DISTRIBUTION, ISOLATION AND POTENTIAL FUNCTIONS OF NEUROPEPTIDE F IN THE EASTERN SUBTERRANEAN TERMITE, *Reticulitermes flavipes* (KOLLAR) (ISOPTERA: RHINOTERMITIDAE)

by

ANDREW BRADLEY NUSS

(Under the Direction of Brian Thomas Forschler)

ABSTRACT

The nervous system and digestive tract of workers, soldiers and alate reproductives of *Reticulitermes flavipes,* the eastern subterranean termite, were examined for neuropeptide F (NPF)-like immunoreactivity using an antibody to *Helicoverpa zea* midgut peptide I (*Hez* MP-I). NPF-like material was observed in approximately 70 cells in the brain and over 75 cells of the ventral nerve cord in all castes. Immunoreactive axons originating from the brain and from 15-25 neurosecretory cells on the foregut occurred over the corpora allata (CC)/corpora cardiaca (CA) complex, salivary glands, foregut, and the anterior half of the midgut. Immunoreactive axons on the rectum originated from the terminal abdominal ganglion. Over 600 NPF-like endocrine cells were counted in the midgut of all castes. A radioimmunoassay with the *Hez* MP-I antibody was used to monitor purification of *R. flavipes* NPF-like material through 8 HPLC steps from an extract of 117,300 workers (350 g). A partial amino acid sequence was determined by Edman degradation, and PCR was used to amplify a corresponding cDNA with sequence from *R. flavipes* head and midgut cDNA. The cDNA sequence codes for the following putative

translated product: VPSVWAKPSDPEQLADTLKYLEELDRFYSQVARPRFa, which was termed *Ref* NPF. This sequence possesses several conserved features common to other invertebrate NPFs and even neuropeptide Y-like peptides. This peptide was chemically synthesized and used in juvenile hormone (JH) synthesis and gut motility bioassays. JH bioassays were performed on *R. flavipes* female brachypterous neotenic corpora allata (CA) incubated in 10^{-6} M NPF or *Dippu*-allatostatin 2 (AST2) 10^{-7} M solutions. *Ref* NPF had no direct effect on JH production by CA, but slightly increased the effect of AST2 on JH inhibition. *Ref* NPF was also ineffective on the CA of the cockroach *Diploptera punctata*. The effect of *Ref* NPF, *Ang* NPF and *Dm* NPF were tested on *R. flavipes* foreguts with an impedance monitor and hindguts with a force transducer. *Ref* NPF, *Ang* NPF and *Dm* NPF did not have an effect on foregut contractions. Hindgut contractions were significantly inhibited with *Dm* NPF, even when stimulated with serotonin or Leucokinin I. *Ref* NPF and *Ang* NPF did not significantly effect hindgut contractions.

INDEX WORDS: termite, immunocytochemistry, nervous system, foregut, midgut, hindgut, neuropeptide

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DEDICATION

I also would like to dedicate this to the numerous teachers I have had over the years for instilling and encouraging my interest in the natural world.

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I can not overstate the importance of the many friends I have made during my time at UGA. These friendships were an unexpected treasure of my graduate school experience. Their support, advice and laughter have kept me going during difficult times and more importantly kept me sane.

TABLE OF CONTENTS

Page

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Many peptides are chemical messengers, or signaling molecules, that occur in both the nervous and endocrine systems and are tasked with coordinating normal bodily function. In the nervous system these may be neurotransmitters in the axons or hormones in neurosecretory cells of the ganglia. The midgut also contains numerous endocrine cells that produce peptide hormones (Orchard et al. 2001). Several of the same peptides produced in the midgut endocrine system are also found in the nervous system, a situation termed the brain-gut endocrine axis.

Discoveries of peptides acting as chemical messengers have increased in recent years aided largely by genomic searches and molecular techniques. As more information accumulates, the importance of brain-gut peptides in insects is increasingly recognized. These discoveries result from physiological or molecular studies of insect model organisms and several groups, such as the termites, have largely been ignored. In this review the neuroendocrine system of insects will be described and related to what is known more specifically pertaining to termites. In particular, the insect RFamide peptides will be reviewed, with a focus on neuropeptide F (NPF).

Cell Communication

Communication between cells in insects is facilitated by three main types of signaling molecules: neurotransmitters, hormones and neuromodulators. Neurotransmitters travel very short distances specifically at the synapse between two nerve cells. Hormones interact with cells distant from the emitting cell and are dispersed by the hemolymph to target cells. These

differences define two major strategies of cell communication in metazoans. Nerve transmission is much faster than the hormonal response and is private, but is an all or nothing response and requires an extensive infrastructure (nerves) to reach the target tissues. The hormonal response is graded, open and does not require nerves for cell communication (Nijhout 1994; Orchard et al. 2001). Somewhat in between these strategies are those cells that release neurohormones to adjacent cells but not at specific synapses. These are referred to as paracrine secretions or neuromodulators (Fujita and Kobayashi 1977; Orchard et al. 2001).

Signaling molecules can be as simple as amino acids, biogenic amines and nitric oxide. More complex molecules such as steroids are also common, but the most diverse signaling molecules are peptides. As messengers, peptides have numerous roles and are distributed in both the nervous and endocrine system (Orchard et al. 2001). Unlike nonpolar hormones which can pass through cell membranes, signaling peptides must effectively be transported "outside" of the cell after they are synthesized through the rough endoplasmic reticulum. These peptides are eventually packaged into vesicles by the Golgi complex (Brown and Lea 1990). The vesicles serve to store the peptides until a stimulus induces cellular mechanisms that result in the exocytotic release of the peptides. Released peptides interact with the receptors on the membranes of target cells. This allows for specificity of the neuropeptide response as only those cells with the appropriate receptors detect the peptide. Activated receptors may set into motion or inhibit signal cascades by phosphorylating, cleaving, or otherwise modifying cellular components that in turn activate other downstream cellular mechanisms (Voet and Voet 2004).

The neuroendocrine system

The insect central nervous system (CNS) includes the cerebral ganglion (brain), ventral ganglia and associated neurons. The CNS is critical in coordinating sensory information and

motor activity in the insect (Chapman 1998). The CNS of termites is organized in the same way as other insects (Richard 1969). The termite brain is composed of the protocerebrum, the deutocerebrum and the tritocerebrum (Thompson 1916). The optic lobes are in the protocerebrum. Also, many neurosecretory cells are concentrated in the pars intercerebralis (PI) and the lateral regions of the protocerebrum (Richard 1969). The deutocerebrum is located ventral to the protocerebrum and contains the antennal lobes. The tritocerebrum, located below the deutocerebrum, is connected to the stomatogastric nervous system through nerves to the frontal ganglion (Thompson 1916).

The retrocerebral complex of the brain consists of the corpora cardiaca (CC) and the corpora allata (CA). In termites, the CC and the CA are paired structures (Lebrun 1983). The CC/CA complex extends posterior to the brain and rests on the esophagus, but is also closely associated with the dorsal aorta (Lebrun 1983). The neurosecretory cells of the PI and lateral neurosecretory cells in the protocerebrum project to the CC via the nervi corporis cardiaci interni (NCC I), and the nervi corporis cardiaci externi (NCC II), respectively (Gillott and Yin 1972). The NCC I and NCC II merge to form the nervi corpori cardiaci (NCC) after exiting the brain but before reaching the CC (Lebrun 1983). A portion of the NCC continues beyond the CC and on to the CA (Noirot 1969).

The ventral nerve cord of termites is composed of the subesophageal ganglion, three thoracic ganglia and six abdominal ganglia. The first five abdominal ganglia are small and correspond to the first five abdominal segments while the terminal abdominal ganglion is a fusion of ganglia seven through eleven. An enlarged median nerve projects from between each of the posterior connectives of the thoracic ganglia and the first five abdominal ganglia (Richard

1969). When enlarged the median nerve is considered a perivisceral organ that facilitates storage and release of hormones from neurosecretory cells of these ganglia (Predel et al. 2004).

The stomatogastric nervous system (SNS) of insects consists of a chain of ganglia, axons and neuroendocrine cells associated with the alimentary tract. The SNS regulates food uptake and transport in the mouth cavity, foregut and midgut (Hartenstein 1997). It also coordinates swallowing of air necessary to split the old cuticle during molting, and for the expansion of the new cuticle (Ayali 2004). The three ganglia of the SNS are the frontal ganglion, the hypocerebral ganglion and the ingluvial (ventricular) ganglia. The frontal ganglion is in connection with the tritocerebrum via the frontal connectives. The frontal ganglion and hypocerebral ganglion are connected by the recurrent nerve and the hypocerebral ganglion and ingluvial ganglia are connected by the esophageal nerves (Ayali 2004). These ganglia may be fused or absent in some species, or the neurons may form a decentralized pattern of cell bodies on the alimentary tract (Hartenstein 1997). The diffuse cell bodies of the ingluvial ganglion are known as the enteric plexus and may be distributed over the surface of the foregut and anterior midgut (Hartenstein 1997). Conversely, the posterior portion of the gut tract is innervated by the proctodeal nerves that originate in the terminal abdominal ganglion. These nerves mesh with the stomatogastric nerves at the midgut/hindgut boundary (Kirby et al. 1984; Sehnal and Žitňan 1996).

The midgut

The insect midgut is the sole digestive tract region lacking a cuticular lining. It is a tubular monolayer of epithelium covered with muscle and nerves. It secretes enzymes and absorbs digested nutrients, similar to the vertebrate intestine (Chapman 1998). The midgut also contains numerous midgut endocrine cells and may be considered the largest endocrine organ in

insects (Brown et al. 1985). These cells are analogous to endocrine cells found in the vertebrate gut (Nishiitsutsuji-Uwo and Endo 1981). Midgut endocrine cells are distributed among the columnar midgut cells and have numerous secretory vesicles containing peptides. These cells originate from regenerative nidi along with the columnar cells in cockroaches (Endo and Nishiitsutsuji-Uwo 1982a; Endo et al. 1983). Several midgut endocrine cell types were described with transmission electron microscopy (TEM) with different morphology and containing vesicles with varying degrees of electron density. The open cell type has a pyramidal shape, with the apical tip reaching to the midgut lumen and the broad basal portion located along the basal lamina (Nishiitsutsuji-Uwo and Endo 1981). A specific stimulus in the midgut lumen perceived by the apical tip stimulates or inhibits the release of peptide contents of secretory vesicles at the basal and lateral surfaces of these midgut endocrine cell types (Endo and Nishiitsutsuji-Uwo 1982b; Brown et al. 1986; Jenkins et al. 1989; Zudaire et al. 1998; Hill and Orchard 2004). The closed cell type has no apical extension to the lumen but may instead release peptides in response to a mechanical stimulus such as stretching of the midgut (Fujita and Kobayashi 1977). The peptides released are thought to act in a paracrine fashion on nearby digestive or muscle cells (Vigna 1986), but may also be distributed by the hemolymph (Jenkins et al. 1989). Immunocytochemical evidence indicates midgut endocrine cells have different distributions in the midgut related to the contents of their vesicles (Brown et al. 1985; Veenstra et al. 1995). Midgut endocrine cells occur in termites but little is known about their function, morphology, or contents of their vesicles (Tokuda et al. 2001).

Several of the peptides found in midgut endocrine cells are also found in the nervous system or neurosecretory cells (Brown and Lea 1990). This circumstance is referred to as the brain-gut endocrine axis. Neuropeptide release by the midgut endocrine cells potentially

activates nerve termini on the midgut surface thereby supplying information about midgut contents to the nervous system (Brown and Lea 1990; Sehnal and Žitňan 1996). This action may induce changes in behavior or other neural controlled functions based on nutritive components in the midgut lumen.

Insect neuropeptides

The first insect neuropeptides were discovered through protein purification of extracts from large amounts of tissue coupled with physiological assays and subsequent amino acid sequencing by Edman degradation and mass spectrometry (Stone et al. 1976; Nachman et al. 1986). Later, immunoassays facilitated further purifications (Duve et al. 1981; Duve et al. 1982; Matsumoto et al. 1989). With the development of gene-cloning technology, neuropeptide cDNA and genes were cloned using degenerate primers based upon neuropeptide amino acid sequences (Nambu et al., 1988; Schneider and Taghert, 1988). More recently, the completion of genome sequencing projects has allowed large-scale homology searches to identify probable neuropeptides (Vanden Broeck 2001; Riehle et al. 2002; Hummon et al. 2006; Li et al. 2008). This was eventually coupled with mass spectroscopy of ganglia extracts to confirm expression (Baggerman et al. 2002). At the forefront of these characterizations was the highly studied insect model organism, *Drosophila melanogaster* (Vanden Broeck 2001). Several families of neuropeptide genes were described in *D. melanogaster* based on known neuropeptide sequences from insects and other organisms (Vanden Broeck 2001). Subsequently, homologous peptide genes were described in the mosquito *Anopheles gambiae*, the honeybee *Apis mellifera* and red flour beetle *Tribolium castaneum* as these genomes became available (Riehle et al. 2002; Hummon et al. 2006; Li et al. 2008). Undoubtedly, homologous genes will be found in other insects as other genomes are sequenced and annotated. One of the major neuropeptide groups

identified is broadly categorized as the RFamide superfamily. This group is widely spread among animal taxa and includes peptides with an arginine-phenylalanine-amide carboxy-(C-) terminus (Dockray 2004). The RFamide classification is primarily structural and was driven in part by early immunohistochemical data using FMRFamide antibodies that recognized several different peptides with an -RFa or -RYa C-terminus (Sehnal and Žitňan 1996). It is unclear if these peptides share a common evolutionary origin (Orchard et al., 2001) but unlikely considering each is encoded by different precursor genes (Riehle et al. 2002). In insects at least five groups occur: FMRFamide-related peptides (FaRPs), myosuppressins (FLRFamides), sulfakinins (HMRFamides), head peptides (HPs)/short neuropeptide Fs (sNPFs), and neuropeptide Fs (NPFs).

FMRFamide was the first RFamide discovered and was isolated from the sunray Venus clam, *Macrocallista nimbosa*, on the basis of its cardioexcitatory activity (Price and Greenberg 1977). FMRFamide related peptides (FaRPs) are defined by the presence of a C-terminal RFamide, a hydrophobic residue in position 3 (from the C-terminus) and an aromatic residue in position 4 (Day and Maule 1999). FaRPs have since been isolated from both vertebrates and additional invertebrates (Orchard et al. 2001). In insects, extended FMRFamides have been identified from dipterans (Orchard et al. 2001) and the American cockroach *Periplaneta americana* (Predel et al. 2004). Up to 24 peptides may be encoded on the *P. americana* FMRFamide gene (Predel et al. 2004).

Leucomyosuppressin (LMS) was first isolated by its ability to inhibit spontaneous contractions in hindguts of the cockroach *Leucophaea maderae* (Holman et al. 1986). This peptide was the first of several myosuppressins (or extended FLRFamides) to be identified from insects. Myosuppressins inhibit foregut, midgut and hindgut contractions in several insect

species (Fujisawa et al. 1993; Lange and Orchard 1997; Fuse and Orchard 1998; Orchard et al. 2001; Aguilar et al. 2004) and may also inhibit ion transport (Lee et al. 1998). This inhibition of gut contractions reduces food intake, presumably through stretch receptors as food accumulates in the foregut (Aguilar et al. 2004). LMS also stimulates an increase in the amount of active amylases and invertases released by the cockroach *Diploptera punctata* midgut (Fuse et al. 1999) and α-amylases by the weevil *Rhychophorus ferrugineus* midgut (Nachman et al. 1997).

The sulfakinins (or HMRFamides) also induce a release of digestive enzymes in *R. ferrugineus* (Nachman et al. 1997). Sulfakinins have a myotropic affect on cockroach hindgut tissue (Nachman et al. 1986; Veenstra 1989) and induce satiety (Wei et al. 2000; Maestro et al. 2001; Meyering-Vos and Muller 2007). The invertebrate sulfakinins share a common (D/E)-(D/E)-Y-(SO_3H)-G-H-(M/L)-RFamide C-terminus with a sulfated tyrosine and are related to the cholecystokinin (CCK) family in vertebrates (Maestro et al. 2001).

Short neuropeptide F has been identified from fruit flies (Vanden Broeck 2001), mosquitoes (Riehle et al. 2002), beetles (Spittaels et al. 1996; Li et al. 2008), and cockroaches (Veenstra and Lambrou 1995). The conserved C-terminus sequence of sNPFs has been described as R(K)-X_1-R-X_2amide where X_1 is Leu, Thr or Pro and X_2 is an aromatic residue such as Phe or Tyr (Mertens et al. 2002). Although this sequence bears some resemblance to "long" NPF, these peptides are encoded on separate genes (Vanden Broeck 2001). Feeding and body size of *D. melanogaster* are influenced by sNPF (Lee et al. 2004). In addition, sNPF inhibits peristaltic activity of the mosquito *Aedes aegypti* midgut (Onken et al. 2004). Led-sNPF I and II may play a role in inhibiting diapause as they are absent from the cephalic ganglia of overwintering Colorado potato beetles, *Leptinotarsa decemlineata* (Huybrechts et al. 2004).

Led-sNPF I also accelerates ovariole development in the locust *Locusta migratoria* (Cerstiaens et al. 1999). Longer NPFs will be discussed in more detail in the next section.

Head peptides resemble sNPF, but the relationship between them is not clear. As yet, head peptides have been isolated from only one species, *Ae. aegypti* (Matsumoto et al. 1989). Although they have considerable sequence similarity to sNPFs, this species possesses an additional complement of neuropeptides encoded on a separate gene that corresponds more directly with known sNPFs (Riehle et al. 2002). It is not known if the head peptides are a result of sNPF gene duplication in this one species or if this represents a more widespread neuropeptide group that has been secondarily lost in other species (Riehle et al. 2002). Head peptides have been shown to inhibit host-seeking behaviors by adult females of *Ae. aegypti* (Brown et al. 1994). They also have the same affect on midgut activity as do the sNPFs (Onken et al. 2004).

Neuropeptide F, Neuropeptide Y, Peptide YY and Pancreatic Polypeptide

The NPY-related superfamily includes neuropeptide Y (NPY), peptide YY (PYY) and pancreatic polypeptide (PP) common to vertebrates and the neuropeptide F (NPF) family in invertebrates (McVeigh et al. 2005). The vertebrate forms are all 36 amino acids in length and share a common PP-fold secondary structure (Larhammar 1996). NPY is found strictly in the nervous system and is the most abundant peptide in the mammalian brain (Larhammar 1996). In the CNS it has a number of functions including stimulation of feeding, hypogonadism, regulation of pituitary secretion, regulation of alcohol consumption and sedation (Pedrazzini et al. 2003), reduction of anxiety, regulation of aggression (Karl and Herzog 2007) and nociception (Hokfelt et al. 2007). NPY is also a vasoconstrictor in the peripheral nervous system (PNS) (Pedrazzini et al. 2003). PP and PYY are primarily expressed in endocrine cells of the digestive system, but PYY also occurs in the nervous system (Berglund et al., 2003). PP inhibits pancreatic secretion,

gall bladder activity and intestinal motility. PYY also induces these effects, induces satiety and inhibits ion transport in the intestine (Berglund et al. 2003).

Invertebrate NPFs are homologs of vertebrate NPY-related peptides. NPFs are between 36 and 40 amino acids in length and share an R-X-R-(F/Y)-amide C-terminus (McVeigh et al. 2005). Most NPFs have a Phe or Tyr residue 10 and 17 residues from the C-terminus, a feature shared with vertebrate PPs (Maule et al. 1991). NPF from the sheep intestinal cestode, *Moniezia expansa,* has an α-helix secondary structure from residues 14 to 31 similar to that in NPY (Miskolzie and Kotovych 2002). However, the lack of a PXXPXXP motif on the N-terminal end of NPFs indicates that they may not share the full PP-fold of vertebrate NPY/PYY/PP peptides (Larhammar 1996).

The first invertebrate NPF was isolated from the flatworm *M. expansa* (Maule et al. 1991). This was followed by the discovery of several other platyhelminth and mollusk NPFs (Leung et al. 1992; Rajpara et al. 1992; Tensen et al. 1998; Dougan et al. 2002; Humphries et al., 2004). The existence of such a peptide in insects was suggested by several immunocytochemical studies with antibodies to mammalian PP and NPY on the CNS and SNS (Duve and Thorpe 1980; El-Salhy et al. 1980; Iwanaga et al. 1981; Duve and Thorpe 1982; Endo et al. 1982; El-Salhy et al. 1983; Myers and Evans 1985; Brown et al. 1986; Iwanaga et al. 1986; Schoofs et al. 1988). After numerous attempts (Duve et al. 1981; Duve et al. 1982; Schoofs et al. 1988; Verhaert et al. 1993), the first insect NPF was identified from *D. melanogaster* (Brown et al. 1999). Sequence identification of other insect NPFs has followed (Stanek et al. 2002), some of which were guided by genomic analysis or bioinformatics approaches (Riehle et al. 2002; Clynen et al. 2006; Hummon et al. 2006). Although several identified insect NPFs clearly are similar to other insect NPFs in length and sequence, they lack the F/Y residues at positions 10 (*Aea* NPF,

Ang NPF and *Cup* NPF) and 17 (*Peh* NPF, *Bom* NPF I, *Aea* NPF and *Cup* NPF) from the C-terminus (Fig. 1.1), a feature preserved in such distant groups as flatworms and vertebrates (McVeigh et al. 2005). Small (5 - 9 amino acids long) invertebrate peptides have also been identified that have an NPF-like C-terminal sequence (Smart et al. 1992; Huang et al. 1998; Schoofs et al. 2001; Sithigorngul et al. 2002; Huybrechts et al. 2005). These are thought to be breakdown products of full-length (36 - 40 amino acid) NPFs. Whether they are indeed the functional forms of the native peptides is not known. Some have been classified as sNPFs, but for the locust and lepidopteran truncated NPFs, discovery of subsequent sequences from related species has revealed full-length NPF amino acid sequences with nearly identical C-termini (Clynen et al. 2006; Joe Crim, unpublished data) (Fig. 1.1).

Synthesis of NPY-related/NPF peptides is conserved through animal lineages (McVeigh et al. 2005). A signal peptide is coded for within the open reading frame of NPY-related/NPF mRNA, followed by the pre-propeptide sequence. As protein synthesis is initiated, the signal peptide is cleaved after the ribosome attaches to a translocon on the ER, allowing synthesis of the propeptide into the ER lumen. The propeptide is cleaved at an N-terminal di- or monobasic residue (Arg or Lys) by a prohormone convertase and the C-peptide is processed at a dibasic cleavage site following a C-terminal Gly (McVeigh et al. 2005). The Gly residue is processed by peptidyl α-hydroxylating monooxygenase (PHM) and peptidyl α-hydroxyglycine α-amidating lyase (PAL) to amidate the C-terminal Tyr/Phe residue (McVeigh et al. 2005). This amidation is critical for interaction with receptors (Eipper et al. 1993). The transcript for *M. expansa* NPF is an exception in that peptide synthesis is halted after the codon for glycine resulting in a product that requires no cleavage but is still amidated (Mair et al. 2000). Another similarity with NPY-like peptides occurs in the form of an intron in the invertebrate NPF gene that corresponds with

an intron that occurs in all vertebrate NPY, PYY and PP peptide genes. This intron is present before the coding region for the penultimate Arg residue, but is variable in length (Mair et al. 2000). The intron lends further support to the idea that the NPY/NPF peptides have a common evolutionary origin, but some invertebrates such as *Schistosoma japonicum* and *S. mansoni* lack this intron (Humphries et al. 2004). Among insects this intron has been noted in *An. gambiae* (Garczynski et al. 2005), *Ae. aegypti, Culex pipiens*, *Pediculus humanus*, *A. mellifera,* and *Bombyx mori* (Joe Crim, personal communication) but is absent in *D. melanogaster* (Joe Crim, personal communication).

NPF signaling

Five receptors have been discovered in mammals that are responsive to the NPY/PPY/PP peptides, and are commonly referred to as Y receptors. These receptors have distinct localizations and different affinities for NPY/PYY/PP. All are G-protein coupled receptors (GPCR) in the rhodopsin family of GPCRs and inhibit adenylyl cyclase which produces cAMP (Pedrazzini et al. 2003). These receptors also stimulate an increase in Ca^{2+} influx (Larhammar 1996). NPF receptors in invertebrates are also closely related GPCRs. The NPF receptor from *D. melanogaster* (NPFR1) was identified through genomic searches and subsequent cloning guided by the *Lymnaea stagnalis* NPF receptor (Tensen et al. 1998) and putative *D. melanogaster* NPY receptor sequences (Li et al. 1992; Garczynski et al. 2002). NPF receptors in *D. melanogaster* occur in the brain, ventral nerve cord and in the midgut of larvae (Garczynski et al. 2002). Subsequently, the *An. gambiae* NPF receptor was identified and characterized using a similar methodology to the *D. melanogaster* NPF receptor isolation (Hill et al. 2002; Garczynski et al. 2005). These receptors are similar to the NPF receptor of *L. stagnalis*. Like the Y receptors, the NPF receptors inhibit cAMP accumulation (Garczynski et al. 2005).

A few functions have been discovered for NPF in insects, and of these several relate to feeding behavior or digestion. Ion transport and motility is inhibited by NPF in *Ae. aegypti* larval midguts (Onken et al. 2004). NPF may also play a role in adult digestion as NPF titers drop in *Ae. aegypti* adult females in the 24 h following a blood meal (Stanek et al. 2002). In larval *D. melanogaster*, consumption of fructose, glucose or aspartame increases the amount of *Dm* NPF transcript and peptide in the subesophageal ganglion (Shen and Cai 2001).

Other NPF studies have used transgenic *D. melanogaster* to study behavioral affects of ablated cells expressing *Dm* NPF or overexpressed *Dm* NPF or NPFR1 genes. *Dm* NPF is not required for normal development in the laboratory, but it instigates food-searching behaviors when larvae are under food-deprived conditions (Wu et al. 2003). *Dm* NPF also promotes feeding on noxious food (Wu et al. 2005b), or under cold conditions when feeding is deleterious (Lingo et al. 2007). These actions indicate that NPF functions as a general "starvation peptide" promoting risky behaviors that will result in the finding of new food resources or acceptance of less preferred resources for the sake of survival. This has similarities to the actions of NPY in vertebrates, which promotes feeding, conserves energy by downregulating certain body systems, and lowers anxiety which permits more risky behaviors for obtaining food (DiBona 2001; Karl and Herzog 2007). This may tie in with the insulin signaling system as the *D. melanogaster* NPF response is partially controlled by *Drosophila* insulin-like peptides (DILPs) 2 and 4, which are in turn regulated by *Drosophila* p70/S6 kinase (dS6K) (Wu et al. 2005a). *Dm* NPF also inhibits the larval excitatory response to glucose and pupation-site searching behavior (Wu et al. 2003).

Transgenic *D. melanogaster* have also been used to explore the role of NPF in adult insects. *Dm* NPF regulates sensitivity of adults to alcohol sedation (Wen et al. 2005). Also, *Dm* NPF expression is partially controlled by the transformer (tra)-dependent sex-determination

pathway in males and results in additional NPF-expressing brain cells compared to females. Ablation of male-specific *Dm* NPF cells leads to an inability to initiate courtship (Lee et al. 2006). Some evidence also suggests that NPF inhibits aggression in adult males (Dierick and Greenspan 2007).

The wide distribution of NPF-like material in the CNS and stomatogastric nervous system of several insects hint at other functions of this peptide that remain to be characterized. NPF neurons in the thoracic ganglia of the tobacco hornworm, *Manduca sexta*, also express receptors for ecdysis-triggering hormone, suggesting NPF may have a role in ecdysis (Kim et al. 2006). Also, there is evidence that NPF inhibits egg release in the desert locust, *Schistocerca gregaria* (Schoofs et al. 2001).

Termite endocrinology

Little is known about the endocrinology of peptide hormones in termites. No information is available regarding NPF in termites. A diuretic hormone has been isolated from a dampwood termite, *Zootermopsis nevadensis* (Baldwin et al. 2001), and peptides similar or identical to *P. americana* cardioaccelerating hormone were isolated from the termites *Mastotermes darwiniensis* (giant northern termite), *Microhodotermes viator* (southern harvester termite), and *Trinervitermes trinervoides* (snouted harvester termite) (Liebrich et al. 1995). A peptide with the same mass as LMS was detected in the CC of *Hodotermes mossambicus* with MALDI-TOF mass spectrometry, but has not been further characterized (Predel et al. 2001). Also, immunostaining with an antibody to pigment-dispersing hormone showed numerous cell bodies and axons in the brain of *Neotermes castaneus* (Sehadova et al. 2003). The production of juvenile hormone (JH) by the CA is regulated by specific peptide hormones namely allatotropin which stimulates JH production and allatostatin which inhibits it (Stay 2000). *Dippu*-allatostatin inhibits JH

production of female and male *Reticulitermes flavipes* (eastern subterranean termite) neotenic CA suggesting a similar peptide occurs in this insect (Yagi et al. 2005). Axons with RFamide-like immunoreactivity have been detected on the surface of the CA in different insect species. SKPANFIRFamide and LMS block the inhibitory effect of allatostatin on JH synthesis in *D. punctata* females on day 6 of the vitellogenic cycle but were ineffective at all other times tested (Stay et al. 2003). *Schistocerca* NPF has not been shown to effect JH production of the CA in the grasshopper *Romalea microptera* (Li et al. 2005).

Ecdysteroids influence reproduction of physogastric termite queens (Noirot and Bordereau 1990) and may reduce the production of JH by the CA (Brent et al. 2005). In contrast to peptide hormones, the functions of JH in termites have been much more extensively studied. JH III is the only form of JH that has been found in termites (Greenberg and Tobe 1985; Park and Raina 2004; Yagi et al. 2005), and it plays a role in reproduction. JH may stimulate vitellogenin production by the fat body for incorporation into the yolk of developing ovarioles (Lüscher 1976; Greenberg and Tobe 1985). An increase in JH titer in *R. flavipes* neotenics was associated with early development of vitellogenic ovarioles (Elliott and Stay 2007). JH titers also are higher in male reproductives but to a much smaller degree than females (Noirot and Bordereau 1990; Yagi et al. 2005).

The differentiation of termite castes is also regulated by JH. Many studies have used JH or JH analogs to determine its role in caste differentiation (Noirot and Bordereau 1990). In lower termites a general model has been proposed where the developmental pathway is determined by JH sensitive periods during the instar. Towards the beginning of the instar, high JH titers abolish reproductive characteristics resulting in workers and soldiers (Yin and Gillott 1975). After this, a second JH sensitive period regulates characteristics of the reproductives

where a high JH titer inhibits expression of alate characters (Nijhout and Wheeler 1982). Closer to the end of the instar, high JH titers induce soldier differentiation (Nijhout and Wheeler 1982).

Although many studies have investigated the role of JH in caste differentiation, little is known about the cellular mechanisms JH activates to manipulate development in termites or how JH titers themselves are regulated. Much evidence suggests that JH levels in workers are modulated by inhibitory pheromones produced by soldiers and reproductives that are passed between nestmates (Lefeuve and Bordereau 1984; Park and Raina 2003; Mao et al. 2005; Park and Raina 2005). In *R. flavipes*, hexamerins with JH-binding capacity also appear to influence caste differentiation prior to JH signal transduction but the dynamics involved are not well understood (Zhou et al. 2006).

Conclusion

Social complexity has limited termites as model organisms for endocrinological studies. A better understanding of termite signaling systems may allow specific control strategies that are alternatives to the current chemical management methods (Gade and Goldsworthy 2003). For instance, knockout of NPF or NPFR1 genes did not adversely affect development of *D. melanogaster* in the laboratory (Wen et al. 2005), but disruption of NPF signaling might severely affect food acquisition and survival in the natural environment. In addition, further knowledge of NPFs in a more primitive social insect group will broaden our understanding of the function of these peptides and the evolutionary mechanisms maintaining them. Termites are particularly interesting in this regard because they represent hemimetabolous insects, a group in which only one full NPF has been identified from an expressed sequence tag database from *L. migratoria* heads (Clynen et al. 2006). Although the sequences and distributions of specific neuropeptides suggest conserved functions among different insect groups, some additional functions may have

been acquired, superseded by other neuropeptides or lost in some species. Comparisons will be particularly interesting in insects related to termites such as cockroaches and orthopterans where substantial neuropeptide research has been performed, but little of it specifically with NPF.

The following chapters of this dissertation attempt to determine the distribution, amino acid sequence and some functions of NPF in *R. flavipes*.

CHAPTER 2

The distribution of NPF-like material in the R. flavipes brain, ventral nerve cord and alimentary tract in workers, soldiers and alates.

An antiserum to *Helocoverpa zea* midgut peptide I (*Hez* MP-I) recognizes NPF-like peptides (Brown et al. 1999). Whether NPF-like peptides occur in *R. flavipes* and in what distribution was examined with whole-tissue immunochemistry employing the *Hez* MP-I antiserum. Caste-specific differences in NPF-like distribution were also determined. The distribution of NPF-like material in both nervous system and midgut endocrine system suggests possible functions of this peptide or peptides.

CHAPTER 3

Identification of R. flavipes NPF and cDNA sequencing.

