate pH 5.2 / 75% ethanol for 2 hours at -20°C. Then, the samples were centrifuged at 14,000 g for 30 min at 4°C, and the pellets were solubilized in 13 μL of sterile DEPC water. Both, sense and antisense DIG-labelled RNA probes were synthesized following the manufacturer's protocol, using the linearized plasmid DNAs as templates for the DIG RNA Labeling Kit (Roche, Indianapolis, IN, USA). After probe synthesis, the RNA concentration in the reaction mix was determined by spectrophotometry at 260 nm in a NanoQuant infinite m200 plate reader (Tecan Systems Inc., San Jose, CA, USA) and visualized in a 1.5 % agarose gel. Additionally, the labeling efficiency of the probes was tested by performing a dot-blot as suggested in the DIG RNA Labeling Kit protocol. Briefly, a DIG-labelled RNA probe provided with the kit (positive control) and the newly synthesized probes were diluted in a range of different concentrations. Then, one microliter of these dilutions was crosslinked in a small piece of nylon membrane (Amersham Hybond-N+ nylon membrane, GE Healthcare Life Sciences, Pittsburgh, PA, USA), which was incubated with 1X Blocking Reagent (Roche, Indianapolis, IN, USA) for 30 min at room temperature. The DIG-labelled RNA probes were detected by incubating the membrane with an anti-

Digoxigenin (DIG) antibody conjugated with alkaline phosphatase (Roche, Indianapolis, IN, USA) for one hour at room temperature, to then develop the colorimetric signal with the BM purple kit (Roche, Indianapolis, IN, USA). The labelling efficiency of the newly synthesized probes was determined by comparing the intensity of the colorimetric signal with the positive control provided in the kit, and only properly labelled probes were used to perform the ISH. By using this method, it was determined that for the sNPF and sNPFR DIG-labeled RNA probes, the concentration obtained per labeling reaction was in average about 100 ng/μL (total yield = 2 μg DIG-labeled RNA probe per reaction).

sNPF and sNPFR in situ hybridization (ISH) in the brain of queens and workers

These ISH were performed following seven different protocols, which were adapted from previous publications in other insect species with the aim to determine the best conditions to successfully perform ISH in fire ant brains. These protocols were designed to work with different preparations of the tissues, such as frozen sections as well as whole mounts. In addition, the probe detection was performed through fluorescence, with and without the use of a "signal amplification system"; or by using a colorimetric reagent. All these protocols are described in detail in Appendix A at the end of this manuscript. As a positive control for these experiments the antisense probe of the sNPFR receptor was included because the location of the sNPFR receptor immunoreactive cells is known in the brain of fire ant queens and workers [50,111].

Data collection and analysis

Worker and queen brains were observed in a Carl Zeiss Axio Imager A1 fluorescent microscope, coupled with an AxioCam MRc color camera (Carl Zeiss) using the settings for fluorescence or transmitted light, depending on the type of detection system used, fluorescence or colorimetric, as indicated above. All the images taken using this microscope were captured and processed using the Axiovision software (version 4.8.2). When using fluorescence, the pictures were enhanced to clearly show the fluorescent signal by modifying the brightness and contrast uniformly in all images with similar settings for negative controls. No additional or partial modifications of images were performed. In addition, high-resolution images and short Z-stacks were obtained for some samples in an OLYMPUS FV1000 confocal microscope located at the Microscopy & Imaging Center on the main campus of Texas A&M University, College Station, Texas. All the images collected with the confocal microscope were analyzed with the Olympus FV10-ASW software (Olympus).

Results

The objective of this chapter was to determine the spatial location of sNPF-expressing cells in the brain of fire ant queens and workers through *in situ* hybridization (ISH). Thus, the first step was to successfully obtain the DIG-labelled RNA probes (sNPF and sNPFR) to then, determine the best conditions for ISH in these tissues. The labeling efficiency of the probes was tested as described above in the Materials and Methods section. A representative image of this test showing the colorimetric signal obtained with different concentrations of the sNPF DIG-labelled RNA probe is shown in Figure 3.1. All synthesized probes were always detectable through this method, indicating that DIG-labelling occurred. Also, the concentrations estimated for the sNPF and sNPFR probes (sense and antisense probes) were always similar. Using these DIG-labelled probes, a total of seven protocols were assayed with variable results, as follows.

The first trial of ISH in the brain of fire ant queens and workers was performed with a protocol designated protocol 1, which was adapted from previous publications in other insect species [113–115] (Appendix A). The results obtained with protocol 1 appeared promising at first inspection, but it was suspicious that the fluorescent signal was only observed near the surface of the brain, but not deep inside (Figure 3.2). With this protocol, the fluorescent signal which supposedly corresponds to the sNPF peptide transcripts appears as fine lines ubiquitously distributed in the brain of workers (Figure 3.2) and queens (Figure B.1 in Appendix B). In the worker brain, these long and fine fluorescent lines were most frequently observed near the mushroom bodies (MB) (Figure

3.2 C, D), but sometimes were also present on the subesophageal ganglion as arborizations (SEG) (Figure 3.2, E) and close to the lobula of the optic lobes (Figure 3.2, F). Negative control samples (using the sense probe) did not show these fluorescent lines (Figure 3.2, G, H). In the queen brain, similar fluorescent signal was observed, but was especially abundant close to the lobula on the optic lobes, as shown in figure B.1. Because the shape of these fluorescent signals was not similar to the clusters of sNPF-expressing cells found in *Drosophila* and honeybee [72,78], a second protocol was tested (protocol 2) to verify the obtained results.

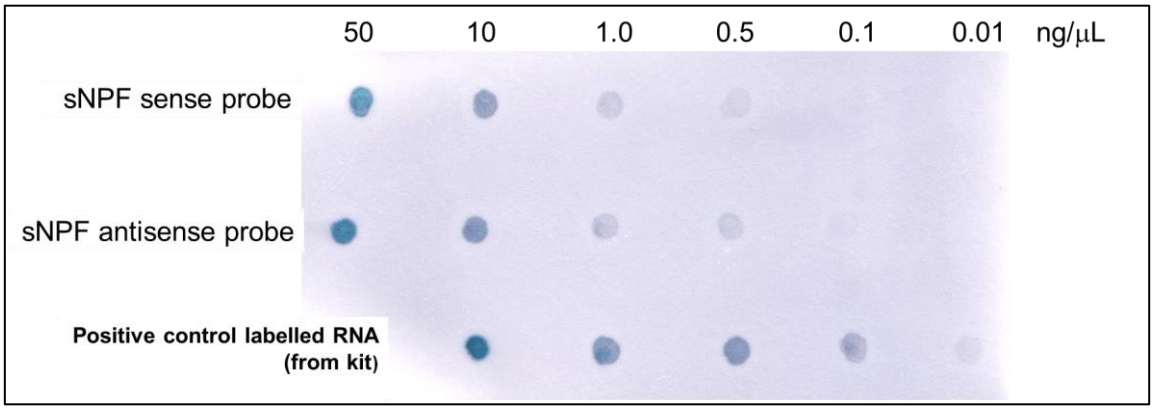
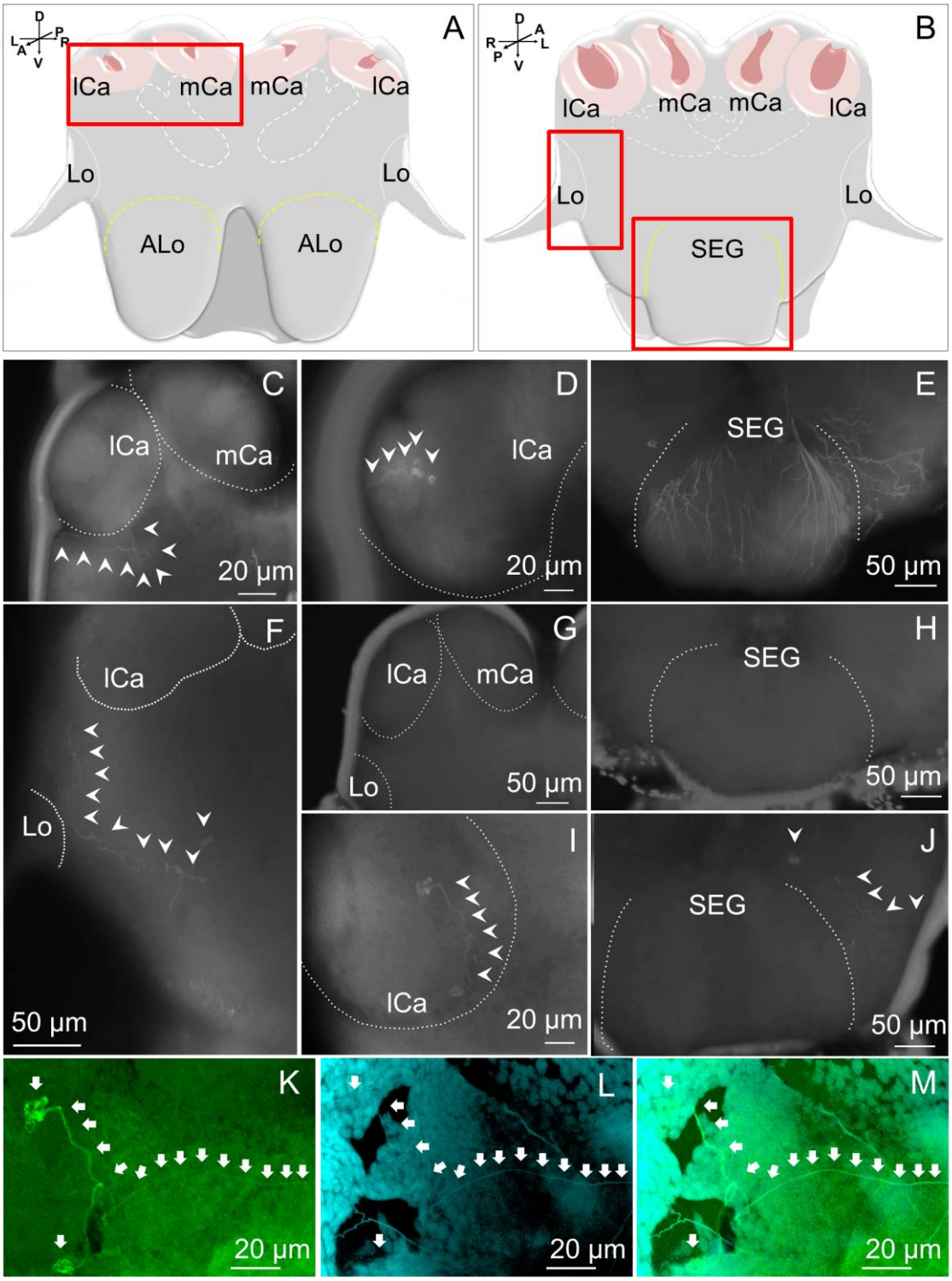


Figure 3.1 Results of labelling efficiency test. This is a representative image of the labelling efficiency of a sNPF DIG-labelled RNA probe synthesized as described above in the Materials and Methods section. The sense and antisense (negative control) probes of the sNPF were detected by colorimetry, and the intensity of the spots of signal was compared with the positive control sample provided with the DIG RNA Labelling kit, to determine their concentration.

Figure 3.2 Results of worker brain *in situ* hybridization for the short neuropeptide F obtained with protocols 1 and 2. A schematic diagram showing the anterior view and the posterior view of the worker brain are shown in (A) and (B), respectively. The red boxes indicate the brain regions from where these pictures were taken. The photos correspond to representative images of the results obtained with ISH protocols 1 and 2 in whole mounted worker brains using the anti-sense sNPF-probe (377 bp). Samples obtained following protocol 1 showed a fluorescent signal that resembles fine lines (indicated with white arrowheads) that were more frequently found below (C) and on top (D) of the lCa of the mushroom bodies; as well as in SEG (E) and close to the lobula in the optic lobe (F). Negative controls do not showed these fluorescent signals in the mentioned areas of the brain (G, H). Similar fluorescent signals were observed in the brain of workers obtained following protocol 2, where fluorescent lines were observed on top of the lCa (I), and near the SEG (J). High resolution images obtained using a confocal microscope revealed that the fluorescent signal corresponding to the sNPF peptide probe (green color, K) follow the same path that auto-fluorescent signal from trachea (cyan color, L) (see white arrows). A picture showing the overlap of both signals is shown in M. ALo = Antennal lobes, lCa = Lateral calyces, mCa = Median calyces, SEG = subesophageal ganglion, Lo = Lobula.


A
lCa
mCa
mCa
lCa
Lo
Lo
ALo
ALo
B
lCa
mCa
mCa
lCa
Lo
Lo
SEG
C
lCa
mCa
20 μm
D
lCa
20 μm
E
SEG
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F
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G
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SEG
50 μm
I
lCa
20 μm
J
SEG
50 μm
K
20 μm
L
20 μm
M
20 μm

In protocol 2, the composition of the hybridization buffer plus the use of highly concentrated probe are supposed to improve the penetration of the probe in deepest layers of the brain. The results obtained using protocol 2 in the brain of queens and workers were very similar to the results obtained using protocol 1 (Figure 3.2; Figure B.1). As observed before, in the worker brain, fine fluorescent lines were found usually close to the MB (Figure 3.2, I) and near the SEG (Figure 3.2, J); and in the queen, fluorescent lines were ubiquitously distributed throughout the brain (Figure B.1), and intense spots of signal were observed in few samples as shown in Figure B.1, C. To examine in more detail the fluorescent signals from samples processed with both protocols, some were selected for confocal microscopy. In these high resolution confocal images, it is clear that these fluorescent lines corresponding to the hypothetical signal from the sNPF peptide probe overlaps with the auto-fluorescent signal given by the trachea, which are abundant in the brain (Figure 3.2 K-M). These results suggest that the fine fluorescent lines obtained with protocols 1 and 2 could correspond to a false positive signal from probe retained in the tracheal system, and not necessarily correspond to the label of sNPF-expressing cells. Because the results obtained with these protocols were inconclusive and the signal most likely artefactual, a third protocol was tested (protocol 3).

Protocol 3 differs from protocols 1 and 2 in the processing of the sample, the composition of the hybridization buffer, and the addition of different amounts of pre-hybridization buffer to the washing steps. All these modifications were supposed to reduce the background/signal ratio to obtain a clearer signal from sNPF-expressing cells.

Also, some brain samples were processed following this protocol and a signal amplification system, which allowed increasing the probe detection sensitivity fourth times (see details in Appendix A). In general, the results obtained with protocol 3 were not better than the results obtained by using protocols 1 and 2, and still the background coming from the tracheae was very intense; however, round spots of fluorescent signal with similar shape to cell clusters were observed in some queen brains (Figure B.2, Appendix B). When using the probe to detect the sNPF peptide transcript, very small spots of signal were observed near the mushroom bodies (Figure B.2 C, D); and with the sNPFR probe (positive control) some fluorescent signal in the superior medial protocerebrum (smP) resembling cluster C7 and also on the SEG, resembling cluster C12 were observed (Figure B.2 E, F respectively). As mentioned before, these cell clusters of sNPFR-immunoreactive cells were identified in a previous study in the fire ant queen brain using an antibody against the sNPF receptor [50]. Even when these fluorescent signals appear to come from clusters of sNPF-expressing cells, when these samples were observed in the confocal microscope, it was clear that these signals came from inside trachea or areas of damaged tissue (Figure B.3). In addition, no difference was observed between samples obtained with and without the signal amplification system (data not shown).

All the data collected from samples obtained following protocol 1, 2 and 3 suggested that one of the main problems could be the penetration of the probe into the brain. With the aim to troubleshoot possible problems with the ability of the probe to enter in the tissues, protocols 4 to 6 were tested using frozen sections of brains instead of

whole mounts. The tissue sections obtained following protocol 4 and 5 were few, because most of them were washed away the glass slide during the ISH process. In addition, the quality of these sections was poor, and most of them were broken (Figure B.4, A-F). Independently of the quality of the tissue sections, in these samples the fact that most of the fluorescent signals observed (in red color) resemble lines of different thickness, suggested that even when using frozen sections a fraction of the probe is trapped by the trachea giving false positives (Figure B.4, A-F). In an attempt to improve the quality of the frozen sections, protocol 6 was performed exactly as described by Lu, *et. al.* in the brain of the ant *Polyrhachis vicina* [116].

Following protocol 6, the quality of the frozen sections improved considerably; however, in these samples almost there is no difference in the fluorescent signal obtained when incubating with the antisense or sense probes (negative control) (Figure B.4, G-H), making very hard to distinguish between possible real signal and false positives. In addition, the patterns of the fluorescent signals obtained were not consistent among different samples, suggesting that most of them could correspond to background.

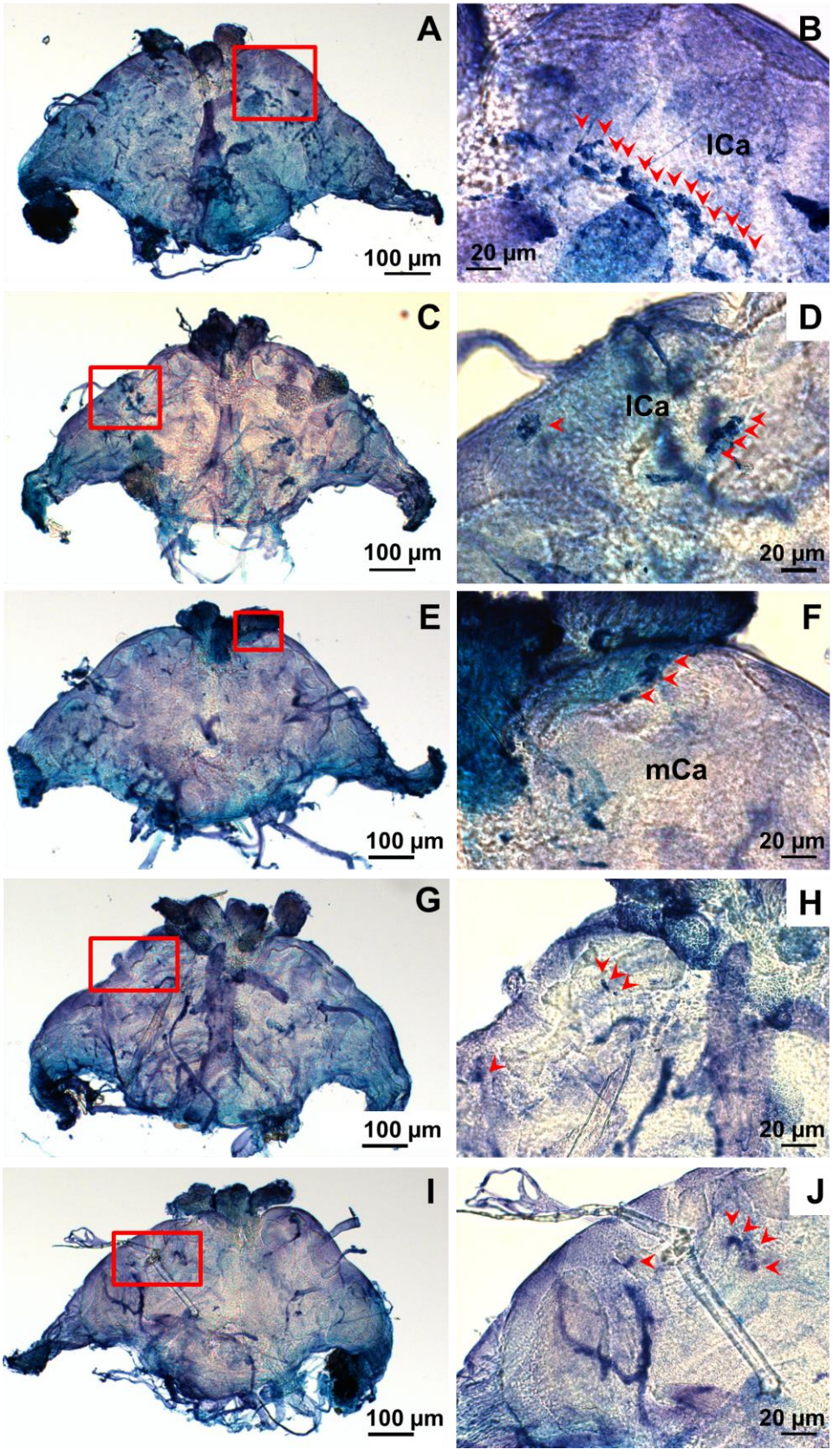
In a final attempt to determine the location of sNPF-expressing cells in the fire ant queen brain, protocol 7 was tested. In this protocol the probes were detected using a colorimetric system instead of fluorescence (for detailed protocol, see Appendix A). In samples incubated with the sense and antisense probes, the results obtained using this protocol showed intense signal (blue) labelling thick tracheas, which are highly abundant on the surface of the brain. Interestingly, round spots of signal near the mushroom bodies were found in all these samples treated with the antisense sNPF peptide probe (Figure

3.3 A-F, red arrowheads). However, in the negative controls (sense sNPF probe) also dark spots of signal were observed randomly in different areas of the brain (Figure 3.3 G-J), making the interpretation of the data more difficult.

Discussion

In situ hybridization (ISH) in one of the most used techniques for gene expression analysis in tissues and it has been applied for a variety of applications, from the discovery of new microorganisms to the identification of mRNA transcript in single cells. However, it is also a technique very difficult to set-up due the large number of variables that can affect the final output. Common difficult steps are the design (length, sequence) and nature of the probe (RNA, DNA), determining the best hybridization conditions (salt concentrations, temperature, concentration of probe, etc), and the appropriate type of tissue analysis (whole mount vs. frozen sections) and the preparation of the tissues (fixation time, permeabilization, RNA integrity, adhesion to slides, etc.) [117]. Until now, there are no published protocols to perform ISH in any tissue of fire ants, but there are eight studies in other ant species where ISH was used in gene expression analysis [116,118–124]; but only two of them describe ISH in the brain of the ants *P. vicina* [116,124].

Figure 3.3 Results of queen brain *in situ* hybridization for the short neuropeptide F obtained with protocol 7. Representative images of the results obtained when using a colorimetric method for probe detection in whole mounted brains of mated queens. (A-F) correspond to samples obtained using the antisense sNPF peptide probe, while (G-J) are negative controls (sense sNPF probe). B, D, F, H, J corresponds to the higher magnification image of the areas enclosed with a red box on A, C, E, G and I, respectively. Red arrowheads in all pictures are indicating the colorimetric spots of signal (blue color).


A
100 μm
B
lCa
20 μm
C
100 μm
D
lCa
20 μm
E
100 μm
F
mCa
20 μm
G
100 μm
H
20 μm
I
100 μm
J
20 μm

Contrary to ants, there are many studies describing ISH protocols in the adult brain of the honeybee *Apis mellifera* [72,125–133], as well as in the adult and larval brain of *D. melanogaster*; and in the latter there are specific ISH protocols for the detection of sNPF transcripts in the adult and larval brain [78,107,134].