To elucidate the amino acid sequence of *R. flavipes* NPF, an extract of *R. flavipes* workers was purified with HPLC, as monitored with a RIA using the *Hez* MP-I antiserum. A partial peptide sequence facilitated amplification of its encoding cDNA with degenerate and specific primers and subsequent sequencing to determine the encoded amino acid sequence. Knowledge of the amino acid sequence allowed chemical synthesis of *Ref* NPF.

CHAPTER 4

Affect of Ref NPF on isolated R. flavipes corpora allata and selected gut regions.

The distribution of NPF-like material in *R. flavipes* described in chapter 2 suggested functions for this peptide in the alimentary tract and CA, among other areas. JH production by the CA can be monitored by a radiochemical assay (Tobe and Pratt 1974; Elliot and Stay 2007). Muscle activity of foreguts and hindguts may be measured by an impedance monitor (Orchard and TeBrugge 2002) or force transducer apparatus (Pascaud et al. 1978; Elia and Orchard 1995). *Ref* NPF, characterized in chapter 3, can be used with these methods to determine possible functions in termites.

The results from these studies provide a better understanding of the neuroendocrine system of *R. flavipes* and improve our knowledge of the NPF family of peptides.

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Table 1.1: Invertebrate NPF-like sequences. [a]Sequence determined by Edman degradation. [b]Sequence determined by cloning. [c]Sequence determined by bioinformatics.

Phylum	Class		Sequence	Reference
Arthropoda (Subphylum Atelocerata)	Hexopoda	*Schistocerca gregaria* NPF	**YSQVARPRFa**	Schoofs et al. 2001[a]
		Locusta migratoria NPF	**AEAQQADGNKLEGLADALKYLQELDRFYSQVARPRFa**	Clynen et al. 2006[c]
		Pediculus humanus NPF	**TSTAETDQRKMKSMAEVLQILQNLDKYYTQAARPRFa**	Crim, unpublished data[c]
		Drosophila melanogaster NPF	**SNSRPPRKNDVNTMADAYKFLQDLDTYYGDRARVRFa**	Brown et al. 1999[a, b]
		Aedes aegypti NPF	**SFTDARPQDDPTSVAEAIRLLQELETKHAQHARPRFa**	Stanek et al. 2002[a, b]
		Culex pipiens NPF	**LTEARPQDDPTSVAEAIRLLQELETKHAQHARPRFa**	Crim, unpublished data[c]
		Anopheles gambiae NPF	**LVAARPQDSDAASVAAAIRYLQELETKHAQHARPRFa**	Riehle et al. 2002[c]
		Bombyx mori NPF I	**LVCMAEAREEGPNNVAEALRILQLLDNYYTQAARPRYa**	Crim, unpublished data[c]
		Bombyx mori NPF II	**QYPRPRRPERFDTAEQISNYLKELQEYYSVHGRGRYa**	Crim, unpublished data[c]
		Helicoverpa zea MP-I	**QAARPRFa**	Huang et al. 1998[a]
		Helicoverpa zea MP-II	**AARPRFa**	Huang et al. 1998[a]
		Apis mellifera NPF	**EPEPMARPTRPEIFTSPEELRRYIDHVSDYYLLSGKARYa**	Hummon et al. 2006[c]
Arthropoda (Subphylum Crustacea)	Branchiopoda	*Daphnia magna* NPF	**DGDVMGGGEGGEMTAMADAIKYLQGLDKVYGQAARPRFa**	Christie et al. 2008[c]
	Malacostraca	*Marsupenaeus japonicus* NPF	**KPDPSQLANMAEALKYLQELDKYYSQVSRPRFa**	Christie et al. 2008[c]
		Penaeus monodon PYF I	**RAFPRFa**	Sithigorngul et al. 2002[a]
		Penaeus monodon PYF II	**YSQVSRPRFa**	Sithigorngul et al. 2002[a]
		Penaeus monodon PYF III	**YAIAGRPRFa**	Sithigorngul et al. 2002[a]
		Penaeus monodon PYF IV	**YSLRARPRFa**	Sithigorngul et al. 2002[a]
Annelida	Clitellata	*Lumbricus rubellus* NPF	**ADGPPVRPDRFRTVAELNKYMADLTEYYTVLGRPRFa**	Crim, unpublished data[c]
Mollusca	Cephalopoda	*Loligo vulgaris* PYF	**YAIVARPRFa**	Smart et al. 1992[a]
	Gastropoda	*Aplysia californica* NPF	**DNSEMLAPPPRPEEFTSAQQLRQYLAALNEYYSIMGRPRFa**	Rajpara et al. 1992[a, b]
		Lymnaea stagnalis NPY	**TEAMLTPPERPEEFKNPNELRKYLKALNEYYAIVGRPRFa**	Tensen et al. 1998[a]
		Helix aspersa NPF	**STQMLSPPERPREFRHPNELRQYLKELNEYYAIMGRTRFa**	Leung et al. 1992[a]
Platyhelminthes	Cestoda	*Moniezia expansa* NPF	**PDKDFIVNPSDLVLDNKAALRDYLRQINEYFAIIGRPRFa**	Maule et al. 1991[a]; Mair et al. 2000[b]
	Trematoda	*Schistosoma japonicum* NPF	**AQALAKLMTLFYTSDAFNKYMENLDAYYMLRGRPRFa**	Humphries et al. 2004 [b, c]
		Schistosoma mansoni NPF	**AQALAKLMSLFYTSDAFNKYMENLDAYYMLRGRPRFa**	Humphries et al. 2004 [b]
	Turbellaria	*Arthurdendyus triangulatus* NPF	**KVVHLRPRSSFSSEDEYQIYLRNVSKYIQLYGRPRFa**	Curry et al. 1992[a]; Dougan et al. 2002[b]

CHAPTER 2

DISTRIBUTION OF NEUROPEPTIDE F-LIKE IMMUNOREACTIVITY IN THE EASTERN SUBTERRANEAN TERMITE, *Reticulitermes flavipes* (ISOPTERA: RHINOTERMITIDAE)[1]

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Abstract

The nervous system and gut of worker, soldier and alate castes of the eastern subterranean termite, *Reticulitermes flavipes* (Kollar) (Isoptera: Rhinotermitidae) were examined for immunoreactivity to an antiserum to *Helicoverpa zea* (Boddie) (Leipidoptera: Noctuidae) MP-I (QAARPRF-NH_2), a truncated form of neuropeptide F. More than 145 immunostained axons and cell bodies were seen in the brain and all ganglia of the ventral nerve cord. Immunoreactive axons exiting the brain projected anteriorly to the frontal ganglion and posteriorly to the corpora cardiaca and corpora allata. In the stomatogastric nervous system, immunoreactive axons were observed over the surface of the foregut, salivary glands, midgut and rectum. These axons originated in the brain and from 15-25 neurosecretory cells on the foregut. Staining patterns were consistent between castes, with the exception of immunostaining observed in the optic lobes of alates. At least 600 immunoreactive endocrine cells were evenly distributed in the midguts of all castes with higher numbers present in the worker caste. Immunostaining of cells in the nervous system and midgut was blocked by preabsorption of the antiserum with *Hez* MP-I but not by a peptide having only the RF-NH_2 in common. This distribution suggests NPF-like peptides coordinate feeding and digestion in all castes of this termite species.

Keywords: immunocytochemistry, nervous system, midgut

1. Introduction

The invertebrate neuropeptide Fs (NPFs) are members of a neuropeptide family that includes three related vertebrate peptides, neuropeptide Y (NPY), peptide YY (PYY), and pancreatic polypeptide (PP) (Brown et al. 1999; Berglund et al. 2003; Pedrazzini et al. 2003; Conlon and Larhammar 2005). Both NPFs and the NPY-related peptides are encoded by homologous genes and processed from propeptides into a bioactive peptide with 36 to 40 amino acids and a Phe or Tyr-NH_2 carboxy (C-) terminus. The first insect NPF was isolated from *Drosophila melanogaster* (Brown et al. 1999) and later, a related one was isolated from the mosquito, *Aedes aegypti* (Stanek et al. 2002). Since then, other insect NPFs have been identified by bioinformatics in the genome databases of *Anopheles gambiae* (Garczynski et al. 2005), and *Apis mellifera* (Hummon et al. 2006), all of which are holometabolous species. To date, NPF has been identified by bioinformatics from only a single hemimetabolous species, *Locusta migratoria* (Clynen et al. 2006). Truncated forms (8 to 10 amino acids) of apparent NPFs have been isolated from the corn earworm, *Helicoverpa zea* (Huang et al. 1998) and the desert locust, *Schistocerca gregaria* (Schoofs et al. 2001).

Immunocytochemical studies of insects provided the first evidence for vertebrate-like peptide hormones in insects. In particular, studies using PP or NPY antisera showed that immunoreactive peptides were localized in specific cells in the nervous system and midgut of cockroaches, crickets, locusts, flies and moths (El-Salhy et al. 1980; Iwanaga et al. 1981; Duve and Thorpe 1982; Endo and Nishiitsutsuji-Uwo 1982b; El-Salhy et al. 1983; Myers and Evans 1985; Brown et al. 1986; Iwanaga et al. 1986; Schoofs et al. 1988). In hindsight, the objective of identifying a gut-specific NPF from an insect was attained with the isolation of the two midgut peptides, *Hez* MP-I (Table 2.1) and –II, from corn earworm larvae, *Helicoverpa zea* (Huang et

al., 1998). Their chromatographic purification was driven with a radioimmunoassay (RIA) using an Arg-Phe-NH_2 antiserum. Subsequently, an antiserum specific to *Hez* MP-I was produced and used in RIAs to monitor isolation of the *D. melanogaster* NPF (Brown et al. 1999). Using immunocytochemistry, the *Hez* MP-I antiserum showed that the NPF was present in relatively few brain neurons and neurosecretory cells and in many midgut endocrine cells in *D. melanogaster* larvae and adults. In vertebrates, the NPY-related peptides also display expression as a brain-gut axis that regulates feeding behavior and digestion (Neary et al. 2005; Cox 2007). Recent studies show that NPFs also affect feeding and digestion in insects. For mosquito larvae, NPF inhibits peristalsis and ion transport of the midgut in vitro (Onken et al. 2004). In *D. melanogaster,* alterations in the gene for NPF and its receptor are associated with specific feeding and food-searching behaviors of larvae particularly under food-deprived conditions (Shen and Cai 2001; Wu et al. 2003; Wu et al. 2005; Lingo et al. 2007).

As a first step to determine whether an NPF exists in termites and if it affects feeding, *Hez* MP-I antibody was used to map and compare the distribution of NPF-like immunoreactivity in the brain, ventral nerve cord and alimentary tract of alate, worker, and soldier castes of the eastern subterranean termite, *Reticulitermes flavipes* (Kollar) (Isoptera: Rhinotermitidae). Different feeding behaviors have been observed among castes of this species. The worker caste feeds directly on wood or other cellulose-rich substrates, whereas the soldier and alate castes rely on food shared by the workers (Laine and Wright 2003). To date, only three peptide hormones have been isolated and structurally characterized from termites: *Zootermopsis nevadensis* diuretic hormone (*Zoone* DH) (Baldwin et al. 2001), *Microhodotermes viator* corpus cardiacum peptide (*Miv*-CC) and *Periplaneta americana* cardioaccelerating hormone (*Pea*-CAH-I) from *Mastotermtes darwiniensis* and *Tritervitermes trinervoides* (Liebrich et al. 1995). The

immunocytochemical distribution of pigment-dispersing hormone was reported in the central nervous system of *Neotermes castaneus* (Sehadova et al. 2003) and *Dippu*-allatostatin-like immunoreactivity in the brain of *R. flavipes* (Yagi et al. 2005). Termites are economically important structural pests worldwide (Su and Scheffrahn 2000), and antagonists of peptide hormones could be used to disrupt the internal signaling systems of pest insects as a potential management tool (Orchard et al. 2001; Gade and Goldsworthy 2003) providing an alternative to the chemistries currently used for termite management.

2. Materials and Methods

2.1 Animals

R. flavipes were collected from 12 different termite-infested logs in Whitehall Forest south of the University of Georgia campus. Workers and soldiers were attracted from logs into PVC tubing containing moistened, rolled cardboard. Termites were transferred to plastic boxes containing moistened slats of pine wood and filter paper and stored at 24˚C in total darkness. Workers and soldiers used for immunocytochemistry were taken between 7 - 66 days after collection. Alates were captured as they emerged from logs in the lab during this species' swarming season (January through April). Alates were either used within one to two days after emergence or male and female pairs were placed together in nesting material for 6 days before use.

2.2 Morphological Measurements

The brain and ventral ganglia of alates ($n \geq 2$), soldiers ($n \geq 6$) and 4th instar or larger workers ($n \geq 5$) were dissected in phosphate buffered saline (PBS), and mounted on microscope slides. Images were taken with a digital camera (JVC America Corp., model KY-F70BU, www.jvc.com) mounted on an Olympus BX60 microscope (www.olympus.com) using Auto-

Montage Pro software (Synoptics Ltd., version 5.01.0005, 2004, www.synoptics.co.uk). Auto-Montage Pro was used to take measurements of length and width for each ganglion and length of ganglial connectives. Diagrams of the caste nervous systems were created from digital images of representative ganglia that were traced in Adobe Photoshop CS (Adobe Systems Inc., version 8.0, 2004, www.adobe.com) for each caste examined.

Alimentary tracts of workers ($n \geq 6$) and soldiers ($n \geq 4$) were dissected, mounted and photographed as above. Tissues were partially unfolded so that the entire alimentary tract could be observed. Length and width of each gut region were measured with Auto-Montage Pro. A generalized diagram of the *R. flavipes* alimentary tract was created from digital images for a description of immunocytochemical data.

2.3 Whole Tissue Immunochemistry

Brain, ventral nerve cord and alimentary tract were dissected from alates, soldiers and 4th instar or larger workers in 4% paraformaldehyde/PBS solution (pH 7.4). Tissues were fixed for 1 h in the paraformaldehyde solution at 4 ºC and then dehydrated in 15 min washes of ethanol/PBS solution in the following series: 30, 50, 70, 100%, and rehydrated in PBS/70% ethanol for 15 min, and PBS for 15 min. Goat serum (5%) with 0.1% Tween 20 (PBS-GS-T) was used to block tissues for 1 hour. *Hez* MP-I antibody (35B, Huang et al. 1998) was diluted 1/800 in PBS-GS-T and incubated with tissues overnight at 4 ºC. Tissues were washed with PBS-GS-T (3 x 30 min) and then incubated with goat-anti rabbit Alexa Fluor 488F® (Molecular Probes, Eugene, OR, USA, 1:2000 in PBS-GS-T, www.probes.com) overnight. Finally, tissues were washed with PBS-T (3 x 30 min), and then mounted on glass slides in a 1:1 PBS/glycerol solution. Controls were performed by preabsorbing primary antibody with FMRF-NH_2 (3.3×10^{-5} M), *Hez* MP-I (7.3×10^{-5} M), *Ang* sNPF I (6.3×10^{-5} M), or *Ang* NPF (1.2×10^{-4} M) (Table 2.1) for 24 h prior to

tissue incubation or by omitting the primary antibody. Specimens were viewed and photographed using the same microscope equipped with epifluorescent optics mentioned above, and at least three of the antiserum and control-treated tissues from individuals of the different castes were observed for the results reported herein.

2.4 Transmission Electron Microscopy

Midguts from worker *R. flavipes* were dissected and fixed according to the procedure of Grube et al. (1997). Briefly, worker midguts were fixed in 2% glutaraldehyde / 0.1 M cacodylate buffer solution (pH 7.0) for 20 h at 4 ˚C. A cacodylate buffer rinse was followed by a secondary fix in 1% OsO_4 (1 h at 4 ˚C). Tissues were washed in 0.1 M cacodylate buffer (15 min at 4 ˚C), H_2O (2x, 15 min at 4 ˚C), ethanol (30, 50, 70, 95, 100, 100, 100%, 15 min each, 4 ˚C), and propylene oxide (15 min at 4 ˚C). Midguts were embedded in Epon 812 resin and polymerized overnight at 65 ˚C. Sections (~40 nm) were stained with uranyl acetate and lead citrate and photographed with a JEOL 100CX II transmission electron microscope.

3. **Results**

3.1 Nervous System - Morphology

The central nervous system in the three castes of *R. flavipes* is comprised of a brain, frontal ganglion, subesophageal ganglion (Thompson 1916), three thoracic ganglia and six abdominal ganglia joined by parallel axon tracts (Figure 2.1). The first five abdominal ganglia are nearly identical in size, but the sixth, a fusion of five terminal ganglia (Richard, 1969), is larger (Table 2.2).

The brain and ventral ganglia of the alate caste were larger than those of workers and soldiers (Table 2.2). Alate brains had well developed optic lobes compared to the reduced optic lobes of soldiers and workers. The subesophageal ganglion was similar in size between soldiers

and alates but smaller in workers. Brain and ventral ganglia proportions were similar in soldiers and workers, but ganglial connectives between the subesophageal ganglion and the first thoracic ganglion were approximately twice as long in the soldiers than those of workers or alates to accommodate the longer head length.

3.2 Brain and Frontal Ganglion

Immunoreactive cells were observed in all regions of the central nervous system of the three castes examined, except for the frontal ganglion. In all castes, strong immunostaining was observed in a pair of neurosecretory cells in the protocerebrum and another pair on the anterior distal margin of the protocerebrum (Figures 2.2, 2.3).

Immunoreactive cell bodies in the brain were counted for each caste (Table 2.3). On average, approximately 70 immunoreactive cells were counted per brain and no significant differences in number of cells were found between castes ($F = 0.03$; df = 2; $P = 0.967$). The number of immunoreactive brain cells varied widely between individuals of a caste. For soldiers the range was 32 to 117 immunoreactive cells and for workers 31 to 94 immunoreactive cells. The proportion of immunoreactive cells in various brain regions was similar within a caste (Table 2.4). Many immunoreactive cells were observed in the tritocerebrum (43% of the immunoreactive brain cells in soldiers and 25% in workers) and in the protocerebrum (30% in soldiers and 51% in workers) (Table 2.4, Figures 2.2, 2.3). In alate brains, immunoreactive axons were observed within the optic lobes (Figure 2.3A) but not in those of workers or soldiers.

Immunoreactive axons were observed on the surface of the corpora cardiaca and the corpora allata of all castes (Figure 2.4), but no immunoreactive cell bodies were observed. The corpora allataof alates were larger and more rounded than those of workers or soldiers.

3.3 Ventral Nerve Cord

In all castes, three brightly stained cells were consistently seen on the ventral surface of the subesophageal ganglion: a pair of cells on the posterior and a single cell on the anterior surface (Figure 2.5A). Although variable, the total number of immunoreactive cells in this ganglion was highest in workers followed by the soldiers and alates (Table 2.3) but differences were not significant ($F = 1.55$; df = 2; $P = 0.251$).

The thoracic ganglia in all castes contained eight brightly stained cells: two cells on the dorsal anterior of the ganglia, and six cells, in groups of three, on the ventral center of the ganglia (Figure 2.5B). The total number of immunoreactive cells counted in thoracic ganglia was similar in all castes (Table 2.3).

Abdominal ganglia 1-5 had from one to eight immunoreactive cells (Table 2.3, Figure 2.5C), but most lacked strong immunoreactivity. Each abdominal ganglion had a single pair of immunoreactive axons that extended along the abdominal body wall, although the exact tissue innervated was not determined. The terminal abdominal ganglion contained an average of 7 brightly stained cells for all castes. No clear distribution pattern for these cells was evident. Workers had more total immunoreactive cell bodies in the terminal abdominal ganglion than soldiers, and soldiers had more than the alates, although these differences were not significant ($F = 1.04$; df = 2; $P = 0.392$) (Table 2.3).

3.4 Stomatogastric Nervous System

Immunoreactive axons were associated with the alimentary tract (Figure 2.6) in all three castes. Immunoreactive axons projected to the frontal ganglion from the tritocerebrum. A single, large, immunoreactive axon tract originating in the frontal ganglion was present on the esophagus (Figure 2.7A). It branched over the surface of the esophagus and salivary (labial)

glands and gland reservoirs (Figure 2.7B) and extended along the esophagus to divide over the crop after the ingluvial ganglion (Figure 2.7B). Five to eight immunoreactive cell bodies were in this ganglion. The axons branched over the crop and ringed the junction of the crop and proventriculus. The proventriculus was covered with immunoreactive axons, and 10 to 20 immunoreactive enteric plexus cell bodies were present on the surface (Figure 2.8A). The two main branches of the esophageal nerve continued down the proventriculus and branched again at the junction of the foregut and midgut. Immunoreactive axons were less numerous from the anterior to the posterior region of the midgut and ended before the hindgut (Figure 2.8A). Immunoreactive axons originating from the 6th abdominal ganglion were observed only on the rectum and junction of the hindgut and rectum (Figure 2.8B).

3.5 Midgut Endocrine System

The midgut of *R. flavipes* is uniformly tube-like with no gastric cecae (Noirot 1995) (Figure 2.6, Table 2.5). The midgut surface is composed of circular nodes (52.2 ± 11.3 μm in diameter [n = 21]) (Figure 2.9B) that contain regenerative nidi and the surrounding mature columnar cells. Regenerative cell nidi were observed at the center of these nodes as revealed by transmission electron microscopy (TEM) (Figure 2.9C). Endocrine cells were identified by the presence of secretory granules near the basal lamina (Figure 2.9C inset). NPF-like endocrine cells were visualized with fluorescence microscopy using the *Hez* MP-I antibody (Figure 2.9A, D). These cells were clearly differentiated from axons by their characteristic bottle shape with a wide base at the basal lamina and an apical extension to the lumen of the gut (Figure 2.9D inset) (Nishiitsutsuji-Uwo and Endo 1981). Two immunoreactive endocrine cells were observed on opposite sides of each node (Figure 2.9D), but the number was not always consistent and as many as five were observed in one node.

Immunostained midguts from caste members were cut longitudinally and placed flat on microscope slides for cell counting. Immunoreactive endocrine cells were evenly distributed throughout the midgut in all castes examined. Workers had more midgut endocrine cells than soldiers or alates, soldiers had more than alates, and female alates had more than male alates. The differences among cell numbers, however, were not significant ($F = 1.20$; df = 2; $P = 0.331$) (workers = 785 ± 165 [n = 7]; soldiers = 703 ± 155 [n = 6]; female alates = 687 ± 34 [n = 2]; male alates = 602 ± 148 [n = 2]).

3.6 Other tissues

No immunostained cells or axons were observed in or associated with the fat body and Malpighian tubules of all castes or the testes and ovaries of two day and six day old male and female alates.

3.7 Preabsorption Controls

No immunoreactivity was observed in the nervous system or gut of the different castes when the antiserum was preabsorbed with *Hez* MP-I, *Ang* sNPF I, *Ang* NPF, or when the primary antibody was omitted. Preabsorbtion with FMRF-NH_2 did not noticeably change immunostaining.

4. **Discussion**

In this study, an NPF-like peptide was localized in numerous specific cells distributed in the both the central nervous system and gut of *R. flavipes* caste members. So distributed, release of this neuropeptide messenger likely integrates feeding behaviors regulated by the nervous system in relation to food content and its digestion and passage in the gut. These putative roles for NPF in termite digestion are supported by studies of dipterans for which NPF regulates

feeding and food-searching behaviors (Shen and Cai 2001; Wu et al. 2003; Wu et al. 2005; Lingo et al. 2007) as well as midgut motility and ion transport (Onken et al. 2004).

The brain and ventral nerve cord of alate, worker and soldier *R. flavipes* contained similar distributions of cells and axons immunoreactive to *Hez* MP-I antibody. Immunoreactive axons leaving the brain entered the frontal ganglion or were associated with the corpora cardiaca/corpora allata complex and the foregut and midgut with branches extending over the surface of the salivary glands. Immunoreactive axons occurred on the rectum. Over 600 immunoreactive midgut endocrine cells were also observed in midguts with higher numbers occurring in the worker caste. The immunoreactive peptide in the central nervous system and midgut endocrine cells likely shares sequence similarity to other NPFs. The *Hez* MP-I antibody used for immunocytochemistry recognizes *Dm* NPF (Brown et al. 1999), and immunostaining in tissues was abolished when the antiserum was preabsorbed by *Hez* MP-I and *Ang* NPF and *Ang* sNPF-I. Together, these results indicate that the *Hez* MP-I antibody has specificity for peptides with an RXRF-NH_2 C-terminus, a shared feature of these three peptides (Table 2.1). Immunostaining in termites was not changed when antiserum was preabsorbed with FMRF-NH_2. This indicates that the *Hez* MP-I antiserum is specific for an NPF-like sequence and does not have a strong affinity to peptides with only an RF-NH_2 C-terminus such as extended FMRF-NH_2, myosuppressins, and sulfakinins.

Immunocytochemistry has been an important tool for describing NPY-related peptides in insects. Detailed immunocytochemical studies with NPF have so far only been reported for holometabolous insects, in particular *D. melanogaster* (Brown et al. 1999), *Ae. aegypti* (Stanek et al. 2002) and *H. zea* (Huang 1996). The brains of *Manduca sexta* also showed PP-like immunoreactivity in cells and in axons that led to the corpora cardiaca and the aorta (El-Salhy et

al. 1983). Patterns of immunostaining in central nervous system and gut among these insects and *R. flavipes* were similar. Paired, immunoreactive, neurosecretory cells were observed in the brain of all of these insects. In *H. zea* and *R. flavipes*, immunoreactivity was observed in cells on the ventral nerve cord ganglia and axons on the surface of the corpora cardiaca. Also, the gut tract of *H. zea, Ae. aegypti,* and *R. flavipes* each exhibited immunoreactive axons at various points.

The distribution of NPF-like peptides in hemimetabolous insects has been described primarily from studies with antisera from vertebrate NPY-related peptides. Immunoreactivity to NPF and NPY antibodies has been reported in brain, subesophageal ganglion and gut of *P. americana* and *S. gregaria*, but a complete description of cell bodies or axon processes is lacking (Zhu et al. 1998). PP-immunoreactivity in *P. americana* (Endo et al. 1982a) and *S. gregaria* (Myers and Evans 1985) and NPY-immunoreactivity in *L. migratoria* central nervous system (Schoofs et al. 1988) was similar to *R. flavipes* NPF-like immunoreactivity, especially with regard to clusters of immunoreactive cell bodies in the central nervous system.

Midgut endocrine cells from several insects are reactive to NPF or NPY/PP antisera. NPF-like immunoreactive midgut endocrine cells in *R. flavipes* share similarities with those observed in *D. melanogaster* (Brown et al. 1999), *Ae. aegypti* (Stanek et al. 2002) and *H. zea* (Huang 1996), although the distribution and number of cells differed. NPY-like immunoreactive midgut endocrine cells have been similarly reported in *L. migratoria* (Schoofs et al. 1988) and *Tramea virginia* (Patankar and Tembhare 2006). PP-immunoreactive midgut endocrine cells occur in *P. americana* (Iwanaga et al. 1981), *Gryllus bimaculatus* (Iwanaga et al. 1986), *Calliphora vomitoria* (Duve and Thorpe 1982) and *Ae. aegypti* (Brown et al. 1986).

The patterns of NPF immunostaining observed in the central nervous system and the gut among alates, workers and soldiers were similar suggesting these peptides may share similar

functions in all three castes. NPFs share an RF-amide C terminus with myosuppressins, extended FMRF-NH_2, sulfakinins, sNPF and head peptides. Many of these RF-amides have myostimulatory or myoinhibitory effects on gut tissues (Fujisawa et al. 1993; Lange and Orchard 1997). Immunoreactive axons on the *R. flavipes* midgut suggest that they may have such a function allowing or preventing pumping of food through the gut. Rings of innervation at the junction of the crop, proventriculus, hindgut and rectum may also influence access of gut contents through gut compartments. Similar patterns of immunoreactivity have been observed in *Ae. aegypti* with other neuropeptides (Veenstra et al. 1995).

The diverse peptides expressed by endocrine cells in the insect midgut are thought to regulate different processes associated with digestion (Brown and Lea 1990; Veenstra et al. 1995). Leucomyosuppressin and Dippu-allatostatin 7 increase secretion of digestive enzymes in the midgut of the cockroach, *Diploptera punctata* (Fuse et al. 1999), and presumably this is mediated by such peptides originating from midgut endocrine cells. To date, there is no evidence that NPF affects digestive enzyme release in the insect midgut, but it does inhibit ion transport in the midgut of *Ae. aegypti* larvae (Onken et al. 2004), where it is present in midgut endocrine cells. NPF released from midgut endocrine cells into the hemolymph (Jenkins et al., 1989) would be carried to other tissues, including the nervous system, where it may stimulate or modulate processes associated with nutrient states. It has been suggested that the receptive termini of axons on the surface of the gut may be activated by peptides released by the midgut endocrine cells, bridging the gap between the gut and nervous system through the brain-gut axis (Sehnal and Zitnan, 1996). The higher number of immunoreactive midgut endocrine cells in worker termites may indicate that this communication plays a role in the differentiation of caste feeding behavior.

Regeneration of columnar and endocrine cells from nidi in the midgut tissue of cockroaches was noted long ago (Endo and Nishiitsutsuji-Uwo 1982b) and more recently reported in termites (Tokuda et al. 2001). We typically observed two or more endocrine cells immunoreactive to *Hez*-MP-I antisera within each nidal cluster, and as many as five were observed. Variation in number of midgut endocrine cells was observed in *L. migratoria*, where between zero and three endocrine cells were observed per regenerative niche (Illa-Bochaca and Montuenga 2006).

This study represents the first description of NPF-like immunoreactivity in termites. Mapping the distribution of an NPF-like peptide in the nervous system and midgut of *R. flavipes* was the first step to understanding its importance. We recently purified the *R. flavipes* NPF from a tissue extract with HPLC, as monitored with the *Hez* MP-I radioimmunoassay (unpublished results) and sequencing and molecular characterization revealed that it is an authentic NPF. Fractions containing this NPF accounted for most of the immunoreactivity in the extract, thus supporting the specificity of the *Hez* MP-I antiserum. The tissue distribution described herein will serve as a guide for the testing of synthetic NPF to discover its function in termites.

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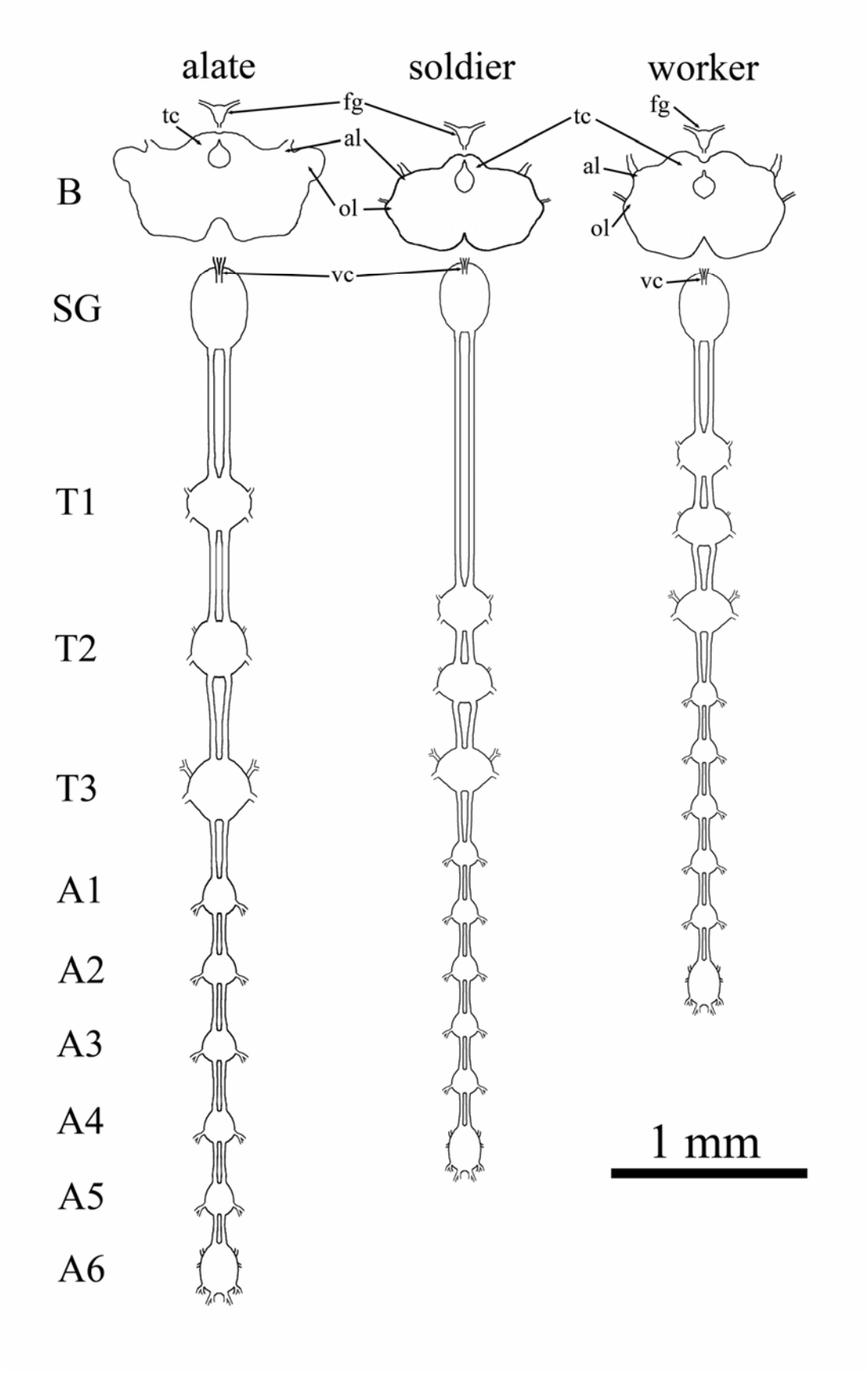
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Figure 2.1. Dorsal view of the brain and ganglia of *Reticulitermes flavipes* alates, soldiers and workers. In this figure brains are displaced to show the subesophageal ganglion located ventrally. Both the ventral connective of the subesophageal ganglion and the frontal ganglion connect to the tritocerebrum of the brain. Abbreviations: al, antennal lobe; fg, frontal ganglion; ol, optic lobe; tc, tritocerebrum; vc, ventral connective; B, brain; SG, subesophageal ganglion; T1-3, thoracic ganglia; A1-6, abdominal ganglia.

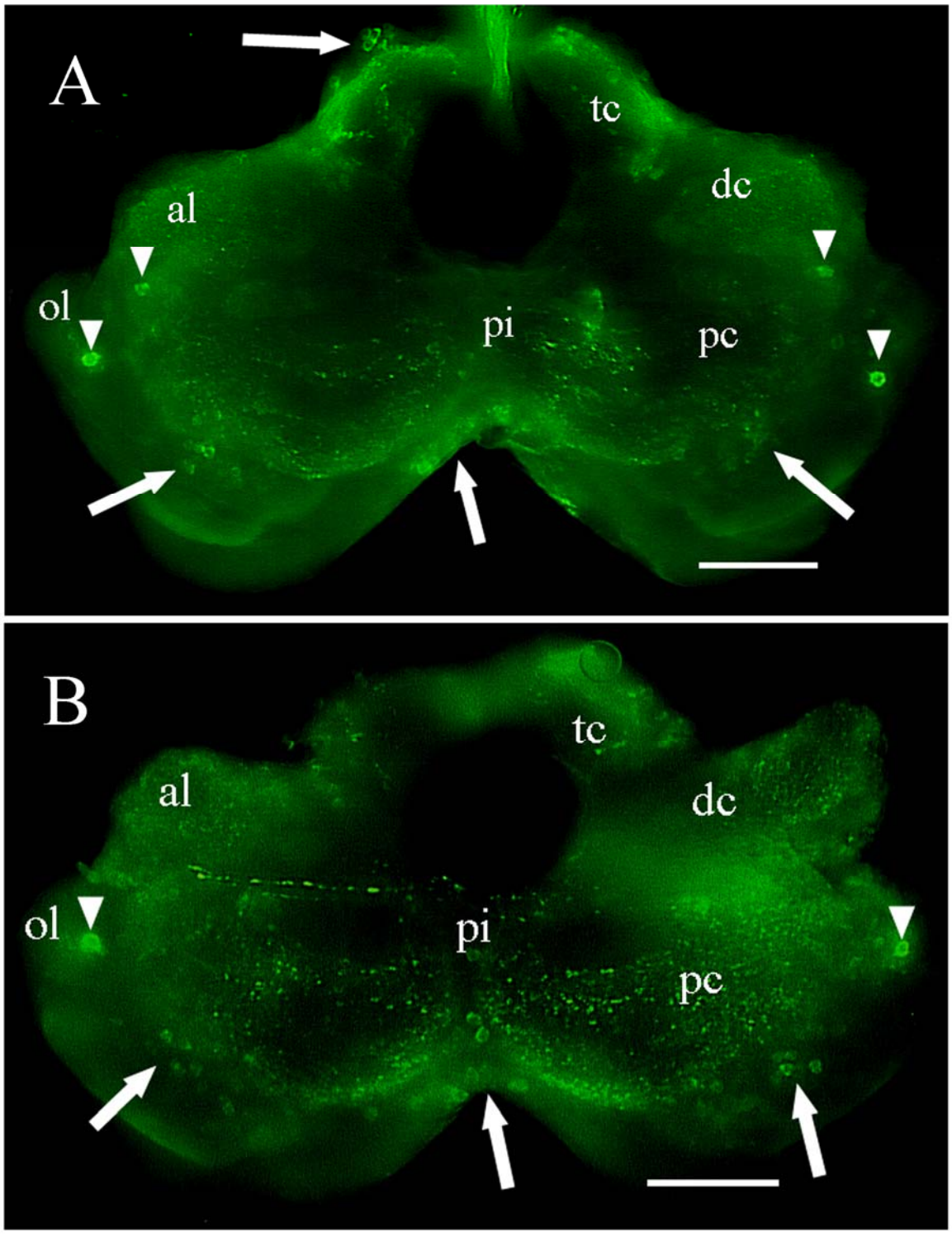
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Figure 2.2. NPF-like immunoreactivity of the brain of a soldier (A) and worker (B) of *Reticulitermes flavipes*. Clusters of immunoreactive cells (arrows) and brightly staining cells (arrowheads) are indicated. Abbreviations: al, antennal lobe; dc, deutocerebrum; op, optic lobe; pc, protocerebrum; pi, pars intercerebralis; tc, tritocerebrum. Bars = 100 μm.

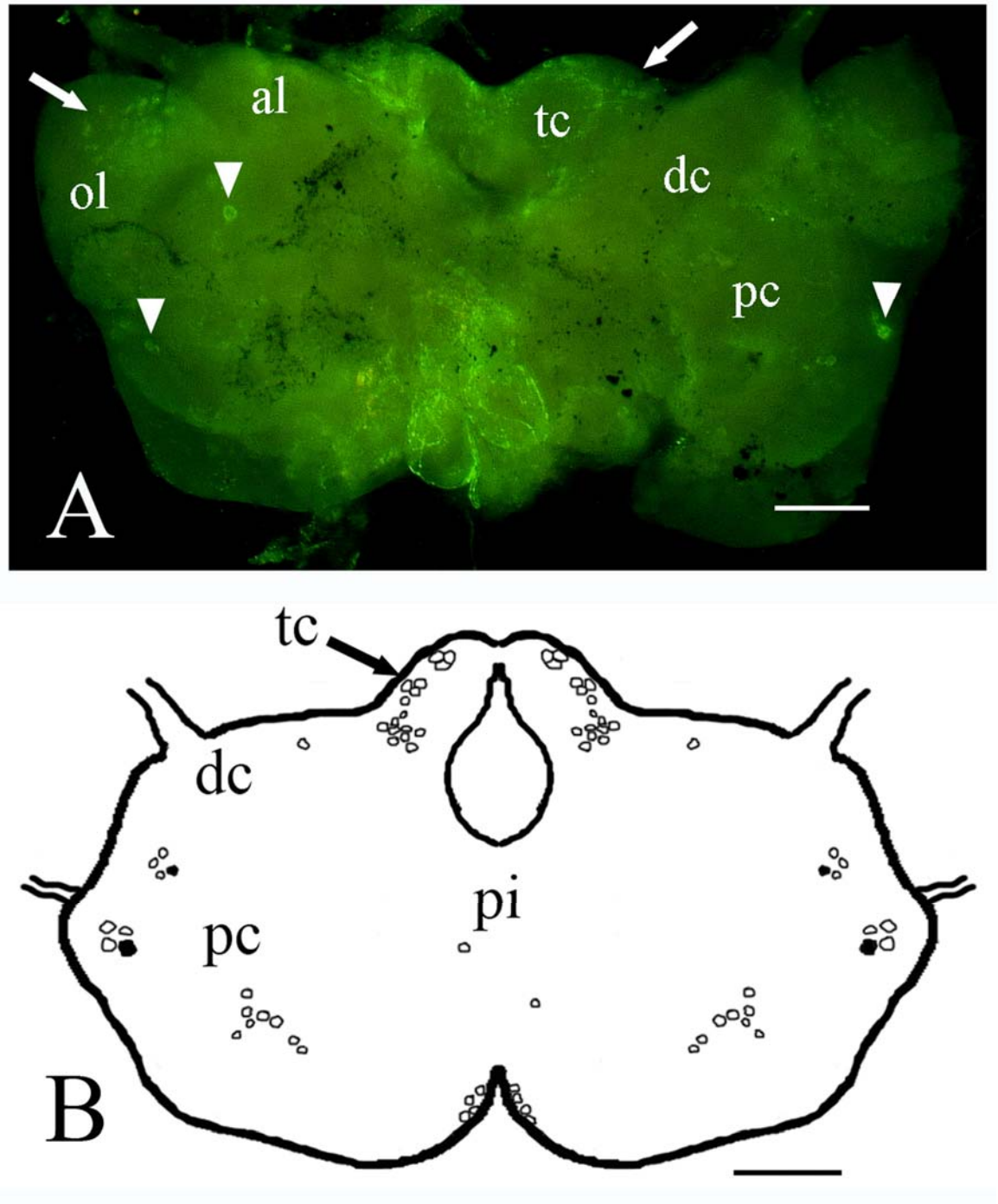

Figure 2.3. (A) NPF-like mmunoreactivity of the female alate brain of *Reticulitermes flavipes*. Clusters of immunoreactive cells (arrows) and brightly staining cells (arrowheads) are indicated. (B) Diagram of immunoreactive cells observed in the brain of a soldier. Clusters of immunoreactive cell bodies occurred in the same regions of alates, workers and soldiers. Brightly immunostaining cell bodies are indicated in black. Abbreviations: al, antennal lobe; dc, deutocerebrum; ol, optic lobe; pi, pars intercerebralis; pc, protocerebrum; tc, tritocerebrum. Bars = 100 µm.

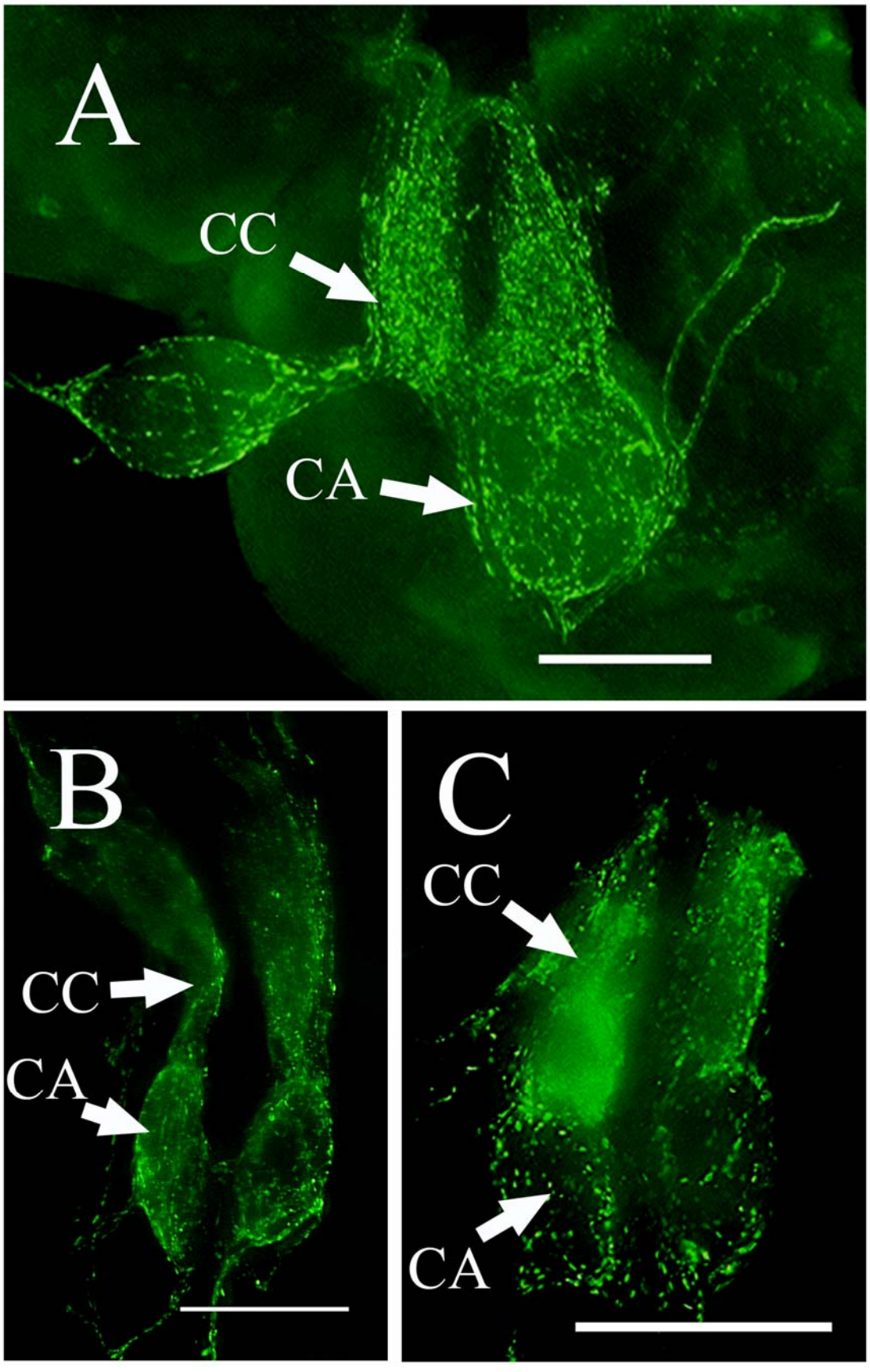
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Figure 2.4. NPF-like immunoreactive axons associated with the corpora cardiaca (CC) and corpora allata (CA) of an alate (A), soldier (B) and worker (C) *Reticulitermes flavipes*. Bars = 100 μm.

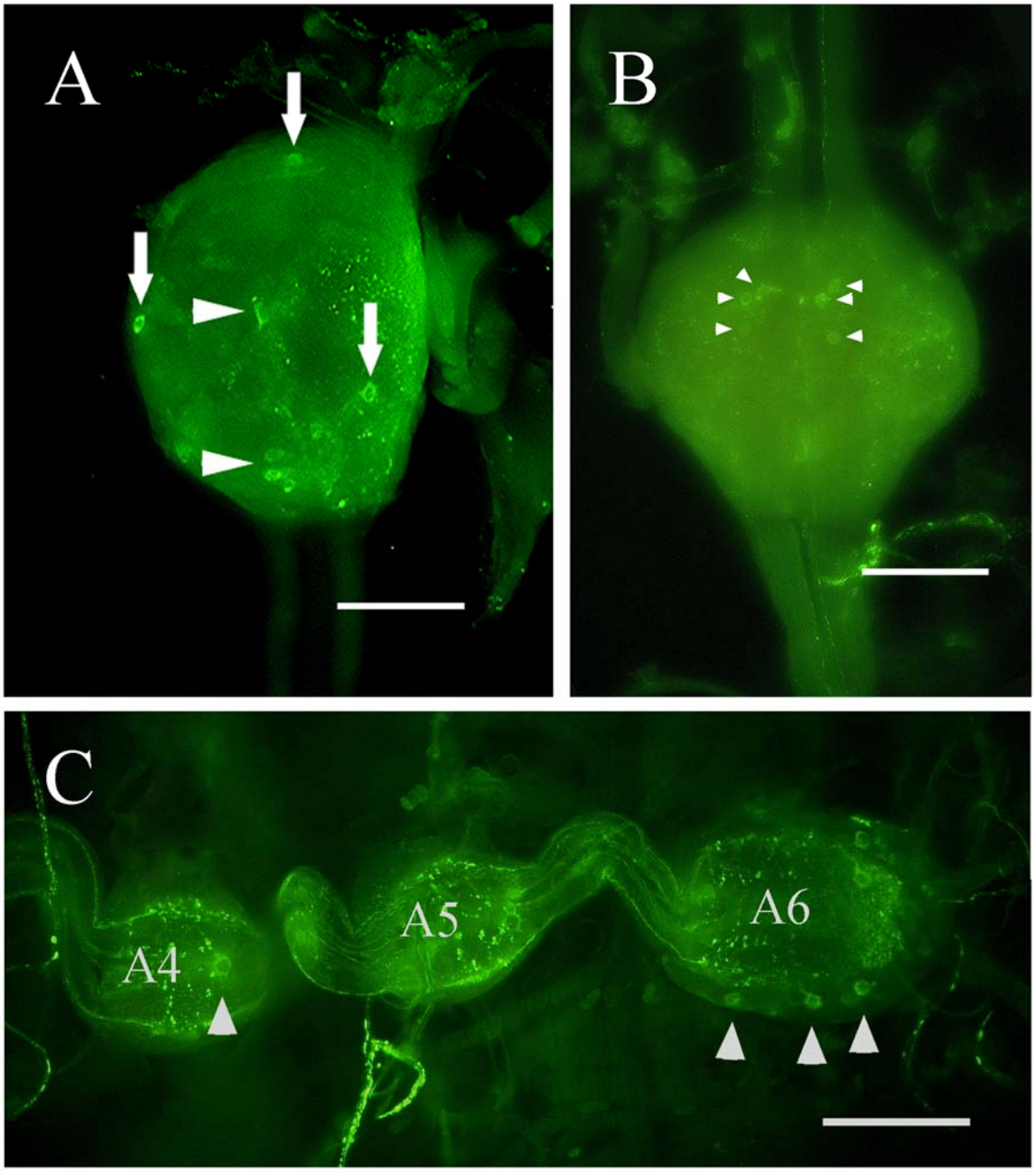
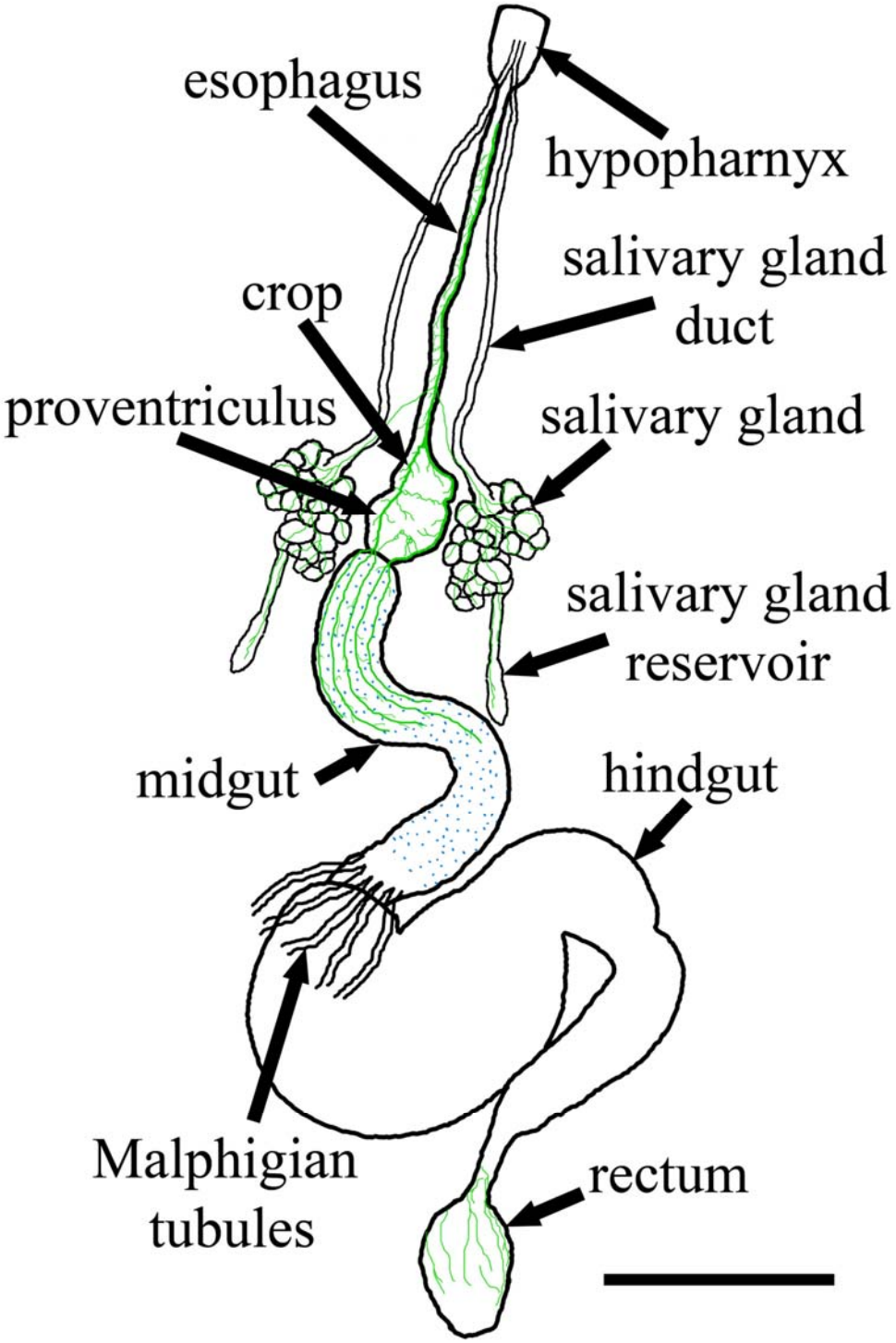


Figure 2.5. NPF-like immunoreactivity of ventral nerve cord ganglia of *Reticulitermes flavipes*. (A) Ventral view of subesophageal ganglion. Bright staining cells (arrows) and additional immunoreactive cells (arrowheads) were observed. (B) Thoracic ganglia. 3 pairs of brightly stained cells were observed consistently (arrowheads). (C) Posterior abdominal ganglia (A4 - A6) with scattered immunoreactive cells (arrowheads). Bars = 100 µm.


esophagus
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duct
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salivary gland
salivary gland
reservoir
midgut
hindgut
Malphigian
tubules
rectum

Figure 2.6. Diagram of expanded *Reticulitermes flavipes* alimentary tract with immunoreactive axon tracts in green and immunoreactive midgut endocrine cells in blue. Bar = 500 μm.

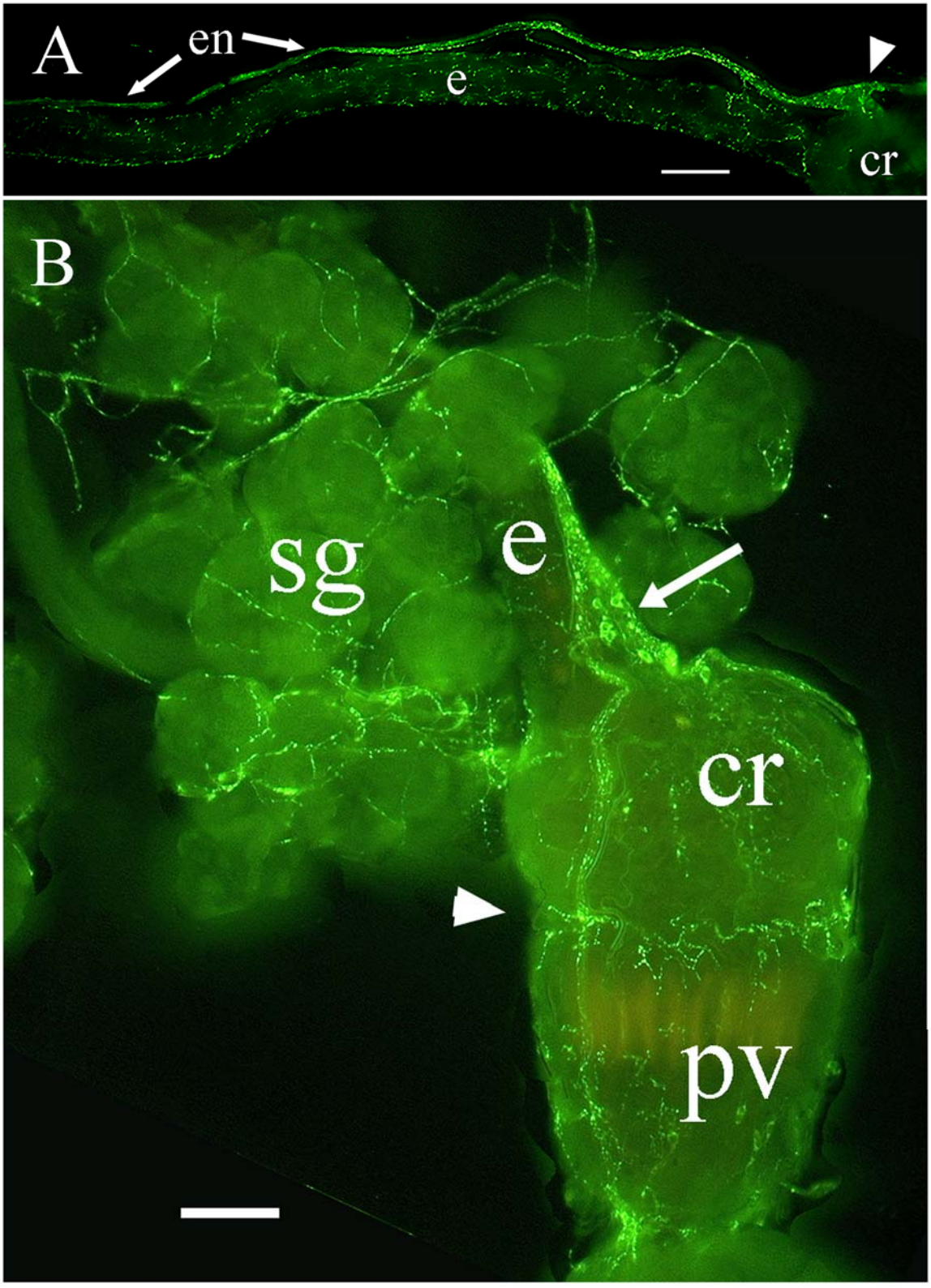

Figure 2.7. NPF-like immunostaining of the foregut of *Reticulitermes flavipes*. (A) Immunoreactive axons on the esophagus and immunoreactivity in the esophageal nerve. This

nerve divides after the ingluvial ganglion (arrowhead) (B) Immunoreactive axons on the crop, proventriculus and salivary glands. The immunoreactive esophageal nerve enters the ingluvial ganglion (arrow) and divides over the crop. Note immunoreactive cells in the ingluvial ganglion. Branches of the nerve ring the junction of crop and proventriculus (arrowhead). Abbreviations: cr, crop; e, esophagus; en, esophageal nerve; sg, salivary glands; pv, proventriculus. Bars = 100 µm.

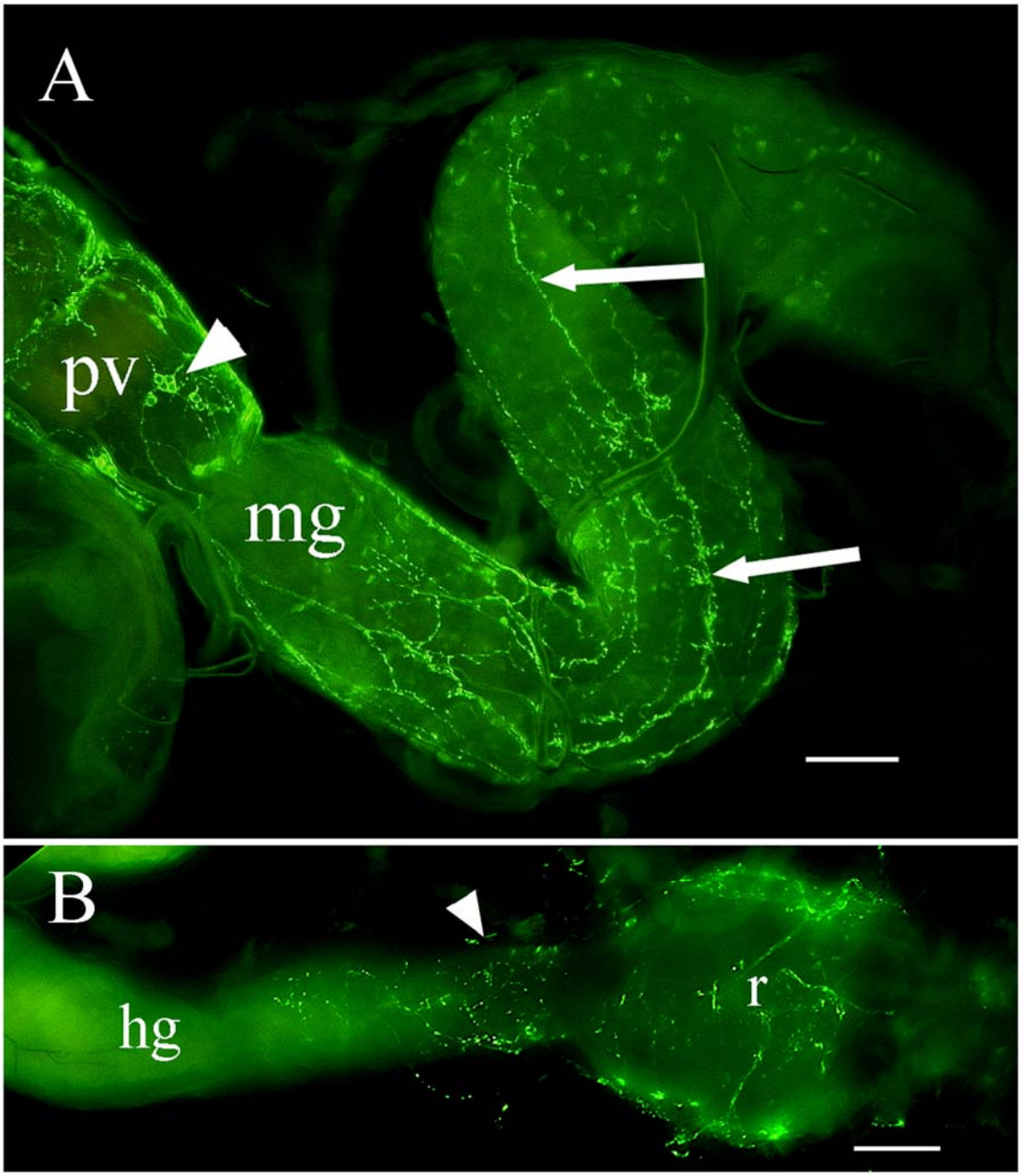

Figure 2.8. (A) NPF-like immunoreactivity of the foregut and midgut of *Reticulitermes flavipes*. Immunoreactive enteric plexus cell bodies on the surface of the proventriculus (arrowhead). Immunoreactive axons on the midgut surface (arrows). (B) Immunoreactive axons on the rectum and posterior hindgut/rectal junction (arrowhead). Abbreviations: hg, hindgut; mg, midgut; pv, proventriculus; r, rectum. Bars = 100 μm.

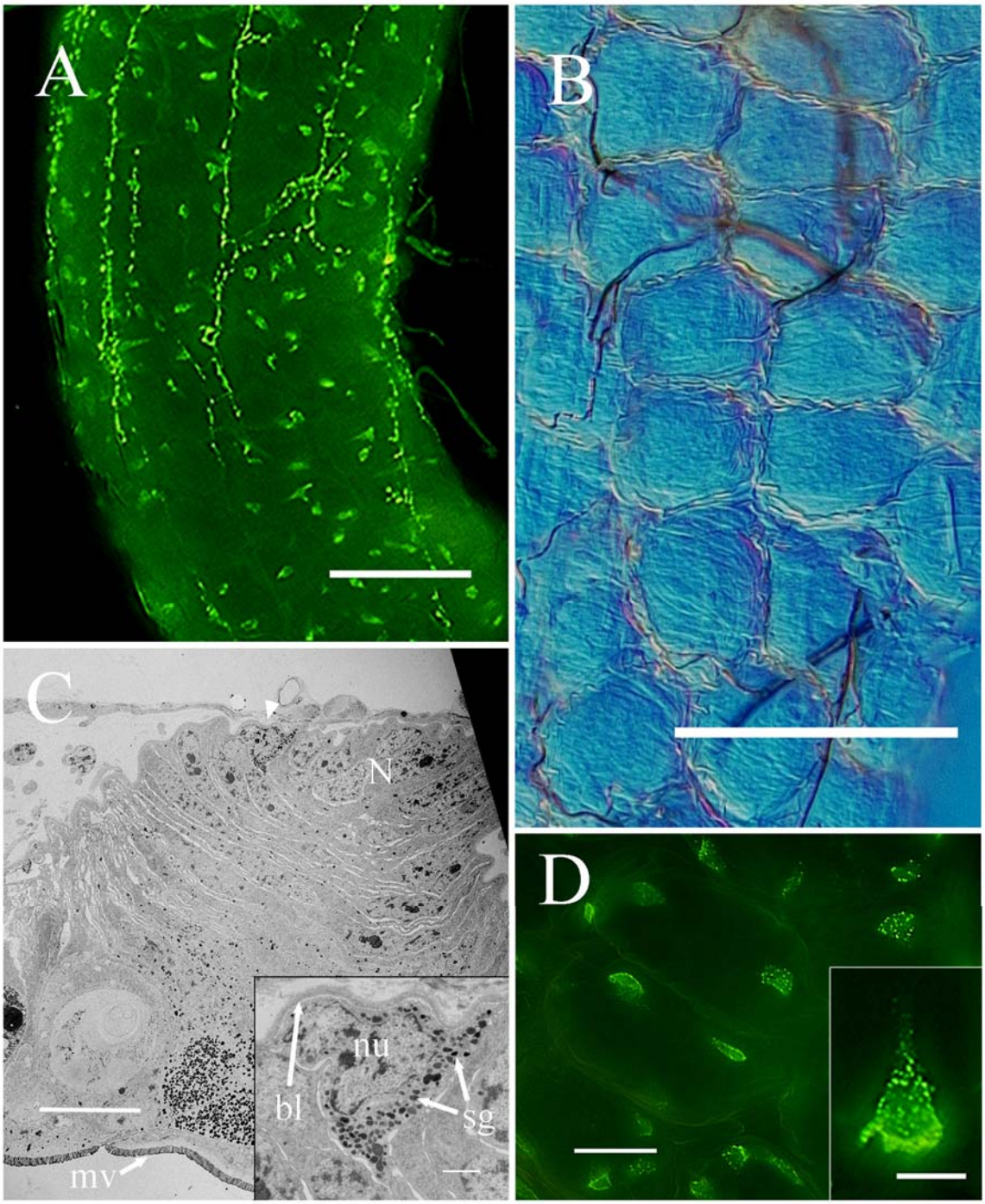

Figure 2.9. (A) Midgut endocrine cells of *Reticulitermes flavipes*. Midgut is oriented anterior (top) to posterior (bottom) Bar = 100 µm. (B) Light microscopy of midgut surface showing the circular nodes. Bar = 100 µm. (C) TEM of midgut cross-section showing a nidus. Midgut is oriented hemolymph side (top) to lumen (bottom). Arrowhead indicates a small portion of an endocrine cell. Bar = 10 µm. Inset: Closeup of endocrine cell. Bar = 1 µm. (D) Immunoreactive endocrine cells. Typically two cells occur per nidal cluster. Bar = 25 µm. Inset: Closeup of

pyramidal immunoreactive endocrine cell. Bar = 10 µm. Abbreviations: bl, basal lamina; mv, microvilli; N, nidus; nu, nucleus; sg, secretory granules.