In this study, we tested seven different protocols trying to establish the best methodology to perform ISH in the brain of fire ants queens and workers. Unfortunately, none of the protocols tested yielded reliable or reproducible results to determine the location of sNPF-expressing cells. From the data collected using protocols 1, 2 and 3 it was difficult to determine which step(s) are inadequate. In all these protocols it appears that most of the sense probe was retained in the trachea present in the brain; consequently, a reduced amount was available to bind the corresponding mRNA transcripts in the tissue, and this probably interfered with its detection. In addition, the high background observed from the probes inside trachea made difficult the identification of real signal indicating the presence of sNPF or sNPFR mRNA transcripts. In an attempt to improve the detection of the probes, a signal amplification system was tested following protocol 3, but because no differences were observed between samples obtained with and without the signal amplification system, it was still unclear at this point if the main problem with this protocol was the penetration of the probe into the brain, or alternatively, the capacity of the sense probes to bind their respective targets. In addition, other technical difficulties which could lead to failed results in these three protocols may arise from the preparation of the brain samples, the permeabilization of the tissues, and the method used for probe detection (fluorescence,

colorimetric). Modifications to improve these steps were performed throughout the seven protocols tested, and it appears that the cause behind all these failed trials could be the design of the probe itself. It is possible that the length and the sequences selected for both probes (sNPF, sNPFR) are not the best to successfully hybridize with the sNPF peptide or the sNPF receptor mRNA transcripts in the tissues.

The nature (RNA, DNA), length and sequence of the probe are very important aspects to consider when performing ISH, but it appears that there are no defined rules in this regard to ensure success with this technique. For example, a previous study in honeybee brain demonstrated the usefulness to perform ISH using several small DNA oligomers simultaneously (each 20 nucleotides in length) to form DNA/RNA hybrids, which are supposed to be more stable than RNA/RNA hybrids [129]. On the other hand, in *D. melanogaster* the sNPF transcripts were successfully detected in the brain through ISH using only one and long DIG-labelled RNA probe of 850 bp in length [78,134]. Longer RNA probes supposedly increase sensitivity and specificity when compared with short probes, but longer probes also have more difficulty penetrating into the tissues than shorter ones [117]. This is a very important aspect to consider in this case because similarly to mammals, the insect brain is a tissue "insulated" by several different layers of glial cells surrounding and supporting neurons [135]. In insects, the brain blood barrier (BBB) has been extensively studied in *D. melanogaster,* and a previous study showed that in living embryos (stage 17) the BBB is extremely effective, even to prevent the penetration of dextran molecules as small as 10 kDa. Based on this characteristic of the brain, and after failing in protocols 1 to 3; we decided to test the ISH utilizing frozen

sections (protocols 4 to 6), with the aim to minimize the chances that our probes are unable to hybridize with their respective targets due to their inability to enter the brain. Unexpectedly, by switching from whole mounts to frozen sections, new technical difficulties arose. The first problem observed was the poor quality of the sections in protocol 4. This could be due in part because the fire ant queen brain is really small (in average, about 900 μm wide, 600 μm in height), thus each frozen section is small to attach to the glass slide; and also, this tissue is extremely soft, facilitating the mechanical damage due any harsh manipulation. In addition, it appears that the tissues were over digested by proteinase K, and this contributed to the increase in tissue damage as well as in the background signal. However, the quality of the tissue sections gradually improved from protocol 4 to 6, as expected. In the results obtained using protocols 4 and 5 (Figure B.3), the fluorescent signal observed corresponds mainly to unspecific background due to unspecific binding to the broken brain tissue and broken trachea. Therefore, it is nearly impossible to determine if some of these signals indeed correspond to the *snpf* transcripts. Contrary to protocols 4 and 5, while samples obtained following protocol 6 had almost no unspecific background, no distinguishable patterns of sNPF-expressing cells were found (Figure B.3), and these results strongly suggest that our designed probes are definitely not able to hybridize with its corresponding mRNA target or alternatively, that the probe detection system is not sensitive enough to detect the *snpf* transcripts.

In our final attempt to obtain a working protocol of ISH in the fire ant brain, we tested protocol 7, were the probe detection is performed with a colorimetric system

(Figure 3.2). The rationale of using this detection method is that it allows manipulating the time of precipitation of the colorimetric substrate for as long as necessary to observe the signal from the DIG-labelled RNA probes. However, this method has the disadvantage that unspecific background also increases. As expected, the signal to noise ratio in these samples was low and the strong background interfered with the identification of a true signal, if any. These results suggested that the short length of the designed probes could be another reason for the difficulty of its detection. Assuming that the probes are DIG-labelled with 100% efficiency, the antisense sNPF and sNPFR probes will have a 23.07% and 33.27% DIG-UTP content, respectively. This indicates that only about one third of the probe could be detected by an anti-DIG antibody, leaving most of the probe undetectable; thus is it reasonable to believe that the methods for probe detection utilized in all the protocols tested could be also impairing the quality of the obtained results. It will be interesting to test in the future if the use of a longer DIG-labelled RNA probe or DNA probe coupled with a more sensitive probe detection method (such as the tyramide signal amplification systems, [136]) could help to identify the sNPF-expressing neurons in the fire ant brain.

In addition to the technical difficulties of stablishing a ISH protocol for the fire ant brain, the mRNA transcript levels of the sNPF are unknown in the brain of queens and workers. Similarly to the honey bee [72], it could be possible that the gene expression of the sNPF is low and limited to just a few cells in the brain, making the detection of its mRNA transcripts even more difficult. Perhaps by determining the gene expression levels of this neuropeptide in the brain through another technique such as

RT-qPCR, it could be possible to find the physiological conditions when the sNPF is highly expressed, to then proceed with the ISH. Alternatively, other gene highly expressed in the brain (i.e., structural proteins such as β-tubulin, or nc82, [137]) could be used simultaneously as a positive control for the ISH.

Conclusions

In conclusion, the location of sNPF-expressing cells in the brain of fire ant queens and workers still remains unknown. Even when different methods were tested in this study trying to find the best conditions for ISH, it appears that the common factor which leaded to failure with all the protocols tested were the length and/or design of the DIG-labelled RNA probes. For each gene target, there are no specific rules in relation to probe length or nucleotide composition which ensures success, and also hybridization temperatures must be empirically adjusted for each probe tested in different tissues. Nevertheless, longer DIG-labelled RNA probes or labelled-DNA probes could be tested in the future to accomplish this objective.

Lacking a previously validated and functional DIG-labelled RNA probe for any gene expressed in the fire ant brain, makes it difficult to identify the real cause of failure when using these protocols. However, some important technical recommendations can be rescued from the experience of testing seven different ISH protocols, which can be useful in the future. For example, when working with whole brains, the permeabilization and hybridization steps require longer times than when working with frozen sections

(see Appendix A for details); but in both cases, the use of 10% dextran sulfate in the hybridization buffer appears to help the penetration of the probes into the tissues. Also, it is important to consider that the use of any signal amplification system will also increase the background on the samples, thus washing periods should be longer. Finally, when performing ISH with frozen sections, the use of silane coated glass slides improves considerably the integrity and the quality of the tissue sections, by allowing a better attachment of the tissues to the glass slide.

CHAPTER IV

SILENCING OF THE sNPF RECEPTOR IN SMALL FIRE ANT COLONIES THROUGH FEEDING OF dsRNA

Introduction

There are two approaches that are commonly used to study the function of a specific gene: through its gain of function (i.e. overexpression, ectopic expression), or through its loss of function (i.e. genetic knock out, silencing through RNAi). These approaches could be used independently of one another, and both allow evaluating how the gain or loss of function of the gene-of-interest (GOI) affects the function of other genes or gene networks. In recent years, the implementation of RNA interference (RNAi) technology in different species of insects has proved its success in the field of functional genomics, and RNAi as a technique is a powerful molecular tool for silencing specific genes to study their physiological role, leading to the discovery of new gene targets that ultimately could be used to implement new strategies of insect pest management in the field [138].

The mechanism for RNAi was discovered in the nematode worm *Caenorhabditis elegans*, in which the administration of a double-stranded RNA (dsRNA) resulted in the silencing of a sequence-specific gene [139]. Since then, many studies had been conducted to understand the intracellular components and pathways involved in RNAi, and now it is believed that these pathways evolved in eukaryotes as a defense

mechanism to silence invading nucleic-acid sequences before they can integrate into the host genome or subvert cellular processes [140]. As mentioned above, RNAi is triggered by long dsRNA which intracellularly is processed by the enzyme Dicer (RNAse III-like enzyme) into small interfering RNA (siRNA) of about 20-25 nucleotides in length. These siRNAs then serve as sequence determinants to guide the RNA-induced silencing complex (RISC) to target single-stranded RNA (ssRNA) molecules, such as messenger RNA (mRNA) for degradation [141].

In social insects such as termites and bees several studies have demonstrated the effectiveness of dsRNA and siRNA to induce gene silencing through the injection or feeding of these molecules at different stages of development of these insects. For example, in honeybees, intra-abdominal injection of dsRNA in newly emerged workers showed a reduction on the mRNA transcript levels of the vitellogenin gene in 96% of the injected bees [142]. Similarly successful results were obtained in other studies by feeding honeybee larvae with dsRNA in their natural diet [143,144]. Another example includes the termites *Reticulitermes flavipes*, in which the injection of siRNA in adult workers successfully silenced the gene expression of two hexamerins [145]; and the feeding of a high-dose of dsRNA was successful to silence the gene expression of an endogenous digestive cellulose enzyme, as well as a caste-regulatory hexamerin storage protein [146]. In contrast, just five studies had been published on RNAi in ants [147–151], and only one of them demonstrate the efficacy of RNAi induced by feeding of dsRNA [151].

In the Florida carpenter ants, *Camponotus floridanus* the feeding of a dsRNA to silence the peptidoglycan recognition protein LB gene (*PGRP-LB*) is effective to trigger

RNAi in adult workers; but this effect was not observed in the larvae [151]. Specifically in fire ants, only two studies have previously tested the effectiveness of dsRNA to trigger RNAi through injection and feeding, respectively. First, it was demonstrated that the injection of dsRNA in queen pupae at the red eye stage is able to significantly knockdown the transcript levels of the vitellogenin receptor gene in their ovaries after 5 days post-emergence, and this silencing effect lasted for at least 5 more days [152]. Second, a more recent study showed that the gene expression of the pheromone biosynthesis activating neuropeptide (PBAN) could be significantly reduced in adult workers by injecting 1 μg/100 nL/ant of dsRNA into their hemocoel, and this silencing effect last up to 72 h post-injection, with a maximum effect after 48 h [147]. In addition, in the same study, the effect of the administration of PBAN-dsRNA through feeding was evaluated in a small assay performed in groups of ten fourth-instar larvae tended by six nurse workers which were fed for 12 days with 1μg/μL of dsRNA in a solution of 10% sucrose. The results showed a significant increase in larval mortality after 20 days post-feeding of dsRNA; which is assumed to be consequence of the RNAi effect, but it is unclear if this assumption is true or not, because there was no reported validation of the knockdown in PBAN gene expression supporting this hypothesis [147].

In the present study, we propose to investigate the role of the sNPF receptor from the perspective of how social behavior (the interaction between members of different castes) could be affected by the knockdown of this gene. To do so, we propose to assess the best conditions for delivery to small laboratory colonies the sNPFR-dsRNA by feeding, with the aim to trigger gene silencing of the sNPF receptor to evaluate its

physiological impact in the queen, workers and fourth instar larvae. As mentioned before, the fourth instar larvae are important members of the colony because they are the only ones able to consume solid protein-rich foods and efficiently digest those proteins, which are later shared by all members of the colony. In this context, our hypothesis is that disrupting the function of the sNPF receptor could be detrimental for colony fitness due to deregulation of the colony nutritional signaling, which ultimately could lead to a reduced reproductive capacity of the queen, affecting colony growth.

Materials and methods

Insects

Newly mated queens were collected after their mating flight on Texas A&M University, west campus, in College Station, Texas (Brazos County) during July and August of 2013. Each individual queen was placed inside small round plastic containers (10 cm diameter) painted with Fluon (Insect-a-slip©, BioQuip products, CA, USA) also set with a small test tube filled with water with a cotton plug on top, that queens used as nest to lay eggs. All queens were maintained in the laboratory at 27 ± 2°C in a 16:8 h light-dark photoperiod. Water was provided *ad libitum*, and a solution of 15% honey-water was supplied daily on a one square inch plastic boat containing a small piece of cotton. After the first workers (minims) emerged (about 21-31 days after the queens were collected), frozen crickets and an artificial solid diet specially formulated for ants [112] were supplied daily as sources of protein. The colonies were allowed to grow for six months in the laboratory, in the conditions described above, until they reached a minimal population of about 1,000 workers before starting with the dsRNA feeding assays. These population sizes were estimated by weighing the colonies and calculating the number of workers by using the following mathematical relationship: 100 mg of workers from these laboratory colonies correspond to 302.22 ± 13 workers.

Vector construction and sNPFR-PCR product for dsRNA production in bacteria

To produce dsRNA in bacteria, PCR products of a fragment of the sNPFR (GenBank: DQ026281.1) and the enhanced green fluorescent protein (EGFP; GenBank: U55763.1) (as negative control) were ligated independently in a L4440 vector (Addgene, Cambrigde, MA, USA). For this, first a fragment of 339 bp in length, corresponding to a short region of the 5' UTR and the first 22 amino acids of the sNPFR ORF was amplified by PCR using the set of primers: sisNPFRi F2, sisNPFRi R2 (Table 3); for the EGFP gene, a 326 bp fragment was amplified using the primers EGFPi F1, EGFPi R1 (Table 3). Both PCR reactions were performed using the Advantage® 2 PCR Enzyme System (Clontech, Mountain View, CA, USA) following the manufacturer's protocol; and templates for these amplifications were plasmid DNA carrying respective cDNAs which were obtained as described in previous studies [48,153]. Then, both PCR products were loaded in a 1.2 % agarose gel, and the band corresponding to the 339 bp (sNPFR) and 326 bp (EGFP) were purified from the gel using the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA, USA) according to the standard protocol. Both purified PCR products were then separately ligated into a T-tailed L4440 vector (Addgene, Cambrigde, MA, USA). The preparation of the T-tailed L4440 vector was performed exactly as described before [154]. Briefly, 20 μg of empty L4440 vector were digested with 300 U of the restriction enzyme *Eco*RV (New England BioLabs, Ipswich, MA, USA) for 3h at 37°C. Then, the enzyme was heat-killed at 68°C for 10 min and the DNA was ethanol precipitated. Once the DNA pellet was solubilized in ultrapure water, the T-tailing reaction was carried out at 37°C for 1 h using 5 mM ddTTP and 30 U of

recombinant terminal deoxynucleotidyl transferase (TdT) (Promega, Madison, WI, USA). The enzyme was heat-killed at 70°C for 10 min, and the resulting DNA was purified using a QIAquick PCR purification kit (QIAGEN, Valencia, CA, USA). Finally, the purified PCR products mentioned above were separately ligated in the T-tailed L4440 vector by incubating with T4 DNA ligase (Promega, Madison, WI, USA) overnight at 4°C. Both recombinant L4440 vectors (carrying the sNPFR and EGFP fragments) were used to transform *E. coli* HT115 (DE3) competent cells (*Caenorhabditis* Genetics Center, Minneapolis, MN, USA) according to the procedure described before [154,155]. These cells are ideal to produce large amounts of dsRNA because they are a RNAse III deficient *E.coli* strain with IPTG-inducible T7 polymerase activity. All these cloning steps were conducted by Dr. Prati Bajracharya in Pietrantonio's laboratory.

dsRNA production in bacteria and purification

To perform the dsRNA feeding assays, large amounts of sNPFR dsRNA and EGFP dsRNA were required. To produce the required amounts of dsRNA, recombinant HT115 (DE3) bacteria carrying the respective constructs for sNPFR and EGFP were grown with agitation overnight at 37°C in LB medium (DIFCO laboratories, Detroit, MI, USA) supplemented with 50 μg/mL of ampicillin (Cayman chemicals, Ann Harbor, MI, USA). Then, the bacterial culture was diluted about 2.5-fold with the same medium until OD_{600}: 0.4 (about 3.2 x 10^8 cells/mL), and dsRNA transcription was induced by adding IPTG (Gold Biotechnology, St. Louis, MO, USA) to a final concentration of 1 mM.

After 5 h of induction at 37°C with agitation, the OD_{600} of the bacterial cultures was about 0.8 (6.4 x 10^8 cells/mL), and 250 mL of these bacterial suspensions (about 1.6 x 10^{11} cells/mL) were transferred to bottles and centrifuged at 10,000 g for 20 min at 4°C. The bacterial pellets obtained were used immediately for dsRNA purification. The dsRNA isolation was performed following a protocol described before [151] with some modifications, as follows: the bacterial pellet from each bottle was resuspended in 40 mL of STET buffer (10 mM Tris-HCl, pH 8.0, 0.1 M NaCl, 1 mM EDTA, 5% [w/v] TRITON® X-100), and this bacterial suspension was divided into two 50 mL Falcon tubes, 20 mL in each. Then, 1 mL of freshly prepared lysozyme (Sigma-Aldrich, St. Louis, MO, USA) was added (10 mg/mL in 10 mM Tris-HCl, pH 8.0) to each tube. The suspensions were incubated in a water bath at 37°C for 40 min with some manual agitation from time to time; and after incubation, one volume (20 mL) of phenol:chlorophorm:isoamyl alcohol (25:24:1) (Thermo Fisher Scientific, New Jersey, USA) was added to each tube and agitated vigorously in a vortex for about 3 min. Then, the solutions were centrifuged at 8,500 g for 45 min at 4°C to separate the phases. After centrifugation, the upper aqueous phase was recovered and placed in a new 50 mL Falcon tube to repeat the extraction with phenol:chlorophorm:isoamyl alcohol (25:24:1) one more time. Finally, the aqueous phase was recovered to a new 50 mL Falcon tube and one volume of 100% isopropanol was added to precipitate the nucleic acids overnight at -20°C. The next day, the tubes were centrifuged at 8,500 g for 45 min at 4°C and the obtained pellets were washed with 70% ethanol, centrifuged again at 8,500 g for 15 min at 4°C; and once the ethanol was discarded they were allowed to air-dry under

the hood for 10-15 min. The dry pellets were resolubilized in DEPC water and immediately treated with Turbo DNAse for 1 h at 37°C (Life Technologies Grand Island, NY, USA) to remove all traces of genomic DNA. Then, 1 μL of RNAse A (Life Technologies Grand Island, NY, USA) was added to each tube and incubated at 37°C for additional 30 min to eliminate the single-stranded RNA (ssRNA). To stop the enzymes' activity and remove all contaminants, the samples were treated with one volume of phenol:chlorophorm:isoamyl alcohol (25:24:1), precipitated with 100% isopropanol and resolubilized in DEPC water as mentioned above. Finally, all the purified dsRNA was stored at -80°C until further use. To check the quality and quantity of these dsRNAs, 1:10 dilutions of these samples were loaded in a 1.5% native agarose gel and ran for 30 min at 100 V using 1X TAE buffer prepared with DEPC water. dsRNA standards (synthesized *in vitro*) at eight different concentrations were included in each gel, to estimate the concentration of the purified dsRNA samples by densitometry using the software ImageJ (National Institutes of Health, Bethesda, MD, USA) (Figure 4.1). This step was required because the presence of small pieces of remaining nucleic acids from the dsRNA purification process from bacteria (which are not the specific dsRNA) increases the readings by spectrophotometry at 260 nm, thus overestimating the dsRNA concentration of the samples.

The synthesis of dsRNA *in vitro* was performed with the MEGAscript® RNAi kit (Life Technologies Grand Island, NY, USA) following the manufacturer's protocol, and using as template a purified PCR product corresponding to the sNFPR or EGFP fragments previously cloned in the L4440 vector as described above, plus the T7

promoters at the end of each fragment (in both directions). This PCR product was obtained using the Advantage® 2 PCR Enzyme System (Clontech, Mountain View, CA, USA), the recombinant L4440 vector with the sNPFR or EGFP inserts (purified plasmid DNA), and the following primers at a final concentration of 200 nM: forward 5' – GGA AGC AAC CTG GCT TAT CG – 3', reverse 5' – ACG TTG TAA AAC GAC GGC CA - 3'. This PCR reaction had a final volume of 50 μL, and the PCR conditions were as follows: 95°C for 1 min (activation) and 35 cycles at 95°C for 30 s, 60°C for 1 min and 68°C for 45 s; followed by a final extension step at 68°C for 1 min.

Colony feeding assay with bacteria expressing dsRNA for sNPFR and EGFP (Trial 1)

Before conducting this trial, a small pilot study was performed to determine the tolerance of the ants to the presence of heat-killed dsRNA producing bacteria in the liquid food. A range of different concentrations of these bacteria (from OD_{600} 0 to 0.8, which is equivalent to 6.4 x 10^8 cells/mL) suspended in a 15% honey/water solution (liquid food) were provided simultaneously to the colonies to determine their preferences. Additionally, a non-choice assay was also performed using a bacterial concentration of OD_{600} = 1.6 (1.28 x 10^9 cells/mL) in 15% honey/water. Bacteria expressing sNPFR dsRNA and EGFP dsRNA were grown, and transcriptional induction of both dsRNAs was performed as described above.

Table 3 DNA primers used for cloning of the short neuropeptide F receptor and enhanced green fluorescent protein fragments in the L4440 vector and for RT-qPCR gene expression analyses

Primer name	**Sequence**	**Amplicon size (bp)**
Primers for cloning the short neuropeptide F receptor (GenBank: DQ026281.1) and enhanced green fluorescent protein (GenBank: U55763.1) fragments in the vector L4440		
sisNPFRi F2	5' GGTGGACTATCATCGTTGTCTCATCGCAC 3'	339
sisNPFRi R2	5' CACAGCCAGATTCGACATCATATCTTGCG 3'	
EGFPi F1	5' GAAGCAGCACGACTTCTTCAAGTCCGCC 3'	326
EGFPi R1	5' GGGTGTTCTGCTGGTAGTGGTCGGCGAG 3'	
Primers for detection of gene expression levels of the short neuropeptide F receptor and ribosomal protein L18 (rpl18) (GenBank: EH413666.1) by RT-qPCR		
sisNPFR-F1Q	5' ACCATCGGATGCAGACAGTTACCA 3'	81
sisNPFR-R1Q	5' GCACAGCTAACAAACAGAGCAGCA 3'	
siRPL18QF-1	5' TACACCGACCACCGATTTCA 3'	80
siRPL18QR-1	5' GATCACGGCGACGCAATT 3'	

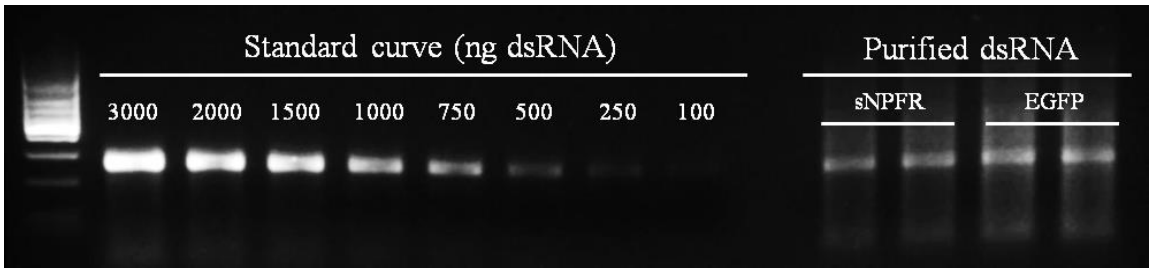


Figure 4.1 Example of quantification of dsRNA by gel densitometry. Representative image of a gel loaded with different concentrations of a standard curve of dsRNA synthesized *in vitro,* to estimate the concentration of dsRNA produced and purified from bacteria as described in Materials and Methods. The intensity of the bands was quantified using the software ImageJ.

After induction of transcription, the bacterial cultures were heat-killed by exposure to 80°C for 20 min, and then allowed to slowly cool down to room temperature, to ensure proper renaturation of the dsRNA. Then, liquid cultures were centrifuged at 10,000 g for 20 min, and bacterial pellets were resuspended in a solution of 15% honey/water to a final OD_{600} = 1.6 (1.28 x 10^9 cells/mL), aliquoted and stored at -80°C for further use. These bacterial suspensions were used as sources of dsRNA in this feeding assay. This assay was performed with a total of twelve small fire ant colonies, similar in size (average 1048 ± 200 workers per colony) and age, which were kept in boxes (7.5 x 3.5 inches). Six of these colonies were fed with heat-killed bacteria expressing sNPFR-dsRNA (target colonies), while the other six were fed with heat-killed bacteria expressing EGFP-dsRNA (control colonies). Each colony was fed daily during 21 days with about 2 mL of their corresponding bacterial suspension treatment, which was provided by soaking cotton placed in a 1 inch square plastic boat. To track consumption by ants, green food coloring (McCormick & Company, Inc. Sparks, MD, USA) was

added to this bacterial suspension at a final ratio of 1:100 v/v (Figure 4.2). In addition, one frozen cricket was added every day as part of their regular diet (protein source).

Colony feeding assay using 1 μg/μL of purified dsRNA in liquid food (Trial 2)

In this trial, fourteen small colonies containing about 200 mg of workers (about 604 ± 1 worker) and 50 mg of brood were used. These colonies were maintained under the same conditions described before for trial 1. Seven colonies were treated with sNPFR-dsRNA and the remaining seven with EGFP-dsRNA (control colonies). All colonies were fed every other day with 50 μL of sNPFR-dsRNA or EGFP-dsRNA diluted in a 10% sucrose solution (liquid food) at a final concentration of 1 μg/μL. This solution was provided inside a 0.2 mL Eppendorf tube to avoid evaporation, and green food coloring (McCormick & Company, Inc. Sparks, MD, USA) was used to track consumption of the liquid in the ants (final ratio of 1:100 v/v). In addition, a cube of about 0.5 cm in length (about 0.4 g) of artificial diet (solid food) was provided every other day as protein source (Figure 4.3, A). Detailed instructions to prepare this solid diet are shown in Appendix C at the end of this manuscript. All colonies were treated with their corresponding purified dsRNA during 21 days. To verify the integrity through time of the dsRNA offered in the provided diet and thus to determine how often the dsRNA must be replaced in the liquid food, leftovers of liquid food after two and three days of contact with the ants and the colony environment were collected and ran in a 1.5% agarose gel to observe the integrity of the dsRNA.

Figure 4.2 Example of a small fire ant colony in trial 1. All colonies used in this trial were placed in a small box (7.5 x 3.5 inches) which contained a tube of water with a cotton plug, and two small plastic boats were crickets (protein source) or a 15% honey/water solution containing dsRNA heat-killed bacteria were provided. Green food coloring was used in the honey/water solution to track the consumption of the liquid in the ants.

Colony feeding assay using 2 μg/μL of purified dsRNA in liquid and solid food (Trial 3)

This trial was performed exactly as trial 2 (described above) except that the final concentration of purified dsRNA used in liquid food (10% sucrose solution with purified dsRNA) was 2 μg/μL, and also dsRNA was provided in the solid food (artificial diet). The preparation of the solid food containing dsRNA was performed as follows: an average of 0.36 ± 0.05 g of solid diet were placed inside a 0.2 mL Eppendorf tube and mixed with 50 μL of liquid food (10% sucrose solution with 2 μg/μL purified dsRNA) until the liquid completely percolate into the solid diet. Green food coloring (McCormick & Company, Inc. Sparks, MD, USA) was used to track consumption (final

ratio of 1:100 v/v) (Figure 4.3, B). Both, liquid and solid foods containing dsRNA were replaced every other day, and the consumption of solid food was determined by weighing the leftover solid food inside the Eppendorf tube at the time it was replaced for fresh solid food.

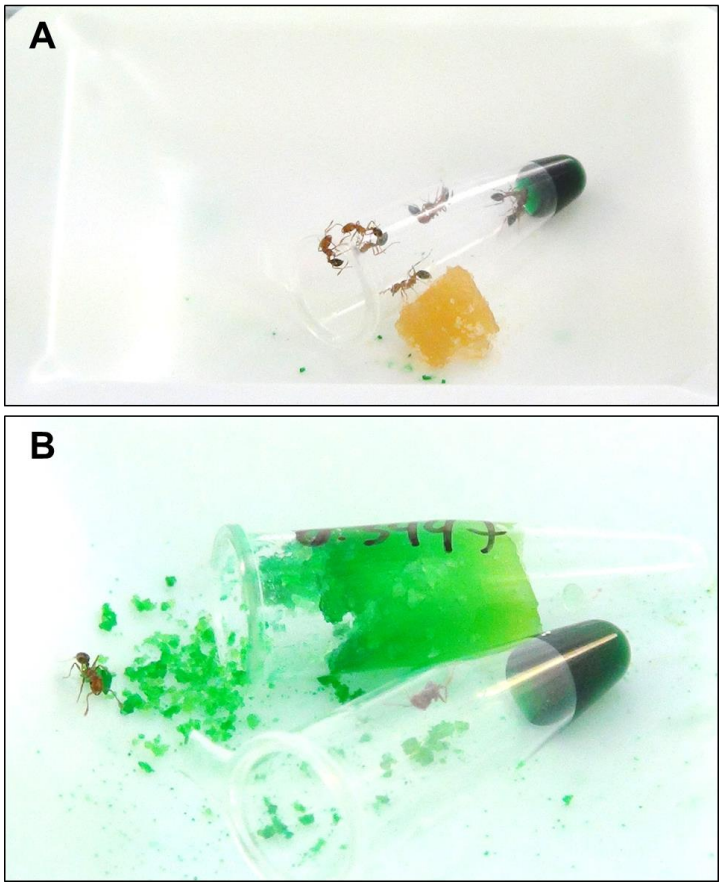


Figure 4.3 Example of how the dsRNA in food sources was provided to the fire ant colonies in trials 2 and 3. In both trials, liquid and solid foods were provided together contained inside a small plastic boat. (A) In trial 2, 50 μL of liquid food carrying purified dsRNA were provided inside a 0.2 mL Eppendorf tube, and a small cube (0.4 g) of artificial diet was given as protein source (solid food). (B) On trial 3, liquid and solid foods were provided inside 0.2 mL Eppendorf tubes. Green food coloring was using in both food sources to track consumption.

Data collection and statistical analysis of dsRNA feeding assays

For all the trials, the behavior of the ants was monitored daily with the aim to identify a characteristic phenotype as a consequence of the sNPFR gene expression knockdown. Photos and videos were used to document important features/behaviors of the colonies during the treatments. Also, the number of dead worker ants was determined daily during the 21 days of treatment, and the survival of workers in all colonies was statistically analyzed with the Kaplan-Meier survival test followed by the Log-Rank test. In addition, the weight of workers and brood were determined before and after the treatments with heat-killed dsRNA producing bacteria (trial 1) or purified dsRNAs (trials 2 and 3), and these results were statistically analyzed using One-way ANOVA. The data collected from the gene expression analyzes in all trials were statistically compared by independent samples t-test. All statistical analyzes were performed in the SPSS Statistics software (IBM Corporation, New York, USA), and differences were considered significant if $P \leq 0.05$ in all cases.

Determination of dsRNA knockdown efficiency in fourth instar larvae, queen and worker tissues by RT-qPCR

In all trials, after 21 days of treatment with dsRNA, queens from all colonies were recovered and dissected. Brain, ovaries, midgut and fat body were individually dissected and stored at -80°C until further use. In trial 1, gene expression analyses were performed in six ovaries of randomly selected queens (three from colonies treated with heat-killed dsRNA producing bacteria, and three from control colonies). In trials 2 and 3,

all 14 midguts and fat bodies of queens from colonies treated with purified sNPFR-dsRNA or EGFP-dsRNA were used for gene expression analyzes; while only 4 brains and ovaries were utilized from each group of queens, respectively. In addition, all brood from all colonies was recovered, and pools of six fourth instar larvae were made and stored at -80°C for gene expression analyzes. Also, worker ants were selected for dissection while they were still alive, to obtain pools of 15 brains which were used for gene expression analyzes. Total RNA of all tissues and fourth instar larvae were isolated using TRIzol® (Life Technologies, Grand Island, NY, USA) following the manufacturer's protocol. Traces of genomic DNA were removed by treating the samples with DNAse I from the TURBO DNA-*free*™ Kit (Life Technologies, Grand Island, NY, USA), and cDNA was synthesized from 500 ng of total RNA using oligo dT and the SuperScript® III First-Strand Synthesis System (Life Technologies, Grand Island, NY, USA) following the standard protocol.

For relative RT-qPCR gene expression analyzes, primers were designed to amplify an 81 bp fragment of the sNPF receptor; and a fragment of 80 bp of the ribosomal protein L18 (RPL18) which was used as reference gene, as described before [156]. The sequence of primers for the *sNPF receptor* (sisNPFR-F1Q, sisNPFR-R1Q) and the *rpl18*gene (siRPL18QF-1, siRPL18QR-1) are shown in Table 3. The RT-qPCRs to detect the *sNPF receptor* and the *rpl18* genes were performed at the same time (same plate), but in separate PCR reactions. The RT-qPCRs for both sets of primers were performed using the *Power* SYBR® Green PCR Master Mix (Life Technologies, Grand Island, NY, USA), 250 nM of each primer (forward and reverse) and 1 μL of diluted

cDNA (1:2) in a final volume of 25 μL per reaction. The cycling conditions were as follows: 95°C for 10 min (activation step) and 45 cycles of 95°C for 15 s, and 60°C for 30 s, followed by a final melting curve analysis, to confirm the specificity of the PCR products. In addition, negative controls (no cDNA) were included each time the RT-qPCR reactions were performed, to monitor possible contaminations with cDNA. RT-qPCR efficiencies for each sample were evaluated using the LinRegPCR software (Heart Failure Research Center, Amsterdam, The Netherlands), and the relative gene expression changes were determined using the $2^{-\Delta\Delta Ct}$ method as described previously [157]. In addition, some of the RT-qPCR products from both sets of primers were loaded in a 1.5% agarose gel to check the correct size of the amplicons (data not shown). Statistical analyzes of the relative gene expression data obtained was done with the independent samples t-test using the SPSS Statistics software (IBM Corporation, New York, USA), and differences were considered to be significant if $P < 0.05$.

Results

Feeding assay of bacteria expressing dsRNA for sNPFR and EGFP (Trial 1)

Previously to start trial 1, a pilot study was performed (as described in the Materials and Methods section) to determine if the ants can tolerate different concentrations of heat-killed dsRNA producing bacteria in their liquid food (15% honey/water solution in this case). The results obtained indicated that none of the bacterial suspensions tested have a repellent effect in the ants (it was considered that perhaps the bacterial odor could be a deterrent for food consumption), and they indistinctly consumed liquid food containing all concentrations of bacteria (data not shown).

In trial 1, the green food coloring added to the 15% honey/water solution (carrier of the heat-killed dsRNA producing bacteria) was observed after three to four days in the gut of workers and larvae (Figure 4.4). These results demonstrate that at least four days of treatment are required for the liquid food to reach most of the members of the colony. To evaluate the effect of the treatment with the heat-killed dsRNA producing bacteria in the workers, their mortality was tracked daily, and an average of 10.27 ± 6.47 ants died every day in colonies treated with EGFP-dsRNA producing bacteria; while 12.45 ± 6.63 died in colonies treated with sNPFR-dsRNA producing bacteria. The survival analysis performed with these data indicated there is no significant differences between control colonies (EGFP-dsRNA) and colonies treated with heat-killed bacteria expressing sNPFR-dsRNA ($P = 0.104$; Figure 4.5).

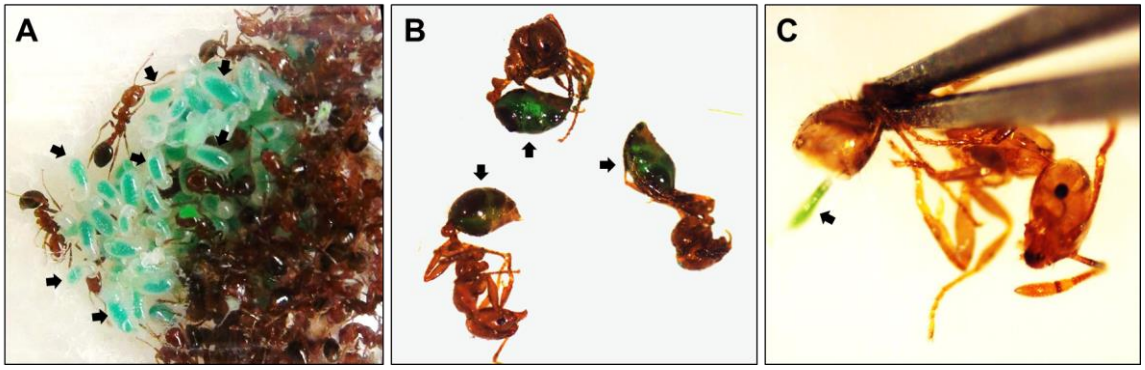


Figure 4.4 Examples of larvae and workers stained with green food coloring. After three to four days of treatment with liquid food with green food coloring it is possible to observe the green dye (black arrows) in the gut of larvae (A), and in the abdomen of the workers (B). When workers were dissected, green liquid food was present in the crop (not shown) and in the midgut, as shown in C (black arrow).

These survival results are concordant with the final weight of total workers measured at the end of the treatments with the heat-killed dsRNA producing bacteria (after 21 days), in which there is no significant difference between the two treatments (Figure 4.6, A). In addition, the final amount of brood was also determined by weight, and even when a slight reduction was observed in colonies treated with sNPFR-dsRNA heat-killed bacteria comparing with the control colonies, this difference is not statistically significant (Figure 4.6, B). Moreover, it is possible that this variation could be due differences in the initial brood weight (before treatment with heat-killed dsRNA producing bacteria) which was not determined by weight or any other accurate measurement.

When examining the colonies searching for visible phenotypes, it was found that after 12 days of treatment a few workers from colonies treated with sNPFR-dsRNA heat-killed bacteria presented an unusual behavior for a few hours before they died. These behaviors were characterized by anomalies in the coordination of their legs and locomotion, and were never observed in workers from colonies treated with EGFP-dsRNA heat-killed bacteria. The abnormal workers were observed crawling with their abdomen folded towards the ventral (inferior) side of the body; while others showed overall sluggishness and different levels of incoordination of legs movements (spread out legs), including difficulty to stand up (data not shown). These observations suggested that these abnormal behaviors could be consequence of the treatment with sNPFR-dsRNA heat-killed bacteria and the knockdown of the sNPFR gene expression in the brain or ganglia, perhaps the terminal abdominal ganglion because more often the posterior legs were more affected. To test this hypothesis, the brain of 15 of these affected workers were collected (while they were still alive) for total RNA isolation and gene expression analysis as described in Materials and Methods. Normal workers from control colonies (EGFP-dsRNA) were included as control. These results showed that the relative gene expression levels of the sNPFR in the brain of normal and abnormal workers are similar (Figure 4.7), giving no support to the proposed hypothesis to explain these abnormal behaviors.

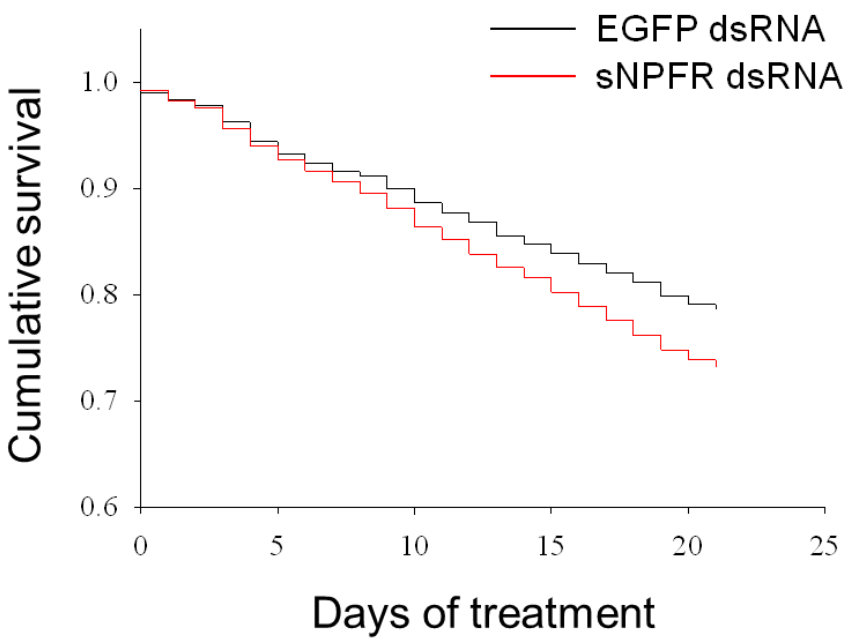


Figure 4.5 Survival curves obtained for workers in trial 1. The curves for worker survival were obtained with the Kaplan-Meier survival analysis after 21 days of treatment with heat-killed producing bacteria as explained in Materials and Methods. No significant differences were found with the log-rank test.

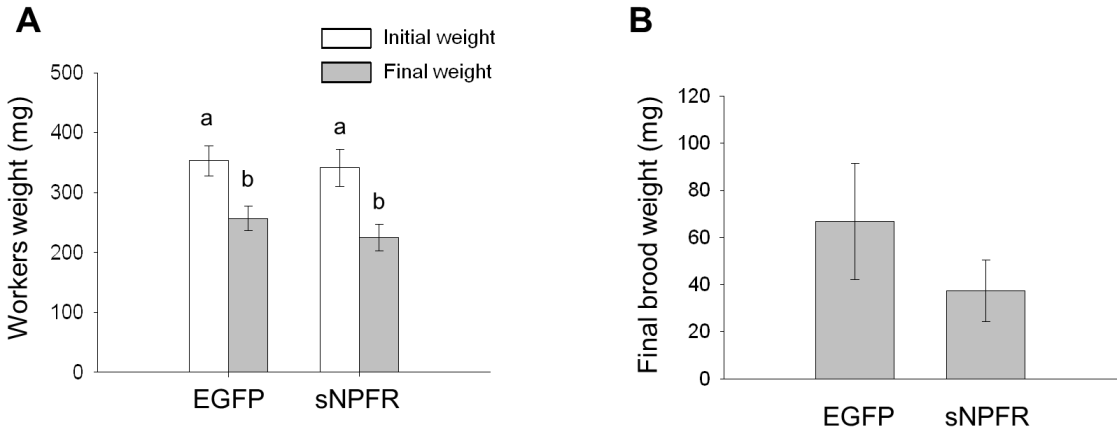


Figure 4.6 Weights of workers and brood in trial 1. The amount of workers and brood in the colony is represented by their total weight. In (A), the average weight of workers before and after 21 days of treatment with heat-killed dsRNA producing bacteria is shown. No significant differences were found between both groups after the treatment. The final weight of brood was determined in colonies treated with heat-killed sNPFR-dsRNA producing bacteria and control colonies after 21 days of treatment. No significant differences were found between both groups (B).

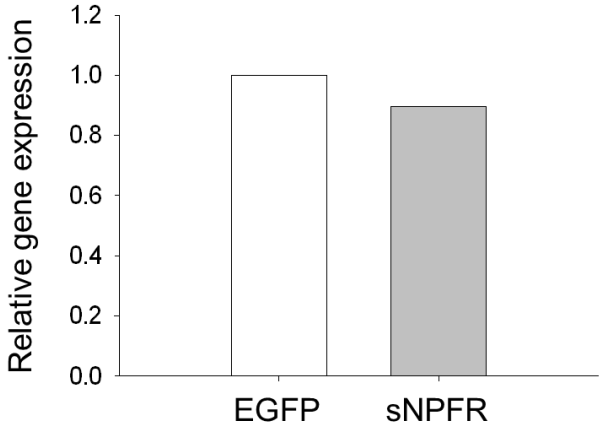


Figure 4.7 Transcript relative expression levels by RT-qPCR in the brain of workers in trial 1. The relative expression of *sNPFR* transcript levels is similar in the brain of normal workers (EGFP, control colonies) and abnormal workers from colonies treated with sNPFR-dsRNA heat-killed bacteria. No significant differences were found.

To determine the effects of the treatment with dsRNA heat-killed bacteria in other members of the colony, gene expression analyses to determine the efficiency of the knockdown of the sNPFR gene were performed in whole fourth instar larvae and the ovaries of some queens. In the fourth instar larvae, it appears that the gene expression levels of the sNPFR are very variable, and no significant differences were found when comparing larvae from control colonies and those treated with sNPFR-dsRNA heat-killed bacteria (Figure 4.8, A). In queens, we found that the expression of the sNFPR in the ovaries is significantly reduced (51.3% decrease. $P = 0.007$) in queens from colonies treated with sNPFR-dsRNA heat-killed bacteria in comparison with queens from control colonies (Figure 4.8, B). These results suggest that using heat-killed dsRNA producing bacteria as a delivery system for RNAi could be effective. However, more replicates will be needed to fully support this hypothesis.

Feeding assay using purified dsRNA (Trial 2 and 3)

The second and third trials were performed to evaluate the effectiveness of RNAi through feeding of purified dsRNA using different food carriers. In trial 2, dsRNA was provided in the carbohydrate source (10% sucrose solution; liquid food), which is mainly consumed by the workers; while in trial 3, dsRNA was provided in both the carbohydrate and the protein source (artificial diet; solid food), which is mainly given to and consumed by the fourth instar larvae and the queen.