Table 2.1: Amino acid sequence of peptides used to preabsorb *Hez* MP-I antiserum.

	Amino acid sequence
Ang NPF	LVAARPQDSDAASVAAAIRYLQELETKHAQHARPRF-NH_2
Hez MP-I	QAARPRF-NH_2
Ang sNPF-I	AVRSPSLRLRF-NH_2
FMRF-NH_2	FMRF-NH_2

Table 2.2: Average length (L), width (W) and length of ganglial connectives (C) of dissected brains and ventral nerve cords of the alate, worker and soldier castes of *R. flavipes* (±SD μm). Number of specimens measured is included in parenthesis. Abbreviations: B, brain; SG, subesophageal ganglion; T1-3, thoracic ganglia; A1-6, abdominal ganglia.

		Alate	Soldier	Worker
Brain	L:	467.9 ± 72.1 (5)	235.8 ± 49.9 (11)	181.3 ± 32.0 (8)
	W:	971.9 ± 132.2 (5)	729.3 ± 36.6 (11)	724.0 ± 60.4 (8)
SG	L:	384.9 ± 38.7 (3)	426.0 ± 30.7 (10)	336.3 ± 14.1 (8)
	W:	289.8 ± 40.3 (3)	283.9 ± 37.4 (10)	255.1 ± 10.9 (8)
	C:	610.0 ± 0.0 (2)	1169.7 ± 172.3 (6)	451.4 ± 36.0 (5)
T1	L:	280.7 ± 24.3 (3)	257.4 ± 17.0 (12)	230.0 ± 7.7 (6)
	W:	316.5 ± 27.1 (3)	275.8 ± 25.6 (12)	262.7 ± 16.5 (6)
	C:	432.6 ± 45.2 (4)	193.1 ± 33.8 (9)	158.0 ± 24.7 (6)
T2	L:	283.6 ± 16.8 (6)	202.6 ± 10.3 (10)	198.8 ± 11.3 (6)
	W:	269.7 ± 43.3 (6)	266.9 ± 14.9 (10)	272.0 ± 24.1 (6)
	C:	430.8 ± 33.4 (5)	174.1 ± 22.5 (8)	195.8 ± 26.4 (5)
T3	L:	297.3 ± 21.2 (6)	217.0 ± 22.2 (10)	207.5 ± 11.3 (6)
	W:	314.0 ± 48.1 (6)	292.5 ± 18.5 (10)	282.2 ± 15.7 (6)
	C:	372.6 ± 68.7 (6)	263.0 ± 20.2 (7)	250.5 ± 24.4 (6)
A1	L:	183.9 ± 26.7 (5)	140.1 ± 12.0 (11)	134.9 ± 11.0 (7)
	W:	150.6 ± 24.2 (5)	148.4 ± 12.8 (11)	140.3 ± 22.4 (7)
	C:	259.2 ± 56.2 (4)	180.0 ± 38.9 (12)	182.4 ± 38.1 (7)
A2	L:	164.3 ± 9.1 (4)	140.9 ± 13.0 (12)	133.9 ± 10.2 (7)
	W:	162.0 ± 10.9 (4)	140.9 ± 19.9 (12)	130.4 ± 13.2 (7)
	C:	257.5 ± 31.6 (5)	191.2 ± 36.3 (12)	165.9 ± 29.2 (7)
A3	L:	171.6 ± 20.2 (5)	134.3 ± 14.5 (12)	126.9 ± 16.8 (7)
	W:	142.0 ± 18.4 (5)	142.4 ± 15.3 (11)	138.7 ± 15.6 (7)
	C:	236.9 ± 39.5 (5)	205.0 ± 35.7 (12)	201.5 ± 37.9 (6)
A4	L:	180.9 ± 14.4 (5)	132.5 ± 17.0 (12)	127.8 ± 12.4 (5)
	W:	132.5 ± 16.6 (5)	131.0 ± 12.9 (12)	127.0 ± 11.2 (5)
	C:	209.6 ± 46.9 (5)	178.2 ± 23.0 (11)	179.8 ± 17.3 (6)
A5	L:	174.5 ± 13.6 (5)	135.4 ± 10.5 (12)	131.7 ± 8.6 (6)
	W:	115.8 ± 21.7 (5)	127.6 ± 11.5 (12)	124.8 ± 9.0 (6)
	C:	194.8 ± 34.3 (4)	159.7 ± 24.9 (10)	162.0 ± 22.4 (5)
A6	L:	245.1 ± 19.9 (4)	220.2 ± 15.6 (10)	233.4 ± 9.0 (5)
	W:	184.9 ± 15.5 (4)	153.0 ± 8.7 (10)	148.8 ± 10.0 (5)

Table 2.3: Average number of immunoreactive NPF-like cells counted in brain and ventral nerve cord of alates, soldiers, and workers (±SD). Number of specimens counted is included in parenthesis.

	Alate	Soldier	Worker
Brain	71.0 ± 5.7 (2)	67.0 ± 27.9 (6)	65.9 ± 23.8 (7)
SG	9.3 ± 6.6 (6)	13.4 ± 6.3 (5)	18.5 ± 11.6 (4)
T1	6.3 ± 2.1 (3)	10.0 ± 2.0 (5)	6.2 ± 3.1 (5)
T2	9.7 ± 4.7 (3)	9.7 ± 2.5 (3)	8.0 ± 2.9 (4)
T3	8.5 ± 3.5 (2)	7.3 ± 1.0 (4)	8.7 ± 2.5 (3)
A1	3.7 ± 3.8 (3)	2.2 ± 1.8 (6)	1.3 ± 1.0 (4)
A2	7.0 ± 5.3 (3)	3.8 ± 2.8 (6)	5.5 ± 2.1 (4)
A3	6.7 ± 4.7 (3)	3.3 ± 2.1 (6)	2.8 ± 2.2 (4)
A4	6.0 ± 1.7 (3)	4.3 ± 1.0 (6)	3.3 ± 1.5 (4)
A5	5.3 ± 1.2 (3)	4.2 ± 2.0 (6)	7.3 ± 1.0 (4)
A6	12.5 ± 2.1 (2)	14.3 ± 5.0 (6)	18.3 ± 6.2 (4)

Table 2.4: Average number of NPF-like immunoreactive cell bodies counted by brain region in soldiers, and workers (±SD). Number of specimens counted is included in parenthesis.

Brain Region	Soldier	Worker
protocerebrum	23.0 ± 11.5 (5)	44.0 ± 11.3 (2)
deutocerebrum	9.8 ± 4.2 (6)	10.0 ± 1.4 (2)
tritocerebrum	32.8 ± 10.5 (5)	22.0 ± 1.4 (2)
pars intercerebralis	11.0 ± 5.7 (6)	11.0 ± 1.4 (2)

Table 2.5: Average length (L), and width (W) of dissected gut tissues of workers and soldiers (±SD μm). Widths were measured on the anterior and posterior portions of the midgut and posterior hindgut (AW and PW, respectively). Number of specimens measured is included in parenthesis. Abbreviations: Hpx, hypopharnyx; Eso, esophagus; FG, foregut; MG, midgut; AHG, anterior hindgut; PHG, posterior hindgut; Rect, rectum.

		Soldier	Worker
Hpx	L:	305.3 ± 21.0 (5)	550.7 ± 27.5 (7)
Eso	L:	3091.5 ± 201.9 (8)	2055.1 ± 112.8 (7)
	W:	106.7 ± 25.4 (9)	84.1 ± 11.5 (7)
FG	L:	788.1 ± 99.5 (11)	666.3 ± 68.7 (8)
	W:	500.4 ± 119.3 (11)	449.8 ± 13.9 (8)
MG	L:	2379.4 ± 277.3 (9)	2596.4 ± 169.5 (7)
	AW:	306.1 ± 27.5 (9)	359.7 ± 25.6 (7)
	PW:	267.6 ± 28.7 (9)	341.3 ± 15.5 (7)
AHG	L:	1745.9 ± 190.5 (9)	2195.3 ± 413.8 (6)
	W:	728.6 ± 150.8 (9)	940.8 ± 154.6 (6)
PHG	L:	1618.9 ± 257.3 (8)	2513.7 ± 285.7 (6)
	AW:	293.8 ± 80.3 (8)	446.2 ± 57.1 (6)
	PW:	128.0 ± 22.4 (8)	193.5 ± 52.0 (6)
Rect	L:	668.8 ± 194.9 (4)	712.0 ± 71.6 (6)
	W:	414.8 ± 100.9 (4)	421.0 ± 82.2 (6)
Total	L:	10209.0 ± 815.6 (4)	11281.8 ± 548.5 (6)

CHAPTER 3

MOLECULAR CHARACTERIZATION OF NEUROPEPTIDE F FROM *Reticulitermes flavipes* (ISOPTERA: RHINOTERMITIDAE)

1. Introduction

Members of the neuropeptide Y (NPY) family of neuropeptides evolved early in animal lineages and are found in chordates, mollusks, platyhelminths, and arthropods (Brown et al., 1999; McVeigh et al., 2005). In mammals these are represented by NPY, peptide YY (PYY), and pancreatic polypeptide (PP) (Berglund et al., 2003). NPY is the most abundant neuropeptide in the mammalian brain, an observation that has inspired numerous studies to determine its functions (Pedrazzini et al., 2003). Several functions are associated with the acquisition of food (Pedrazzini et al., 2003). Homologous neuropeptides, termed neuropeptide Fs (NPFs), are characterized for invertebrates (McVeigh et al., 2005). NPF also has a role in the acquisition of food in *Drosophila melanogaster* but few other functions are known in insects (Wu et al., 2003, 2005).

The first full-length insect NPF was isolated from extracts of *D. melanogaster* using an antiserum to *Helicoverpa zea* midgut peptide I (*Hez* MP-I) (Huang et al., 1998; Brown et al., 1999), an antiserum with low specificity for FMRFamide (Huang et al., 1998). Subsequently, NPFs were isolated and sequenced from other dipteran species (Riehle et al., 2002; Stanek et al., 2002; Garczynski et al., 2005). The use of bioinformatic techniques has elucidated NPF sequences in mosquitoes (Garczynski et al., 2005), locusts (Clynen et al., 2006), and honeybees (Hummon et al., 2006). The presence of potentially truncated NPF sequences has been

demonstrated from extracts of *H. zea* (Huang et al., 1998) and *Schistocerca gregaria* (Schoofs et al., 2001). Attempts to determine NPF from the *Tribolium castanaeum* genome database have so far been unsuccessful (Li et al., 2008).

The presence of NPF-like material in the eastern subterranean termite, *Reticulitermes flavipes*, was demonstrated by immunocytochemistry with the *Hez* MP-I antiserum (Chapter 2). The current study continues this investigation by reporting the purification and partial amino acid sequencing of an NPF from an extract of the worker caste. Degenerate primers based on this sequence were used to isolate the cDNA sequence of the full *R. flavipes* NPF transcript. Little is known about termite signaling peptides, and this is the first member of the RFamide superfamily of neuropeptides to be described in termites.

2. Materials and Methods

2.1. Whole body peptide extraction

An estimated 117,300 field-collected *R. flavipes* workers (numbers estimated by weight) weighing 349.3 g were separated from logs collected from Whitehall Forest (East Whitehall Rd., Athens, GA) and frozen at -80 ºC. Termites were boiled in 500 ml 3% acetic acid in batches of 100 g for 10 min. Extracts were centrifuged at 4 ºC for 15 min at 10,000 rpm. Supernatants were pooled, frozen at -80 ºC and lyophilized to reduce volume. Lipids were removed by mixing with 400 ml hexane in a separatory funnel. After settling, the non-lipid phase was drained. The non-lipid phase was loaded onto Varian Mega Bond Elut C_{18} columns (60 cc / 10 g, Varian Co., Harbor City, CA) by a 'Baker'-10 Extraction System (J.T. Baker Chemical Co., Phillipsburg, NJ) and step eluted with 5% CH_3CN in 0.1% TFA, 10% CH_3CN in 0.1% TFA, 80% CH_3CN in 0.1% TFA, and 100% CH_3CN.

2.2. Radioimmunoassay

A radioimmunoassay procedure modified slightly from Huang et al., 1998 was used to monitor chromatography fractions (see below) for immunoreactivity. Aliquots (1-600 termite equivalents) of each fraction were lyopholized then resuspended with 220 µl RIA buffer (0.05 M Tris-HCl, pH 7.2; 0.1% bovine serum albumin; 0.02% sodium azide). Synthetic *Hez* MP-I peptide with an amino-terminal Tyr was radiolabeled with Na I^{125} by the chloromine T method and purified by high performance liquid chromatography (HPLC) ([I^{125}]YQAARPRFa) (Dr. Stephen Garczynski, University of Georgia, Athens, GA, USA). Radiolabeled peptide (8000-9000 cpm/tube; 100 µl), antiserum (*Hez* MP-I 35B, diluted 1/70000; 100 µl) and QAARPRFa standards or aliquots from fractions (100 µl) were combined in individual 6 x 50 mm borosilicate glass culture tubes (Kimble Glass Inc.) (300 µl total volume). Tubes were incubated overnight (18-24 h) at 4 ˚C. Unbound peptide was precipitated with 100 µl dextran-coated charcoal and calf serum solution per tube. Tubes were vortexed, centrifuged (1500*g*, 10 min at 4 ˚C), and the supernatant aspirated. Remaining pellets were counted using a gamma counter (Cobra™ II AutoGamma, Packard Instrument Company).

Linear regression was used to create a standard line from the bound/free ratios and log fmol of the standard tubes. Amounts of peptide in the HPLC samples were extrapolated from the standard line.

2.3. High performance liquid chromatography (HPLC)

Reverse phase HPLC (RP-HPLC) on a Beckman chromatography 126/166 (pump/detector) unit was used to isolate immunoreactive material for structural characterization. For the first step, material in the 80% C_{18} passed termite extract step was lyophilized and redissolved with HPLC grade reagents. Six different columns were used over eight steps to

separate peptides from this sample (Fig 3.1). The ion pairing agent in each step was either heptafluorobutyric acid (HFBA) or trifluoroacetic acid (TFA). Steps containing TFA were monitored at 206 or 215 nm and steps containing HFBA or CH_3COONH_4 were monitored at 275 nm. Aliquots were taken from all fractions at each step and monitored for immunoreactivity by RIA. Sample *'B'* (Fig 3.1) was purified to homogeneity yielding a single peak of activity (Fig. 3.2).

2.4. Peptide analysis

The purified sample *'B'* was analyzed by matrix-assisted laser desorption ionization - time of flight (MALDI-TOF) mass spectrometry (Jan Pohl, Emory University School of Medicine and Winship Cancer Institute, Atlanta, GA, USA). A partial amino acid sequence of sample *'B'* was obtained by Edman degradation (Dr. Jan Pohl, Emory University School of Medicine and Winship Cancer Institute, Atlanta, GA, USA).

2.5. NPF cDNA

Five termite worker midguts and ten heads were dissected separately into RNAlater® (Ambion, Inc.) and left overnight at 4 ºC. Worker head and midgut cDNA libraries were generated from total RNA isolated following the procedure from an RNeasy® kit (Qiagen Inc., 2005) with oligo d(T). Degenerate forward primers for amplification of NPF cDNA were designed based on the partial amino acid sequence obtained from Edman degradation N-terminal sequencing of sample *'B'* (Table 3.1: Retic 1.1, 2, and 3.2) (Integrated DNA Technologies, Coralville, IA).

A touchdown PCR procedure was used with primers Retic 1.1 / NotI d(T) (Table 3.1) using head and midgut cDNA (10 cycles; 94 ºC, 20 s; 65 ˚C [-1 ºC each cycle], 20 s; 72 ºC, 1 min) (Table 3.2). Resulting products were then amplified by additional PCR cycles (30 cycles;

94 ºC, 20 s; 56 ºC, 20 s; 72 ºC, 1 min). Nested primer sets were used for a second round of PCR on products generated from touchdown PCR for both head and midgut: Retic 2 / NotI, and Retic 3.2 / NotI (40 cycles; 94 ºC, 20 s; 61 ºC, 20 s; 72 ºC, 1 min) (Table 3.2). Products were visualized on a 1 % agarose gel containing ethidium bromide (EtBr), and individual bands were extracted and gel purified using a GenElute™ Minus EtBr Spin Column (Sigma®). Two bands were amplified by PCR, run on an agarose gel and purified. One additional purified product was cloned into *Escherichia coli* (Sigma TOPO4™ vector kit) and grown for 24 h at 37 ºC. Plasmids were purified from growing colonies using a Nucleospin Plus Plasmid Miniprep Kit (Qiagen®). The two PCR products and plasmids were then sequenced (Molecular Cloning Laboratories, San Francisco, CA).

Sequenced PCR products from the touchdown PCR procedure were aligned by hand forming a consensus sequence of 350 bp. Reverse primers were designed near the 3' end of the resulting sequence (Table 3.1: NPF1, NPF2, NPF Rev). To obtain the 5' sequence, 5' RACE was used. Primer NPF1 (Table 3.3) was mixed with two tubes with different sets of total RNA (head and midgut, isolated as above) and heated to 70 ºC for 2 min. Reverse transcriptase (Advantage RT for PCR) and deoxynucleotides (dATP, dCTP, dGTP, and dTTP) were added to the mixture and cDNA was made by incubating at 42 ºC for 1 h then heating to 94 ºC for 5 min. Resulting cDNA was purified (High Pure PCR Product Purification Kit, Roche®). Purified cDNA was combined with dATP in TdT Reaction buffer (0.2 M potassium cacodylate, 25 mM Tris-HCl, 0.25 mg/ml bovine serum albumen), incubated for 3 min at 94 ºC then chilled on ice. A poly A tail was added to the 3' end of the cDNA strands by adding terminal transferase in 5 mM $CoCl_2$ solution (Terminal transferase, recombinant, Roche®) and incubating at 37 ºC for 30 min. Terminal transferase was inactivated by heating to 70 ºC for 10 min then chilling on ice. The

resulting product was PCR amplified using NPF2 / Not d(T) (10 cycles; 94 ºC, 20 s; 59.8 ºC, 20 s; 72 ºC, 40 s; then 10 cycles; 94 ºC, 20 s; 59.8 ºC, 20 s; 72 ºC, 40 s + 20 s additional each subsequent cycle). A nested primer set (NPF Rev / NotI) was used to further amplify DNA (35 cycles; 94 ºC, 20 s; 60.5 ºC, 20 s; 72 ºC, 1.5 min). Products were separated by polyacrylamide gel electrophoresis, and 5 bands were excised and purified as before. Bands were cloned (Strataclone™ PCR cloning kit), plasmids purified from three clones (Qiagen®) and sequenced forwards and backwards (Molecular Cloning Laboratories, San Francisco, CA). Assembly of a consensus sequence using Clustal W resulted in a sequence of 443 bp.

To confirm the NPF sequence, a specific primer was designed for the 5' end (Table 3.1: UPNPF 1) and PCR was performed on head and midgut cDNA with NPF Rev as the reverse primer (35 cycles; 94 ºC, 20 s; 60.5 ºC, 20 s; 72 ºC, 1.5 min). Resulting products were cloned. A total of ten clones (5 from head cDNA template, five from midgut cDNA template) were purified and sequenced forwards and backwards. Sequences were aligned to establish a consensus sequence.

The sequence obtained from both head and midgut cDNA samples was identical. The sequence was 541 bp and contained an open reading frame (ORF) of 88 amino acids (Fig. 3.3). The cDNA organization was similar in structure to other NPFs with a signal peptide, mature NPF region and a carboxy- (C-) peptide. SignalP analysis (Bendtsen et al., 2004) predicted a signal peptide cleavage site before Lys_{28}. Based on the organization of other insect NPF pre-propeptides, the C-peptide is presumably cleaved at the dibasic Lys-Arg site following Gly_{58}. Gly_{58} is a putative amide donor to the terminal Phe residue.

2.6. Alignments and cladograms

The translated *R. flavipes* NPF (*Ref* NPF) cDNA sequence was aligned with Clustal X using insect and other known invertebrate NPF sequences obtained from GenBank and new NPF sequences discovered by bioinformatics (Joe Crim, personal communication) (Table 3.4). For cladograms, invertebrate NPF sequences and a subset of insect NPF sequences were first aligned with Clustal X then trimmed to equal lengths and to remove gaps (Fig. 3.4). Trees were generated from this data based on Jukes-Cantor distance analysis (GCG).

3. Results

3.1. Isolation of *R. flavipes* NPF

The 80% C_{18} passed termite extract step contained an estimated 3.76 nmol of NPF-like material. After 8 HPLC steps, the material was isolated as a single peak (Fig. 3.1). The purified sample *'B'* (Fig. 3.2) contained 8.4 pmol of immunoreactive material as estimated by RIA. Eighteen Edman degradation cycles were performed on sample *'B'* before the sequencing was terminated due to multiple residue uncertainties. The partial amino acid sequence was obtained from the N-terminus of sample *'B'* was: XPSDPEQLADTLXYLQEL.

3.2 Alignments and cladograms

The NPF sequences from 10 insects and 10 non-insect invertebrates are now known and were compared to *Ref* NPF (Table 3.4). An (A/G)-(R/K)-X_1-R-(F/Y)-amide carboxy- (C-) terminus occurs in all NPFs (Fig. 3.4A, B and C). Two hydrophobic residues, most commonly Leu, occur in all NPFs at positions 13 and 16 from the C-terminus. Partial homologies include aromatic amino acids at positions 10 and 17 from the C-terminus, with the exception of the mosquitoes (*Aea* NPF, *Ang* NPF, and *Cup* NPF), *Peh* NPF, *Bom* NPF2 and *Dam* NPF (Fig. 3.4A, B and C). This feature is shared with vertebrate PPs (Maule et al., 1991). An aromatic residue

commonly occurs at 9 residues from the C-terminus with the exceptions of mosquitoes and turbellarians (Fig. 3.2A, B and C). Pro frequently occurs in position 3 from the C-terminus, with exceptions in some insects (*Dm* NPF, *Apm* NPF and *Bom* NPF2) and annelids (*Lur* NPF).

In the cladogram with all NPFs, arthropod NPFs typically formed a separate clade except for *Apm* NPF and *Bom* NPF2 (Fig. 3.6B). All mollusk NPFs formed a clade as did the trematode and turbellarian NPFs. The insect cladogram contained the following clades: *Cup* NPF, *Aea* NPF and *Ang* NPF; *Ref* NPF and *Lom* NPF; *Peh* NPF and *Bom* NPF1; *Apm* NPF and *Bom* NPF2 (Fig. 3.6A).

4. Discussion

The present study determined a partial amino acid isolation and full cDNA sequence for NPF from *R. flavipes*. The discovery of *Ref* NPF confirms the presence of NPF-like material in *R. flavipes* suggested by a previous immunocytochemical investigation (Chapter 2). The *Ref* NPF sequence also has several characteristics of known insect and other invertebrate NPFs. Among insects, most full-length NPFs have been isolated from holometabolus insects whereas hemimetabolus insects are only represented by *Lom* NPF (Clynen et al., 2006) and a putative *Peh* NPF (Joe Crim, personal communication).

The *Hez* MP-I antibody was a useful tool to isolate the previously unknown *R. flavipes* NPF sequence. The *Hez* MP-I sequence is highly similar to the semi-conserved C-terminal portion of known insect NPFs. In contrast, the *Dm* NPF antibody was used to monitor the isolation of *Aea* NPF (Stanek et al., 2002). This antigen is a much longer molecule that has much less overall similarity to other insect NPFs. We initially attempted to use *Dm* NPF antibody for purification but no immunoreactivity was detected. Synthetic *Ref* NPF was not immunoreactive with *Dm* NPF antibody, but was detected by the *Hez* MP-I antibody (Chapter 4).

The epitopes in the *Hez* MP-I antibody are made to a shorter, more conserved NPF sequence while the *Dm* NPF sequence is atypical, even in the C-terminal region. The *Dm* NPF antibody may be more suitable for purifying dipteran NPFs (Stanek et al., 2002).

SignalP analysis (Bendtsen et al., 2004) of *Ref* NPF cDNA predicted signal peptide cleavage between residues Ala_{27} and Lys_{28} (Fig 3.3). This feature corresponds to the Edman degradation data in which the first ambiguous amino acid would be Lys_{28} followed by Pro_{29} and so on, with eventual processing resulting in a 30 amino acid long peptide. This is shorter than the conventional 36-39 amino acid NPF length typical in other insects (Clynen et al., 2006). It is possible that the NPF we isolated was partially degraded during HPLC purification at the susceptible basic site (Lys_{28}). The other immunoreactive fractions from the initial extract may constitute a longer, intact form of NPF, possibly one that is the conventional 36 amino acid length.

The cannonical amino acid residues of NPFs are conserved in the *Ref* NPF sequence. The trend among invertebrate NPFs for strong C-terminal sequence conservation (Brown et al, 1999) also occurs in this peptide. The amino acid sequence of *Ref* NPF was most similar to *Lom* NPF (58% identical, 83% conserved) (Fig. 3.6A). The C-peptides of these two sequences were also highly similar sharing 50% identical amino acid residues (Fig. A.4, Appendix A). The similarities between these taxa were not surprising considering that termites are more closely related to locusts than any other species for which NPF has been sequenced. Also of interest are *Schistocerca gregaria* NPF (*Scg* NPF) (Schoofs et al., 2001), *Maj* NPF and *Penaus monodon* PYF II (*Pem* PYF II) (Sithigorngul et al., 2002), which are virtually identical to the C-terminus of *Ref* NPF and *Lom* NPF (Table 1.1, Chapter 1).

Ref NPF exhibits numerous homologies with "ancestral" vertebrate NPY, PYY and PP sequences and conserved amino acids among known vertebrate members of these peptides (Fig. 3.2D, E) (Larhammar, 1996). Curiously, *Ref* NPF exhibits homologies near the N-terminus in contrast to other insects. Vertebrate members of the NPY family contain a common PXXPXXP motif near the N-terminus which is partially conserved in *Ref* NPF (Fig. 3.2C, D).

An intron found in several NPF genes has been cited as evidence for a NPY-NPF homology and was noted in *Moe* NPF (Figure 1A in Mair et al., 2000). Among vertebrates the genes for NPY, PPY and PP, each contain an intron of variable size in the region that corresponds to the penultimate Arg residue in the coding sequence (Mair et al., 2000). This intron has since been noted in *An. gambiae* (Garczynski et al., 2005), *Ae. aegypti*, and *A. mellifera* (Joe Crim, personal communication) but was not found in *D. melanogaster* (Brown et al., 1999). An additional 111 bp cDNA product in the *Ref* NPF sequence occurred in the same position as the intron in other NPF and NPY-related genes (Fig. A.1, Appendix A). Whether or not this product comprises an actual intron was not resolved in our study.

The cladograms generated with NPF sequences placed closely related species together, but did not cluster all insects together (Fig. 3.4B). However, this analysis did indicate the divergence of *Apm* NPF and *Bom* NPF1 amino acid sequences from other insect NPFs. *Dm* NPF and *Moe* NPF were also divergent and did not cluster strongly with any group, somewhat surprising considering the analysis included other dipterans and flatworms. This may partially be a result of the small number of species sampled.

Knowledge of the amino acid sequence of *Ref* NPF allows chemical synthesis of *Ref* NPF for use in bioassays to determine the role of this peptide in termites. The immunocytochemical distribution of an NPF-like peptide in axons on the corpora allata (Chapter 2) suggests

that *Ref* NPF has an affect on JH synthesis. The presence of NPF-like material in axons on the foregut and midgut also indicate action on these tissues, possibly in the regulation of gut motility.

5. References

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Purification steps of *Ref* NPF

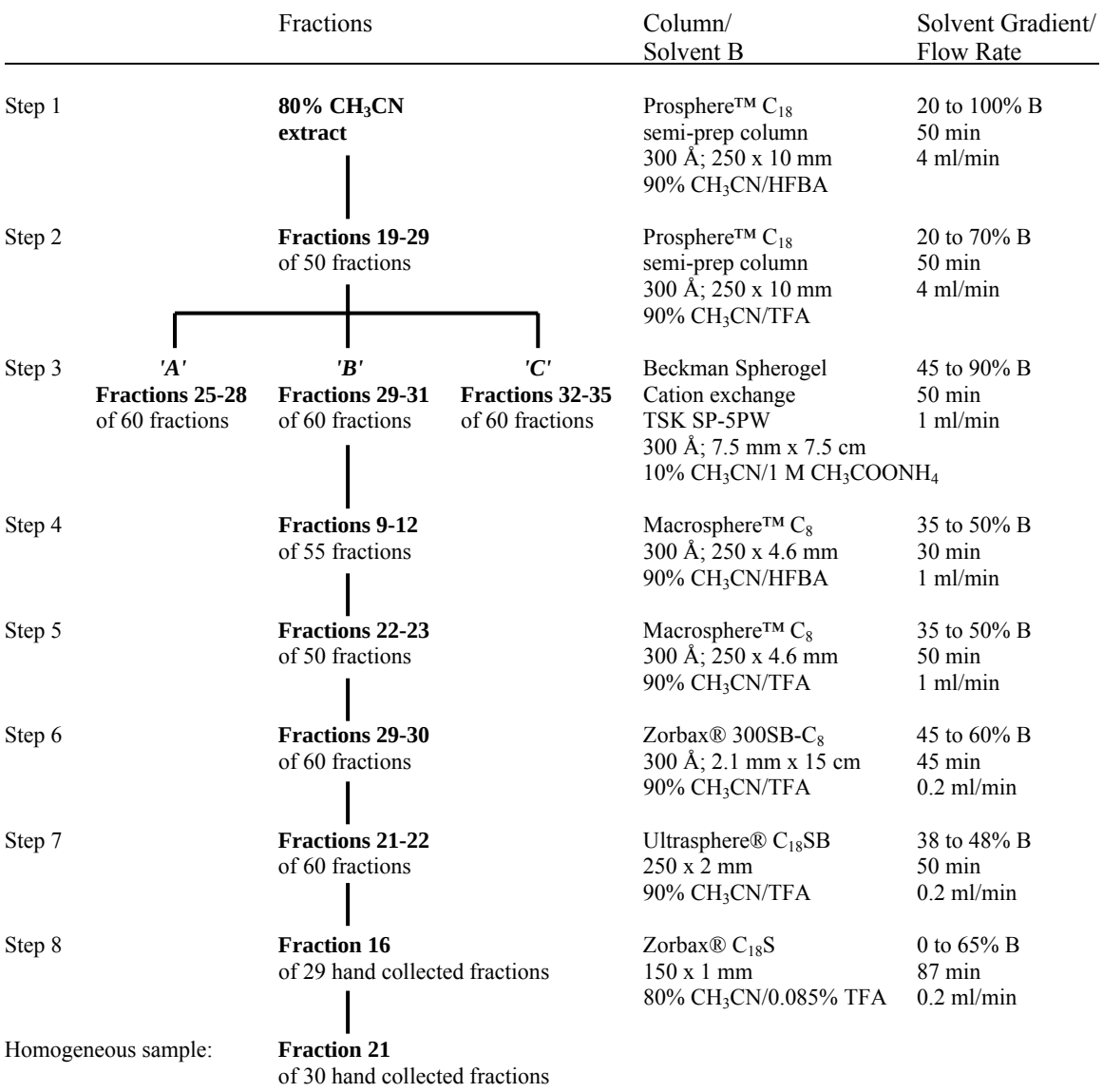


Figure 3.1: Flowchart of *Ref* NPF purification. Immunoreactive fractions indicated in bold were pooled . Composition of solvent B is listed. TFA and HFBA were at 0.1% unless otherwise noted. Solvent A contained water and the ion pairing agent except for step 3 where solvent A was 0.02 M CH_3COONH_4 with 10% CH_3CN.