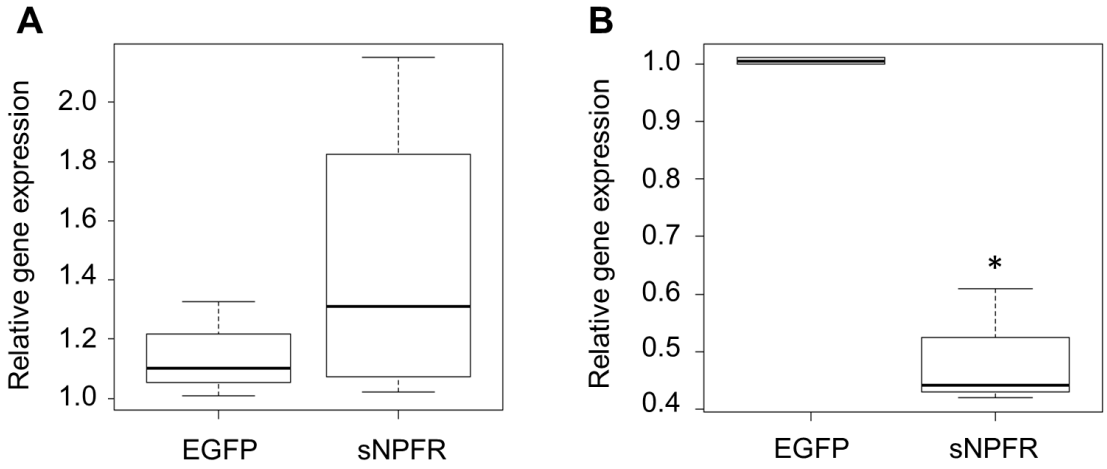


Figure 4.8 Transcript relative expression levels by RT-qPCR in fourth instar larvae and queen tissues in trial 1. Boxplots showing the sNPFR gene expression levels in (A) fourth instar larvae and (B) queen ovaries are shown. There are no statistical significant differences for larvae from both groups (n = 4, each); but the *sNPFR* gene expression was significantly decreased in ovaries of queens from colonies treated with sNPFR-dsRNA heat-killed bacteria (n = 3) in comparison with ovaries of queens from control colonies (n = 2).

Before starting these trials, the stability of the dsRNA in the liquid food was determined. Thus, leftover liquid food that had been 1.5 days in contact with the ants was collected and kept in the same conditions of the colonies for an extra half (two days) and 1.5 more days (three days). Then, these samples were run in an agarose gel as described in Material and Methods. We found that a fraction of the dsRNA remains intact even after three days of exposure to the ants and the colony environment (Figure 4.9); based on these results, all liquid foods in the trials were replaced every other day, therefore continuously providing dsRNA minimally degraded.

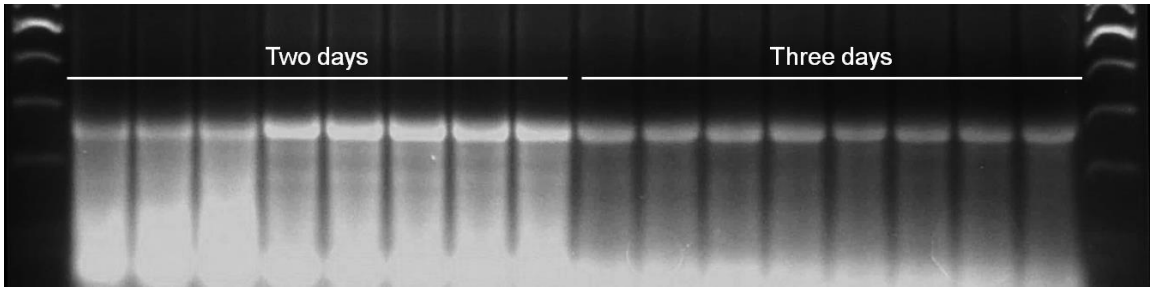


Figure 4.9 dsRNA stability after contact with worker ants. Leftovers of liquid food containing dsRNA were collected from different colonies after 1.5 daysof contact with the worker ants These leftovers were kept in the same environment for an extra half (two days, left side in the picture) and 1.5 more days (three days, right side in the picture) and ran in an agarose gel. In this gel, the dsRNA can be easily observed even after two (left panel) and three days (right panel) of contact with the ants (bright upper band); some degradation appears to have occurred after three days.

Similarly to trial 1, workers survival on trials 2 and 3 was not altered by the treatment with purified sNPFR-dsRNA in comparison with workers from control colonies (Figure 4.10), and this result is concordant with the final total weight of workers measured after the 21 days of treatment (Figure 4.11 A, C). In trial 2, we found a significant decrease in the total amount of brood, in both groups, EGFP-dsRNA (control) and sNPFR-dsRNA treated colonies, when comparing their initial and final amounts of brood after 21 days of treatment. However, when comparing only the final amount of brood between both groups, they were similar, and no significant differences of the treatment were found (Figure 4.11, B). Interestingly, in trial 3 we found a significant decrease (51.7% in average; $P = 0.0045$) in the amount of brood from colonies treated with sNPFR-dsRNA after 21 of treatment when comparing with EGFP-

dsRNA treated colonies (Figure 4.11, D). This result could be related to an observed increase in mortality of the fourth instar larvae after 5 days of treatment; but no survival analyses were performed on fourth instar larvae only to effectively prove these observations.

In trial 3, dsRNA was provided in the solid food, therefore, with the aim to evaluate if the administration of sNPFR-dsRNA in solid food affects (decreases) the foraging behavior for protein-rich food we tried to estimate the amount of solid food consumed. The consumption of solid food was determined by measuring the weight of the leftover solid diet (with dsRNA) every other day. Curves showing this consumption in individual colonies for a period of 10 days are shown in figure 4.12.

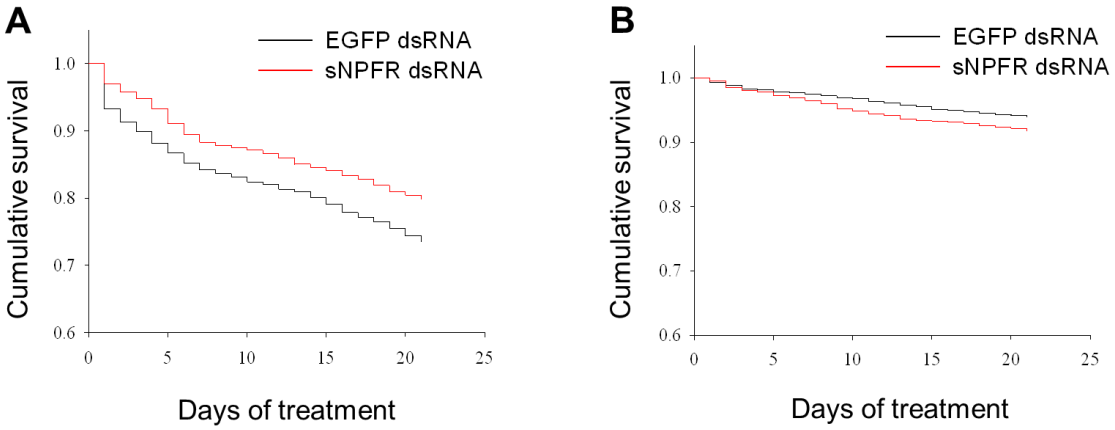


Figure 4.10 Survival curves obtained for workers in trials 2 and 3. The curves for worker survival were obtained with the Kaplan-Meier survival analysis after 21 days of treatment with 1 µg/µL of purified dsRNA provided in liquid food only (trial 2, A); and 2 µg/µL of purified dsRNA provided in liquid and solid food (trial 3, B). In both trials, no significant differences between treatments were found with the log-rank test.

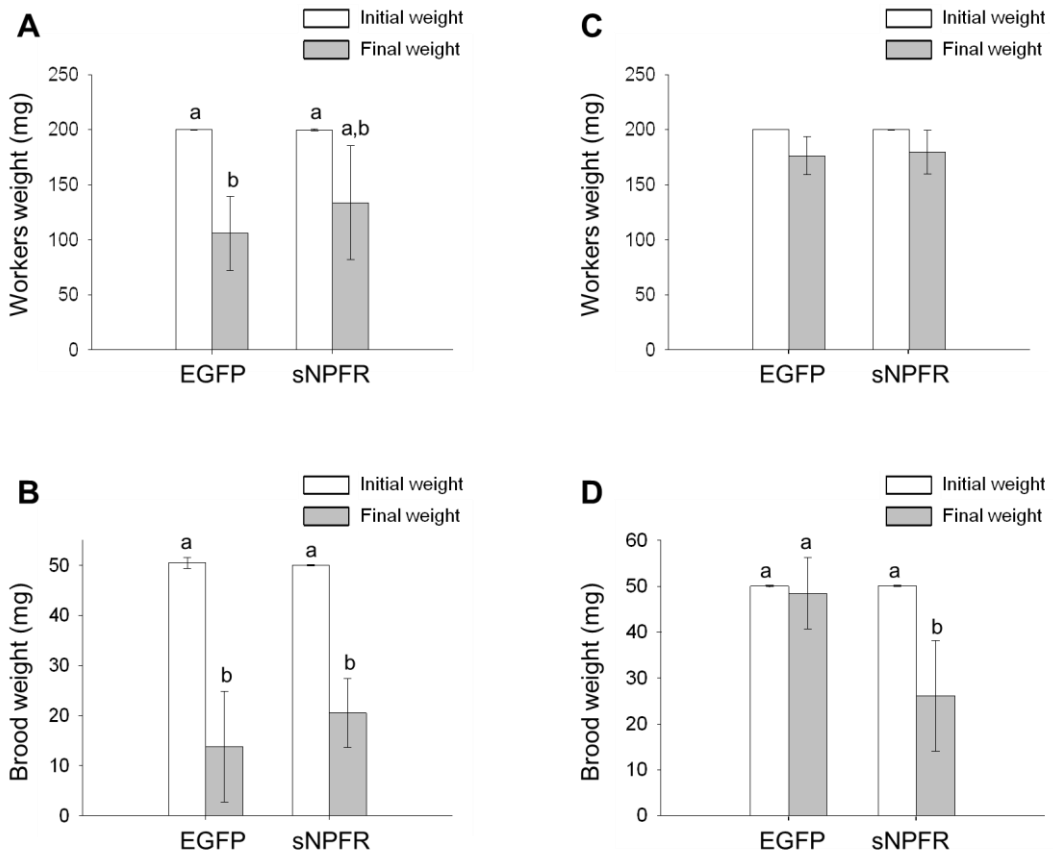


Figure 4.11 Weights of workers and brood in trials 2 and 3. The weight of workers and brood before and after 21 days of treatment in trials 2 (A, B) and 3 (C, D) are shown. The final mass of workers is similar in colonies treated with sNPFR-dsRNA and their corresponding control colonies (EGFP-dsRNA) in trials 2 (A) and 3 (C). No significant differences were found. In trial 2, the final amount of brood in both control (EGFP-dsRNA) and sNPFR-dsRNA treated colonies was significantly reduced in comparison with their respective initial amount of brood (B). In trial 3, there was no significant differences between the initial and final amount of brood in control colonies (treated with EGFP-dsRNA); but the amount of brood was significantly reduced in colonies treated with sNPFR-dsRNA in comparison with control colonies, after 21 days of treatment (D).

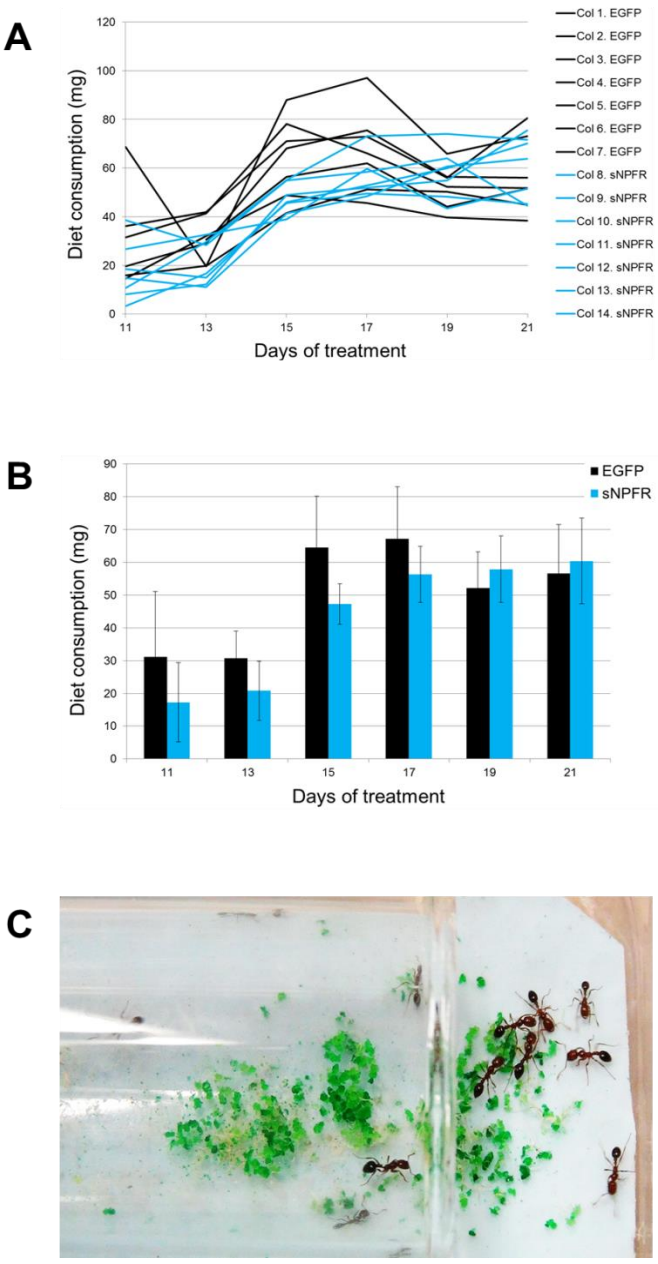


Figure 4.12 Estimation of consumption of solid food in colonies from trial 3. A timeline of the solid food consumption for individual colonies from trial 3 is shown in (A). The same data is shown in (B), where the bars represent the mean for each treatment group per day (error bars correspond to the standard deviation from the mean). No significant differences were found between EGFP-dsRNA (black bars) and sNPFR-dsRNA (blue bars) treated colonies. In (C), a group of workers is nearby a pile of small pieces of solid diet accumulated at the opening of the glass tube were water only is provided, indicating that ants carried the solid food away from the source.

These results indicate that colonies treated with EGFP-dsRNA and sNPFR-dsRNA consumed similar amount of solid food, and no significant differences were found between both groups (Figure 4.12); however, these results could be affected by the fact that the ants tend to accumulate small pieces of solid food in different areas of their habitat (box), as shown in figure 4.12, C.

To evaluate the gene silencing efficiency of the treatment with purified sNPFR-dsRNA on trials 2 and 3, gene expression analyses were performed in the fourth instar larvae and different tissues of individual queens, as described in Materials and Methods. In the fourth instar larvae, the relative transcript levels of the *sNPFR* are quite variable, especially among colonies treated with sNPFR-dsRNA (Figure 4.13 A, B). In trial 2, a significant increase in the sNPFR relative transcript level was found in larvae from colonies treated with sNPFR-dsRNA (Figure 4.13 A); while in trial 3, no significant differences were found between larvae from control and sNPFR-dsRNA treated colonies (Figure 4.13 B). In queens, the relative transcript levels of the sNPFR in the brain, ovary and fat body were similar in tissues of queens from control and sNPFR-dsRNA treated colonies. No significant differences were found between both treatment groups in these tissue samples in both trials 2, and 3 (Figure 4.13 C-H). In trial 2, although, it appears that there is a decrease in sNPFR gene expression in the midgut of queens from some of the colonies treated with sNPFR-dsRNA; this effect is not observed in all of them, thus the variability in sNPFR gene expression in these group of samples is high; and no significant differences were found between the relative transcript levels of the sNPFR in the midgut of queens from control and sNPFR-dsRNA treated colonies (Figure 4.13, I).

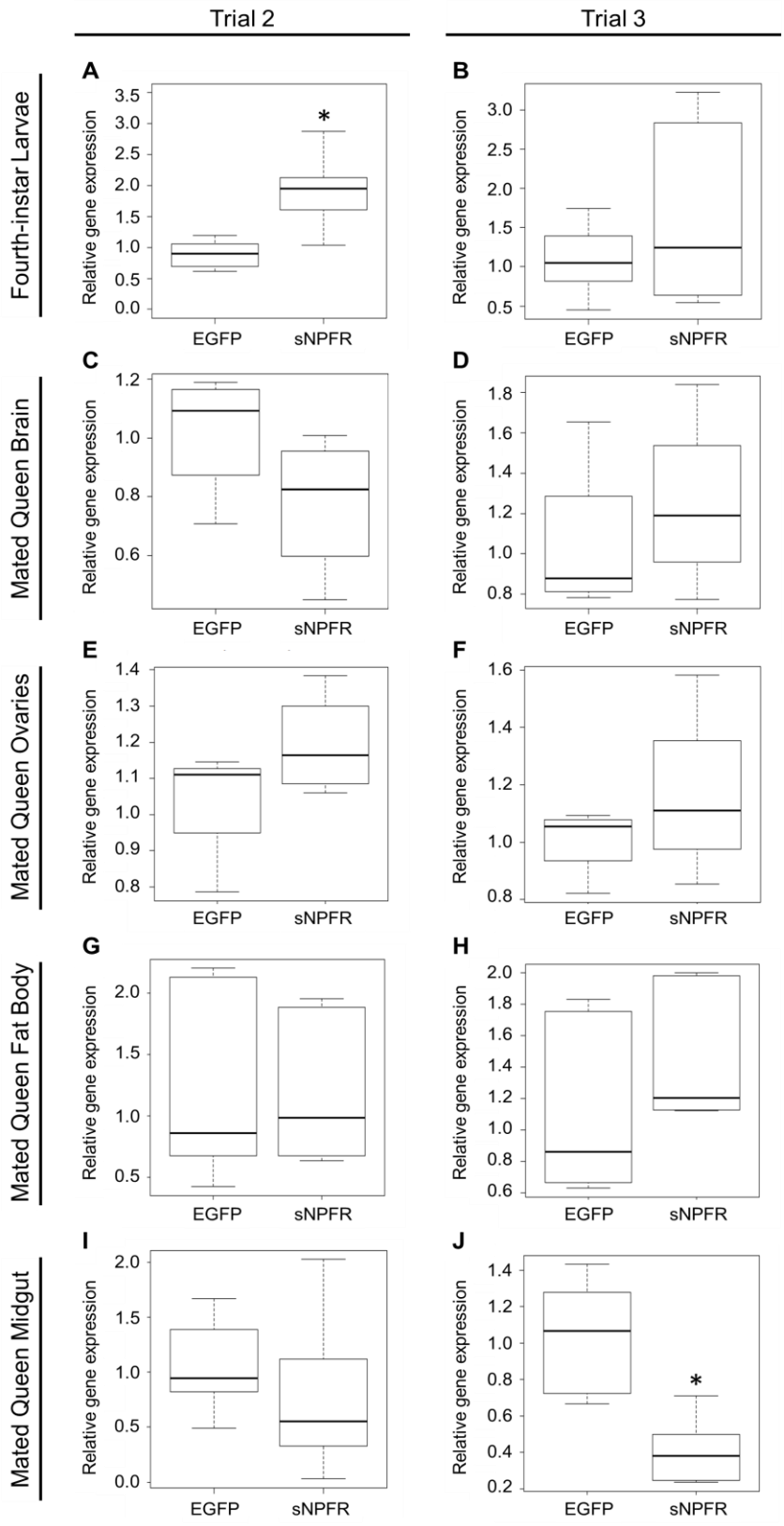
In contrast, in trial 3, the sNPFR gene expression in the midgut of queens treated with sNPFR-dsRNA was significantly reduced by 61.3% in average (*P = 0.0006*) in comparison with the sNPFR gene expression in the midgut of queens treated with EGFP-dsRNA (control colonies) (Figure 4.13, J).

In workers from trials 2 and 3, no gene expression analyses were performed because it appeared that the treatment with sNPFR-dsRNA did not have any effect over their behavior or mortality rate (Figure 4.10). However, these ants were frozen and stored for future analyses.

Discussion

This study is the first attempt of RNAi through feeding using heat-killed dsRNA expressing bacteria in fire ant colonies. This system for dsRNA delivery was first used in the worm *C. elegans* with excellent results [154], and recently was tested in the Colorado potato beetle, *Leptinotarsa decemlineata*, with successful results after six days of treatment with both: a dsRNA producing bacteria, and *in vitro* synthesized dsRNA [155]. One of the main challenges we faced in embarking in this study was that social insects such as the fire ant feed by sharing the liquid food via trophallaxis between all the members of the colony; thus, is nearly impossible to determine what is the dose of dsRNA consumed by each ant in the colony. In addition, high concentrations of dsRNA must be used because the effect of dilution among members of the colony of the single doses collected by a specific individual due to their feeding behavior.

Figure 4.13 Transcript relative expression levels by RT-qPCR in fourth instar larvae and queen tissues in trials 2 and 3. On trial 2, the relative transcript abundance of the sNPFR was significantly increased in larvae from colonies treated with sNPFR-dsRNA (A); while no significant differences were found between larvae from control and sNPFR-dsRNA treated colonies in trial 3 (B). In queens, the relative transcript abundance of the sNPFR was determined in the brain (trial 2, C. Trial 3, D), ovaries (trial 2, E. Trial 3, F), fat body (trial 2, G. Trial 3, H), and the midgut (trial 2, I. Trial 3, J). No significant differences were found in the tissues of queens from colonies treated with sNPFR-dsRNA when comparing with queens from control colonies (C-I); with exception of the midgut on trial 3, where a significant decrease on the sNPFR gene expression was found in queens from colonies treated with sNPFR-dsRNA (J). Trial 2: queen midguts EGFP n = 7; sNPFR n = 7. Trial 3: EGFP n = 6; sNPFR n = 7.


Trial 2
Trial 3
Fourth-instar Larvae
Mated Queen Brain
Mated Queen Ovaries
Mated Queen Fat Body
Mated Queen Midgut
A
B
C
D
E
F
G
H
I
J
Relative gene expression
EGFP
sNPFR
*

An additional problem in all trials was the fast evaporation of water from liquid and solid foods at the temperature required to maintain the fire ants colonies (27 ± 2°C); thus constant monitoring and replacement of both food sources was required. However, by providing liquid and solid foods inside 0.2 mL Eppendorf tubes (as in trial 3, Figure 4.3 B) the evaporation of water was considerably reduced in both food sources.