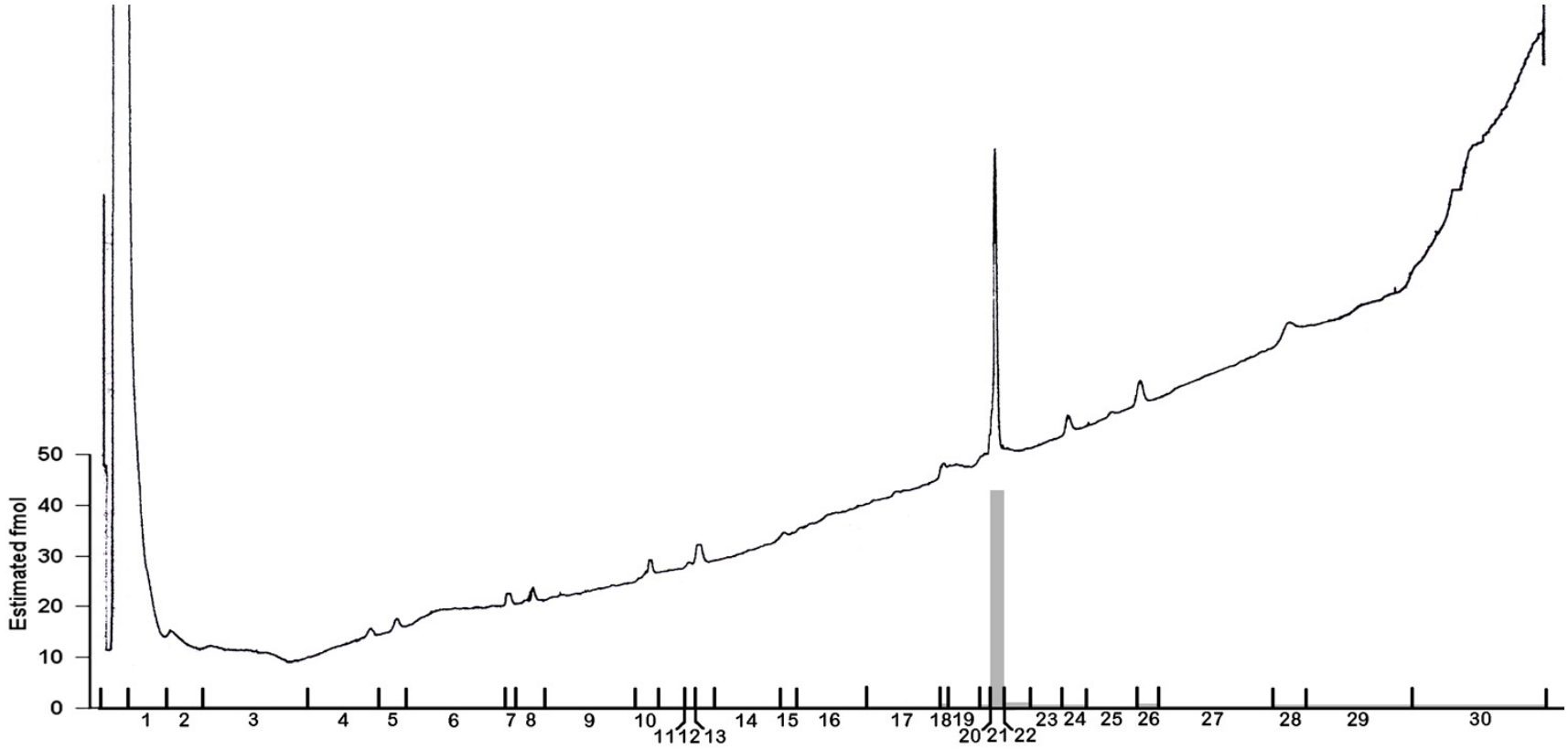


Figure 1: Chromatograph (λ = 215 nm) of the final step of purification of sample *B* of *Reticulitermes flavipes* worker extract by RP-HPLC (step 8) using a $C_{18}S$ column with CH_3CN and TFA. The amount of NPF-like material in each fraction was estimated by radioimmunoassay using *Hez* MP-I antibody on material equivalent to 600 workers (gray bars).

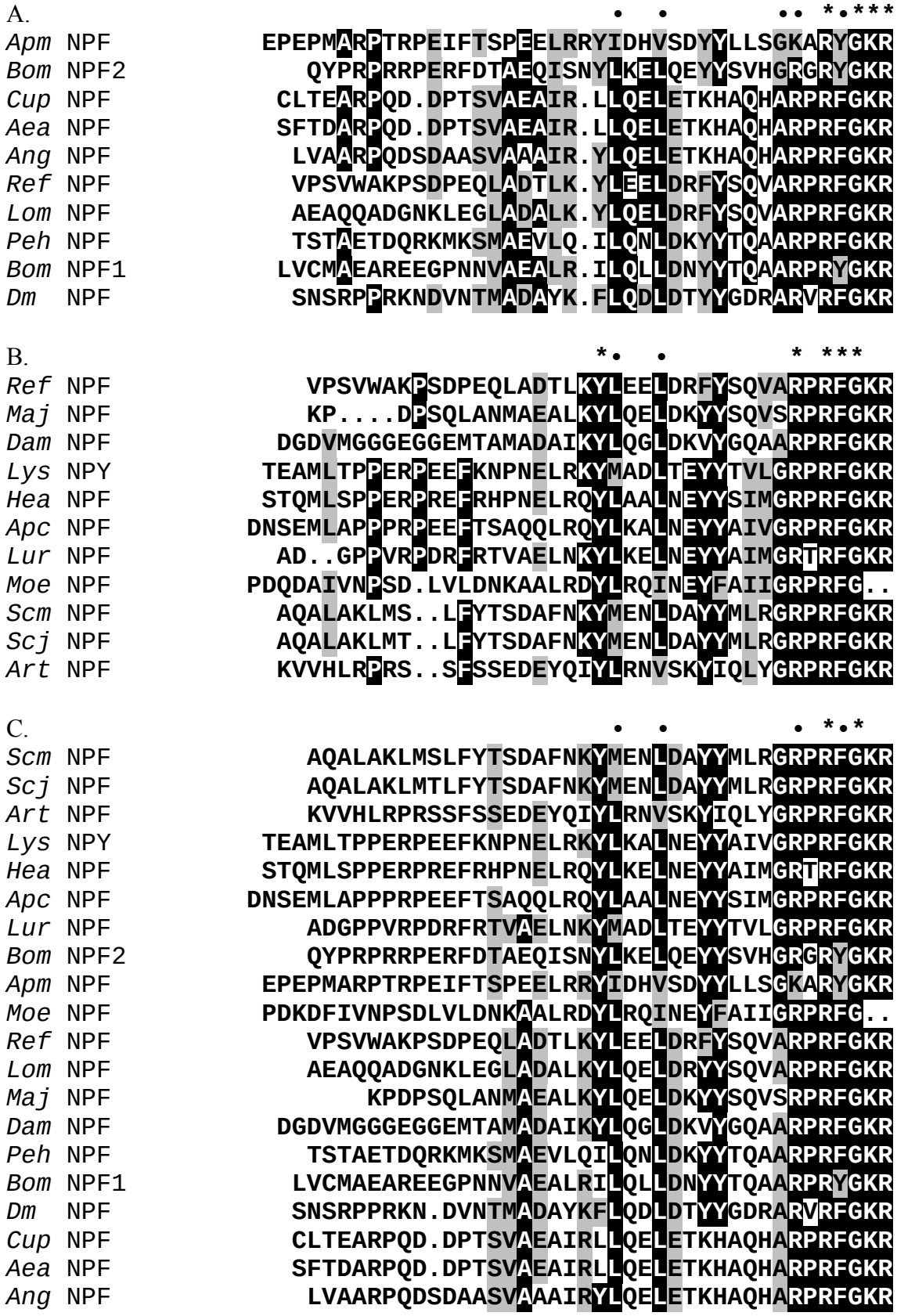
```
  1   GACGTACACGACGGACTTTGTTGCCTGAACGCTAGCAGCAGGAAGTCGCAGCTCGCCACA
 61   GCCATCTACACGTCAGGTCATCGACGTTCACTTTCTGAAAAGAAGGTCACGTGATTCACC

121   ATGCAGAACTTCCATTTTTGGCTTCTTGTGTTGGGATGTGCCCTCATCTTCGTCCCTAGT
       M  Q  N  F  H  F  W  L  L  V  L  G  C  A  L  I  F  V  P  S
       1                                                        20
181   ATCGTTCCCAGTGTCTGGGCCAAACCCTCCGACCCCGAGCAACTGGCGGACACCCTCAAG
       I  V  P  S  V  W  A  K  P  S  D  P  E  Q  L  A  D  T  L  K
                                                                40
241   TATCTGGAAGAATTGGATCGCTTTTACTCCCAGGTCGCCAGGCCAAGGTTTGGCAAGAGG
       Y  L  E  E  L  D  R  F  Y  S  Q  V  A  R  P  R  F  G  K  R
                                                                60
301   GCAGAACTGAGACCCGTCACTGAACAAGAGGCCGCTCCTGATGATTCCTCTGACAGTCTT
       A  E  L  R  P  V  T  E  Q  E  A  A  P  D  D  S  S  D  S  L

361   TGGCGGCAGTTTGCCAGCAGAAGGTGAAAGACTTTAGACGTCACGTGGTCAACCAAAAAC
       W  R  Q  F  A  S  R  R  *

421   CGCAATTCCCAACAATTACAGTGTTACTATTTGTTTCTTTAACCTTCGTCTTCAGAAACA
481   CATTGTAATTTATTATTGTAACACGAAACTGCATTAAGAATTAAATAAAAATTTTCGGCA
541   AATCTCTT
```

Figure 3.3: Nucleotide sequence and amino acid sequence for the prepropeptide of *Reticulitermes flavipes* NPF. Putative signal peptide is in bold, and the putative mature 36 amino acid long peptide is underlined. The sequence is presumably processed at Gly_{58} forming an amide at the C-terminus. The C-peptide is in italics.


A.
Apm NPF EPEPMARPTRPEIFTSPEELRRYIDHVSDYYLLSGKARYGKR
Bom NPF2 QYPRPRRPERFDTAEQISNYLKELQEYYSVHGRGRYGKR
Cup NPF CLTEARPQD.DPTSVAEAIR.LLQELETKHAQHARPRFGKR
Aea NPF SFTDARPQD.DPTSVAEAIR.LLQELETKHAQHARPRFGKR
Ang NPF LVAARPQDSDAASVAAAIR.YLQELETKHAQHARPRFGKR
Ref NPF VPSVWAKPSDPEQLADTLK.YLEELDRFYSQVARPRFGKR
Lom NPF AEAQQADGNKLEGLADALK.YLQELDRFYSQVARPRFGKR
Peh NPF TSTAETDQRKMKSMAEVLQ.ILQNLDKYYTQAARPRFGKR
Bom NPF1 LVCMAEAREEGPNNVAEALR.ILQLLDNYYTQAARPRYGKR
Dm NPF SNSRPPRKNDVNTMADAYK.FLQDLDTYYGDRARVRFGKR
B.
Ref NPF VPSVWAKPSDPEQLADTLKYLEELDRFYSQVARPRFGKR
Maj NPF KP....DPSQLANMAEALKYLQELDKYYSQVSRPRFGKR
Dam NPF DGDVMGGGEGGEMTAMADAIKYLQGLDKVYGQAARPRFGKR
Lys NPY TEAMLTPPERPEEFKNPNELRKYMADLTEYYTVLGRPRFGKR
Hea NPF STQMLSPPERPREFRHPNELRQYLAALNEYYSIMGRPRFGKR
Apc NPF DNSEMLAPPPRPEEFTSAQQLRQYLKALNEYYAIVGRPRFGKR
Lur NPF AD..GPPVRPDRFRTVAELNKYLKELNEYYAIMGRTRFGKR
Moe NPF PDQDAIVNPSD.LVLDNKAALRDYLRQINEYFAIIGRPRFG..
Scm NPF AQALAKLMS..LFYTSDAFNKYMENLDAYYMLRGRPRFGKR
Scj NPF AQALAKLMT..LFYTSDAFNKYMENLDAYYMLRGRPRFGKR
Art NPF KVVHLRPRS..SFSSEDEYQIYLRNVSKYIQLYGRPRFGKR
C.
Scm NPF AQALAKLMSLFYTSDAFNKYMENLDAYYMLRGRPRFGKR
Scj NPF AQALAKLMTLFYTSDAFNKYMENLDAYYMLRGRPRFGKR
Art NPF KVVHLRPRSSFSSEDEYQIYLRNVSKYIQLYGRPRFGKR
Lys NPY TEAMLTPPERPEEFKNPNELRKYLKALNEYYAIVGRPRFGKR
Hea NPF STQMLSPPERPREFRHPNELRQYLKELNEYYAIMGRTRFGKR
Apc NPF DNSEMLAPPPRPEEFTSAQQLRQYLAALNEYYSIMGRPRFGKR
Lur NPF ADGPPVRPDRFRTVAELNKYMADLTEYYTVLGRPRFGKR
Bom NPF2 QYPRPRRPERFDTAEQISNYLKELQEYYSVHGRGRYGKR
Apm NPF EPEPMARPTRPEIFTSPEELRRYIDHVSDYYLLSGKARYGKR
Moe NPF PDKDFIVNPSDLVLDNKAALRDYLRQINEYFAIIGRPRFG..
Ref NPF VPSVWAKPSDPEQLADTLKYLEELDRFYSQVARPRFGKR
Lom NPF AEAQQADGNKLEGLADALKYLQELDRYYSQVARPRFGKR
Maj NPF KPDPSQLANMAEALKYLQELDKYYSQVSRPRFGKR
Dam NPF DGDVMGGGEGGEMTAMADAIKYLQGLDKVYGQAARPRFGKR
Peh NPF TSTAETDQRKMKSMAEVLQILQNLDKYYTQAARPRFGKR
Bom NPF1 LVCMAEAREEGPNNVAEALRILQLLDNYYTQAARPRYGKR
Dm NPF SNSRPPRKN.DVNTMADAYKFLQDLDTYYGDRARVRFGKR
Cup NPF CLTEARPQD.DPTSVAEAIRLLQELETKHAQHARPRFGKR
Aea NPF SFTDARPQD.DPTSVAEAIRLLQELETKHAQHARPRFGKR
Ang NPF LVAARPQDSDAASVAAAIRYLQELETKHAQHARPRFGKR

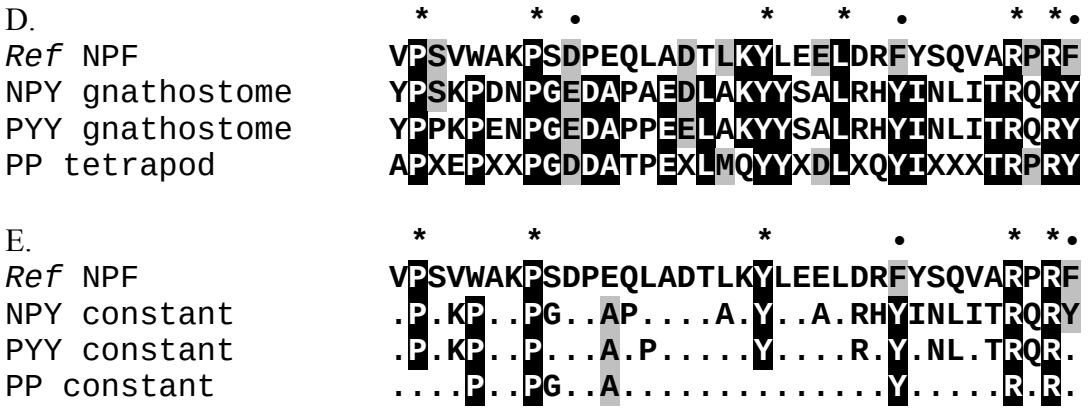


Figure 3.4: A-C: Clustal X alignment of 35-40 amino acid long NPF sequences. A: Alignment of insect NPFs. B: Alignment of non-insect invertebrate NPFs with *Ref* NPF. C: Alignment of all invertebrate NPF sequences. D-E: Manual alignment of *Ref* NPF with vertebrate NPY-like amino acid sequences. D: Alignment of *Ref* NPF with "ancestral" NPY, PYY and PP. E: Alignment of *Ref* NPF with constant amino acids of NPY, PYY and PP (Larhammar, 1996). Majority identical. Majority conserved. ***** All residues identical. • All residues conserved.

A.

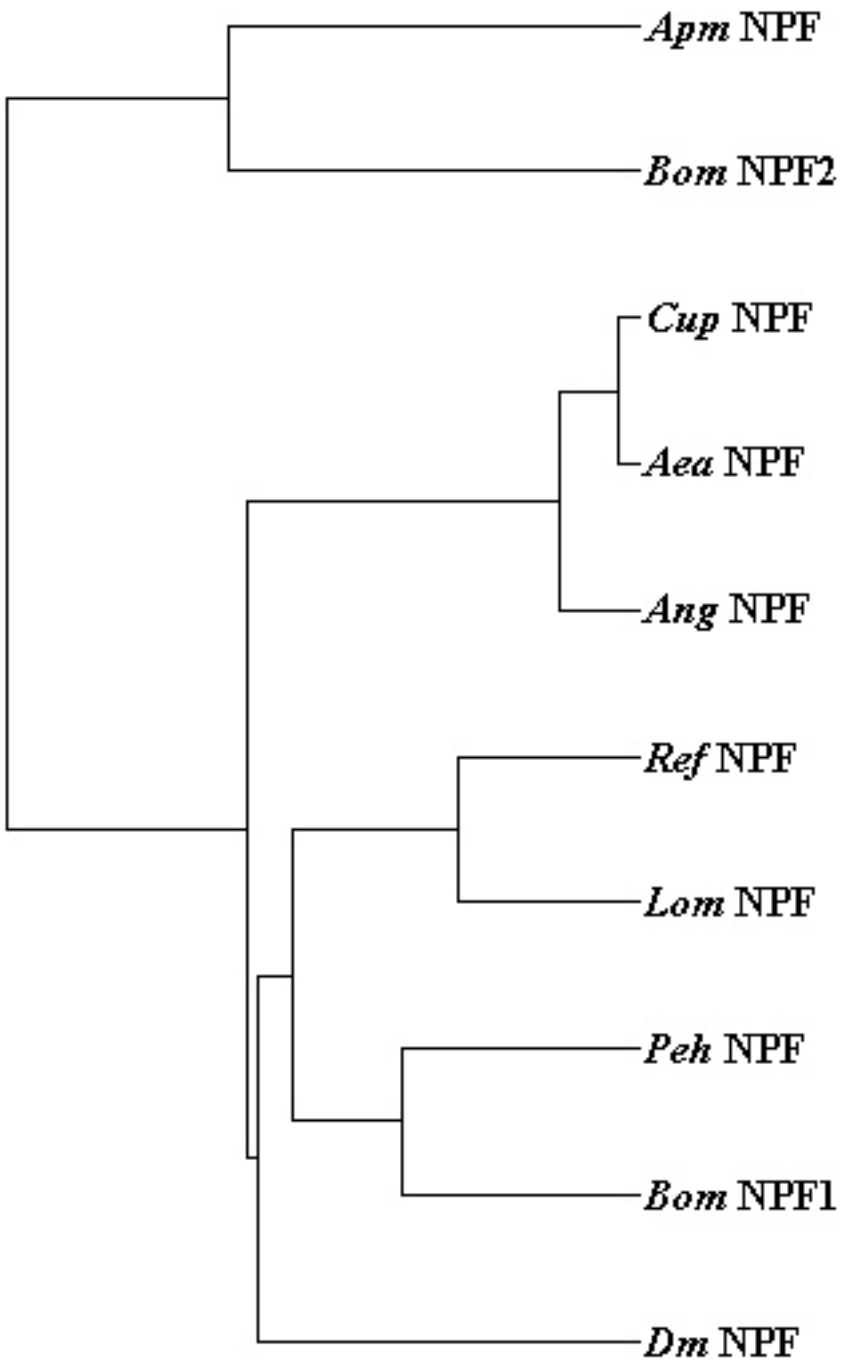
Aea NPF **TDARPQDDPTSVAEAIRLLQELETKHAQHARPRFGKR**
Cup NPF **AEARPQDDPTSVAEAIRLLQELETKHAQHARPRFGKR**
Ang NPF **VAARPQDDAASVAAAIRYLQELETKHAQHARPRFGKR**
Ref NPF **PSVWAKPDPEQLADTLKYLEELDRFYSQVARPRFGKR**
Lom NPF **EAQQADGKLEGLADALKYLQELDRYYSQVARPRFGKR**
Peh NPF **STAETDQKMKSMAEVLQILQNLDKYYTQAARPRFGKR**
Bom NPF2 **QYPRPRRERFDTAEQISYLKELQEYYSVHGRGRYGKR**
Bom NPF1 **CMAEAREEPNNVAEALRILQLLDNYYTQAARPRYGKR**
Apm NPF **PMARPTREIFTSPEELRYIDHVSDYYLLSGKARYGKR**
Dm NPF **NSRPPRKDVNTMADAYKFLQDLDTYYGDRARVRFGKR**

B.

Maj NPF **VAEAKPDPQLANMAEALKYLQELDKYYSQVSRPRFG**
Dam NPF **DVMGGGEGEMTAMADAIKYLQGLDKVYGQAARPRFG**
Scm NPF **AQALAKLMLFYTSDAFNKYMENLDAYYMLRGRPRFG**
Scj NPF **AQALAKLMLFYTSDAFNKYMENLDAYYMLRGRPRFG**
Moe NPF **DFIVNPSDVLDNKAALRDYLRQINEYFAIIGRPRFG**
Lys NPF **MLTPPERPEFKNPNELRKYLKALNEYYAIVGRPRFG**
Hea NPF **MLSPPERPEFRHPNELRQYLKELNEYYAIMGRTRFG**
Art NPF **KVVHLRPRSFSSEDEYQIYLRNVSKYIQLYGRPRFG**
Apc NPF **MLAPPPRPEFTSAQQLRQYLAALNEYYSIMGRPRFG**
Apm NPF **PMARPTRPIFTSPEELRRYIDHVSDYYLLSGKARYG**
Ref NPF **VPSVWAKPDPEQLADTLKYLEELDRFYSQVARPRFG**
Peh NPF **TSTAETDQKMKSMAEVLQILQNLDKYYTQAARPRFG**
Lur NPF **ADGPPVRPRFRTVAELNKYMADLTEYYTVLGRPRFG**
Lom NPF **AEAQQADGKLEGLADALKYLQELDRYYSQVARPRFG**
Dm NPF **NSRPPRKNDVNTMADAYKFLQDLDTYYGDRARVRFG**
Cup NPF **LTEARPQDDPTSVAEAIRLLQELETKHAQHARPRFG**
Bom NPF2 **QYPRPRRPRFDTAEQISNYLKELQEYYSVHGRGRYG**
Bom NPF1 **VCMAEAREGPNNVAEALRILQLLDNYYTQAARPRYG**
Ang NPF **LVAARPQDDAASVAAAIRYLQELETKHAQHARPRFG**
Aea NPF **FTDARPQDDPTSVAEAIRLLQELETKHAQHARPRFG**

Figure 3.5: Trimmed NPF sequences for Jukes-Cantor distance analysis (GCG). A. Insect NPFs. B. All NPFs.

A.


Apm NPF
Bom NPF2
Cup NPF
Aea NPF
Ang NPF
Ref NPF
Lom NPF
Peh NPF
Bom NPF1
Dm NPF

B.

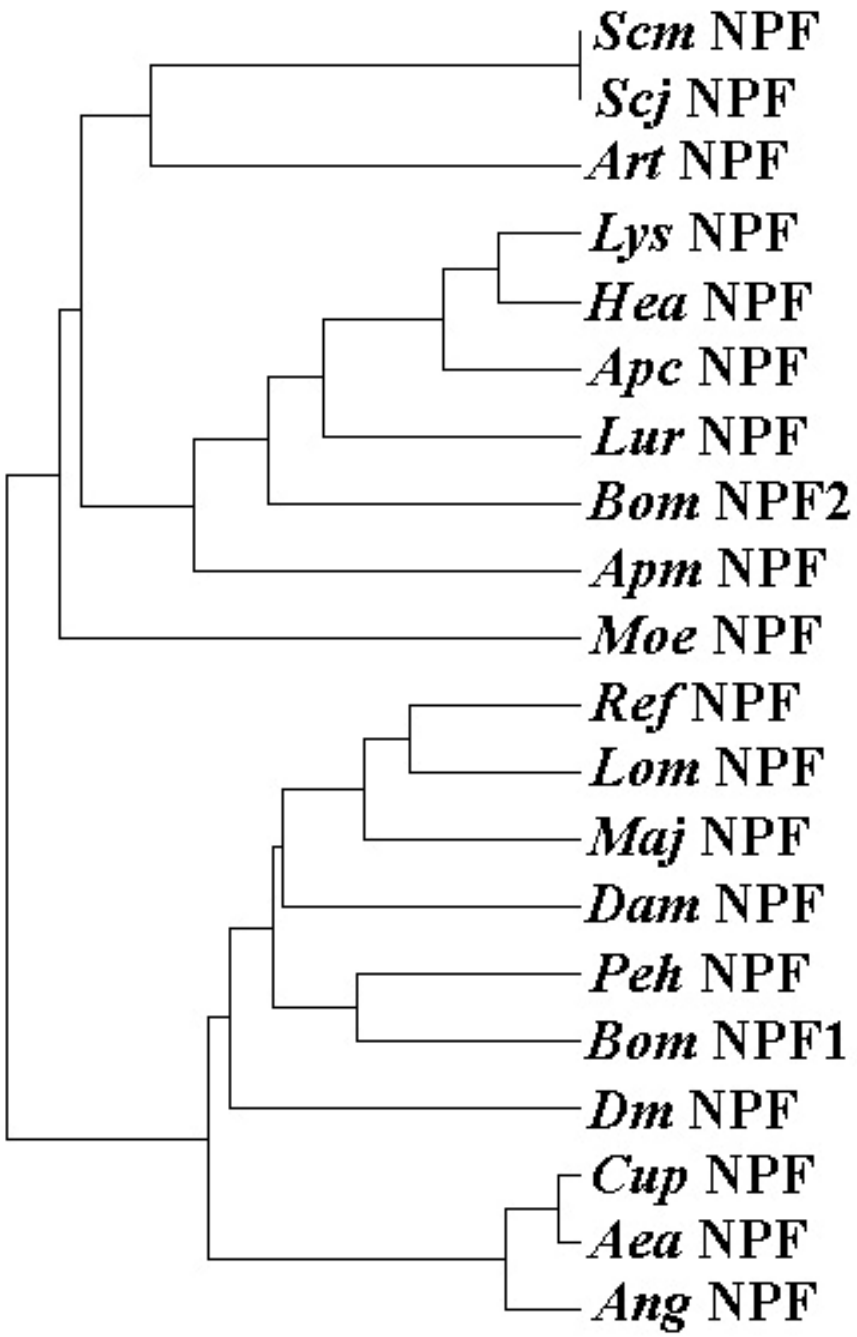


Figure 3.6: Cladograms of NPF sequences. NPF sequences were aligned with Clustal X and trimmed (Fig. 3.5). The dendrogram was constructed by Jukes-Cantor distance analysis of the sequences within GCG. A. Insect NPFs. B. All NPFs.

Table 3.1: Primer sequences used to isolate *Ref* NPF cDNA sequence. Sequences in bold indicate degenerate primers based upon the partial *R. flavipes* NPF amino acid sequence. Letters below each primer are the corresponding amino acids. Nucleic acid abbreviations: I = inosine; N = A, G, C, T; R = A, G; H = A, C, T; B = C, G, T; Y = C, T.

Forward primers / corresponding amino acids
Retic 1.1: **5'-CCIAGYGAYCCNGARC-3'** **P S D P E Q**
Retic 2: 5'-GARCAGYTBGCHGAYAC-3' **E Q L A D T**
Retic 3.2: 5'-GAYCCHGARCAGYTBGC-3' **D P E Q L A**
UPNPF1: 5'-ACACGACGGACTTTGTTGCC-3'
Reverse primers
NotI: 5'-AACTGGAAGAATTCGCGGCCGCAGGAA-3'
NotI d(T): 5'-AACTGGAAGAATTCGCGGCCGCAGGAAT$_{(18)}$-3'
NPF 1: 5'-GCCGAAAATTTTTATTTAATTCTTAATGC-3'
NPF 2: 5'-CAATGTGTTTCTGAAGACGAAGG-3'
NPF Rev: 5'-CACTGTAATTGTTGGGAATTGCGG-3'

Table 3.2: Nested PCR reactions used to isolate 3' sequences from head and gut cDNA. Final products were either cloned (H1.2) or amplified by PCR (H2.2 and G2) before sequencing.

Template	Forward primer / corresponding amino acids	Reverse primer	Resulting product
Head cDNA round 1	Retic 1.1: 5'-CCIAGYGAYCCNGARC-3' P S D P E Q	Not I d(T)	H1
H1 round 2	Retic 2: 5'-GARCAGYTBGCHGAYAC-3' E Q L A D T	Not I	H1.2 (cloned)
H1 round 2	Retic 3.2: 5'-GAYCCHGARCAGYTBGC-3' D P E Q L A	Not I	H2.2 (PCR amplified)
Midgut cDNA round 1	Retic 1.1: 5'-CCIAGYGAYCCNGARC-3' P S D P E Q	Not I d(T)	G2
G2 round 2	Retic 2: 5'-GARCAGYTBGCHGAYAC-3' E Q L A D T	Not I	G2 (PCR amplified)

Table 3.3: 5' RACE. cDNA synthesis and PCR amplification of *Ref* NPF. Final products were cloned and sequenced.

Template	Forward primer	Reverse primer	Resulting product
Head/midgut total RNA	-no forward primer-	NPF 1	*Ref* NPF cDNA
NPF cDNA round 1	Not I d(T)	NPF 2	*Ref* NPF 5' cDNA
NPF cDNA round 2	Not I	NPF Rev	*Ref* NPF 5' cDNA

Table 3.4: Abbreviations and accession numbers of NPF sequences used for cladograms.

Species	Abbreviation	Accession numbers	Reference
Locusta migratoria NPF	*Lom* NPF	CO854418	Clynen et al. 2006
Reticulitermes flavipes NPF	*Ref* NPF		This study
Pediculus humanus NPF	*Peh* NPF		Crim, unpublished data
Drosophila melanogaster NPF	*Dm* NPF	AF117896	Brown et al. 1999
Aedes aegypti NPF	*Aea* NPF	AF474405	Stanek et al. 2002
Culex pipiens NPF	*Cup* NPF		Crim, unpublished data
Anopheles gambiae NPF	*Ang* NPF	AY579077	Riehle et al. 2002
Bombyx mori NPF I	*Bom* NPF1		Crim, unpublished data
Bombyx mori NPF II	*Bom* NPF2		Crim, unpublished data
Apis mellifera NPF	*Apm* NPF	GB16364-RA	Hummon et al. 2006
Daphnia magna NPF	*Dam* NPF	EG565358	Christie et al. 2008
Marsupenaeus japonicus NPF	*Maj* NPF	CI998017	Christie et al. 2008
Lumbricus rubellus NPF	*Lur* NPF		Crim, unpublished data
Aplysia californica NPF	*Apc* NPF	M98854	Rajpara et al. 1992
Lymnaea stagnalis NPY	*Lys* NPY	AJ238276	Tensen et al. 1998
Helix aspersa NPF	*Hea* NPF	AAB24383	Leung et al. 1992
Moniezia expansa NPF	*Moe* NPF	AJ242779	Maule et al. 1991; Mair et al. 2000
Schistosoma japonicum NPF	*Scj* NPF	AY533028	Humphries et al. 2004
Schistosoma mansoni NPF	*Scm* NPF	AY662954	Humphries et al. 2004
Arthurdendyus triangulatus NPF	*Art* NPF	AAB22842	Curry et al. 1992; Dougan et al. 2002

CHAPTER 4

THE EFFECT OF *Reticulitermes flavipes* NEUROPEPTIDE F ON JUVENILE HORMONE SYNTHESIS AND GUT MOTILITY

1. Introduction

Neuropeptide F (NPF) occurs widely among invertebrates and is homologous to neuropeptide Y (NPY), pancreatic polypeptide (PP), and peptide YY (PYY) found in vertebrates (McVeigh et al. 2005). Several NPFs have been discovered in insects and other invertebrates (Maule et al. 1991; Leung et al. 1992; Rajpara et al. 1992; Tensen et al. 1998; Brown et al. 1999; Dougan et al. 2002; Riehle et al. 2002; Stanek et al. 2002; Humphries et al. 2004; Clynen et al. 2006; Hummon et al. 2006) (Table 1.1, Chapter 1). The amino acid sequence of an additional NPF, *Ref* NPF, was recently isolated and structurally characterized from the eastern subterranean termite, *Reticulitermes flavipes* (Chapter 3).