The results obtained in trial 1 showed that the ingestion of the heat-killed sNPFR-dsRNA producing bacteria is not affecting significantly the mortality of either workers or larvae; however, after this first trial still remained unclear if the dsRNA contained in the bacteria was able to trigger RNAi or not because just few samples were recovered to verify the knockdown of the sNPFR gene by RT-qPCR. An interesting observation from this trial was the abnormal locomotor behavior of some workers from colonies treated with heat-killed sNPFR-dsRNA producing bacteria. As mentioned before in the results section, we suspected that these alterations could be somehow related to the knockdown of the sNPFR in the brain of these workers, but the preliminary results obtained do not support this hypothesis. However, we did not investigate if the sNPFR gene expression was successfully silenced in any other tissues of these workers, thus it is also feasible that these behaviors could be consequence of the knockdown of the sNPFR in the abdominal ganglia, which also is regulating movement through the release of neurotransmitters in the neuromuscular junction [100].

The gene expression analysis performed in whole fourth instar larvae in trial 1 revealed interesting effects of the treatment with the heat-killed sNPFR-dsRNA producing bacteria. In the larvae from control colonies (treated with heat-killed EGFP-

dsRNA producing bacteria) the sNPFR expression levels are quite similar among samples from all colonies, presenting a minimal standard deviation; but in larvae from colonies treated with sNPFR-dsRNA producing bacteria there is considerably more variability in the sNPFR gene expression levels, which in some samples reached the double comparing with larvae from control colonies (Figure 4.8). Interestingly, this effect was also observed in larvae treated with purified sNPFR-dsRNA from trial 2 and 3, suggesting that the fourth instar larvae is sensitive to the treatment with sNPFR-dsRNA (purified or in bacteria), and this may be triggering dysregulation of the sNPF/sNPFR signaling system to consecutively lead to a feedback mechanism that may cause overexpression of the sNPFR gene in some of these larvae as a compensatory mechanism for the loss of function (Figure 4.8 A; Figure 4.13 A, B). A similar response was observed in *B. dorsalis*, where continuously feeding of dsRNA triggered the over-expression of the target gene [158]. However, an additional and more detailed study will be necessary to explain these unexpected results.

The results of the gene expression analysis in queens from trial 1 were promising: the administration of the heat-killed sNPFR-dsRNA producing bacteria in the liquid food significantly silenced the gene expression of the sNPFR in the ovary (Figure 4.8). However, more replicates will be necessary to unequivocally verify this statement. The sNPFR (as protein) is present in mid- and early-oogenesis oocytes, but not in oocytes at the late-oogenesis stage [50], suggesting that this receptor could be involved in the regulation of nutrients uptake/nutrient sensing inside the eggs to promote their development. Unfortunately, we did not investigate if this knockdown of the sNPFR is

having any consequence over the reproductive capacity of these queens; for example by impairing egg development, however, the lesser weight of brood in trial 3 makes it still possible to speculate that some ovarian silencing may occur although it was not detectable in trial 3. Unfortunately, the role of the sNPFR in the ovary still remains unclear.

The results of gene expression analyses in trial 2 indicate that the concentration of purified dsRNA provided in liquid food only (1 μg/μL) was not enough to silence either the sNPFR in any queen tissue, or in the fourth instar larvae. This could be due to dsRNA concentration dilution caused by trophallaxis or by degradation of the dsRNA in the ants digestive system. However, in trial 3 we demonstrated that by providing a higher concentration of purified dsRNA (2 μg/μL) in liquid as well as solid food is effective to induce RNAi in the midgut of queens, but not in other tissues (Figure 4.13). All queens from colonies treated with purified sNPFR-dsRNA in trial 3 survived, therefore, it is reasonable to think that the role of the sNPFR in the midgut is not indispensable for their survival; however, we did not investigate if the silencing of the sNPFR in the midgut has any effect in food digestion (*i.e.*, secretion of digestive enzymes), absorption of nutrients and metabolism, or if it affects the function of the neurosecretory cells present in the midgut. In addition to the knockdown of the sNPFR in the queen midgut, in trial 3 an effect over the brood was also observed, and after 21 days of treatment a significant decrease in the final brood weight was found in sNPFR-dsRNA treated colonies when comparing with the final brood weight of control colonies (Figure 4.11, D). We observed that after 5 days of treatment, there was an apparent decrease in number of the fourth

instar larvae (data not shown) which was unexpected, and therefore not properly measured. It is interesting that this only occurred on trial 3, were dsRNA was added to the solid food, which is preferably consumed by the fourth-instar larvae (as mentioned before, this is the only member of the colony that can feed upon solid foods).This observation suggested that maybe the silencing of the gene expression of the sNPFR in these larvae could be the cause of mortality, but gene expression analyses in pools of the survivor fourth-instar larvae from sNPFR-dsRNA treated colonies did not showed a decrease in the transcript levels of the receptor, thus it is unclear if dsRNA is able to trigger gene silencing in these larvae. However, larvae of other younger instars were not tested by RT-qPCR to determine the efficacy of the RNAi; thus, if the treatment with sNPFR-dsRNA is inducing mortality in younger larvae, this also could explain the decrease in brood weight after 21 days of treatment. On the other hand, it could be possible that less final brood weight could be a consequence of some indirect effect of the silencing of the sNPFR gene expression in the queen midgut. For example, less availability of nutrients may decrease the amount of eggs produced in the ovary, and ultimately will decrease the amount of brood in the colony [159].

Several studies have shown that neuroendocrine cells in the midgut are able to secrete several neuropeptides that act locally as hormones, or that can be secreted into the hemolymph to regulate other tissues [160]. Previous RT-PCR experiments showed that in fire ants the sNPFR transcript is expressed in the midgut [48], but the spatial localization of this receptor in this tissue is unknown, thus adding more difficulty to understand its role in this organ. In this regard, a previous study performed in the

American cockroach, *Periplaneta americana* shed some light over the function of the sNPF/sNPFR signaling pathway in the midgut. In these roaches, the sNPF is released into the hemolymph from the corpora cardiaca and acts on the midgut as a hormone to control feeding behavior during starvation by inhibiting the α-amylase, protease and lipase activities in the midgut [161]. In addition, in these cockroaches was also observed that their locomotor activity is about 2.4 times higher in starved conditions, and these results are associated with changes in the concentration of sNPF in the hemolymph. It was recently proven that the sNPF injection increased the locomotor activity in these roaches (promoting foraging), thus in *P. americana* the sNPF is involved in the regulation of feeding, digestion and locomotion [162].

In this study, we did not investigate why the administration of purified dsRNA through feeding (trials 2 and 3) is not able to trigger a systemic RNAi response, but there are several possible explanations for the results obtained. First, it is possible that the dsRNA given in any food source is not able to enter into the hemolymph to target other tissues, due the degradation of a large fraction of it in the midgut. Alternatively, if the dsRNA is able to reach the hemolymph from the midgut and is quickly degraded by RNAses [163], this also will prevent gene silencing in other tissues. Additionally, it may be possible that both events happen simultaneously. Mechanisms of dsRNA degradation have been studied in other insects species such as the pea aphid (*Acyrthosiphon pisum*), where the presence of RNAses (able to degrade dsRNA) in their hemolymph and salivary secretions was demonstrated, allowing them to completely eliminate exogenous dsRNAs from their bodies in less than 5 hours [164].

Differences in the effectiveness of sNPFR silencing were found in different queen organs when comparing the results from gene expression analyses in queens from all trials. These differences may be attributed to the concentration of dsRNA provided and the delivery method, as follows: When using the heat-killed sNPFR-dsRNA producing bacteria it is possible to silence the gene expression of the sNPFR in the ovary; while in trials 2 and 3 this effect is not observed, and in the latter the silencing effect is restricted to the midgut only. This suggests that the dsRNA enclosed in the dead bacteria is able to enter into the hemolymph, reach the ovaries and trigger RNAi. It is possible that the bacterial cell/wall "cover" could add some protection to the dsRNA from degradation by RNAses in the environment and the digestive system of the ants (i.e. salivary glands secretions) increasing the probability of sNPFR-dsRNA uptake by epithelial cells to then enter into the hemolymph, after the digestion of these bacteria in the midgut. However, further studies will need to be performed to test this hypothesis. This could be easily tested by feeding the ants with a dsRNA labelled with any fluorophore (i.e., fluorescein) to then track its distribution from the midgut to the hemolymph, or other organs [165].

In model organisms such as *C. elegans* and *D. melanogaster*, several studies had investigated and identified the molecular components of the machinery involved in the development of RNAi responses. In *C. elegans*, a protein denominated SID-1 (Systemic RNA Interference Deficiency-1) is required to trigger systemic RNAi responses. SID-1 is predicted to encode a 776 amino acids protein with 11 transmembrane domains which is supposed to facilitate the transport (uptake) of dsRNA into the cells to trigger RNAi

[166]. Interestingly, no *sid* gene orthologs had been found in different insect species, including *D. melanogaster*, which congruently has no robust systemic RNAi responses. Data from *in silico* analysis on the presence of putative *sid-1* orthologs in insects showed that these proteins have more similarity with the *tag-130* genes than with the *sid-1* genes of *C. elegans* [167]. In the worm, *tag-130* genes are not involved in systemic RNAi, and these results suggested the existence of an alternative dsRNA uptake pathway in insects. These alternative pathways had been well studied in *Drosophila* S2 cells. Initially, it was observed that that these cells develop RNAi when soaked in medium with dsRNA [168], and now is known that the scavenger receptors SR-C1 and Eater, are responsible for more than 90% of uptake of dsRNA by these cells [169]. Similarly, other proteins such as the vacuolar H^+ ATPase, and orthologs of rsd-3 (RNAi spreading defective 3) among others, appears to have a role facilitating RNAi responses in S2 cells [170]. The rsd-3 protein exhibit an epsin N-terminal homology (ENTH) domain found commonly in vesicle trafficking proteins [171].

A detailed study focusing on the identification of different components involved in the facilitation of systemic RNAi responses in fire ants is needed to explain our results obtained in the dsRNA feeding assays. Nevertheless, the most remarkable aspect of our discovery is that opens the possibility to target the health of the queen through the silencing of specific genes in the midgut, and this may lead in the future to the development of a new and more specific alternative for fire ant population control.

Conclusions

Three different methods of dsRNA delivery in the food supply were assessed in small fire ant laboratory colonies by using heat-killed dsRNA-producing bacteria or purified dsRNA at two different concentrations. Results of the tree trials showed no impact of the treatment on worker mortality or on the production of visible phenotypes with exception of few workers found in trial 1 which presented impaired locomotion and incoordination of their legs.

In queens the silencing of the sNPFR could be achieved in the ovary by using directly the heat-killed dsRNA-producing bacteria in the liquid food, or alternatively a silencing effect could be observed in the midgut when feeding the colonies with 2 μg/μl of purified dsRNA provided in liquid and solid foods. In the fourth instar larvae no receptor silencing was observed when analyzing whole bodies, but we believed the significant decrease in the final weight of brood in trial 3 could be somehow a consequence of the treatment with sNPFR-dsRNA if not directly, indirectly by affecting the queen midgut for less overall queen nutrition and therefore promoting less vitellogenin in eggs and less healthy brood. Alternatively it is possible that some of the dsRNA in the queen hemolymph could have been transferred to the eggs promoting embryo death upon egg laying, but this has not been investigated

CHAPTER V

SUMMARY AND CONCLUSIONS

In this thesis, we first investigated the localization of cells expressing the sNPF receptor and peptide in the brain of queens and workers. Second, we evaluated different conditions to perform dsRNA feeding assays in small colonies with the goal to knockdown the gene expression of the sNPFR simultaneously in all members of the colony, to elucidate its role in fire ant physiology.

In the first chapter of this manuscript we performed immunolocalization of sNPFR-expressing cells in the brain of all worker subcastes. Workers from different subcastes (major, medium and minors) perform different tasks, which are loosely associated with their size and age. Also, their behavior is affected by the presence of brood, which acts as a modulator of the colony' nutritional requirements. We hypothesized that the short neuropeptide F (sNPF) signaling system (NPY-like) could be involved in mechanisms of worker division of labor by sensing or responding to colony nutritional status. Thus, we investigated the expression of the short neuropeptide F receptor (sNPFR) in the brain and subesophageal ganglion (SEG) of workers from colonies with and without brood. Across worker subcastes a total of 9 clusters of immunoreactive sNPFR cells were localized in the brain and the subesophageal ganglion (SEG); some of these cells were similar to those observed previously in the queen [50]. Worker brain sNPFR cell clusters were found in the protocerebrum near mushroom bodies, in the central complex and in the lateral horn. Other sNPFR immunoreactive

cells were found at the edge of the antennal lobes. Across subcastes, we observed both a constant and a differential pattern of sNPFR-clusters, with a higher number of sNPFR cells found in minor than in major workers. Those sNPFR cells detected in all worker subcastes appear to be involved in olfaction or SEG functions. The differential expression of clusters in subcastes suggests that sNPFR signaling is involved in regulating behaviors associated with specific subcastes and thus, division of labor. Some sNPFR cells appear to be involved in nutrient sensing and/or brood care, feeding behavior and locomotion. In colonies without brood, workers showed a lower cluster number, and an overall reduced sNPFR signal. Thus, overall our results suggest the sNPF signaling system is a candidate for the neurobiological control of worker division of labor and sensing brood presence, perhaps correlating with protein requirements and availability.

With the long term goal of identifying the neuronal networks involved in the sNPF/sNPFR signaling pathway, we attempted to localize the sNPF-expressing cells in the brain of queens and workers through *in situ* hybridization (ISH), to further construct a full map of neurons in the brain which are responsible for the synthesis of the peptide, and/or responsive (sensitive) to its activity through the activation of the sNPFR. To achieve this goal, we develop a DIG-labelled sNPF probe (antisense) able to bind the mRNA transcripts of the sNPF in the brain tissues, and tested seven different ISH protocols. Unfortunately, our results did not showed clearly and consistently the location of these sNPF-expressing cells in the queen and worker brain; thus their location is still unknown.

Finally, we wanted to evaluate the function of the sNPFR from the point of view of the social interactions between members of all castes of the colony and the brood. For this, our methodology consisted in evaluating different conditions to effectively deliver dsRNA through feeding, with the goal to induce the gene silencing of the sNPFR simultaneously in all members of the colony. We tested three methods which include the administration of a heat-killed dsRNA producing bacteria in the liquid food (trial 1); and the use of two different concentrations of purified dsRNA provided in the liquid food only (trial 2), or in the liquid and solid food at a higher concentration (trial 3). We obtained different results from these three trials, which suggested that the efficacy of the dsRNA-delivery depends on the concentration of dsRNA provided and its "status" (purified vs. contained in heat-killed bacteria), and the food source used as a carrier (liquid vs. solid food).

In trial 1, no phenotypes or behavioral alterations related to foraging behavior were observed in workers, but some workers from colonies treated with heat-killed sNPFR-dsRNA producing bacteria presented alterations in their locomotion and coordination of their body parts for a few hours before their death; however, survival analyses did not detect significant differences between all workers from these colonies when compared with control colonies (treated with heat-killed EGFP-dsRNA producing bacteria). This is in agreement with the final amount of workers and brood quantified after the treatment with heat-killed dsRNA producing bacteria, were not statistically significant changes were found. Gene expression analyses demonstrate that in the conditions of this trial 1, no gene silencing effect was observed in the fourth instar

larvae, but there is a significant decrease of the sNPFR transcript levels in the ovaries of queens.

In trial 2, no statistically significant differences were found between colonies treated with purified sNPFR-dsRNA or EGFP-dsRNA in any of the parameters evaluated, with exception of an increase in the sNPFR transcript levels in the fourth instar larvae from colonies treated with purified sNPFR-dsRNA, which was unexpected. It appears the concentration of dsRNA provided was too low to cause significant gene silencing and workers feeding on the honey solution containing the dsRNA may not provide an effective dose to the larvae or queen when only the carbohydrate source contains the dsRNA. On the contrary, on trial 3, we found a significant decrease in the final brood weight after the treatment with purified sNPFR-dsRNA, and also a significant decrease on the sNPFR transcript levels in the midgut of queens treated with purified sNPFR-dsRNA. From these results we concluded that gene silencing could be achieved in the midgut of queens by providing a high concentration of purified dsRNA in liquid and solid foods simultaneously, but no systemic RNAi response could be triggered by oral delivery of dsRNA.

REFERENCES

[1] Ross KG, Keller L (1995) Ecology and evolution of social organization: insights from fire ants and other highly eusocial insects. Annu Rev Ecol Syst 631-656.

[2] Ascunce MS, Yang CC, Oakey J, Calcaterra L, Wu WJ, Shih CJ, Goudet JA, Ross KG, Shoemaker D (2011) Global invasion history of the fire ant *Solenopsis invicta*. Science 331: 1066-1068.

[3] Gotzek D, Ross KG (2007) Genetic regulation of colony social organization in fire ants: an integrative overview. Q Rev Biol 82: 201-226.

[4] Lofgren CS, Banks WA, Glancey BM (1975) Biology and control of imported fire ants. Annu Rev Entomol 20: 1-30.

[5] NAPIS (National Agricultural Pest Information System) (2014) Survey status of red imported fire ant (IFA) *Solenopsis invicta* 2011 to present. Pest Tracker. http://pest ceris purdue edu/map php?code=ISASAZA#, accessed July 2014.

[6] Pimentel D, Zuniga R, Morrison D (2005) Update on the environmental and economic costs associated with alien-invasive species in the United States. Ecol Econ 52: 273-288.

[7] Porter SD, Savignano DA (1990) Invasion of polygyne fire ants decimates native ants and disrupts arthropod community. Ecology 71: 2095-2106.

[8] Wojcik DP, Allen CR, Brenner RJ, Forys EA, Jouvenaz DP (2001) Red imported fire ants: Impact on biodiversity. Am Entomol 47: 16-23.

[9] Lasota JA, Dybas RA (1991) Avermectins, a novel class of compounds: Implications for use in arthropod pest control. Annu Rev Entomol 36: 91-117.

[10] Lofgren CS, Williams DF (1982) Avermectin B_1a: Highly potent inhibitor of reproduction by queens of the red imported fire ant (Hymenoptera: Formicidae). J Econ Entomol 75: 798-803.

[11] Oi DH, Williams DF, Pereira RM, Horton PM, Davis TS, Hyder AH, Bolton HT, Porter SD (2008) Combining biological and chemical controls for the management of red imported fire ants (Hymenoptera: Formicidae). Am Entomol. 54: 46-55.

[12] Williams DF, Oi DH, Porter SD, Pereira RM, Briano JA (2003) Biological control of imported fire ants (Hymenoptera: Formicidae). Am Entomol 49: 150-163.

[13] Oi DH, Valles SM (2009) Fire Ant control with entomopathogens in the USA. In: Use of microbes for control and eradication of invasive arthropods. Springer Netherlands. pp. 237-257.

[14] Yang CC, Yu YC, Valles SM, Oi DH, Chen YC, Shoemaker DW, Wu WJ, Shih CJ (2010) Loss of microbial (pathogen) infections associated with recent invasions of the red imported fire ant *Solenopsis invicta*. Biol Invasions 12: 3307-3318.

[15] Wilson EO (1978) Division of labor in fire ants based on physical castes (Hymenoptera: Formicidae: Solenopsis). J Kansas Entomol Soc 51: 615-636.

[16] Mirenda JT, Vinson BS (1981) Division of labour and specification of castes in the red imported fire ant *Solenopsis invicta* buren. Anim Behav 29: 410-420.

[17] Petralia RS, Vinson SB (1979) Developmental morphology of larvae and eggs of the imported fire ant, *Solenopsis Invicta*. Ann Entomol Soc Am 72: 472-484.

[18] Petralia RS, Vinson SB (1978) Feeding in the larvae of the imported fire ant *Solenopsis invicta*: Behavior and morphological adaptations. Ann Entomol Soc Am 71: 643-648.

[19] Howard DF, Tschinkel W (1981) The flow of food in colonies of the fire ant, *Solenopsis invicta*: a multifactorial study. Physiol Entomol 6: 297-306.

[20] Cassill DL, Tschinkel W (1999) Regulation of diet in the fire ant, *Solenopsis invicta*. J Insect Behav 12: 307-328.

[21] Vinson SB (1997) Invasion of the red imported fire ant (Hymenoptera: Formicidae): spread, biology, and impact. Am Entomol 43: 23-39.

[22] Wheeler DE (1986) Developmental and physiological determinants of caste in social hymenoptera: evolutionary implications. Amer Nat 128: 13-34.

[23] Smith CR, Toth AL, Suarez AV, Robinson GE (2008) Genetic and genomic analyses of the division of labour in insect societies. Nat Rev Genet 9: 735-748.

[24] Ometto L, Shoemaker D, Ross KG, Keller L (2011) Evolution of gene expression in fire ants: The effects of developmental stage, caste, and species. Mol Biol Evol 28: 1381-1392.

[25] Manfredini F, Lucas C, Nicolas M, Keller L, Shoemaker D, Grozinger CM (2014) Molecular and social regulation of worker division of labour in fire ants. Mol Ecol 23: 660-672.

[26] Sarda S, Zeng J, Hunt BG, Soojin VY (2012) The evolution of invertebrate gene body methylation. Mol Biol Evol 29: 1907-1916.

[27] Glastad KM, Hunt BG, Yi SV, Goodisman MAD (2011) DNA methylation in insects: on the brink of the epigenomic era. Insect Mol Biol 20: 553-565.

[28] Hunt BG, Glastad KM, Soojin VY, Goodisman MA (2013) Patterning and regulatory associations of DNA methylation are mirrored by histone modifications in insects. Genome Biol Evol 5: 591-598.

[29] Glastad KM, Chau LM, Goodisman MA (2015) Chapter Seven: Epigenetics in social insects. Adv Insect Physiol 48: 227-269.

[30] Shi YY, Huang ZY, Zeng ZJ, Wang ZL, Wu XB, Yan WY (2011) Diet and cell size both affect queen-worker differentiation through DNA methylation in honey bees (*Apis mellifera*, Apidae). PLoS ONE 6: e18808.

[31] Bonasio R, Li Q, Lian J, Mutti N, Jin L, Zhao H, Zhang P, Wen P, Xiang H, Ding Y, Jin Z, Shen S, Wang Z, Wang W, Wang J, Berger S, L, Liebig, A, Zhang G, e, Reinberg D (2012) Genome-wide and caste-specific DNA methylomes of the ants *Camponotus floridanus* and *Harpegnathos saltator*. Curr Biol 22: 1755- 1764.

[32] Smith CR, Mutti NS, Jasper WC, Naidu A, Smith CD, Gadau JA (2012) Patterns of DNA methylation in development, division of labor and hybridization in an ant with genetic caste determination. PLoS ONE 7: e42433.

[33] Wurm Y, Wang J, Riba-Grognuz O, Corona M, Nygaard S, Hunt BG, Ingram KK, Falquet L, Nipitwattanaphon M, Gotzek D (2011) The genome of the fire ant *Solenopsis invicta*. PNAS 108: 5679-5684.