Immunoreactive NPF-like material was observed in cells and axons throughout the nervous system and the alimentary tract of *R. flavipes* using an antiserum to a truncated form of NPF in corn earworm, *Helicoverpa zea*, designated as *Hez* MP-I (Chapter 2). In particular, NPF-like material was observed in axons originating in the brain to form a network on the surface of the corpora allata (CA). The CA produces juvenile hormone (JH), and neotenic *R. flavipes* females have increased rates of JH synthesis that correlate with vitellogenesis and yolk uptake by oocytes (Elliott and Stay 2007). JH putatively functions to stimulate vitellogenin production by the fat body in termites (Lüscher 1976; Greenberg and Tobe 1985) and JH III treatments increase the expression of two vitellogenin genes in worker *R. flavipes* (Scharf et al. 2005). JH synthesis

rates decline in *R. flavipes* neotenics as oocytes reach maturity (Elliott and Stay 2007). Allatostatins reduce the rate of JH synthesis in the CA of *R. flavipes* neotenics (Yagi et al. 2005). The A-type allatostatins used to demonstrate inhibition of JH synthesis in termites also decrease JH synthesis in cockroaches and crickets but are not effective in other insect groups to date (Stay and Tobe 2007). The cycle of JH synthesis in relation to egg maturation and effects of allatostatins are well known in the cockroach *Diploptera punctata* (Rankin and Stay 1984; Tobe et al. 2000; Stay et al. 2003). FLRFamide prevented inhibition of JH synthesis in female *D. punctata* CA by *Dippu*-allatostatin 2 (*Dippu* AST-2) at day 6 after mating demonstrating that RFamides influence JH production by the CA (Stay et al. 2003). In locusts, *Schistocerca gregaria* NPF (*Scg* NPF) stimulates egg development, an observation that may be due to an increase in JH or ecdysone production (Schoofs et al. 2001). JH synthesis by the CA in the grasshopper *Romalea microptera* was not influenced by *Scg* NPF (Li et al. 2005), but it is unknown if such results apply to other insects.

NPF-like material also was observed in axons, cell bodies and endocrine cells on the alimentary tract of *R. flavipes* (Chapter 2). The foregut surface contained numerous axons originating from brain cells and from cell bodies on the foregut. Axon processes extended from the foregut over the anterior midgut. The midgut also contained numerous NPF-like midgut endocrine cells. The anterior hindgut lacked NPF-like material, and the rectum had NPF-like axons over the surface likely from the terminal abdominal ganglion (Chapter 2). Comparable immunostaining patterns have been observed on the alimentary tracts of *Drosophila melanogaster* (Brown et al. 1999), *H. zea* (Huang 1996), the mosquito *Aedes aegypti* (Brown et al. 1986; Stanek et al. 2002), the blue bottle fly, *Calliphora vomitoria* (Duve and Thorpe 1982), the American cockroach, *Periplaneta americana* (Iwanaga et al. 1981; Endo et al. 1982), the

dragonfly, *Tramea virginica* (Patankar and Tembhare 2006), the cricket, *Gryllus bimaculatus* (Iwanaga et al. 1986) and the locust *Locusta migratoria* (Schoofs et al. 1988) with antisera to NPF, NPY, and PP. For these insects, the roles of NPF in the digestive system have not been thoroughly investigated. Of interest, NPF inhibits ion transport and motility in the midgut of *Ae. aegypti* larvae (Onken et al., 2004) and affects feeding behavior in *D. melanogaster* larvae (Shen and Cai 2001).

In this study we synthesized *Ref* NPF for use in bioassays to determine its function. Both *Ref* NPF 30 and *Ref* NPF 36 were made because the actual length of the native peptide in *R. flavipes* has not been confirmed (Chapter 3). The distribution of NPF-like material in axons on the CA of *R. flavipes* suggests that this peptide may influence the production of JH. JH synthesis in the CA of *D. punctata* may also be affected by *Ref* NPF. Also, NPF-like material in axons and endocrine cells of the alimentary tract of *R. flavipes* indicates NPF affects motility of the gut. We have examined these tissues with bioassays to test possible actions of *Ref* NPF.

2. Materials and Methods

2.1. *Ref* NPF synthesis

The peptides KPSDPEQLADTLKYLEELDRFYSQVARPRF-NH_2 (30 amino acids: '*Ref* NPF 30') and VPSVWAKPSDPEQLADTLKYLEELDRFYSQVARPRF-NH_2 (36 amino acids: '*Ref* NPF 36') were synthesized with Fmoc chemistry in an Applied Biosystems 431A Peptide Synthesizer and precipitated with ether (Dr. Kevin Clark, University of Georgia, Athens, GA). Peptides were purified with high performance liquid chromatography on a Prosphere™ C_{18} column (gradient program: 20% B, 5 min, 20-40% B, 5 min, 40-55% B, 30 min, 55-100% B, 5 min; 4 ml/min; monitored at 275 nm). MALDI-TOF mass spectrometry confirmed the identity of the NPFs (Mass Spectrometry Facility, University of Georgia).

Other signaling molecules were synthesized at the University of Georgia (*Dm* NPF: SNSRPPRKNDVNTMADAYKFLQDLDTYYGDRARVRFamide; *Ang* NPF: LVAARPQDSDAASVAAAIRYLQELETKHAQHARPRFamide) or obtained from the lab in which work was performed (University of Iowa: *Dippu* AST-2; University of Toronto: Leucokinin I (LK I), SchistoFLRFamide and serotonin).

2.2. Juvenile Hormone Biosynthesis Assay

2.2.1 Animals

Reticulitermes flavipes neotenics were collected from a termite-infested log obtained from Whitehall Forest south of the University of Georgia campus. Moistened, rolled cardboard inside PVC tubing was placed next to the log in a metal tray as the log dried. Termites moved to the moistened cardboard and were collected. Neotenics emerging from the log were maintained in groups of 12 - 50 with 1,500 workers in 17 x 12 x 6.5 cm plastic boxes (Tri-State Plastics, Inc.) with moistened sand/vermiculite mixture and pine slats for 2 - 4 weeks before assays. Termites were transported to the lab of Barbara Stay at the University of Iowa, (Iowa City, IA) and used for assays. Only female brachypterous, non-physogastric neotenics were present in sufficient numbers to perform assays at the time of the investigation.

Diploptera punctata were collected from cultures maintained at the University of Iowa. Newly molted adult virgin females were taken from this collection and isolated for six days prior to dissection.

2.2.2 Juvenile hormone biosynthesis assay

Neotenics were immobilized on ice and weighed before dissections. To access the CA, the mandibles were removed and the head capsule was cut along each side starting at the posterior, and then was removed from the body. The ventral head cuticle was pinned with two

minuten pins on left and right sides, and the dorsal cuticle pulled open and pinned exposing the brain and mandibular muscles. The esophagus with attached CC and CA was removed. CA diameter was measured with an ocular micrometer.

D. punctata females were immobilized on ice then secured to a dissecting dish with clay. A v-shaped incision was made in the pronotum to expose the neck. The cuticle was cut and peeled back from the neck and the tracheae were removed. The exposed esophagus was gently pulled from the head exposing the CA, which were removed with forceps.

CC/CA pairs from neotenic termites were transferred to borosilicate test tubes with a droplet of TC 199 in the bottom. Cockroach CA pairs were separated and placed in individual borosilicate tubes with a droplet of TC 199 medium. After 30 to 45 min at room temperature, the media was aspirated to eliminate stored methionine in the CA and replaced with 47 µl of 50 µM ^{14}C methionine (56 mCi, in TC 199). ^{14}C methionine solution (47 µl) was added to two control tubes containing no CA to establish background radiation. Tubes were covered with parafilm to prevent evaporation, and tissues were incubated at 27 ºC on a shaker in a plexiglass box for 3 h. Afterwards, tissues were removed with a wire loop and 300 µl isooctane was added. Tubes were vortexed, centrifuged for 5 min, and 200 µl supernatant was taken and added to 5 ml scintillation fluid in glass vials. Vials were shaken and counted in a gamma counter. Background counts were made over a 10 min period and samples were counted for 5 min each. Amount of JH produced by tissue incubation was calculated as pmol JH per pair of CA/CC per hour (pmol/pair/h) for neotenics or pmol JH per CA (pmol/h) for cockroaches.

Ref NPF (0.425 mg) was dissolved in 10 µl dimethylformamide (DMF), then 90 µl of 60% acetonitrile (CH_3CN) for a 10^{-3} M solution. This stock was further diluted with 60% CH_3CN to 10^{-5}, 10^{-6} and 10^{-7} M. *Dippu* AST-2 was dissolved water and diluted to 10^{-7} and 10^{-8}

M. All dilutions were dried under nitrogen (N_2) for 10 min then dissolved in an equal volume of TC 199 medium with ^{14}C methionine (56 mCi).

The effect of *Ref* NPF on JH production by CC/CA was tested by *Ref* NPF 36 (10^{-7} or 10^{-6} M) or *Ref* NPF 30 (10^{-6} M) added to 47 µl ^{14}C methionine solution and incubated with termite or cockroach CA for 3 h as above. The effect of *Ref* NPF 36 on JH synthesis inhibition of *R. flavipes* CA by *Dippu* AST-2 was also tested. *Dippu* AST-2 alone (10^{-8} - 10^{-7} M) and *Dippu* AST-2 with *Ref* NPF 36 (10^{-5}, 10^{-7} M) were assayed. An equal number of control CA incubated without peptide were used to establish baseline JH synthesis each day tests were performed.

2.3. Gut motility assays

2.3.1 Impedance Monitor

Reticulitermes flavipes were obtained as described above and transported to the lab of Ian Orchard at University of Toronto at Mississauga (Mississauga, ON) where assays were performed. Workers between the 3rd and 5th instar were dissected in 100 µl *S. gregaria* saline (Wei et al., 2000) by cutting the head in half transversely and pulling the gut tract out through the abdomen with sharpened forceps. The midgut was cut posterior to the stomodeal valve. The foregut was then transferred to 100 µl fresh saline in a Sylgard coated dish where the esophagus and anterior midgut were secured with single minuten pins. Electrodes of the impedance converter were carefully maneuvered within 300 µm of the foregut at the crop-proventriculus junction. The electrodes were attached to an impedance converter (Model 2991, UFI, Morro Bay, CA) powered by a 9 volt battery and set to alternating current mode. The output signal was sent to a linear chart recorder (Linear 1200, Barnstead International) recording at 0.1 V and 2 cm/min.

Foreguts were equilibrated for an initial 15 min period, followed by a 5 min period to establish background activity. Every 5 min, 100 µl saline was exchanged to insure aeration of tissue. Foreguts were exposed to different concentrations and combinations of peptides or serotonin for 5 min, after which the tissue was rinsed four times and re-equilibrated in fresh saline for 5 min. Up to four incubations with peptide or serotonin were performed per single foregut preparation.

2.3.2 Force Transducer

The gut tracts of *R. flavipes* workers were dissected as above. The midgut was cut anterior to the pyloric valve, and a hair was tied around the junction of the midgut and hindgut. The gut was then transferred to 100 µl fresh saline in a Sylgard coated dish, and cuticle surrounding the rectum was secured to the bottom of the dish with four minuten pins. The other end of the hair was tied to a miniature force transducer (Aksjeselapet Mikro-Elektronikk, Norway). Force transducer readings were recorded with AcqKnowledge software (Version 3.5.3, ©1998, BIOPAC Systems Inc.). Hindguts were equilibrated and incubated with peptides or serotonin, as described above.

2.4. Data analysis

Means of JH production rates for treated and control CA were compared using Tukey's multiple comparison procedure ($\alpha = 0.05$) (SAS 9.1, © 2002-2003, SAS Institute Inc., Cary, NC, USA). Frequency and amplitude of force transducer readings were quantified with Clampfit software (©2005, Molecular Devices Corporation). Readings were calculated over a 4.5 min time span within each 5 min incubation period. Means of percent increase of frequency and amplitude by serotonin and LK I were tested to see if they were significantly different from zero using a one-tailed t-test ($\alpha = 0.05$) (Microsoft® Office Excel, 2003). Means of percent inhibition

of frequency and amplitude by *Dm* NPF were also compared using Tukey's multiple comparison procedure ($\alpha = 0.05$) (SAS 9.1, © 2002-2003, SAS Institute Inc., Cary, NC, USA).

3. Results

3.1 Synthetic *Ref* NPFs

Masses of synthetic *Ref* NPF 30 and 36 purified as major peaks by HPLC were 3610.7 Da and 4250.1 Da respectively, corresponding to their predicted masses of 3612.0 Da and 4251.8 Da. After *Ref* NPF 30 and 36 did not dissolve in water alone so dimethylformamide (DMF) was used to dissolve the peptides which were then diluted with solutions for bioassays. Radioimmunoassays (RIA - see Chapter 3) with *Hez* MP-I and *Dm* NPF antibodies were performed to confirm that *Ref* NPF 30 and 36 were in solution. Lyophilized *Ref* NPF 30 and 36 were re-hydrated in 10 µl dimethylformamide (DMF) and diluted in RIA buffer from 0.1 fmol to 10 pmol with *Hez* MP-I or *Dm* NPF used as standards for comparisons.

The *Hez* MP-I antibody in RIA had similar affinities for both forms of *Ref* NPF (Fig. 4.1A). *Hez* MP-I antibody bound to *Hez* MP-I with approximately 10-fold greater affinity than the synthetic termite peptides. Little or no *Ref* NPF bound to the *Dm* NPF antibody, even at the highest concentrations (Fig. 4.1B).

3.2 JH assays

Ref NPF 36 alone at 10^{-6} M did not significantly affect JH synthesis rates of CA from *R. flavipes* female neotenics over that of control CA (Table 4.1) ($F = 0.15$; df = 1, 28; $P = 0.7011$). As a control, *Dippu* AST-2 significantly reduced JH synthesis rates over that of control CA at both 10^{-8} ($F = 10.22$; df = 1, 10; $P = 0.0095$) and 10^{-7} M ($F = 27.51$; df = 1, 12; $P = 0.0002$) resulting in 65 ± 8 % and 83 ± 5 % inhibition, respectively. Inhibition of JH production was increased approximately 10% when 10^{-7} M *Dippu* AST-2 was combined with *Ref* NPF 36 (10^{-7}

and 10^{-5} M), although these differences were not significant ($F = 1.09$; df = 2, 18; $P = 0.3577$) (Table 4.1). A similar response was obtained when 10^{-8} M *Dippu* AST-2 was combined with *Ref* NPF 36, but only at the 10^{-5} M concentration ($F = 0.79$; df = 2, 15; $P = 0.4709$). Neotenic weight did not affect JH synthesis rate from CA. All neotenic CA were between 0.10 and 0.15 mm in diameter. JH synthesis rates declined in control neotenics over the week that CA assays were performed. All JH synthesis rates were low in the neotenics used in this experiment (control rates 1.08 - 1.55 pmol/pair/h) in comparison to previous studies (control rate 11.77 ± 0.68 pmol/pair/h [Elliott and Stay, 2007]).

The rates of JH synthesis in *D. punctata* CA were unaffected by *Ref* NPF 36 (10^{-7}, 10^{-6} M) compared to control CA (Table 4.2) ($F = 0.24$; df = 5, 20; $P = 0.9411$).

3.3 Gut motility assays

3.3.1 Impedance monitor

Foregut contractions were complex, but contraction in different regions was distinguished by careful observation. The esophagus, crop, crop-proventriculus junction, proventriculus, and stomodeal valve all exhibited independent movements that were detectable by the impedance monitor. Specific placement of the electrodes discriminated crop-proventriculus junction contractions from the other types of contractions, primarily by their much greater amplitude (Fig. 4.2).

Serotonin stimulated all types of foregut activities with the exception of stomodeal valve contractions. The duration of crop-proventriculus junction contractions varied by dose. Initially 10^{-7} M serotonin induced a burst of crop-proventriculus junction contractions lasting approximately 45 s (Fig. 4.3). This was followed by no crop-proventriculus junction activity for approximately 1 min after which crop-proventriculus junction was active again for another 45 -

60 s. Higher concentrations of serotonin (5 x 10^{-7} and 10^{-6} M) sustained crop-proventriculus junction contractions throughout the entire 5 min monitoring period (Fig. 4.2). The 4x saline wash abolished all crop-proventriculus junction contractions. LK I did not noticeably stimulate foregut contractions at 10^{-7} of 10^{-6} M (Fig. 4.4).

Ref NPF 36 and *Dm* NPF did not measurably affect foregut tissue by themselves or stimulation of foregut tissue by serotonin (Figs. 4.2, 4.3, 4.5). SchistoFLRFamide (10^{-6} M) was effective at inhibiting crop-proventriculus junction contractions when introduced with serotonin (10^{-6} M and 10^{-7} M) (Fig. 4.6).

Attempts to monitor contractions of *R. flavipes* midguts with the impedance monitor were unsuccessful. Noise from contractions of the stomodeal and pyloric valves obscured peristaltic contractions of the midgut.

3.3.2 Force transducer

Both serotonin and LK I induced hindgut contractions over the activity of background and saline washes (Table 4.3, Figs. 4.7, 4.8). Serotonin increased the frequency and amplitude of contractions at 10^{-7} and 10^{-6} M, but not at 10^{-8} M (Table 4.3). Contractions induced by serotonin were much more frequent (Fig. 4.7) and, upon close observation, appeared to be primarily from the circular muscles as waves of contractions radiated along the hindgut. LK I significantly increased both the amplitude and frequency of hindgut contractions over background and saline wash activities at all concentrations. LK I-induced contractions appeared to affect the longitudinal muscles, and contractions were stronger (greater amplitude) and more regularly spaced than serotonin-induced contractions (Figs. 4.7, 4.8). LK I induced contractions at a lower concentration (10^{-9} M) than serotonin (10^{-7} M) (Table 4.3).

Ref NPF 30 and 36 did not inhibit spontaneous (Fig. 4.9) or serotonin-induced contractions (Table 4.4), but the frequency and amplitude increased over baseline activity for both *Ref* NPF 30 and *Ref* NPF 36. *Ang* NPF also induced a slight increase in the frequency and amplitude of serotonin-induced contractions as well as those induced by LK I (Table 4.4). In contrast, *Dm* NPF (10^{-7} - 10^{-6} M) reduced the frequency of hindgut contractions induced by serotonin and LK I in a dose-dependent manner (Fig. 4.10). The amplitude of serotonin-induced contractions was reduced by 10^{-6} M *Dm* NPF (Fig. 4.11).

Activities of dissected hindguts attached to the force transducer were variable. Almost all hindguts exhibited spontaneous contractions during the equilibration period, but activity ceased before a baseline reading on gut activity was made. Hindguts that sustained spontaneous contractions during the baseline reading were incubated with 10^{-8}, 10^{-7} or 10^{-6} M concentrations of *Dm* NPF. This peptide reduced the frequency of spontaneous contractions in a dose-dependent manner, but only reduced the amplitude of contractions at the 10^{-6} M concentration (Figs. 4.10, 4.11).

4. Discussion

4.1 JH assays

Only non-physogastric neotenics were used in the JH synthesis assays partially explaining the low JH synthesis rates observed. A reason for why only non-physogastric individuals were available at the time of the assays may be that the health of the termites used in the CA assays was poor. Mites were seen clinging to the bodies of termites, dead workers and soldiers were observed, and the remaining live termites responded feebly to disturbance (compared to newly field-collected termites that immediately run for a sheltered portion of the collection when the box is disturbed). There were clearly egg-laying individuals in the

containers as piles of eggs were noted when extracting the neotenics suggesting the termites were sufficiently healthy in the recent past. A possible explanation is that transport from Georgia to Iowa disturbed the neotenics to a point where JH synthesis was downregulated. Conversely, low JH synthesis rates of *R. flavipes* neotenics are associated with the maturation of eggs or early development of ovarioles (Elliot and Stay 2007). The presence of eggs with non-physogastric neotenics may indicate that egg-laying was not continuous and that neotenics used were between egg-laying cycles. The egg-laying rate of *R. flavipes* in captivity is also limited by the size of arenas used for containment (Grube and Forschler 2004) which may also explain low JH synthesis rates. The low JH synthesis rates of the study termites may have obscured the effect of *Ref* NPF in this experiment. However, the cockroaches used in our experiment were healthy and developmentally more uniform, but JH synthesis rate was also not affected by *Ref* NPF.

Ref NPF may not influence the CA in *R. flavipes*. It is possible that the NCC I and II, which contain NPF-like material in *R. flavipes* (Chapter 2), do not release it directly into the CA, but instead continue to other targets down the postallatal nerve. Preliminary immunocytochemical studies with the *Hez* MP-I antiserum on sections of worker termites showed immunoreactive axons on the surface of the CA, but not penetrating the tissue. Similar observations were made using a more general antiserum to RFamide (Barbara Stay, personal communication). Also, the truncated *Scg* NPF did not influence JH synthesis by the CA in *R. microptera* (Li et al., 2005).

4.2. Gut motility assays

The activity of serotonin, LK I, *Dm* NPF and SchistoFLRFamide used in this study indicate that regulation of gut motility in *R. flavipes* is similar to that in other insects. Contractions at the crop-proventriculus junction of *R. flavipes* foreguts were stimulated by

serotonin and inhibited by SchistoFLRFamide, thus indicating that foregut preparations used in this experiment were viable and receptive to biogenic amines and other peptides. Serotonin has been used as a stimulator of gut tissue to show inhibition of contractions by peptides (Onken et al. 2004). SchistoFLRFamide is classified with myosuppressin-type peptides which are inhibitory on foregut contractions in cockroaches (Aguilar, et al. 2004).

Hindgut contractions of *R. flavipes* were stimulated by both LK I and serotonin. As with serotonin, LK I has been used as a stimulator of hindgut tissue for inhibition assays (Sarkar et al. 2003). Inhibition of spontaneous and induced hindgut contractions by *Dm* NPF (Figs 4.7, 4.8, 4.10, 4.11) suggested the presence of NPF receptors in this region. This was unexpected because no NPF-like axons were detected on the anterior hindgut in *R. flavipes* (Chapter 2). A network of NPF-like axons does occur on the surface of the hindgut of other insects such as *H. zea* (Huang 1996) and *Rhodnius prolixus* (Gonzalez and Orchard 2008). In termites, NPF may be carried to hindgut NPF receptors by the hemolymph from release sites such as the CC, neurosecretory cells or midgut endocrine cells (Jenkins et al., 1989). However, results from bioassays using heterologous neuropeptides must be viewed with caution. *Dm* NPF contains conserved structural elements that also occur in *Ref* NPF, but the overall amino acid similarity between the two peptides is low (36% identical, 61% conserved) (Chapter 3). The possibility exists that *Dm* NPF is acting on receptors different from the putative termite NPF receptor.

4.3 Solubility of *Ref* NPF 30 and 36

Lack of *Ref* NPF activity on tissues investigated in this study was unexpected considering *Ref* NPF is a native peptide in *R. flavipes*. Solubility problems with *Ref* NPF 30 and 36 suggest that this peptide did not fold correctly after synthesis and purification with HPLC. Although *Ref* NPF 30 and 36 were recognized by the *Hez* MP-I RIA (Fig. 4.1A) and material of the correct

size was detected by MALDI-TOF, these only confirm the properties that the peptide was of the correct composition and that the antigenic portion of the *Ref* NPF was exposed to the corresponding *Hez* MP-I epitopes. It does not confirm correct folding of *Ref* NPF which would be required for interaction with termite NPF receptors. Unfortunately there was insufficient native peptide remaining after the purification and Edman degradation of NPF-like fractions (Chapter 3) to examine the activity of this material on the CA and alimentary tract tissues.

Only one of three NPF-like fractions was purified to homogeneity and sequenced during the initial *Ref* NPF characterization (Chapter 3). The other two fractions may be degradation products of *Ref* NPF. Smaller fragments of *Ref* NPF may be required for interaction with receptors or another NPF with a different sequence may occur in termites (Appendix A). For instance, two different NPF sequences were detected in the genome of the silkworm, *Bombyx mori* (Joe Crim, personal communication).

4.4. Conclusion

In the past, purification of peptides was coupled with bioassays of known activity. For example, the first FMRFamide was isolated from the Sunray Venus clam, *Macrocallista nimbosa* based on its activity on the heart of the clam, *Mercenaria mercenaria* and on the radula protractor muscle of the whelk, *Busycon contrarium* (Price and Greenburg, 1977). As the use of antisera became a widespread tool for describing peptide distribution and for peptide purification, it was predicted that discovery of peptides might outpace prior knowledge of their function (Brown and Lea, 1990). Immunoassays have allowed us to isolate *Ref* NPF in such a fashion. The paucity of bioactivity information pertaining to NPFs makes the assignment of functions to NPF in *R. flavipes* difficult.

The inability to establish activity of *Ref* NPF on CA or gut tissue was unexpected. Solubility problems suggest that the *Ref* NPF we used may not be in the correct conformation for interaction with receptors. Our knowledge of NPF functions is very sparse especially in insects other than *D. melanogaster*. Few studies have attempted to test NPF on isolated tissue preparations (Onken et al., 2004; Li et al. 2005). It is possible that *Ref* NPF acts as a neuromodulator and its effects were more subtle and not detectable by the assays we used. Despite the unclear activity of synthetic *Ref* NPF, ancillary information was gained in this experiment by demonstrating myostimulators and myoinhibitors that are potent in other insects are also effective in termites.

Other tissues besides those examined in this study such as the salivary glands and midgut may be affected by NPF. For instance, NPF function in the termite midgut may be similar to the ion-transport inhibition and midgut motility inhibition activity recorded in mosquitoes (Onken et al., 2004). The brain-gut distribution of NPF may also indicate that NPF functions in the proposed cross-talk between the nervous system and gut (Brown and Lea, 1990). Behavioral effects of NPF are unknown in insects outside of *D. melanogaster*. In the future, injection of NPF or NPF-antagonists into termites may reveal some of the behavioral aspects under the control of NPF in *R. flavipes*. Also, techniques such as RNAi may facilitate downregulation of NPF in termites allowing observation of behavior at low NPF levels.

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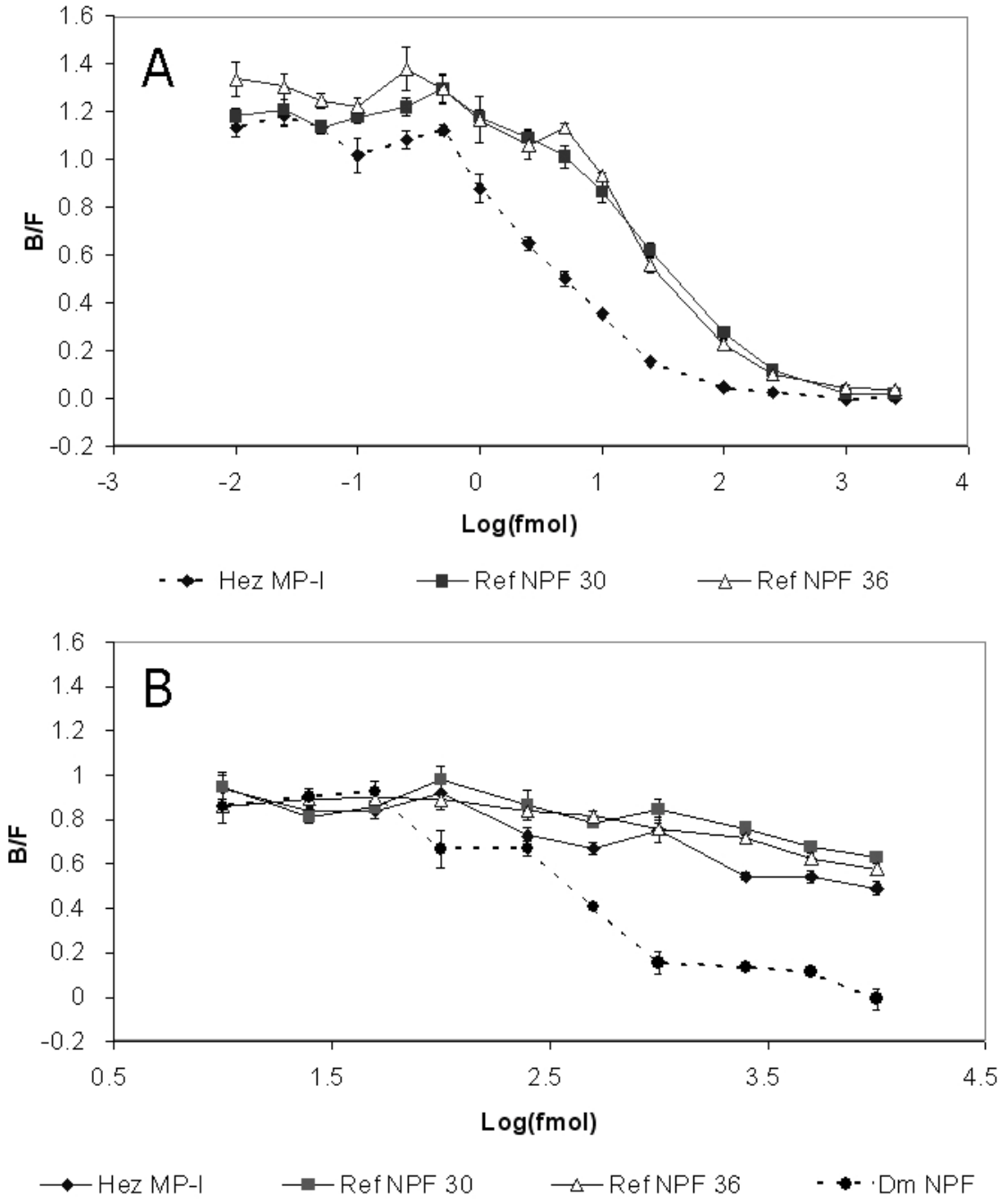


Figure 4.1: Cross-reactivity of antisera to synthetic peptides as determined by radioimmunoassay. Reactivity is expressed as a bound/free (B/F) ratio of the tested peptide (± SEM) versus the log dose of the peptides. A) *Hez* MP-I antiserum. B) *Dm* NPF antiserum.

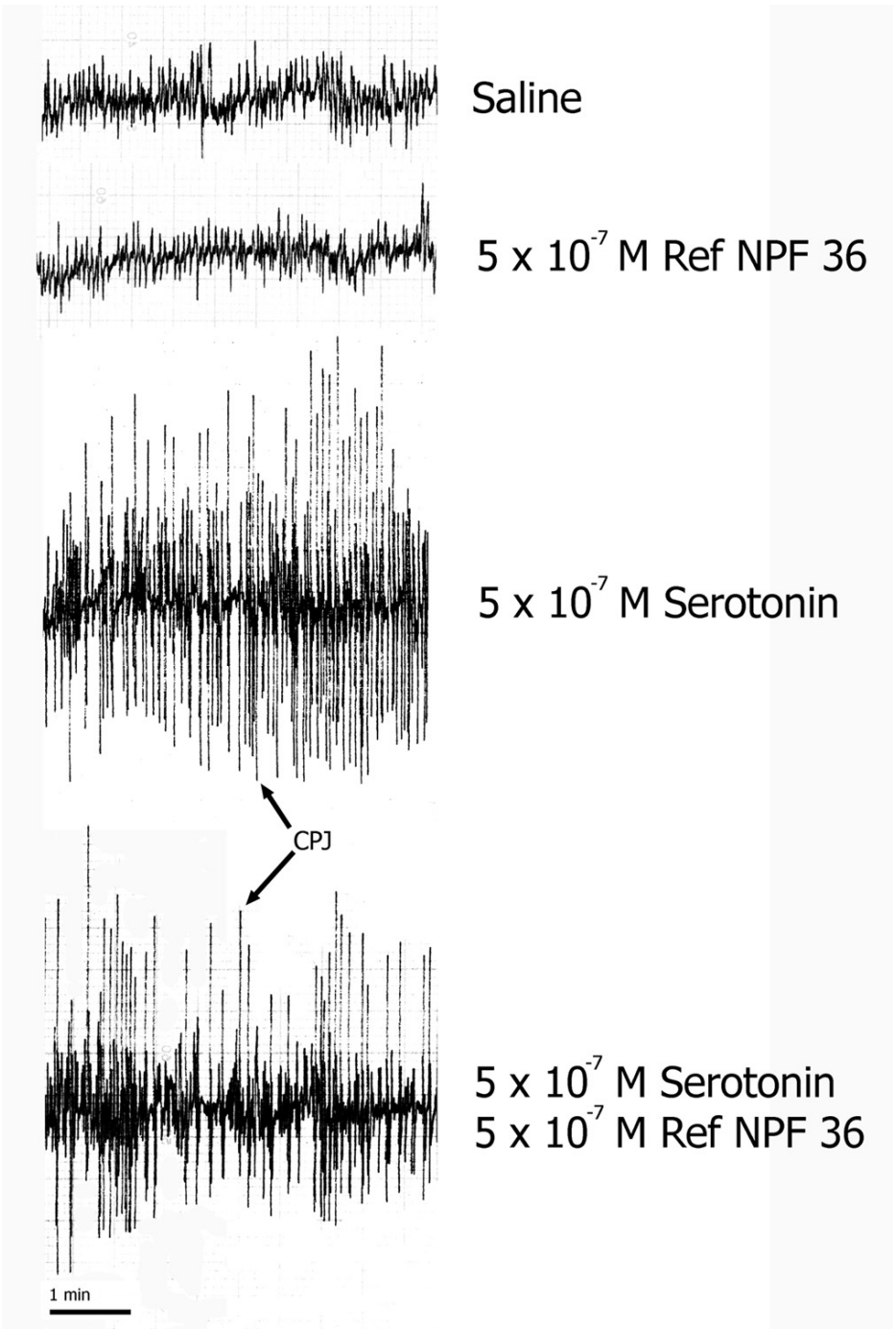


Figure 4.2: Impedance monitor readings of *Reticulitermes flavipes* crop-proventricular junction (CPJ) contractions in response to 5 x 10^{-7} M *Ref* NPF and 5 x 10^{-7} M serotonin.