[34] Elango N, Hunt BG, Goodisman MA, Soojin VY (2009) DNA methylation is widespread and associated with differential gene expression in castes of the honeybee, *Apis mellifera*. PNAS 106: 11206-11211.

[35] Ross KG, Fletcher DJ (1985) Genetic origin of male diploidy in the fire ant, *Solenopsis invicta* (Hymenoptera: Formicidae), and its evolutionary significance. Evolution 888-903.

[36] Glastad KM, Hunt BG, Yi SV, Goodisman MAD (2014) Epigenetic inheritance and genome regulation: is DNA methylation linked to ploidy in haplodiploid insects? P Roy Soc B-Biol Sci 281.

[37] Badisco L, Van Wielendaele P, Vanden Broeck J (2013) Eat to reproduce: a key role for the insulin signaling pathway in adult insects. Front Physiol 4: 202.

[38] Barker JF (1978) Neuroendocrine regulation of oocyte maturation in the imported fire ant *Solenopsis invicta*. Gen Comp Endocr 35: 234-237.

[39] Vargo EL, Laurel M (1994) Studies on the mode of action of a queen primer pheromone of the fire ant *Solenopsis invicta*. J Insect Physiol 40: 601-610.

[40] Chen ME, Lewis DK, Keeley LL, Pietrantonio PV (2004) cDNA cloning and transcriptional regulation of the vitellogenin receptor from the imported fire ant, *Solenopsis invicta* Buren (Hymenoptera: Formicidae). Insect Mol Biol 13: 195-204.

[41] Tu MP, Yin CM, Tatar M (2002) Impaired ovarian ecdysone synthesis of *Drosophila melanogaster* insulin receptor mutants. Aging Cell 1: 158-160.

[42] Tu MP, Yin CM, Tatar M (2005) Mutations in insulin signaling pathway alter juvenile hormone synthesis in *Drosophila melanogaster*. Gen Comp Endocr 142: 347-356.

[43] Lee KS, Kwon OY, Lee JH, Kwon K, Min KJ, Jung SA, Kim AK, You KH, Tatar M, Yu K (2008) *Drosophila* short neuropeptide F signalling regulates growth by ERK-mediated insulin signalling. Nat Cell Biol 10: 468-475.

[44] Nässel DR, Wegener C (2011) A comparative review of short and long neuropeptide F signaling in invertebrates: Any similarities to vertebrate neuropeptide Y signaling? Peptides 32: 1335-1355.

[45] Nässel DR, Winther AME (2010) *Drosophila* neuropeptides in regulation of physiology and behavior. Prog Neurobiol 92: 42-104.

[46] Lee KS, You KH, Choo JK, Han YM, Yu K (2004) *Drosophila* short neuropeptide F regulates food intake and body size. J Biol Chem 279: 50781-50789.

[47] Dillen S, Zels S, Verlinden H, Spit J, Van Wielendaele P, Broeck JV (2013) Functional characterization of the short neuropeptide F receptor in the desert locust, *Schistocerca gregaria*. PLoS ONE 8: e53604.

[48] Chen ME, Pietrantonio PV (2006) The short neuropeptide F-like receptor from the red imported fire ant, *Solenopsis invicta* Buren (Hymenoptera: Formicidae). Arch Insect Biochem Physiol 61: 195-208.

[49] Nagata S, Matsumoto S, Nakane T, Ohara A, Morooka N, Konuma T, Nagai C, Nagasawa H (2012) Effects of starvation on brain short neuropeptide F-1,-2, and-3 levels and short neuropeptide F receptor expression levels of the silkworm, *Bombyx mori*. Front Endocrinol 3.

[50] Lu HL, Pietrantonio PV (2011) Immunolocalization of the short neuropeptide F receptor in queen brains and ovaries of the red imported fire ant (*Solenopsis invicta* Buren). BMC Neurosci 12: 57.

[51] Tschinkel W (1998) The reproductive biology of fire ant societies. Bioscience 593-605.

[52] Tschinkel, Walter (2006) The Fire Ants. Cambrigde,Mass: The Belknap Press of Harvard University Press.

[53] Langkilde T (2009) Invasive fire ants alter behavior and morphology of native lizards. Ecology 90: 208-217.

[54] Ligon RA, Siefferman L, Hill GE (2011) Invasive fire ants reduce reproductive success and alter the reproductive strategies of a native vertebrate insectivore. PLoS ONE 6.

[55] Wilder SM, Barnum TR, Holway DA, Suarez AV, Eubanks MD (2013) Introduced fire ants can exclude native ants from critical mutualist-provided resources. Oecologia 172: 197-205.

[56] LeBoeuf AC, Benton R, Keller L (2013) The molecular basis of social behavior: models, methods and advances. Curr Opin Neurobiol 23: 3-10.

[57] Gronenberg W, Heeren S, Holldobler B (1996) Age-dependent and task-related morphological changes in the brain and the mushroom bodies of the ant *Camponotus floridanus*. J Exp Biol 199: 2011-2019.

[58] Muscedere ML, Traniello JF (2012) Division of labor in the hyperdiverse ant genus *Pheidole* is associated with distinct subcaste-and age-related patterns of worker brain organization. PLoS ONE 7: e31618.

[59] Mysore K, Subramanian KA, Sarasij RC, Suresh A, Shyamala BV, VijayRaghavan K, Rodrigues V (2009) Caste and sex specific olfactory glomerular organization and brain architecture in two sympatric ant species *Camponotus sericeus* and *Camponotus compressus* (Fabricius, 1798). Arth Struct & Dev 38: 485-497.

[60] Mysore K, Shyamala BV, Rodrigues V (2010) Morphological and developmental analysis of peripheral antennal chemosensory sensilla and central olfactory glomeruli in worker castes of *Camponotus compressus* (Fabricius, 1787). Arth Struct & Dev 39: 310-321.

[61] Gronenberg W (1999) Modality-specific segregation of input to ant mushroom bodies. Brain Behav Evol 54: 85-95.

[62] Gronenberg W, Holldobler B (1999) Morphologic representation of visual and antennal information in the ant brain. J Comp Neurol 412: 229-240.

[63] Ehmer B, Gronenberg W (2004) Mushroom body volumes and visual interneurons in ants: comparison between sexes and castes. J Comp Neurol 469: 198-213.

[64] Seid MA, Goode K, Li C, Traniello JF (2008) Ageand subcasterelated patterns of serotonergic immunoreactivity in the optic lobes of the ant *Pheidole dentata*. Dev Neurobiol 68: 1325-1333.

[65] Giraldo YM, Patel E, Gronenberg W, Traniello JF (2013) Division of labor and structural plasticity in an extrinsic serotonergic mushroom body neuron in the ant *Pheidole dentata*. Neurosci Lett 534: 107-111.

[66] Dolezal AG, Johnson J, Holldobler B, Amdam GV (2013) Division of labor is associated with age-independent changes in ovarian activity in *Pogonomyrmex californicus* harvester ants. J Insect Physiol 59: 519-524.

[67] Libbrecht R, Keller L (2013) Genetic compatibility affects division of labor in the Argentine ant *Linepithema humile*. Evolution 67: 517-524.

[68] Simola DF, Ye C, Mutti NS, Dolezal K, Bonasio R, Liebig JA, Reinberg D, Berger SL (2013) A chromatin link to caste identity in the carpenter ant *Camponotus floridanus*. Genome Res 23: 486-496.

[69] Nässel DR (1993) Neuropeptides in the insect brain: a review. Cell Tissue Res 273: 1-29.

[70] Mertens I, Meeusen T, Huybrechts R, De Loof A, Schoofs L (2002) Characterization of the short neuropeptide F receptor from *Drosophila melanogaster*. Biochem Bioph Res Co 297: 1140-1148.

[71] Wu Q, Wen T, Lee G, Park JH, Cai HN, Shen P (2003) Developmental control of foraging and social behavior by the *Drosophila* neuropeptide Y-like system. Neuron 39: 147-161.

[72] Ament SA, Velarde RA, Kolodkin MH, Moyse D, Robinson GE (2011) Neuropeptide Y-like signalling and nutritionally mediated gene expression and behaviour in the honey bee. Insect Mol Biol 20: 335-345.

[73] Brockmann A, Annangudi SP, Richmond TA, Ament SA, Xie F, Southey BR, Rodriguez-Zas SR, Robinson GE, Sweedler JV (2009) Quantitative peptidomics reveal brain peptide signatures of behavior. PNAS 106: 2383-2388.

[74] Cook SC, Eubanks MD, Gold RE, Behmer ST (2011) Seasonality directs contrasting food collection behavior and nutrient regulation strategies in ants. PLoS ONE 6: e25407.

[75] Broeck JV (2001) Neuropeptides and their precursors in the fruitfly, *Drosophila melanogaster*. Peptides 22: 241-254.

[76] Garczynski SF, Brown MR, Crim JW (2006) Structural studies of *Drosophila* short neuropeptide F: Occurrence and receptor binding activity. Peptides 27: 575-582.

[77] Hauser F, Cazzamali G, Williamson M, Blenau W, Grimmelikhuijzen CJ (2006) A review of neurohormone GPCRs present in the fruitfly *Drosophila melanogaster* and the honey bee *Apis mellifera*. Prog Neurobiol 80: 1-19.

[78] Nässel DR, Enell L, Santos J, Wegener C, Johard H (2008) A large population of diverse neurons in the *Drosophila* central nervous system expresses short neuropeptide F, suggesting multiple distributed peptide functions. BMC Neurosci 9: 90.

[79] Petralia RS, Sorensen AA, Vinson SB (1980) The labial gland system of larvae of the imported fire ant, *Solenopsis invicta* Buren. Cell Tissue Res 206: 145-156.

[80] Whitfield CW, Cziko AM, Robinson GE (2003) Gene expression profiles in the brain predict behavior in individual honey bees. Science 302: 296-299.

[81] Page Jr RE, Rueppell O, Amdam GV (2012) Genetics of reproduction and regulation of honey bee (*Apis mellifera* L.) social behavior. Annu Rev Genet 46: 97.

[82] Toth AL, Kantarovich S, Meisel AF, Robinson GE (2005) Nutritional status influences socially regulated foraging ontogeny in honey bees. J Exp Biol 208: 4641-4649.

[83] Toth AL, Robinson GE (2007) Evo-devo and the evolution of social behavior. TIG 23: 334-341.

[84] Sorensen AA, Busch T, Vinson SB (1983) Behaviour of worker subcastes in the fire ant, *Solenopsis invicta*, in response to proteinaceous food. Physiol Entomol 8: 83-92.

[85] Miller Iii DG, Ratnieks FLW (2001) The timing of worker reproduction and breakdown of policing behaviour in queenless honey bee (*Apis mellifera* L.) societies. Insectes Soc 48: 178-184.

[86] Dobrin SE, Fahrbach SE (2012) Rho GTPase activity in the honey bee mushroom bodies is correlated with age and foraging experience. J Insect Physiol 58: 228-234.

[87] Lutz CC, Rodriguez-Zas SL, Fahrbach SE, Robinson GE (2012) Transcriptional response to foraging experience in the honey bee mushroom bodies. Dev Neurosci 72: 153-166.

[88] O'Donnell S, Donlan NA, Jones TA (2004) Mushroom body structural change is associated with division of labor in eusocial wasp workers (*Polybia aequatorialis*, Hymenoptera: Vespidae). Neurosci Lett 356: 159-162.

[89] Kapan N, Lushchak V, Luo J, Nässel DR (2012) Identified peptidergic neurons in the *Drosophila* brain regulate insulin-producing cells, stress responses and metabolism by coexpressed short neuropeptide F and corazonin. Cell Mol Life Sci 69: 4051-4066.

[90] Sinakevitch I, Niwa M, Strausfeld NJ (2005) Octopamine-like immunoreactivity in the honey bee and cockroach: Comparable organization in the brain and subesophageal ganglion. J Comp Neurol 488: 233-254.

[91] Kahsai L, Winther ÃsM (2011) Chemical neuroanatomy of the *Drosophila* central complex: distribution of multiple neuropeptides in relation to neurotransmitters. Journal of Comparative Neurology 519: 290-315.

[92] Kahsai L, Carlsson MA, Winther AME, Nässel DR (2012) Distribution of metabotropic receptors of serotonin, dopamine, GABA, glutamate, and short neuropeptide F in the central complex of *Drosophila*. Neuroscience 208: 11-26.

[93] Kahsai L, Martin JR, Winther AME (2010) Neuropeptides in the *Drosophila* central complex in modulation of locomotor behavior. J Exp Biol 213: 2256-2265.

[94] Seid MA, Wehner R (2008) Ultrastructure and synaptic differences of the boutons of the projection neurons between the lip and collar regions of the mushroom bodies in the ant, *Cataglyphis albicans*. J Comp Neurol 507: 1102-1108.

[95] Griss C, Simpson SJ, Rohrbacher J, Rowell CHF (1991) Localization in the central nervous system of larval *Manduca sexta* (Lepidoptera: Sphingidae) of areas responsible for aspects of feeding behaviour. J Insect Physiol 37: 477-482.

[96] Paul J, Gronenberg W (2002) Motor control of the mandible closer muscle in ants. J Insect Physiol 48: 255-267.

[97] Mitchell BK, Itagaki H, Rivet MP (1999) Peripheral and central structures involved in insect gustation. Microsc Res Tech 47: 401-415.

[98] Melcher C, Pankratz MJ (2005) Candidate gustatory interneurons modulating feeding behavior in the *Drosophila* brain. PLoS Biol 3(9): e305 .

[99] Audsley N, Weaver RJ (2009) Neuropeptides associated with the regulation of feeding in insects. Gen Comp Endocrinol 162: 93-104.

[100] Choi MY, Raina A, Vander Meer RK (2009) PBAN/pyrokinin peptides in the central nervous system of the fire ant, *Solenopsis invicta*. Cell Tissue Res 335: 431-439.

[101] Sorensen AA, Vinson SB (1981) Quantitative food distribution studies within laboratory colonies of the imported fire ant, *Solenopsis invicta* Buren. Insectes Soc 28: 129-160.

[102] Sorensen AA, Mirenda JT, Vinson SB (1981) Food exchange and distribution by three functional worker groups of the imported fire ant *Solenopsis invicta* Buren. Insectes Soc 28: 383-394.

[103] Chen W, Shi W, Li L, Zheng Z, Li T, Bai W, Zhao Z (2013) Regulation of sleep by the short neuropeptide F (sNPF) in *Drosophila melanogaster*. Insect Biochem Molec 43: 809-819.

[104] Kaneko Y, Hiruma K (2014) Short neuropeptide F (sNPF) is a stage-specific suppressor for juvenile hormone biosynthesis by corpora allata, and a critical factor for the initiation of insect metamorphosis. Dev Biol 393: 312-319.

[105] Shang Y, Donelson NC, Vecsey CG, Guo F, Rosbash M, Griffith LC (2013) Short neuropeptide F is a sleep-promoting inhibitory modulator. Neuron 80: 171-183.

[106] Bajracharya P, Lu HL, Pietrantonio PV (2014) The red imported fire ant (*Solenopsis invicta* Buren) kept Y not F: Predicted sNPY endogenous ligands deorphanize the short NPF (sNPF) receptor. PLoS ONE 9(10): e109590 .

[107] Johard H, Yoishii T, Dircksen H, Cusumano P, Rouyer F, Helfrich-Förster C, Nässel DR (2009) Peptidergic clock neurons in *Drosophila*: Ion transport peptide and short neuropeptide F in subsets of dorsal and ventral lateral neurons. J Comp Neurol 516: 59-73.

[108] Boerjan B, Cardoen D, Bogaerts A, Landuyt B, Schoofs L, Verleyen P (2010) Mass spectrometric profiling of neuropeptides in the worker honeybee, *Apis mellifera*. Neuropharmacology 58: 248-258.

[109] Schmitt F, Vanselow JT, Schlosser A, Kahnt J, Rossler W, Wegener C (2015) Neuropeptidomics of the carpenter ant *Camponotus floridanus*. J Proteome Res 14: 1504-1514.

[110] Carlsson MA, Enell LE, Nässel DR (2013) Distribution of short neuropeptide F and its receptor in neuronal circuits related to feeding in larval *Drosophila*. Cell a nd tissue research 353: 511-523.

[111] Castillo P, Pietrantonio PV (2013) Differences in sNPF receptor-expressing neurons in brains of fire ant (*Solenopsis invicta* Buren) worker subcastes: Indicators for division of labor and nutritional status? PLoS ONE 8(12): e83966.

[112] Dussutour A, Simpson SJ (2008) Description of a simple synthetic diet for studying nutritional responses in ants. Insect Soc 55: 329-333.

[113] Ibanez F, Hancock J, Tamborindeguy C (2014) Identification and expression analysis of aquaporins in the potato psyllid, *Bactericera cockerelli*. PLoS ONE 9(10): e111745 .

[114] Pietrantonio PV, Jagge C, Keeley LL, Ross LS (2000) Cloning of an aquaporin-like cDNA and *in situ* hybridization in adults of the mosquito *Aedes aegypti* (Diptera: Culicidae). Insect Mol Biol 9: 407-418.

[115] Pietrantonio PV, Jagge C, McDowell C (2001) Cloning and expression analysis of a 5HT7-like serotonin receptor cDNA from mosquito *Aedes aegypti* female excretory and respiratory systems. Insect Mol Biol 10: 357-369.

[116] Lu S-M, Zhao Z, Li K, Zhang Y-L, Xi G-S (2011) Cloning and expression analysis of a muscarinic cholinergic receptor from the brain of ant, *Polyrhachis vicina*. Arch Insect Biochem Physiol 78: 46-60.

[117] Wilcox JN (1993) Fundamental principles of *in situ* hybridization. J Histochem Cytochem 41: 1725-1733.

[118] Khila A, Abouheif E (2009) *In situ* hybridization on ant ovaries and embryos. Cold Spring Harbor Protocols 2009: db-rot5250.

[119] Khila A, Abouheif E (2010) Evaluating the role of reproductive constraints in ant social evolution. Philos Trans R Soc Lond B Biol Sci 365: 617-630.

[120] Khila A, Abouheif E (2008) Reproductive constraint is a developmental mechanism that maintains social harmony in advanced ant societies. PNAS 105: 17884-17889.

[121] Shbailat SJ, Khila A, Abouheif E (2010) Correlations between spatiotemporal changes in gene expression and apoptosis underlie wing polyphenism in the ant *Pheidole morrisi*. Evol Dev 12: 580-591.

[122] Shbailat SJ, Abouheif E (2013) The wing-patterning network in the wingless castes of Myrmicine and Formicine ant species is a mix of evolutionarily labile and non-labile genes. J Exp Zool B Mol Dev Evol 320: 74-83.

[123] Okada Y, Miyazaki S, Miyakawa H, Ishikawa A, Tsuji K, Miura T (2010) Ovarian development and insulin-signaling pathways during reproductive. differentiation in the queenless ponerine ant *Diacamma sp.* J Insect Physiol 56: 288-295.

[124] Ouyang X, LI C, Wei S, Hou L, XI G (2012) Expression of estrogen related receptor gene in the brain of different caste of *Polyrhachis vicina.* J Shaanxi Norm Univ (Nat Sci Ed) 3: 015.

[125] Beggs KT, Hamilton IS, Kurshan PT, Mustard JA, Mercer AR (2005) Characterization of a D2-like dopamine receptor (AmDOP3) in honey bee, *Apis mellifera.* Insect Biochem Mol Biol 35: 873-882.

[126] Blenau W, Erber J, Baumann A (1998) Characterization of a dopamine D1 receptor from *Apis mellifera*: cloning, functional expression, pharmacology, and mRNA localization in the brain. J Neurochem 70: 15-23.

[127] Grohmann L, Blenau W, Erber J, Ebert PR, Strunker T, Baumann A (2003) Molecular and functional characterization of an octopamine receptor from honeybee (*Apis mellifera*) brain. J Neurochem 86: 725-735.

[128] Kamikouchi A, Takeuchi H, Sawata M, Natori S, Kubo T (2000) Concentrated expression of Ca2+/calmodulin-dependent protein kinase II and protein kinase C in the mushroom bodies of the brain of the honeybee *Apis mellifera* L. J Comp Neurol 417: 501-510.

[129] McNeill MS, Robinson GE (2015) Voxelbased analysis of the immediate early gene, c-jun, in the honey bee brain after a sucrose stimulus. Insect Mol Biol 24: 377-390.

[130] Takeuchi H, Yasuda A, Yasuda-Kamatani Y, Sawata M, Matsuo Y, Kato A, Tsujimoto A, Nakajima T, Kubo T (2004) Prepro-tachykinin gene expression in the brain of the honeybee *Apis mellifera*. Cell Tissue Res 316: 281-293.

[131] Thany SH, Lenaers G, Crozatier M, Armengaud C, Gauthier M (2003) Identification and localization of the nicotinic acetylcholine receptor alpha3 mRNA in the brain of the honeybee, *Apis mellifera*. Insect Mol Biol 12: 255-262.

[132] Velarde RA, Sauer CD, Walden O, Fahrbach SE, Robertson HM (2005) Pteropsin: A vertebrate-like non-visual opsin expressed in the honey bee brain. Insect Biochem Mol Biol 35: 1367-1377.

[133] Zannat MT, Locatelli F, Rybak J, Menzel R, Leboulle G (2006) Identification and localisation of the NR1 sub-unit homologue of the NMDA glutamate receptor in the honeybee brain. Neurosci Lett 398: 274-279.

[134] Johard HA, Enell LE, Gustafsson E, Trifilieff P, Veenstra JA, Nässel DR (2008) Intrinsic neurons of *Drosophila* mushroom bodies express short neuropeptide F: relations to extrinsic neurons expressing different neurotransmitters. J Comp Neurol 507: 1479-1496.

[135] Freeman MR, Doherty J (2006) Glial cell biology in *Drosophila* and vertebrates. Trends Neurosci 29: 82-90.

[136] Van Gijlswijk RP, Zijlmans HJ, Wiegant J, Bobrow MN, Erickson TJ, Adler KE, Tanke HJ, Raap AK (1997) Fluorochrome-labeled tyramides: use in immunocytochemistry and fluorescence in situ hybridization. J Histochem Cytochem 45: 375-382.

[137] Ito K, Shinomiya K, Ito M, Armstrong JD, Boyan G, Hartenstein V, Harzsch S, Heisenberg M, Homberg U, Jenett A (2014) A systematic nomenclature for the insect brain. Neuron 81: 755-765.

[138] Kim YH, Issa MS, Cooper AM, Zhu KY (2015) RNA interference: Applications and advances in insect toxicology and insect pest management. Pest Biochem Physiol 120: 109-117.

[139] Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC (1998) Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. Nature 391: 806-811.

[140] Plasterk RHA (2002) RNA Silencing: The Genome's Immune System. Science 296: 1263-1265.