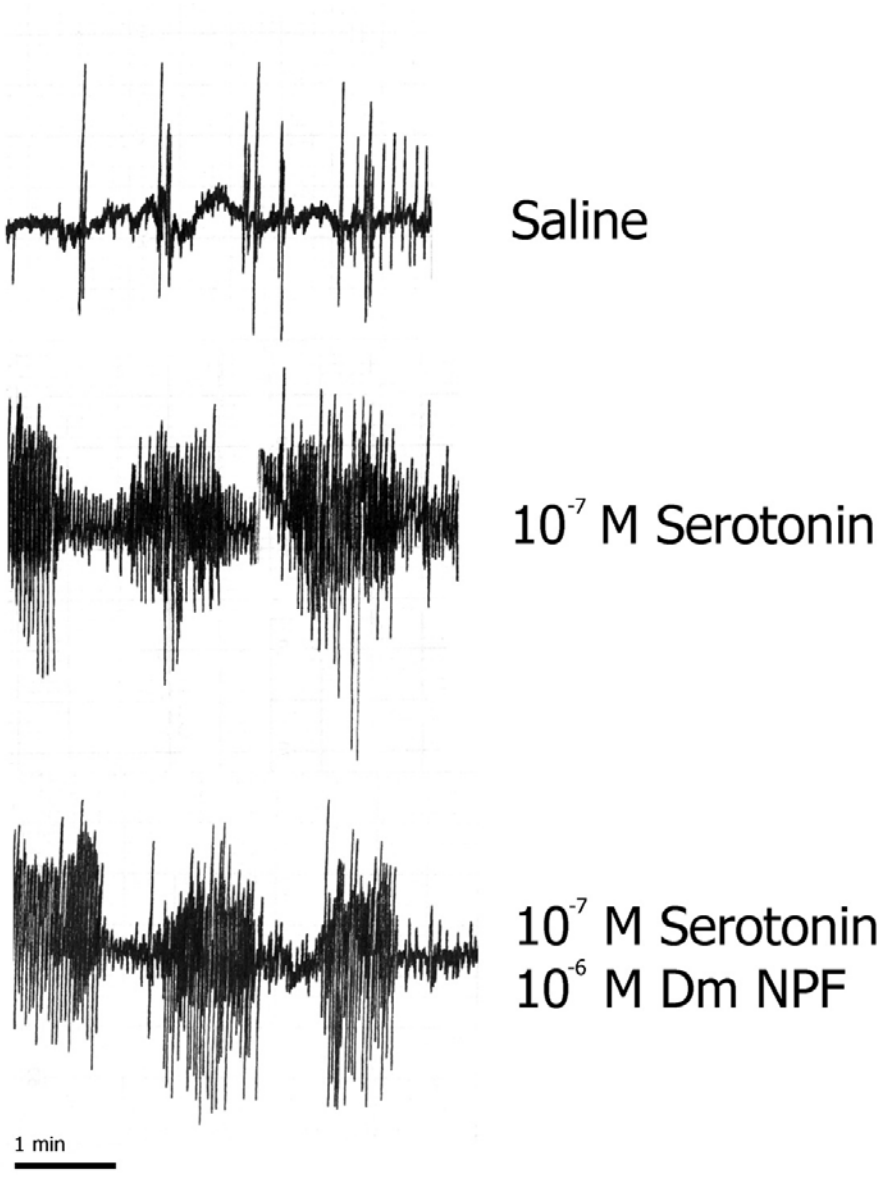


Figure 4.3: Impedance monitor readings of *R. flavipes* crop-proventriculus junction contractions in response to 10^{-7} M serotonin and 10^{-6} M *Dm* NPF.

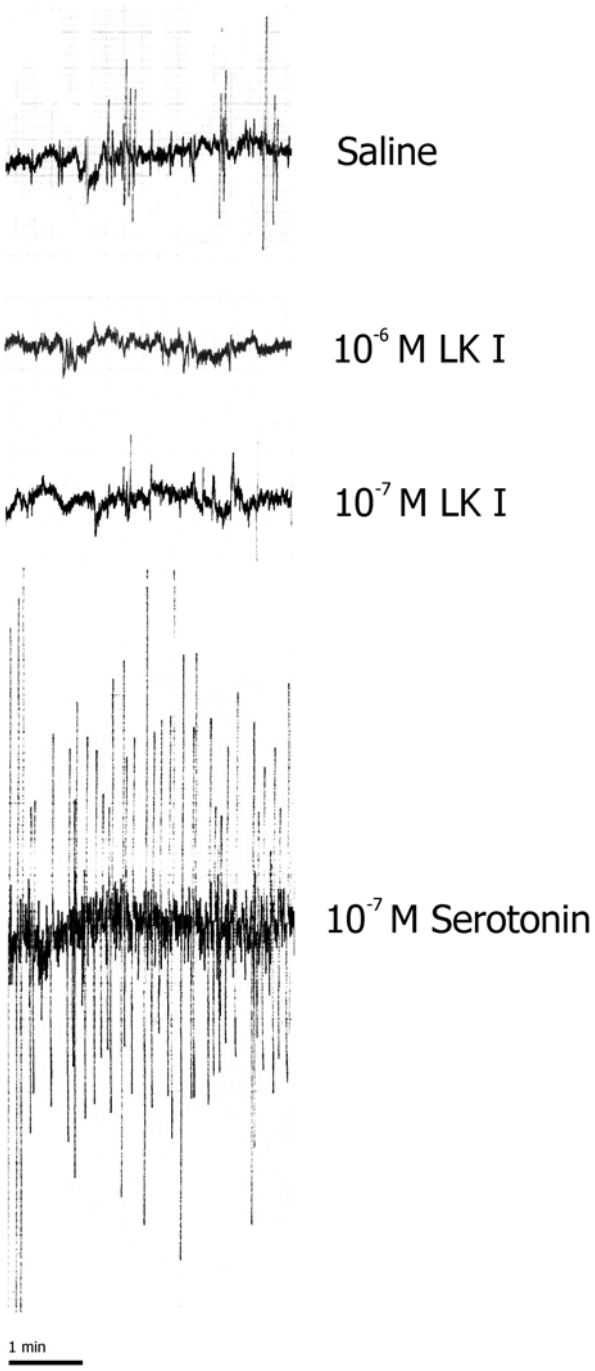


Figure 4.4: Impedance monitor readings of *R. flavipes* crop-proventriculus junction contractions in response to 10^{-6} and 10^{-7} M LK I. Tissue viability at the end of the trial was demonstrated by stimulation with 10^{-7} M serotonin.

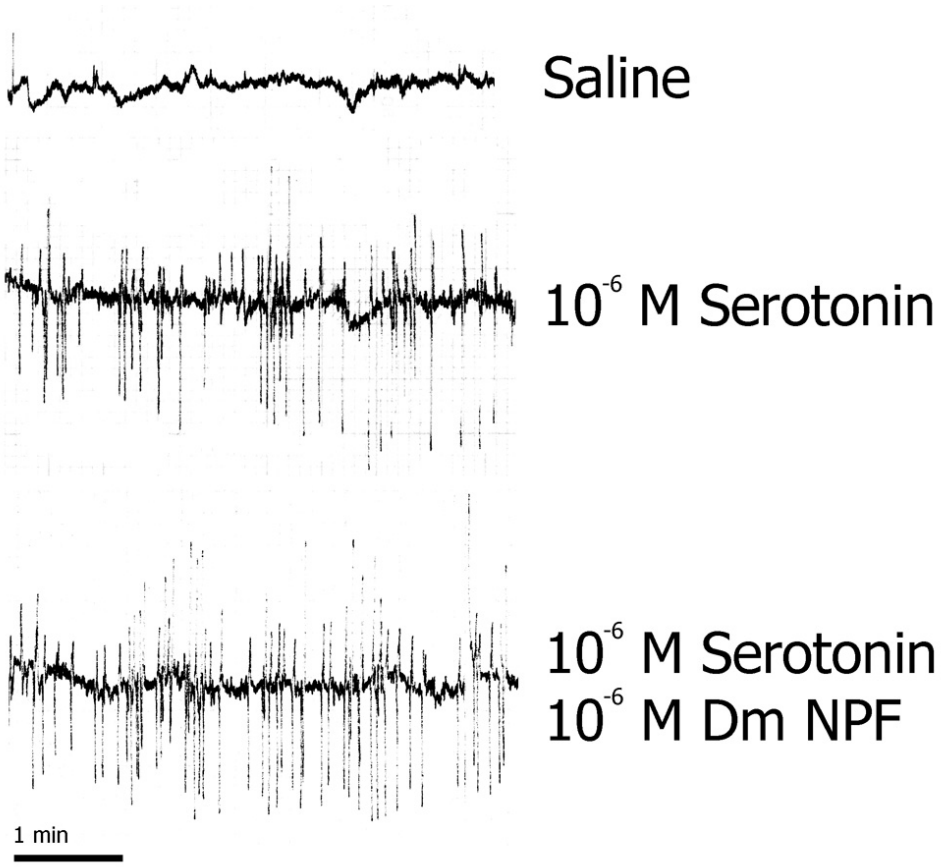


Figure 4.5: Impedance monitor readings of *R. flavipes* crop-proventriculus junction contractions in response to 10^{-6} M serotonin and 10^{-6} M *Dm* NPF.

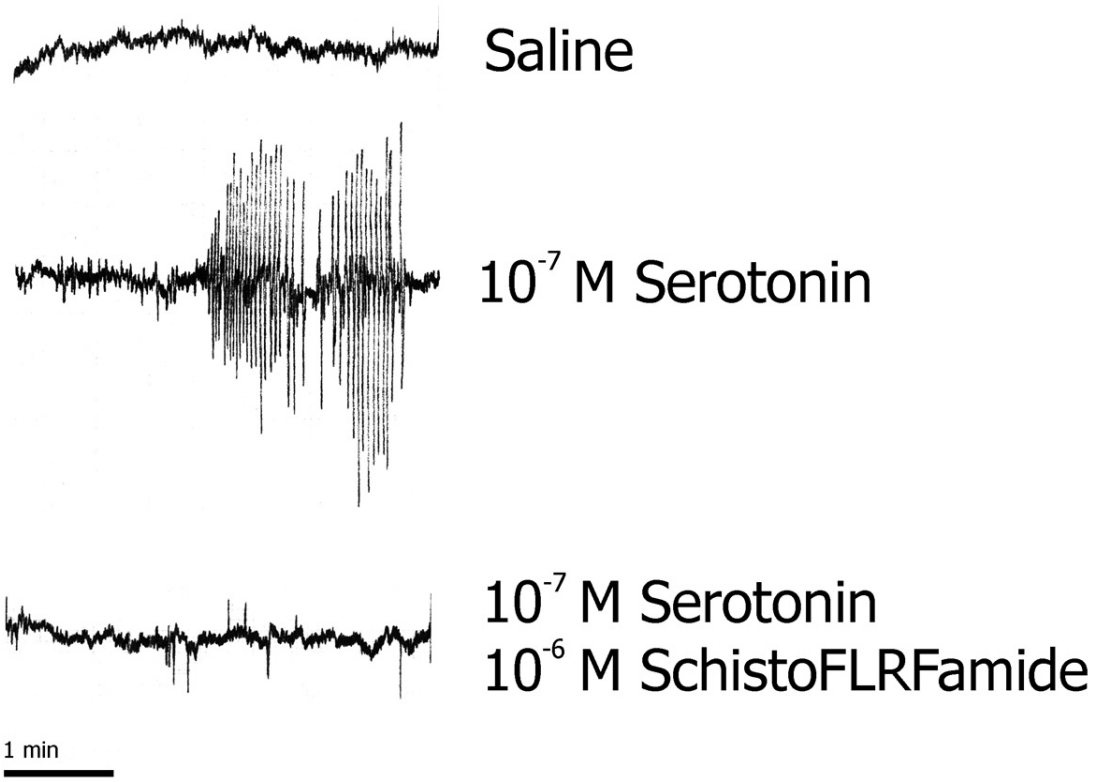


Figure 4.6: Impedance monitor readings of *R. flavipes* crop-proventriculus junction contractions in response to 10^{-7} M serotonin and 10^{-6} M SchistoFLRFamide.

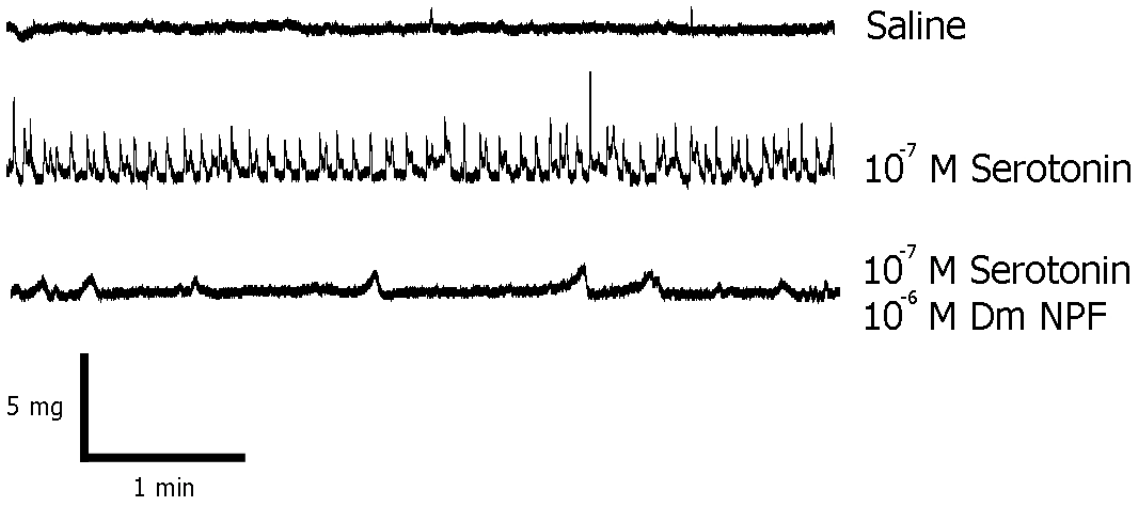


Figure 4.7: Force transducer readings of *R. flavipes* hindgut contractions in response to 10^{-7} M serotonin and 10^{-6} M *Dm* NPF.

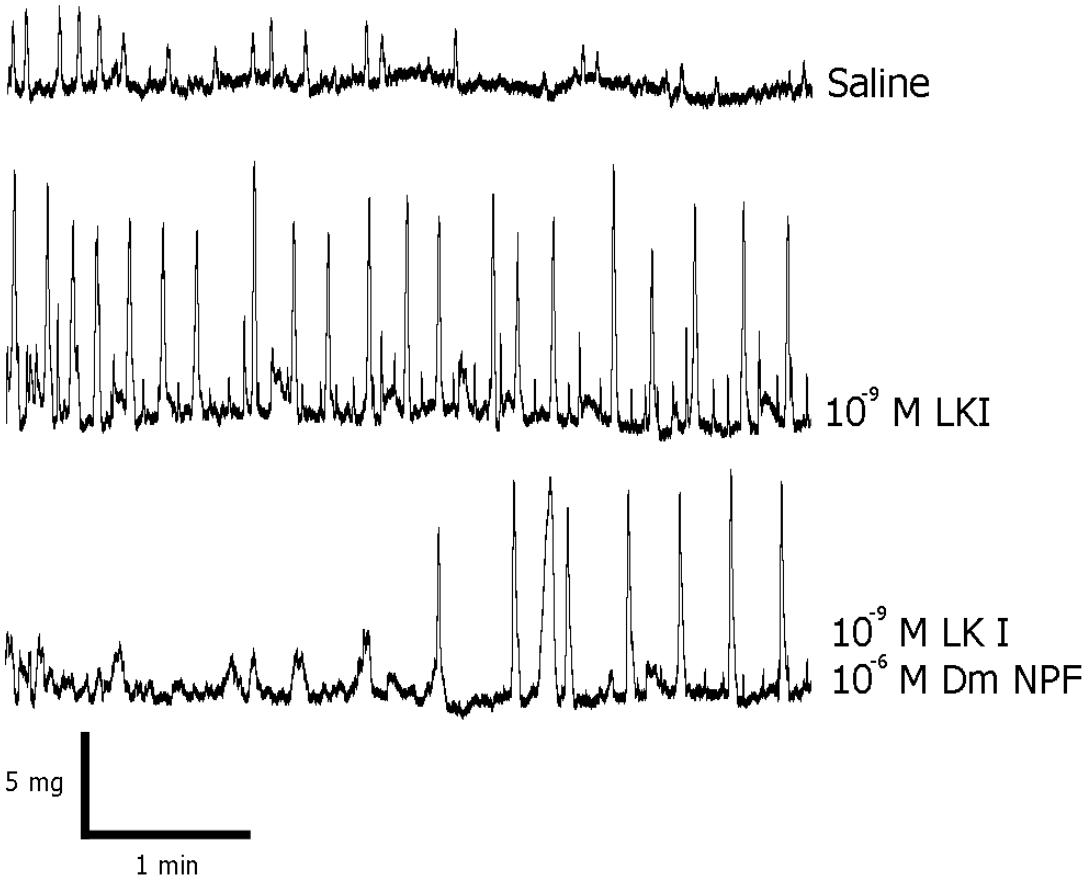


Figure 4.8: Force transducer readings of *R. flavipes* hindgut contractions in response to 10^{-9} M LK I and 10^{-6} M *Dm* NPF.

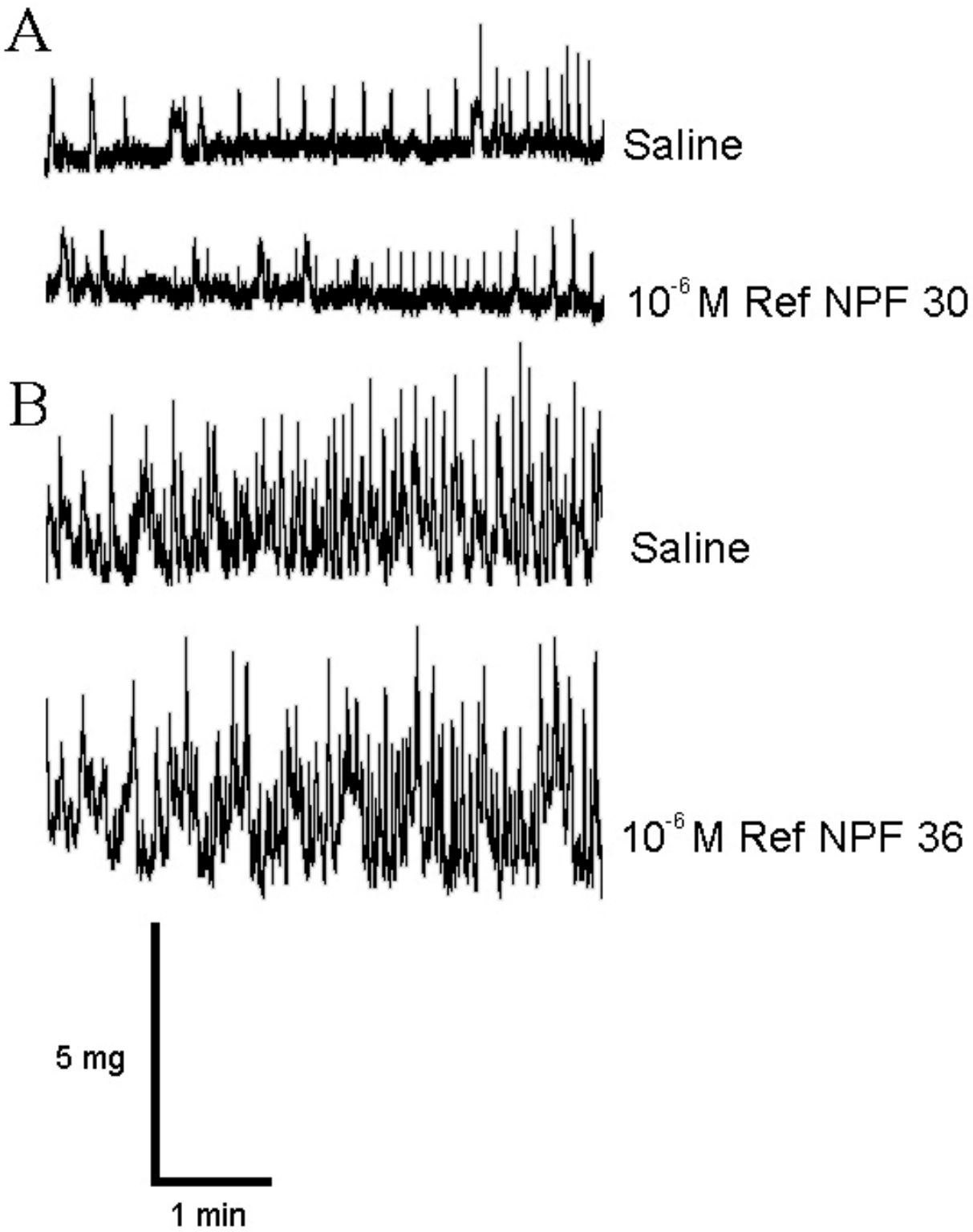
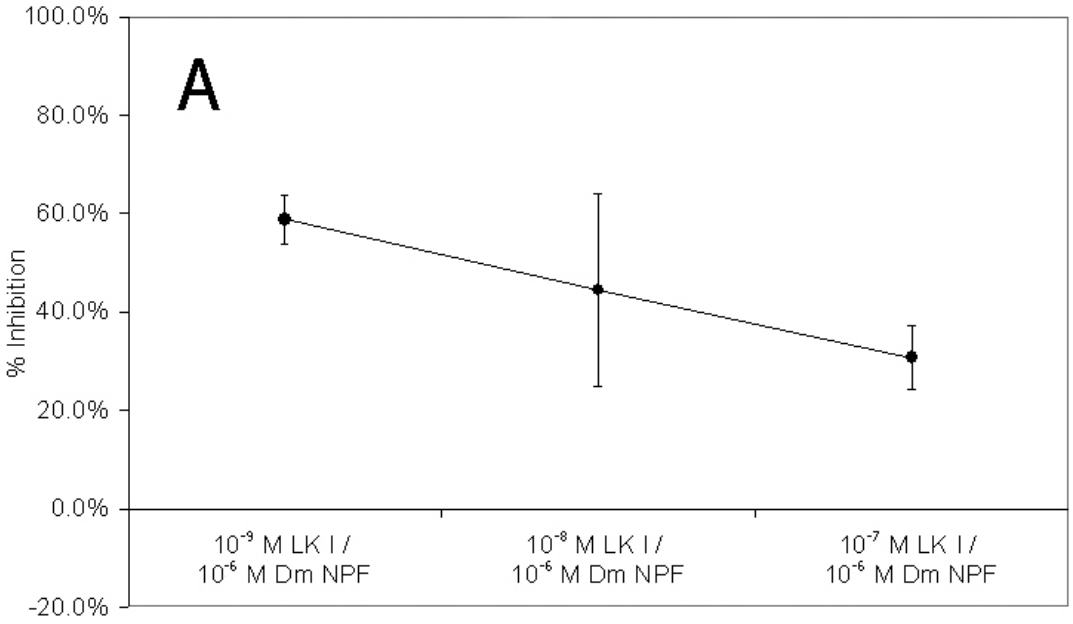
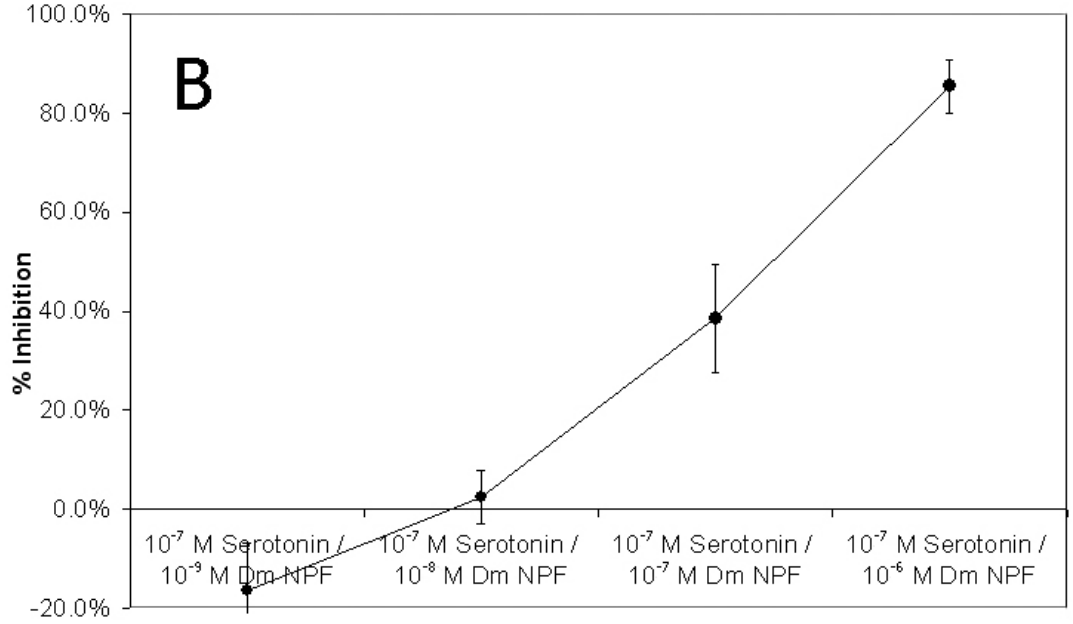


Figure 4.9: Force transducer readings of *R. flavipes* spontaneous hindgut contractions in response to 10^{-6} M *Ref* NPF 30 (A) and 10^{-6} M *Ref* NPF 36 (B).


A
100.0%
80.0%
60.0%
40.0%
20.0%
0.0%
-20.0%
% Inhibition
10-9 M LK I / 10-6 M Dm NPF
10-8 M LK I / 10-6 M Dm NPF
10-7 M LK I / 10-6 M Dm NPF


B
100.0%
80.0%
60.0%
40.0%
20.0%
0.0%
-20.0%
% Inhibition
10-7 M Serotonin / 10-9 M Dm NPF
10-7 M Serotonin / 10-8 M Dm NPF
10-7 M Serotonin / 10-7 M Dm NPF
10-7 M Serotonin / 10-6 M Dm NPF

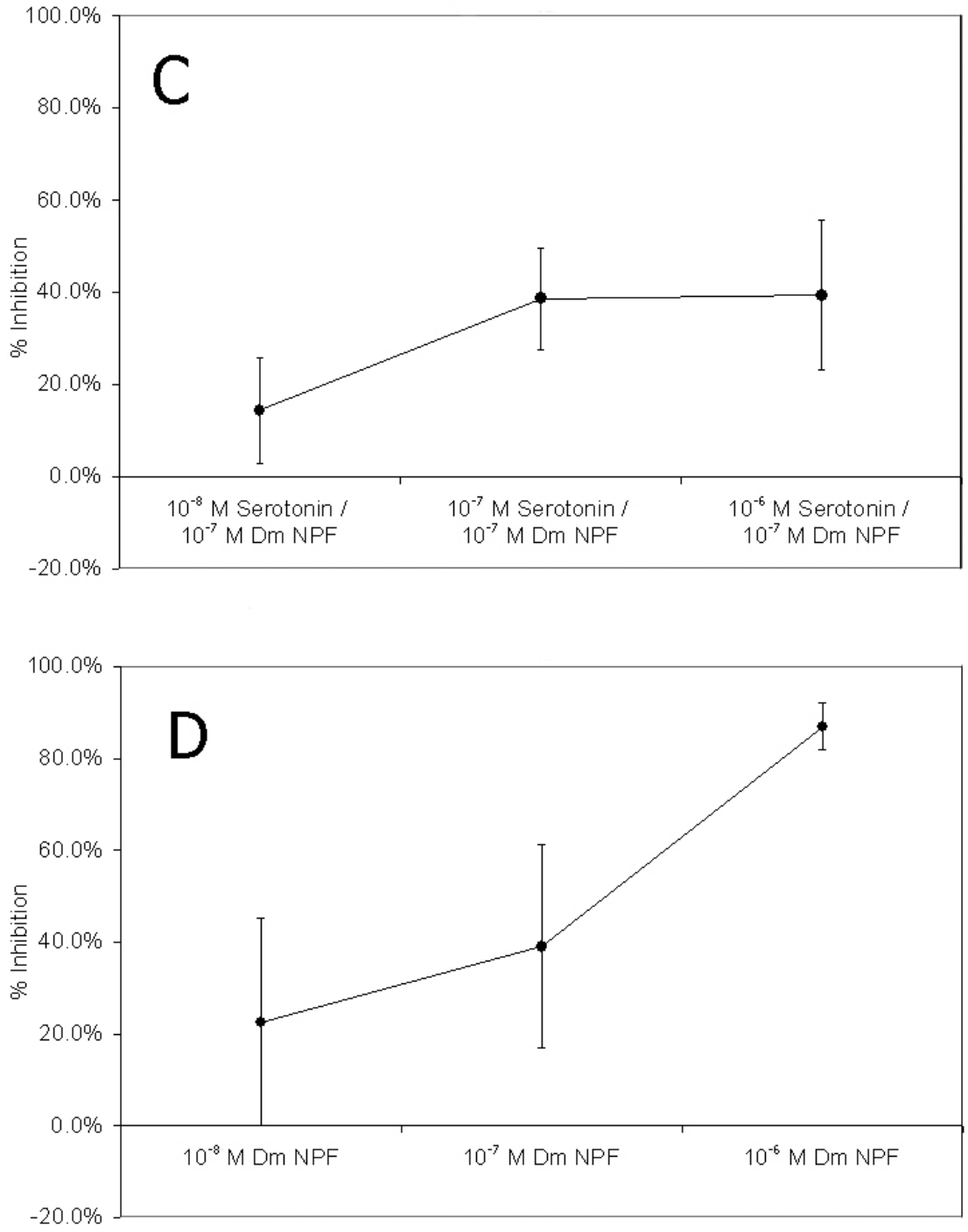
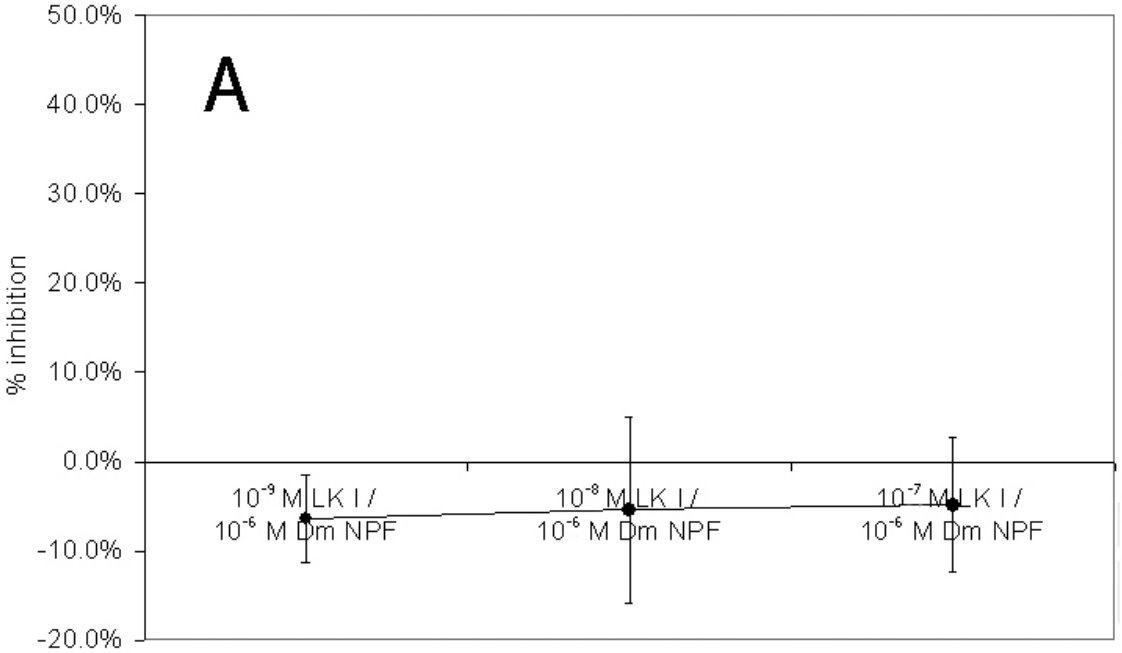
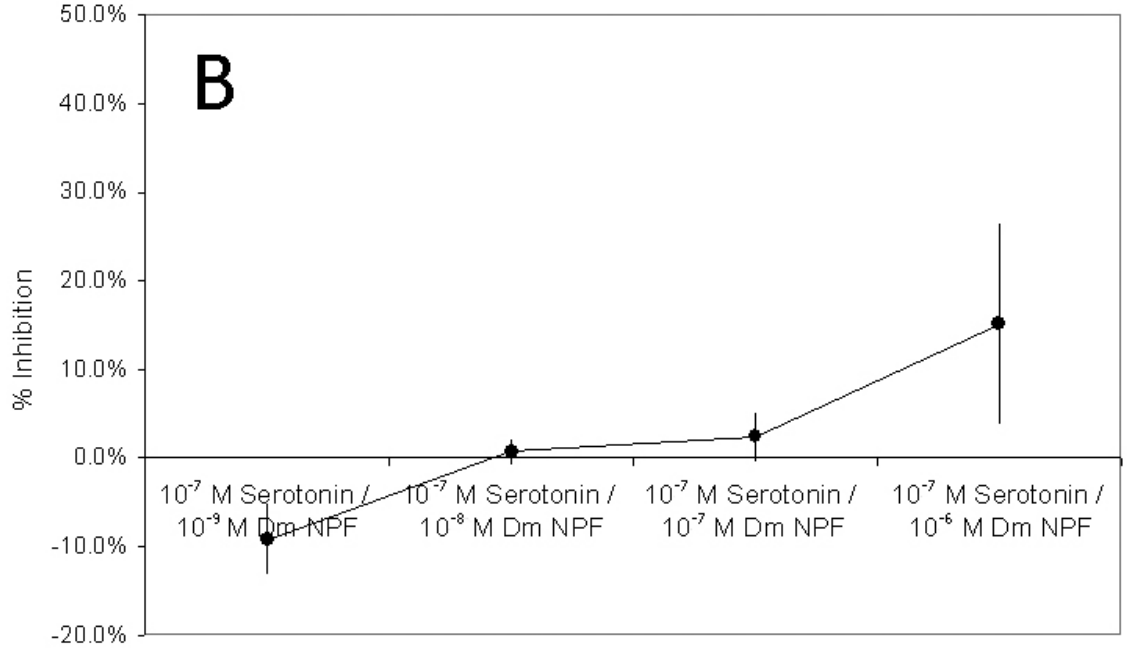


Figure 4.10: Average percentage frequency inhibition (± SEM) of spontaneous, serotonin- or LK I -induced hindgut contractions by *Dm* NPF. Five hindguts were incubated twice for each data point. A) 10^{-6} M *Dm* NPF with LK I. B) 10^{-7} M serotonin with *Dm* NPF. C) 10^{-7} M *Dm* NPF with serotonin. D) Spontaneous contractions with *Dm* NPF.