[141] Meister G, Tuschl T (2004) Mechanisms of gene silencing by double-stranded RNA. Nature 431: 343-349.

[142] Amdam GV, Simoes ZL, Guidugli KR, Norberg K, Omholt SW (2003) Disruption of vitellogenin gene function in adult honeybees by intra-abdominal injection of double-stranded RNA. BMC biotechnology 3: 1.

[143] Nunes FMF, Simoes ZL (2009) A non-invasive method for silencing gene transcription in honeybees maintained under natural conditions. Insect Biochem Mol Biol 39: 157-160.

[144] Wolschin F, Mutti NS, Amdam GV (2011) Insulin receptor substrate influences female caste development in honeybees. Biol lett 7: 112-115.

[145] Zhou X, Oi FM, Scharf ME (2006) Social exploitation of hexamerin: RNAi reveals a major caste-regulatory factor in termites. PNAS 103: 4499-4504.

[146] Zhou X, Wheeler MM, Oi FM, Scharf ME (2008) RNA interference in the termite *Reticulitermes flavipes* through ingestion of double-stranded RNA. Insect Biochem Mol Biol 38: 805-815.

[147] Choi MY, Vander Meer RK, Coy M, Scharf ME (2012) Phenotypic impacts of PBAN RNA interference in an ant, *Solenopsis invicta*, and a moth, *Helicoverpa zea*. J Insect Physiol 58: 1159-1165.

[148] Choi MY, Vander Meer RK (2012) Ant trail pheromone biosynthesis is triggered by a neuropeptide hormone. PLoS ONE 7: e50400.

[149] Lu, Hsiao Ling (2012) Vitellogenin receptor and neuropeptide receptors involved in reproduction of the red Imported fire ant (*Solenopsis invicta* Buren) [dissertation].

[150] Miyazaki S, Okada Y, Miyakawa H, Tokuda G, Cornette R, Koshikawa S, Maekawa K, Miura T (2014) Sexually dimorphic body color is regulated by sex-specific expression of *yellow* gene in Ponerine Ant, *Diacamma Sp.* PLoS ONE 9: e92875.

[151] Ratzka C, Gross R, Feldhaar H (2013) Systemic gene knockdown in *Camponotus floridanus* workers by feeding of dsRNA. Insect Soc 60: 475-484.

[152] Lu HL, Vinson SB, Pietrantonio PV (2009) Oocyte membrane localization of vitellogenin receptor coincides with queen flying age, and receptor silencing by RNAi disrupts egg formation in fire ant virgin queens. FEBS J 276: 3110-3123.

[153] Kwon H, Lu HL, Longnecker MT, Pietrantonio PV (2012) Role in diuresis of a calcitonin receptor (GPRCAL1) expressed in a distal-proximal gradient in renal organs of the mosquito *Aedes aegypti* (L.). PLoS ONE 7(11): e50374 .

[154] Kamath RS, Ahringer J (2003) Genome-wide RNAi screening in *Caenorhabditis elegans*. Methods 30: 313-321.

[155] Zhu F, Xu J, Palli R, Ferguson J, Palli SR (2011) Ingested RNA interference for managing the populations of the Colorado potato beetle, *Leptinotarsa decemlineata.* Pest Manag Sci 67: 175-182.

[156] Cheng D, Zhang Z, He X, Liang G (2013) Validation of reference genes in *Solenopsis invicta* in different developmental stages, castes and tissues. PLoS ONE 8: e57718.

[157] Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR the 2^{-ddCt} method. Methods 25: 402-408.

[158] Li X, Zhang M, Zhang H (2011) RNA interference of four genes in adult *Bactrocera dorsalis* by feeding their dsRNAs. PLoS ONE 6: e17788.

[159] Wheeler D (1996) The role of nourishment in oogenesis. Annu Rev Entomol 41: 407-431.

[160] Lehane, M. and Billingsley, P. (2012) Biology of the insect midgut. Springer Science & Business Media.

[161] Mikani A, Wang QS, Takeda M (2012) Brain-midgut short neuropeptide F mechanism that inhibits digestive activity of the American cockroach, *Periplaneta americana* upon starvation. Peptides 34: 135-144.

[162] Mikani A, Watari Y, Takeda M (2015) Brain-midgut cross-talk and autocrine metabolastat via the sNPF/CCAP negative feed-back loop in the American cockroach, *Periplaneta americana*. Cell Tissue Res 1-16.

[163] Garbutt JS, Belles X, Richards EH, Reynolds SE (2013) Persistence of double-stranded RNA in insect hemolymph as a potential determiner of RNA interference success: evidence from *Manduca sexta* and *Blattella germanica*. J Insect Physiol 59: 171-178.

[164] Christiaens O, Swevers L, Smagghe G (2014) DsRNA degradation in the pea aphid (*Acyrthosiphon pisum*) associated with lack of response in RNAi feeding and injection assay. Peptides 53: 307-314.

[165] Swevers L, Smagghe G (2012) Use of RNAi for control of insect crop pests. In: Arthropod-Plant Interactions. Springer. pp. 177-197.

[166] Winston WM, Molodowitch C, Hunter CP (2002) Systemic RNAi in *C. elegans* requires the putative transmembrane protein SID-1. Science 295: 2456-2459.

[167] Tomoyasu Y, Miller SC, Tomita S, Schoppmeier M, Grossmann D, Bucher G (2008) Exploring systemic RNA interference in insects: a genome-wide survey for RNAi genes in *Tribolium*. Genome Biol 9: R10.

[168] Saleh MC, van Rij RP, Hekele A, Gillis A, Foley E, O'Farrell PH, Andino R (2006) The endocytic pathway mediates cell entry of dsRNA to induce RNAi silencing. Nature Cell Biol 8: 793-802.

[169] Ulvila J, Parikka M, Kleino A, Sormunen R, Ezekowitz RA, Kocks C, Ramet M (2006) Double-stranded RNA is internalized by scavenger receptor-mediated endocytosis in *Drosophila* S2 cells. J Biol Chem 281: 14370-14375.

[170] Huvenne H, Smagghe G (2010) Mechanisms of dsRNA uptake in insects and potential of RNAi for pest control: a review. J Insect Physiol 56: 227-235.

[171] Grishok A (2005) RNAi mechanisms in *Caenorhabditis elegans*. FEBS letters 579: 5932-5939.

[172] Lehmann R, Tautz D (1994) *In Situ* Hybridization to RNA. In: Academic Press Inc., editors. *Drosophila melanogaster:* Practical uses in cell and molecular biology. pp. 576-597.

APPENDIX A

IN SITU HYBRIDIZATION PROTOCOLS FOR FIRE ANT QUEENS AND WORKERS BRAIN

1. Overview

Several protocols were tested for *in situ* hybridization in the brain of fire ant queens and workers. These protocols were designed to work with frozen sections or whole mounted tissue samples. Different concentrations of probes were tested (1, 3, 9, 15, 25, 30, 50, 60, and 100 ng/μL), as well as different hybridization temperatures (40, 45, 50 and 55°C.). In addition, it is important to mention that protocols 1, 2 and 3 were tested in brains of alate (virgin) queens and workers of all subcastes, but protocols 4 to six were tested only in alate (virgin) queen brains. Protocol 7 was tested only in mated queens.

2. List of reagent brands and catalog numbers.

- 50% ultrapure sterile dextran solution. Bioworld. Cat.number: 40400040-1.
- Acetic acid. EM Science. Cat.number: AX0073-9.
- Bovine Serum Albumin (BSA). Gold Biotechnology. Cat.number: A-420-10.
- CHAPS hydrate. Sigma-Aldrich. Cat.number: C9426.
- Deionized Formamide. Sigma-Aldrich. Cat.number: F9037.
- Denhardt's solution. Sigma-Aldrich. Cat.number: 30915.
- Diethylpyrocarbonate (DEPC). Gold Biotechnology. Cat.number: D-340-25.

- Ethylene Diamine Tetraacetic Acid (EDTA). Sigma-Aldrich. Cat.number: E5134.
- Ethylene Glycol Tetraacetic Acid (EGTA). Fisher Scientific. Cat.number: 02783-100.
- Fisherbrand™ Superfrost™ Slides. Fisher Scientific. Cat.number: 22-034980.
- Fisherbrand™ Cover Glasses: Rectangles (22x30, 0.16 to 0.19 mm thick). Fisher Scientific. Cat.number: 12-544-A.
- Fisherbrand™ Cover Glasses: Rectangles (24x60, 0.13 to 0.17 mm thick). Fisher Scientific. Cat.number: 48393106.
- Glycine. Sigma-Aldrich. Cat.number: 50046.
- Heparin. Alfa Aesar. Cat.number: A16198.
- Maleic acid. Sigma-Aldrich. Cat.number: M0375.
- Monobasic potassium phosphate. EM Science. Cat.number: PX1565-1.
- Paraformaldehyde. Sigma-Aldrich. Cat.number: P6148.
- Potasium chloride. Spectrum Chemical MFg group. Cat.number: P1250.
- Silane coated glass slides. LabScientific, inc. Cat. number: 7801W.
- Proteinase K. Life Technologies. Cat.number: 25530-015.
- Sodium chloride. Baker, J. T. Cat.number: 3624-05.
- Sodium citrate. EM Science. Cat.number: SX0445-1.
- Sodium phosphate dibasic. AMRESCO. Cat.number: 0404.
- Total yeast tRNA. Life Technologies. Cat.number: AM7119.
- Trizma hydrochloride. Sigma-Aldrich. Cat.number: T3253.
- Tween 20. Fisher Biotech. Cat.number: BP337-100.
- Ultrapure salmon sperm DNA. Life Technologies. Cat.number: 15632-011.
- Vectashield antifade mounting medium with DAPI. Vector Laboratories. Cat.number: H-1200.

3. Protocol 1.

This protocol was design based on the general procedure described by *Ibanez, et. al* [113]; but following the long hybridization time and fluorescent detection of the probes as described by *Pietrantonio et. al.* [114,115].

3.1 Buffers and other reagents needed:

- DEPC treated ultrapure water (to prepare all buffers and solutions)
- 10X PBS pH 7.4 (1.36 M NaCl; 26.8 mM KCl; 101.4 mM Na_2HPO_4; 17.6 mM KH_2PO_4)
- 20X SSC pH 7.0 (3 M NaCl; 300 mM sodium citrate)
- 20% (v/v) acetic acid
- Dissection buffer (1X PBS; 50 mM EGTA; 50 mM EDTA)
- Fixation buffer (1X PBS, 4% (w/v) Paraformaldehyde)
- Pre-hybridization buffer (50% (v/v) Formamide; 5X SSC; 1 mg/ml Total yeast RNA; Heparin 10 mg/ml; 0.1% (v/v) Tween 20)
- MABT buffer (100 mM maleic acid, 150 mM NaCl, 0.1% (v/v) Tween 20, pH 7.5)
- Blocking buffer (MABT buffer + 2% BSA).

3.2 Procedure:

- Dissection of tissues: Dissect tissues in dissection buffer, and fix using fixation buffer for 2 hours at 4°C. Wash the tissues with 1X PBS to remove the fixative.
- Antigen retrieval: Digest with 20 µg/ml proteinase K in pre-warmed 1X PBS for 20 min at room temperature. Wash the tissues with 1X PBS to remove the proteinase K.
- Permeabilization: Immerse tissues in ice cold 20% (v/v) acetic acid for 20 sec. This will permeabilize the cells to allow access to the probe and the antibody.
- Prehybridization: incubate the tissues in pre-hybridization buffer for 2 h at the desired hybridization temperature (50°C were used with the sNPF and sNPFR probes).
- Preparation of the probes: Dilute the probes to the desired concentrations with pre-hybridization buffer (1, 3, 9 and 15 ng/µL were tested with this protocol). Heat for 70°C for 2 min on a PCR block. This will dehybridize the DIG-labelled RNA probe. Chill on ice immediately to prevent re-hybridization.
- Hybridization: Add 50 to 100 µl of diluted probe to each sample (ensure the entire sample is covered). Incubate in the hybridization chamber at the desired hybridization temperature for 56 h (50°C were used with the sNPF and sNPFR probes).
- Washing step 1: wash off unbound probe by incubating the tissues for 1 h in 2X SSC buffer at 37°C.

- Washing step 2: wash with 0.2X SSC for 1 h at 37°C.
- Washing step 3: Wash twice in MABT buffer for 30 min at room temperature.
- Blocking step: add 200 µl blocking buffer to each sample. Block for 2 hours at room temperature.
- Probe detection: remove the blocking buffer and add the anti-DIG-Rodamine Fab fragments antibody (Roche, Indianapolis, IN, USA) in a 1:10 dilution in blocking buffer (final concentration: 20 µg/mL). Incubate overnight at 4°C in the dark.
- Washing: Wash tissues 6 times with MABT buffer, 30 minutes for each wash, at room temperature.
- Mounting: Place the tissues on glass microscope slides (Fisherbrand™ Superfrost™ Slides, Fisher Scientific, Pittsburgh, PA, USA) with mounting media with DAPI for visualization of nuclei (Vectashield, Vector Laboratories, Burlingame, CA, USA). Microscope cover glasses used were 0.16 to 0.19 mm in thickness (Fisherbrand™ Cover Glasses, Fisher Scientific, Pittsburgh, PA, USA). The slides can be stored at -20°C in the dark for several weeks.

4. Protocol 2.

This protocol is the same as protocol 1, except for the hybridization buffer used contained: 50% formamide, 5X SSC, 140 mM NaCl, 10 mM KH_2PO_4, 0.1 mg/mL Glycogen, 100 µg/mL ultrapure salmon sperm DNA, 0.1% (v/v) Tween 20. Pre-hybridization and hybridization steps were carried out at the same

temperature, which were: 45°C, 50°C, or 55°C. In addition, the probes were used at a concentration of 50, and 100 ng/μL. These modifications were adapted from a previous publication in *D. melanogaster*, as described by *Nässel et.al.* [78].

5. Protocol 3.

This protocol was adapted from Lehmann and Diethard, 1994 [172].

5.1 Buffers and other reagents needed:

- DEPC treated ultrapure water (to prepare all buffers and solutions)
- 10X PBS pH 7.4 (1.36 M NaCl; 26.8 mM KCl; 101.4 mM Na_2HPO_4; 17.6 mM KH_2PO_4)
- 1X PBST (1X PBS, 0.1% (v/v) Tween 20)
- 20X SSC pH 7.0 (3 M NaCl; 300 mM sodium citrate)
- Dissection buffer (1X PBS; 50 mM EGTA; 50 mM EDTA)
- Fixation buffer (1X PBS, 4% (w/v) Paraformaldehyde, 50 mM EGTA, 0.1% (v/v) Tween 20)
- Pre-hybridization buffer (50% (v/v) formamide, 5X SSC, 1X Denhardt's solution, 100 μg/mL ultrapure salmon sperm DNA, 100 μg/mL Heparin, 0.1% (v/v) CHAPS, 0.1% (v/v) Tween 20)

- MABT buffer (100 mM maleic acid, 150 mM NaCl, 0.1% (v/v) Tween 20, pH 7.5)
- Blocking buffer ((MABT + 2% BSA).

5.2 Procedure:

- Dissection of tissues: dissect the ant brains in dissection buffer and immediately fix with freshly prepared fixation buffer for 1.5 h at room temperature. Wash the tissues with PBST 5 times for 5 min to remove the fixative.
- Antigen retrieval: Digest with 30 µg/ml proteinase K in pre-warmed PBST for 30 min at 37°C. Wash the tissues for 2 min with PBST containing 2 mg/mL glycine to remove the proteinase K. Then, wash again with PBST 4 x 5 min.
- Post-fixation: Fix all the tissues for 30 min at room temperature with fixation buffer. Remove the fixative by washing with PBST 5 times for 5 min.
- Incubate the samples with 50% pre-hybridization buffer / 50% PBST for 10 min at the desired hybridization temperature (45°C, 50°C, and 55°C were tested with the sNPF and sNPFR probes).
- Pre-hybridization: incubate the tissues in pre-hybridization buffer for 2 h at the desired hybridization temperature (45°C, 50°C, and 55°C were tested with the sNPF and sNPFR probes).
- Preparation of the probes: Dilute the probes to the desired concentrations with pre-hybridization buffer (15, 25, 30, 50 and 60 ng/µL were tested with this

protocol). Heat for 75°C for 4 min on a PCR block. This will dehybridize the DIG-labelled RNA probe. Chill on ice immediately to prevent re-hybridization.

- Hybridization: Add 50 to 100 µl of diluted probe to each sample (ensure the entire sample is covered). Incubate in the hybridization chamber at the desired hybridization temperature for 56 h (45°C, 50°C, and 55°C were tested with the sNPF and sNPFR probes).
- Washing step 1: wash off unbound probe by incubating the tissues with 400 µL of warmed pre-hybridization buffer for 30 min at 37°C. Repeat this washing step three more times.
- Washing step 2: mix 50% of pre-hybridization buffer + 50% 2X SSC and wash the samples for 30 min at room temperature, twice.
- Washing step 3: mix 25% of pre-hybridization buffer + 75% 2X SSC and wash the samples for 30 min at room temperature, twice.
- Washing step 4: Wash twice in 2X SSC buffer for 30 min at room temperature.
- Washing step 5: Wash twice in MABT buffer for 30 min at room temperature.
- Blocking step: add 200 µl blocking buffer to each sample. Block for 2 hours at room temperature.
- Probe detection: remove the blocking buffer and add the anti-DIG-Rodamine Fab fragments antibody (Roche, Indianapolis, IN, USA) in a 1:16 dilution in blocking buffer (final concentration: 12.5 µg/mL). Incubate overnight at 4°C in the dark. Alternatively, in this step it is possible to use a system for amplification of the signal. To do so, after removal of the blocking buffer incubate the samples

overnight at 4°C with a Biotin-SP (long spacer) IgG fraction monoclonal mouse anti-digoxin antibody (Jackson ImmunoResearch, West Grove, PA, USA) diluted with blocking buffer (final concentration: 2.2 μg/mL). Then, wash the tissues 6 times with MABT buffer for 30 minutes for each wash at room temperature. Finally, incubate with Streptavidin-Alexa Fluor® 488 conjugate (Life Technologies, Grand Island, NY, USA) diluted 1:200 for 1 hour at room temperature. To remove unbound fluorescent conjugate, wash again 3 times with MABT buffer for 15 minutes at room temperature to then mount the samples in microscope slides.

- Washing: Wash tissues 6 times with MABT buffer, 30 minutes for each wash, at room temperature.
- Mounting: Place the tissues on glass microscope slides (Fisherbrand™ Superfrost™ Slides, Fisher Scientific, Pittsburgh, PA, USA) with mounting media with DAPI for visualization of nuclei (Vectashield, Vector Laboratories, Burlingame, CA, USA).). Microscope cover glasses used were 0.16 to 0.19 mm in thickness (Fisherbrand™ Cover Glasses, Fisher Scientific, Pittsburgh, PA, USA). The slides can be stored at -20°C in the dark for several days.

6. Protocol 4.

This protocol is a modification of the protocol 3 described above. After dissection of the tissues, the step of slide preparation was added accordingly with the protocol described by Ament, *et. al*, 2001 [72].

6.1 Buffers and other reagents needed:

- DEPC treated ultrapure water (to prepare all buffers and solutions)
- 10X PBS pH 7.4 (1.36 M NaCl; 26.8 mM KCl; 101.4 mM Na_2HPO_4; 17.6 mM KH_2PO_4)
- 1X PBST (1X PBS, 0.1% (v/v) Tween 20)
- 20X SSC pH 7.0 (3 M NaCl; 300 mM sodium citrate)
- 20% (v/v) acetic acid
- Dissection buffer (1X PBS; 50 mM EGTA; 50 mM EDTA)
- Fixation buffer (1X PBS, 4% (w/v) Paraformaldehyde)
- Pre-hybridization buffer (50% (v/v) formamide, 5X SSC, 1X Denhardt's solution, 100 μg/mL ultrapure salmon sperm DNA, 100 μg/mL Heparin, 0.1% (v/v) CHAPS, 0.1% (v/v) Tween 20)
- MABT buffer (100 mM maleic acid, 150 mM NaCl, 0.1% (v/v) Tween 20, pH 7.5)
- Blocking buffer ((MABT + 2% BSA).

6.2 Procedure:

- Dissection of tissues: dissect the ant brains in dissection buffer, and place them on top of little pieces of aluminum foil. Add a little drop of embedding media on top of the tissue and freeze it immediately in liquid nitrogen.
- Slides preparation: cut the frozen tissues with a thickness of 10 µm, in the cryostat (Leica 1850, Leica Biosystems Inc, Buffalo Grove, IL, USA) located in the Biological science building at Texas A&M University, College Station, Texas. Use the cryostat at -19°C to cut the samples. Place all the sections in Superfrost™ Plus Microscope Slides (Thermo Fisher Scientific). Dry slides at room temperature for 1 min and store them at -80°C until use.
- Drying of the slides: remove sections from the -80°C freezer and thaw for 10 min at room temperature. Dry the slides for an additional 10 min at 50°C.
- Fixation: fix the sections with freshly made fixation buffer for 10 min at room temperature. To remove the fixative, wash the slides with 1X PBS for 3 min, twice.
- Antigen retrieval: digest the tissues with 20 µg/ml proteinase K in pre-warmed 50 mM Tris-HCl for 10 min at room temperature. Wash the slides with 1X PBS for 3 min, twice.
- Permeabilization: Immerse the slides in ice cold 20% (v/v) acetic acid for 1 min to permeabilize the cells.

- Pre-hybridization: Incubate the samples with pre-hybridization buffer for 1 h at the desire hybridization temperature (45°C, 50°C, and 55°C were tested with the sNPF and sNPFR probes).
- Preparation of the probes: Dilute the probes with pre-hybridization buffer (1.0, 1.5, 3.0, and 5.0 ng/mL were tested with this protocol). Heat at 75°C for 4 min to denature the probes. Immediately chill on ice for at least 5 min to prevent re-hybridization.
- Hybridization: Incubate the slides with the probes overnight at the desired hybridization temperature (45°C, 50°C, and 55°C were tested with the sNPF and sNPFR probes).
- Washing step 1: wash off unbound probe by incubating the tissues for 30 min in 2X SSC buffer at room temperature, twice.
- Washing step 2: wash with 0.2X SSC buffer for 30 min at room temperature, twice.
- Washing step 3: Wash twice in MABT buffer for 30 min at room temperature.
- Blocking step: add blocking buffer and incubate for 1 h at room temperature.
- Probe detection: discard the blocking buffer. Add the Biotin-SP (long spacer) IgG fraction monoclonal mouse anti-digoxin antibody (Jackson Immunoresearch, West Grove, PA, USA) diluted in blocking buffer (final concentration: 2.2 μg/mL). Incubate for 1 h at room temperature.
- Washing: Wash tissues for 15 min x 3 times with PBST at room temperature.

- Signal amplification step: Incubate the samples with the Streptavidin-Alexa Fluor® 488 conjugate (Life Technologies, Grand Island, NY, USA) diluted 1:300 in PBS for 1 hour at room temperature.
- Washing: Wash tissues for 15 min x 3 times with PBST at room temperature. After the final wash, allow the slides to dry at room temperature for two minutes.
- Mounting: Samples were mounted using mounting media with DAPI for visualization of nuclei. Microscope cover glasses used were 0.13 to 0.17 mm in thickness (Fisherbrand™ Cover Glasses, Fisher Scientific, Pittsburgh, PA, USA). Store the slides at -20°C in the dark.

7. Protocol 5:

This protocol correspond to the one described by Takeuchi, *et. al.* [130], and was followed without modifications, except by a shorter incubation time with proteinase K (5 min instead of 15). Pre-hybridization and hybridization steps were carried out at the same temperature, which were: 45°C, 50°C, or 55°C for both, sNPF and sNPFR probes. In addition, both probes were used at a concentration of 1.0, 1.5 and 3.0 ng/mL. Silane coated slides were used as recommended by the author (Labscientific, inc, Livingston, NJ, USA).

8. Protocol 6:

This protocol correspond to the one described by Lu, *et. al.* [116], and was followed without modifications. Pre-hybridization and hybridization steps were carried out at the same temperature, which were: 45°C, 50°C, or 55°C for both, sNPF and sNPFR probes. In addition, both probes were used at a concentration of 1.0, 1.5 and 3.0 ng/mL.

9. Protocol 7:

This protocol was developed as a combination of some steps of protocol 3 and the protocol described by Takeuchi, *et. al* [130].

9.1 Buffers and other reagents needed:

- DEPC treated ultrapure water (to prepare all buffers and solutions)
- 10X PBS pH 7.4 (1.36 M NaCl; 26.8 mM KCl; 101.4 mM Na_2HPO_4; 17.6 mM KH_2PO_4)
- 1X PBST (1X PBS, 0.1% (v/v) Tween 20)
- Dissection buffer (1X PBS; 50 mM EGTA; 50 mM EDTA)
- Fixation buffer (1X PBS, 4% (w/v) Paraformaldehyde, 50 mM EGTA, 0.1% (v/v) Tween 20)

9.2 Procedure:

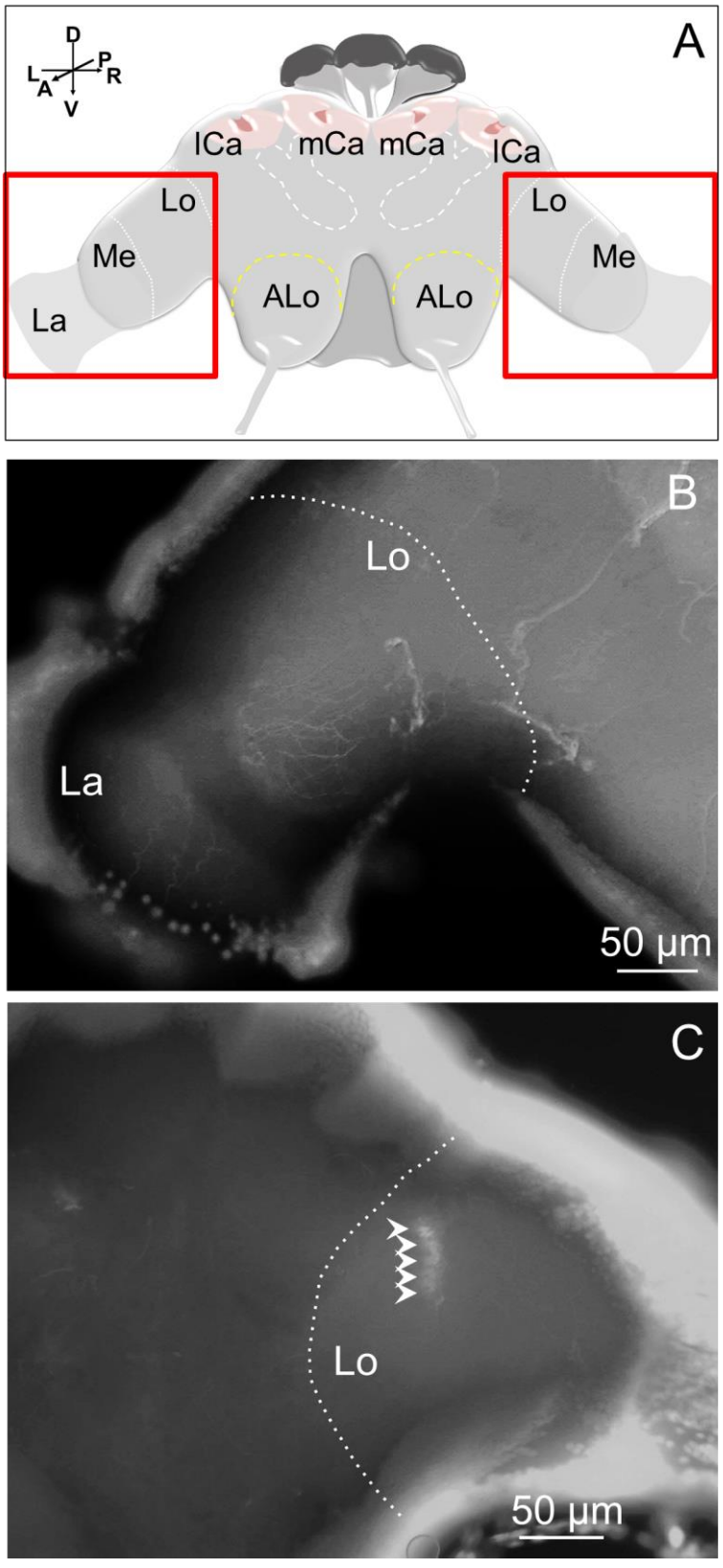
- Dissection of tissues: dissect the ant brains in dissection buffer and immediately fix with freshly prepared fixation buffer for 1.5 h at room temperature. Wash the tissues with PBST 5 times for 5 min to remove the fixative.
- Antigen retrieval: Digest with 30 µg/ml proteinase K in pre-warmed PBST for 30 min at 37°C. Wash the tissues for 2 min with PBST containing 2 mg/mL glycine to remove the proteinase K. Then, wash again with PBST 4 x 5 min.
- Post-fixation: Fix all the tissues for 30 min at room temperature with fixation buffer. Remove the fixative by washing with PBST 5 times for 5 min.
- Hybridization and probe detection: dilute the probes to a final concentration of 15 ng/µl using the hybridization buffer described by Takeuchi, *et. al* [130], and then incubate the brains with the hybridization buffer for 48 hours at 40°C. Proceed with the washing steps and develop the colorimetric signal using the reagents and following the protocol mentioned before [130].

APPENDIX B

SUPPLEMENTARY FIGURES FOR CHAPTER III

These figures are mentioned along the text in chapter III.

Figure B.1 *In situ* hybridizations of the short neuropeptide F obtained with protocols 1 and 2 in the brain of queens. A schematic diagram showing the anterior view of the queen brain is shown in (A). The red boxes enclosed the left and right optic lobes of the brain which correspond to the photos shown in (B) and (C), respectively. These are representative images of the results obtained with ISH protocols 1 and 2 in whole mounted queen brains using the anti-sense sNPF-probe. In queen brains processed following protocol 1, fine fluorescent lines were observed in different areas of the brain, especially close to the optic lobes likely representing trachea (A).Similar results were obtained following protocol 2; but some samples also showed some round spots of fluorescent signal as shown in B (white arrowheads), which are consistent with neurons. ALo = antennal lobes, lCa = lateral calyces, mCa = median calyces, SEG = subesophageal ganglion, La = lamina, Me = medula, Lo = lobula.


A
D
L
A
P
R
V
lCa
mCa
mCa
lCa
Lo
Me
La
ALo
ALo
Lo
Me
B
Lo
La
50 µm
C
Lo
50 µm

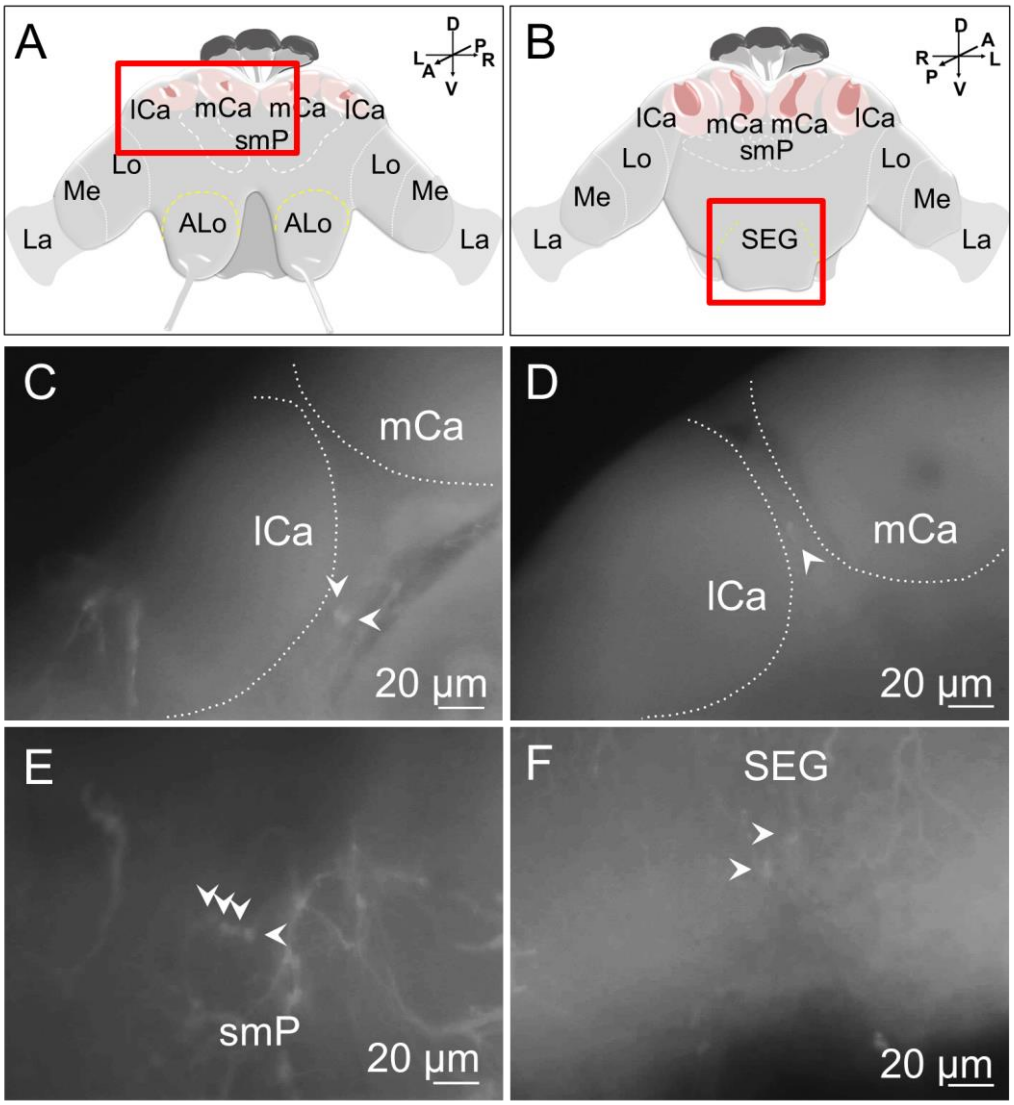
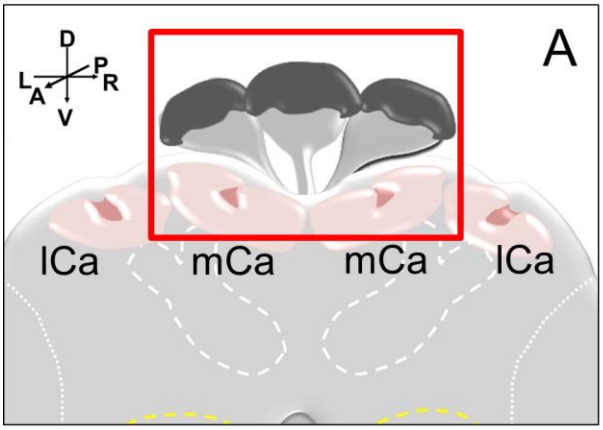
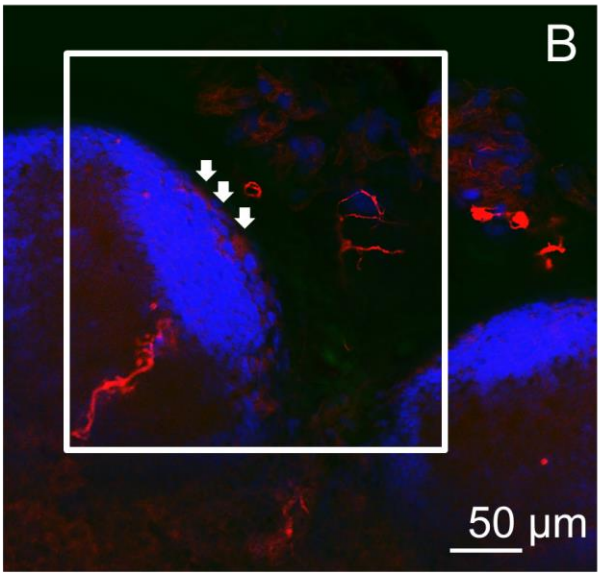


Figure B.2 *In situ* hybridizations of the short neuropeptide F and its receptor obtained with protocol 3 in the brain of queens. A schematic diagram showing the anterior and posterior view of the queen brain is shown in (A) and (B), respectively. The red boxes are indicating the areas of the brain from where the showed photos were taken. These images are representative of the results obtained with ISH protocol 3 using whole mounted queen brains. Round spots of fluorescent signal similar to cell-clusters were observed when using the anti-sense sNPF-probe, especially below the lCa of the mushroom bodies (C), or in between the lCa and mCa, (D). In samples obtained when using the anti-sense sNPFR-probe, a fluorescent signal resembling cluster C7 in the smP (E) and cluster C12 in the SEG (F) were found. ALo = antennal lobes, lCa = lateral calyces, mCa = median calyces, smP = superior medial protocerebrum, SEG = subesophageal ganglion, La = lamina, Me = medula, Lo = lobula.

Figure B.3 *In situ* hybridizations of the short neuropeptide F obtained with protocol 3 in the brain of queens. (A) A schematic of the queen brain showing the top region of the mushroom bodies, from where the pictures in (B) and (C) were taken (specific area is enclosed in red box). High resolution images were captured in a confocal microscope at a magnification of 20X (B); while (C) correspond to the higher magnification image (40X) of the region enclosed in the white box in (B). Arrowheads are pointing to cells present in the border of the mushroom body medial calyx which exhibit a faint red signal in their cytoplasm that may correspond to signal from the sNPF antisense probe. Intense fluorescent red lines are broken pieces of trachea. lCa = lateral calyces, mCa = median calyces.


A
D
L
A
P
R
V
lCa
mCa
mCa
lCa


B
50 μm

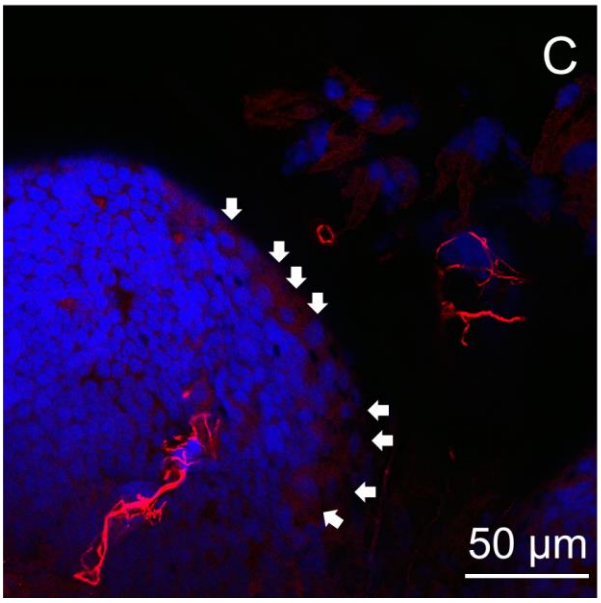
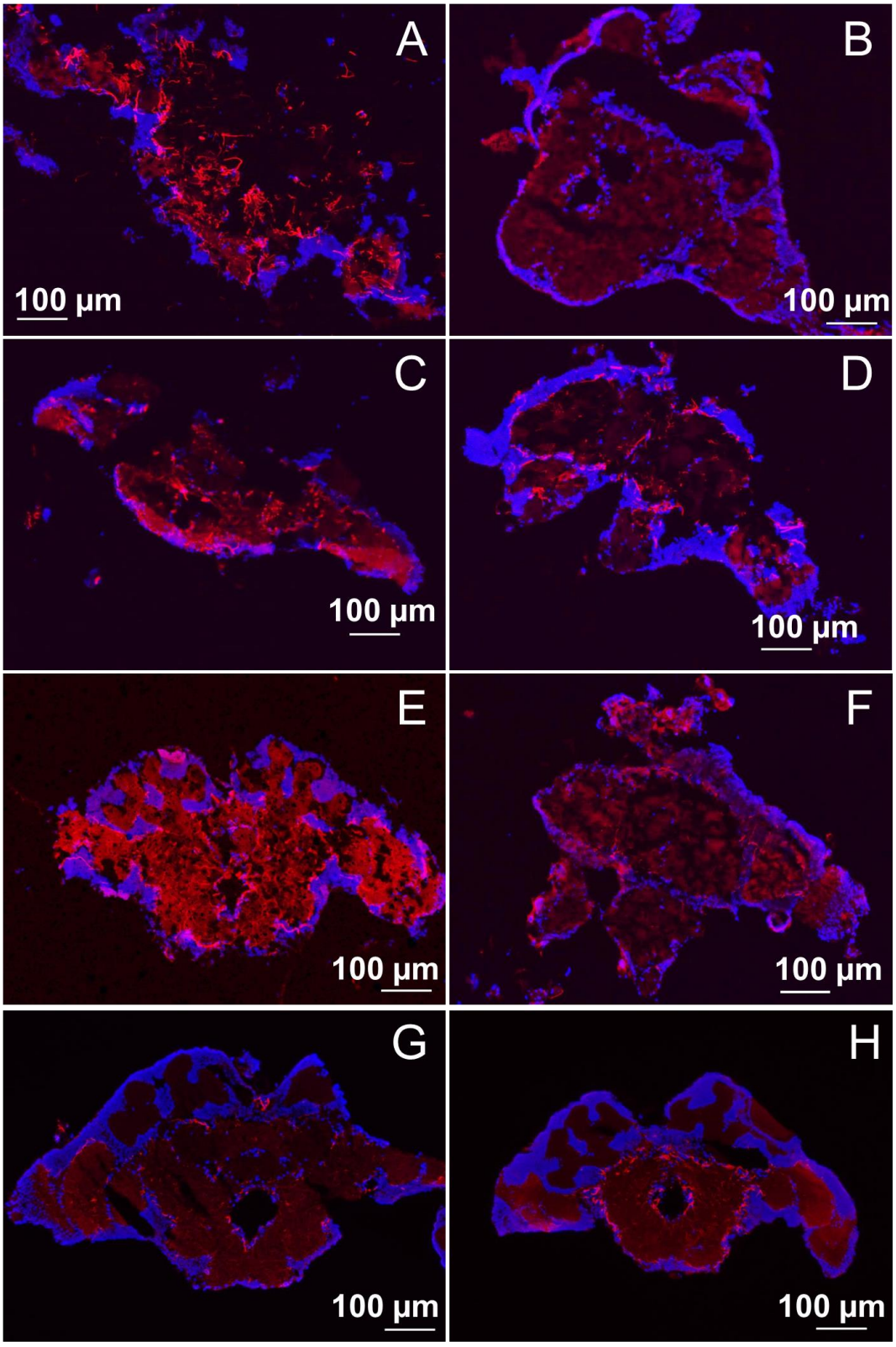

C
50 μm

Figure B.4 *In situ* hybridizations of the short neuropeptide F and its receptor obtained with protocols 4 to 6 in frozen sections of queen brains. Representative images of the results obtained following protocol 4 when using the antisense sNPF peptide probe and its negative control (sense sNPF peptide probe) are shown in (A), (B), respectively. For the same protocol, the image of a slice of brain treated with the antisense sNPFR probe is shown in (C); while its negative control (sense sNPFR probe) is shown in (D). Similar results were obtained when following protocol 5, using the antisense (E) and sense (F) sNPF peptide probes. Note that when using protocol 6 the brain sections are less damaged in comparison with brain sections from protocols 4 and 5, as shown in (G) (antisense sNPFR probe) and (H) (sense sNPFR probe).


A
100 µm
B
100 µm
C
100 µm
D
100 µm
E
100 µm
F
100 µm
G
100 µm
H
100 µm

APPENDIX C

INSTRUCTIONS TO PREPARE ARTIFICIAL SOLID DIET FOR FIRE ANTS

This artificial solid diet was adapted from the diet described by Dussutour and Simpson, 2008 [112].

- Ingredients:

12.0 g	Agar	(Fisher Scientific. Cat. No. BP1423)
17.5 g	Whole egg powder	(Augason Farms, Salt Lake city, UT, USA)
17.5 g	Whey protein isolate (unflavored)	(Jay Robb Enterprises Inc, Carlsbad, CA, USA)
17.5 g	Calcium caseinate	(Acros Organics. Cat. No. 419491000)
82.5 g	White granulated sugar	(H-E-B brand)
1.00 g	Vanderzant vitamin mixture for insects	(Sigma-Aldrich. Cat. No. V1007)
0.50 g	Methyl 4-hydroxybenzoate	(Sigma-Aldrich. Cat. No. H6654)

- Procedure:

1) Weight all the ingredients EXCEPT the agar, and place them together in a big plastic beaker. Add 125 mL of ultrapure water and stir with a silicone spatula until obtain a homogeneous solution without clumps (it will be thick).

2) Weight the agar and place it in a big pyrex-glass beaker. Add 250 mL of ultrapure water and heat in the microwave in intervals of 1 min until the agar is

completely dissolved. Stir with a silicone spatula between heating intervals. Let cool at room temperature for few minutes, until the agar is not extremely hot.

3) Pour the agar into the plastic beaker and mix thoroughly until obtain a homogeneous solution without clumps. Pour about 10-12 mL of this mixture in sterile Petri dishes under the hood (this volume will fill almost completely the Petri dish). Let the mixture set under the hood for about one hour.

4) Once the diet gets solidified, cover the Petri dish with its lid, seal it with parafilm on the borders, and store it inside a Ziploc bag in the frigde (+4°C) until further use. This diet will last for about one and a half month sealed in the fridge.