A
50.0%
40.0%
30.0%
20.0%
10.0%
0.0%
-10.0%
-20.0%
% inhibition
10-9 M LK I / 10-6 M Dm NPF
10-8 M LK I / 10-6 M Dm NPF
10-7 M LK I / 10-6 M Dm NPF


B
50.0%
40.0%
30.0%
20.0%
10.0%
0.0%
-10.0%
-20.0%
% Inhibition
10-7 M Serotonin / 10-9 M Dm NPF
10-7 M Serotonin / 10-8 M Dm NPF
10-7 M Serotonin / 10-7 M Dm NPF
10-7 M Serotonin / 10-6 M Dm NPF

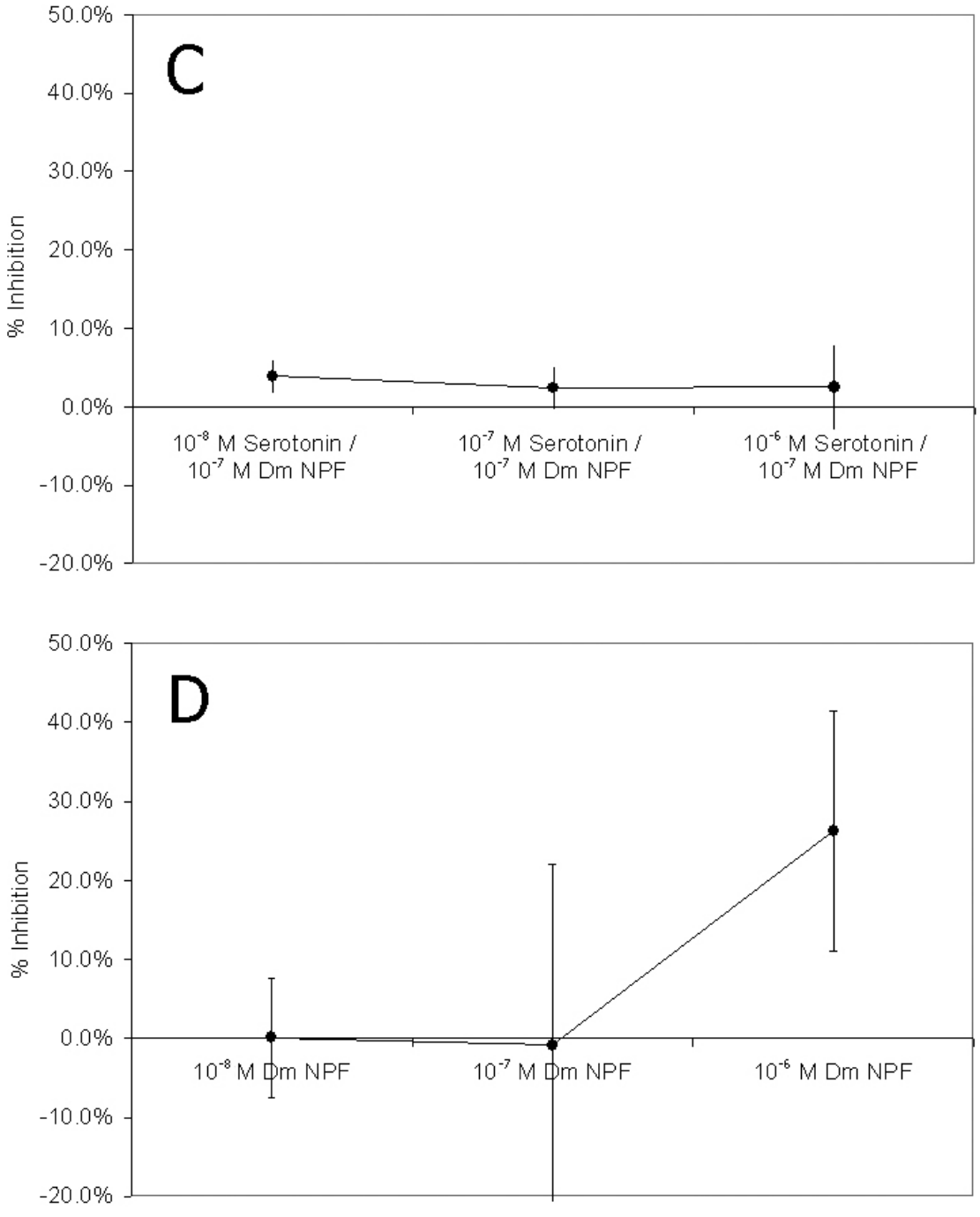


Figure 4.11: Average percentage amplitude inhibition (± SEM) of spontaneous, serotonin- or LK I -induced hindgut contractions by *Dm* NPF. Five hindguts were incubated twice for each data point. A) 10^{-6} M *Dm* NPF with LK I. B) 10^{-7} M serotonin with *Dm* NPF. C) 10^{-7} M *Dm* NPF with serotonin. D) Spontaneous contractions with *Dm* NPF.

Table 4.1: JH synthesis rates (pmol/pair/h ±SEM) by *Reticulitermes flavipes* CA incubated with *Ref* NPF (10^{-7}, 10^{-5} M), AST 2 (10^{-8}, 10^{-7} M) or a combination of the two. Control CA were incubated without peptides.

Treatment	Control pmol/pair/h	n	Treatment pmol/pair/h	n	% Inhibition
10^{-6} *Ref* NPF 36	1.55 ± 0.12	15	1.48 ± 0.13	15	4 ± 9
10^{-6} *Ref* NPF 30	1.08 ± 0.20	6	0.94 ± 0.14	6	13 ± 13
10^{-7} M *Dippu* AST-2	1.33 ± 0.18	7	0.22 ± 0.06	7	83 ± 5
10^{-7} M *Dippu* AST-2 / 10^{-7} *Ref* NPF 36	1.33 ± 0.18	7	0.11 ± 0.05	7	92 ± 4
10^{-7} M *Dippu* AST-2 / 10^{-5} *Ref* NPF 36	1.33 ± 0.18	7	0.13 ± 0.06	7	90 ± 5
10^{-8} M *Dippu* AST-2	1.08 ± 0.20	6	0.38 ± 0.09	6	65 ± 8
10^{-8} M *Dippu* AST-2 / 10^{-7} *Ref* NPF 36	1.08 ± 0.20	6	0.39 ± 0.05	6	64 ± 4
10^{-8} M *Dippu* AST-2 / 10^{-5} *Ref* NPF 36	1.08 ± 0.20	6	0.28 ± 0.07	6	74 ± 6

Table 4.2: JH synthesis rates (pmol/h ±SEM) in individual *Diploptera punctata* CA. Treated CA were compared to the untreated control CA from the same individual.

Treatment	Control pmol/h	Treatment pmol/h	n
Control	12.76 ± 2.54	14.41 ± 3.11	4
10^{-6} *Ref* NPF 36	13.74 ± 1.65	13.40 ± 1.92	4
10^{-7} *Ref* NPF 36	12.59 ± 2.70	15.67 ± 2.32	5

Table 4.3: Percent increase of frequency and amplitude of hindgut contractions over background and saline washes when incubated for 5 min with serotonin or LK I (±SEM). Each hindgut was washed 4x in saline between incubations. Statistically significant measurements: *p < 0.05, **p < 0.02.

Treatment	Average % frequency	Average % amplitude	n
10^{-8} M serotonin	896 ± 560	42.4 ± 68.1	5
10^{-7} M serotonin	**1475 ± 431	**52.2 ± 20.8	20
10^{-6} M serotonin	*590 ± 225	**21.9 ± 5.0	5
10^{-9} M LK I	*505 ± 196	*34.1 ± 14.1	5
10^{-8} M LK I	**1724 ± 493	**77.5 ± 22.2	5
10^{-7} M LK I	**615 ± 190	**153.1 ± 47.0	5

Table 4.4: Percent inhibition of amplitude and frequency of hindgut contractions by SchistoFLRFa, *Ang* NPF, and *Ref* NPF 30 and 36 over serotonin- and LK I-induced contractions.

Treatment	% amplitude inhibition	% frequency inhibition	n
10^{-7} M serotonin / 10^{-7} M *Ref* NPF 36	-33.1	-39.6	1
10^{-7} M serotonin / 10^{-7} M *Ref* NPF 30	-19.6	-57.1	1
10^{-7} M serotonin / 10^{-6} M *Ang* NPF	-11.8	-16.2	1
10^{-7} M LK I / 10^{-6} M *Ang* NPF	3.4	-38.4	1
10^{-7} M serotonin / 10^{-6} SchistoFLRFa	-2.4	16.8	1

CHAPTER 5

CONCLUSIONS

As described within this work, the distribution of NPF-like material was visualized in three different castes of the eastern subterranean termite, *Reticulitermes flavipes* with whole-tissue immunocytochemistry using an antibody to *Helicoverpa zea* MP-I (Huang et al. 1998; Brown et al. 1999). All castes contained similar proportions of immunoreactive cells in the brain and ventral nerve cord. Immunoreactive cells also occurred on the surface of the foregut and had immunoreactive axons that together with immunoreactive axons from the brain formed a network over the corpora allata (CC)/corpora cardiaca (CA) complex, salivary glands, foregut, and anterior half of the midgut. An immunoreactive axon network was also observed over the rectum, originating from the terminal abdominal ganglion. Over 600 immunoreactive midgut endocrine cells were also observed in all castes.

The distribution of NPF-like material in *R. flavipes* suggests that NPF has several roles that are not limited by caste specialization. The distribution was similar to the NPF distribution in *Helicoverpa zea* (Huang 1996), *Drosophila melanogaster* (Brown et al. 1999) *Aedes aegypti* (Stanek et al. 2002) and *Rhodnius prolixus* (Gonzalez and Orchard, 2008) as described with immunocytochemistry using antibodies to *Hez* MP-I or *Dm* NPF. Similar patterns of immunoreactivity have also been detected in the nervous system or midgut endocrine cells of *Periplaneta americana* (Iwanaga et al. 1981; Endo et al. 1982; Zhu et al. 1998), *Schistocerca gregaria* (Myers and Evans 1985; Zhu et al. 1998), *Locusta migratoria* (Schoofs et al. 1988), *Gryllus bimaculatus* (Iwanaga et al. 1986), *Tramea virginia* (Patankar and Tembhare 2006),

Calliphora vomitoria (Duve and Thorpe 1982) and *Aedes aegypti* (Brown et al. 1986) with antisera to pancreatic polypeptide (PP) or neuropeptide Y (NPY). The distribution of NPF-like material in both midgut endocrine cells and axons on the gut tract suggest that NPF may be a signaling molecule that provides information to the nervous system about the contents of the gut through the brain-gut axis (Brown and Lea, 1990; Sehnal and Žitňan, 1996). Regulation of gut motility may also be a function of NPF as suggested by its association with the alimentary tract. Midgut motility of *Ae. aegypti* is inhibited by NPF (Onken et al. 2004). NPF is also believed to play a role in feeding, but evidence for this in insects has only so far been demonstrated in *D. melanogaster* (Wu et al. 2003; Wu et al. 2005b; Lingo et al. 2007).

The observation of NPF-like material in *R. flavipes* led us to isolate NPF from this insect using high performance liquid chromatography monitored by radioimmunoassay using the *Hez* MP-I antibody. This approach was used over conventional cDNA isolation by design of degenerate primers to conserved peptide regions because NPF amino acid sequences, outside of key conserved residues, are variable (Garczynski et al. 2005). Purification and partial amino acid sequencing of a termite extract allowed degenerate primer design which facilitated discovery of the *R. flavipes* NPF (*Ref* NPF) cDNA sequence. Translation of the cDNA sequence confirmed the identity of *Ref* NPF as an authentic NPF by the possession of aromatic amino acids at positions 10 and 17 from the carboxy- (C-) terminus and an (R/K)-X_1-R-(F/Y)-amide C-terminus (McVeigh et al. 2005). The pre-propeptide sequence was also similar to structure of known NPFs with the inclusion of a signal peptide, mature NPF region and a carboxy- (C-) peptide with a Gly residue for C-terminal amidation (McVeigh et al., 2005). Signal P analysis (Bendtsen et al., 2004) predicted a signal peptide cleavage site that would result in a 30 amino acid long mature peptide. However, most insect NPFs are at least 36 amino acids in length. The

uncertainty in signal peptide cleavage led us to synthesize both 30 and 36 amino acid long forms of *Ref* NPF (*Ref* NPF 30 and *Ref* NPF 36, respectively) for use in bioassays. The sequence of the 36 amino acid long form was: VPSVWAKPSDPEQLADTLKYLEELDRFYSQVARPRFa

The use of previously unreported invertebrate NPF sequences generously provided by Joe Crim (personal communication) along with *Ref* NPF and other published NPF sequences (Table 3.4, Chapter 3) provided a data set that was aligned and subjected to cladistic analysis. *Ref* NPF most closely resembled *Lom* NPF (Clynen et al. 2006) and had strong C-terminal similarity with crustacean *Penaeus monodon* PYF II (Sithigorngul et al. 2002) and *Marsupenaeus japonicus* NPF (Christie et al. 2008) sequences.

The effect of NPF was also tested on isolated tissue preparations, an approach that up until this point had only been attempted on *Ae. aegypti* midguts (Onken et al. 2004) and the CA of *Romalea microptera* (Li et al. 2005) for this particular signaling peptide. Juvenile hormone (JH) synthesis of *R. flavipes* CA from neotenics is reduced by another peptide, allatostatin (Yagi et al. 2005), but *Ref* NPF 30 or 36 did not significantly alter JH synthesis rates of test subjects. Tests with *Ref* NPF 36 on the CA of the cockroach *Diploptera punctata* were also negative.

The contractions of isolated *R. flavipes* worker foreguts were examined with the use of an impedance monitor. Serotonin stimulated foregut contractions that were in turn inhibited by SchistoFLRFamide. *Ref* NPF 30 or 36 did not exert a noticeable effect on the foregut, nor did *Dm* NPF or *Ang* NPF. A force transducer was used to monitor the response of the *R. flavipes* worker hindgut to peptides and serotonin. Both serotonin and Leucokinin I stimulated hindgut contractions. These contractions were significantly inhibited by *Dm* NPF but were not significantly affected by solutions of *Ref* NPF 30, *Ref* NPF 36 or *Ang* NPF.

Future studies should solve the *Ref* NPF solubility problems that interfered with bioassays. Also, determination of whether *Ref* NPF 30, *Ref* NPF 36 or both occur as active signaling molecules should be resolved. When the active form of *Ref* NPF is determined, the bioassays in this study could be repeated to confirm or refute the lack of activity observed in this study. Other tissues such as the salivary glands and midgut may also be evaluated. The effect of *Ref* NPF on behavior could be investigated by injection of *Ref* NPF or NPF-antagonists into termites. RNAi might also be employed to downregulate *Ref* NPF and observe the effects of reduced peptide synthesis.

5.1 References

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APPENDIX A

ADDITIONAL *Reticulitermes flavipes* (ISOPTERA: RHINOTERMITIDAE) NPF-LIKE cDNA PRODUCTS AND OPEN READING FRAME ALIGNMENTS

1. Introduction

1.1 Additional *Ref* NPF PCR product

Vertebrate neuropeptide Y (NPY), peptide YY (PYY) and pancreatic polypeptide (PP) represent gene duplication either by whole genome duplication or local gene duplication (Larhammar, 1996). Neuropeptide F (NPF), the invertebrate homolog of NPY, may also have duplicate members in select groups as suggested by the presence of two NPF-like sequences in *Bombyx mori* (Joe Crim, personal communication). Two amplified PCR products were observed approximately 450 and 550 bp in length (Fig. A.1) while determining the NPF sequence of *Reticulitermes flavipes* (Chapter 3). These products occurred both when head or midgut cDNA were used as the template. Translation of the shorter sequence corresponds to the amino acid sequence of the NPF peptide extract (Chapter 3, Fig 3.1). It was unclear whether the second product was a false PCR product or amplification of an actual mRNA transcript.

1.2 Cladistic analysis

Cladistic analysis of NPF amino acid sequences was previously performed (Chapter 3) but the short sequence of NPFs (36-40 amino acids) provided a limited data set for comparisons between different taxa. We investigated the possibility of using the open reading frames (ORFs) of insect NPF sequences for a stronger comparison.

2. Materials and Methods

2.1 Sequence

Procedures were identical to those in Chapter 3 for extraction of RNA, PCR, cloning and sequencing. The forward primer UPNPF 1 and the reverse primer NPF Rev (Chapter 3, Table 3.1) were used to amplify head and midgut cDNA (35 cycles; 94 ºC, 20 s; 60.5 ºC, 20 s; 72 ºC, 1.5 min). Products were cloned and 3 clones were purified and sequenced.

2.2 Alignment

Insect NPF ORFs were obtained (Chapter 3, Table 3.4) and aligned with Clustal X. A subset of insect ORFs excluding *Bom* NPF and *Apm* NPF was aligned and trimmed (Fig. A.4). A tree was generated from the subset data based on Jukes-Cantor distance analysis (GCG). Subsets of related taxa from this tree and between *Bom* NPF and *Apm* NPF were aligned with Clustal X.

3. Results

3.1 Sequence

The cloned sequences were aligned as in Chapter 3. The sequences contained an additional 111 bp in comparison to the *Ref* NPF transcript (Fig A.2). The addition was located between nucleotides 288 and 289 of the cDNA sequence and was flanked by 3' and 5' AG and GT nucleotides, respectively. This corresponds to the region before the Arg_{56} residue in the translated sequence.

3.2 Alignment

Homology of ORF sequences was poor when compared across all insects (Fig. A.3). Subgroup alignments of ORFs from insects that clustered together in the cladogram revealed

homologous regions (Fig. A.4). The C-peptides of *Ref* NPF and *Lom* NPF were highly similar sharing 50% identical amino acid residues (Fig. A.4).

4. Discussion

The extra cDNA sequence is an intriguing discovery because it occurs in a region that contains an intron in some other invertebrate NPFs (Garczynski et al., 2005, Mair et al., 2000; Joe Crim, personal communication) and vertebrate NPY, PYY and PP sequences (Mair et al., 2000). Also, the extra sequence codes for a peptide that contains several characteristic NPF features. Further experimentation will be required to determine if this product is actually expressed or if it is an incomplete splice product.

The conservation of the C-peptide residues in *Ref* NPF and *Lom* NPF is particularly strong on the carboxy and amino ends of the C-peptide. The similarities between these species were not surprising considering that termites are more closely related to locusts than any other species for which NPF has been sequenced. No strong similarity was noted in the signal peptides of these sequences. Conservation of amino acid sequence was also evident when comparing mosquito sequences and again the signal peptide was more divergent than the C-peptide. Divergence of NPF signal peptides and C-peptides between closely related species has been reported for *Schistosoma japonicum* and *S. mansoni*, even though the mature NPFs differ by only one amino acid (Humphries et al., 2004).

5. References

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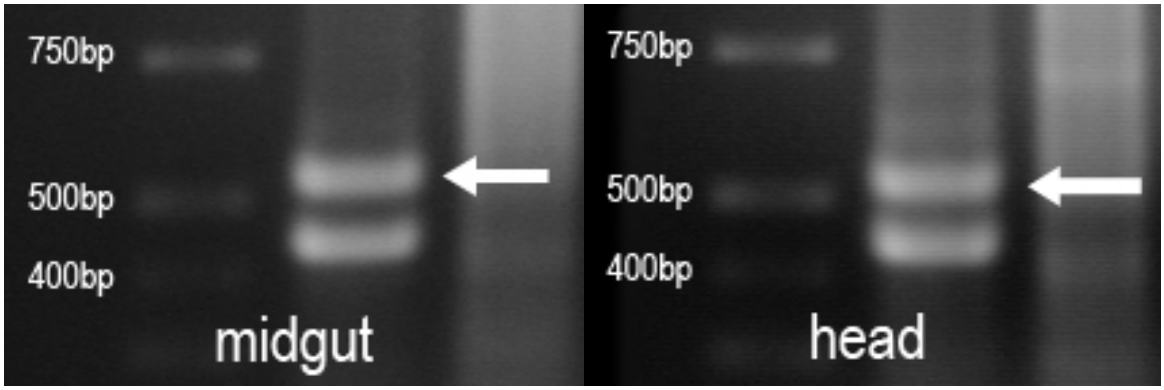


Figure A.1: Midgut and head cDNA PCR amplified with UPNPF1 and NPF Rev primers. Arrows indicate the bands cloned and sequenced to generate the sequence in Fig. A.2.

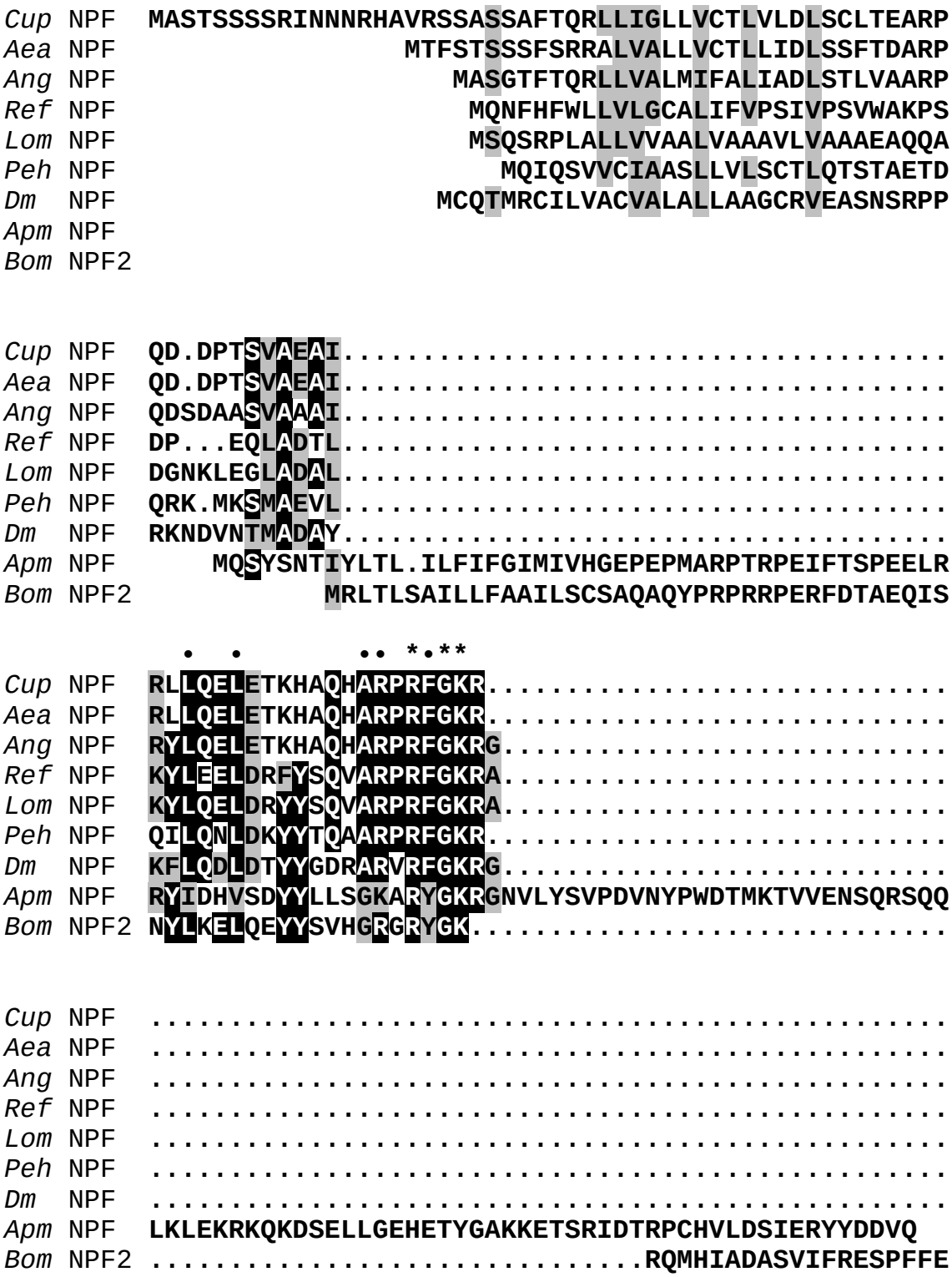
```
  1 GACGTACACGACGGACTTTGTTGCCTGAACGCTAGCAGCAGGAAGTCGCAGCTCGCCACA
 61 GCCATCTACACGTCAGGTCATCGACGTTCACTTTCTGAAAAGAAGGTCACGTGATTCACC
                                                      *  F  T

121 ATGCAGAACTTCCATTTTTGGCTTCTTGTGTTGGGATGTGCCCTCATCTTCGTCCCTAGT
     M  Q  N  F  H  F  W  L  L  V  L  G  C  A  L  I  F  V  P  S
     1                                                          20
181 ATCGTTCCCAGTGTCTGGGCCAAACCCTCCGACCCCGAGCAACTGGCGGACACCCTCAAG
     I  V  P  S  V  W  A  K  P  S  D  P  E  Q  L  A  D  T  L  K
                                                                40
241 TATCTGCAAGAATTGGATCGCTTTTACTCCCAGGTCGCCAGGCCAAGACCGCGCAGCGAG
     Y  L  E  E  L  D  R  F  Y  S  Q  V  A  R  P  R_ P  R  S  E
                                                                60
301 ACTGGGCGGAACCACGAGCTCTCCAAGATGGAGAAAGCTCTCAGAATGCTGCAACTCCAA
     T  G  R  N  H  E  L  S  K  M  E  K  A  L  R  M  L  Q  L  Q
                                                                80
361 GAATTGGATCGCTTTTACTCTCCGCGCTCCCGGCCGAGGTTTGGCAAGAGGGCAGAACTG
     E  L  D  R  F  Y  S  P  R  S  R  P  R  F  G  K  R  A  E  L
                                                               100
421 AGACCCGTCACTGAACAAGAGGCCGCTCCTGATGATTCCTCTGACAGTCTTTGGCGGCAG
     R  P  V  T  E  Q  E  A  A  P  D  D  S  S  D  S  L  W  R  Q
                                                               120
481 TTTGCCAGCAGAAGGTGAAAGACTTTAGACGTCACGTGGTCAACCAAAAACCGCAATTCC
     F  A  S  R  R  *

541 CAACAATTACAGTGTTACTATTTGTTTCTTTAACCTTCGTCTTCAGAAACACATTGTAAT
601 TTATTATTGTAACACGAAACTGCATTAAGAATTAAATAAAAATTTTCGGCAAATCTCTT
```

Figure A.2: Nucleotide sequence and amino acid sequence for the prepropeptide of *Reticulitermes flavipes* NPF containing an additional 111 bp. Putative signal peptide is in bold, and the putative mature peptide is underlined. Translation of the putative insert region is highlighted in gray. The putative splice site is highlighted in black.

A.


Cup NPF MASTSSSSRINNNRHAVRSSASSAFTQRLLIGLLVCTLVLDLSCLTEARP
Aea NPF MTFSTSSSFSRRALVALLVCTLLIDLSSFTDARP
Ang NPF MASGTFTQRLLVALMIFALIADLSTLVAARP
Ref NPF MQNFHFWLLVLGCALIFVPSIVPSVWAKPS
Lom NPF MSQSRPLALLVVAALVAAAVLVAAAEAQQA
Peh NPF MQIQSVVCIAASLLVLSCTLQTSTAETD
Dm NPF MCQTMRCILVACVALALLAAGCRVEASNSRPP
Apm NPF
Bom NPF2
Cup NPF QD.DPTSVAEAI
Aea NPF QD.DPTSVAEAI
Ang NPF QDSDAASVAAAI
Ref NPF DP...EQLADTL
Lom NPF DGNKLEGLADAL
Peh NPF QRK.MKSMAEVL
Dm NPF RKNDVNTMADAY
Apm NPF MQSYSNTIYLTL.ILFIFGIMIVHGEPEPMARPTRPEIFTSPEELR
Bom NPF2 MRLTLSAILLFAAILSCSAQAQYPRPRRPERFDTAEQIS
Cup NPF RLLQELETKHAQHARPRFGKR
Aea NPF RLLQELETKHAQHARPRFGKR
Ang NPF RYLQELETKHAQHARPRFGKRG
Ref NPF KYLEELDRFYSQVARPRFGKRA
Lom NPF KYLQELDRYYSQVARPRFGKRA
Peh NPF QILQNLDKYYTQAARPRFGKR
Dm NPF KFLQDLDTYYGDRARVRFGKRG
Apm NPF RYIDHVSDYYLLSGKARYGKRGNVLYSVPDVNYPWDTMKTVVENSQRSQQ
Bom NPF2 NYLKELQEYYSVHGRGRYGK
Cup NPF
Aea NPF
Ang NPF
Ref NPF
Lom NPF
Peh NPF
Dm NPF
Apm NPF LKLEKRKQKDSELLGEHETYGAKKETSRIDTRPCHVLDSIERYYDDVQ
Bom NPF2 RQMHIADASVIFRESPFFE

```
Cup NPF  ......GYLQPASYGQDEQEVNLYLNR
Aea NPF  ......SYLNPAGYGQDEQEDDWQDSTFTR
Ang NPF  ......GYLNPAIFGQDEQEVDWQDSTFSR
Ref NPF  ......EL.........................................
Lom NPF  ......EL.........................................
Peh NPF  ........................NYGGLDERLMVRNSYQFLSVQAKQ
Dm  NPF  ......SLMDILRNHEMDNINLGKNANNGG...................
Apm NPF
Bom NPF2 HSLNEDGLLKKFGYK


Cup NPF
Aea NPF
Ang NPF
Ref NPF  ............RPVTEQEAAPDD.SSDSLWRQFASRR
Lom NPF  ............RPDVVDDVIPEEMSADKFWRRFARRR
Peh NPF  FDNDNKLITESGRGTV
Dm  NPF  .........EFARGFNEEEIF
Apm NPF
Bom NPF2
```

Figure A.3: Clustal X alignment of translated insect NPF open reading frames (ORFs). ▉ Majority identical. ▒ Majority conserved. * All residues identical. • All residues conserved.

```
Cup NPF  SAFTQRLLIGLLVCTLVLDLSCLTEARPQDTSVAEAIRLLQELETKHAQH
Aea NPF  SSFSRRALVALLVCTLLIDLSSFTDARPQDTSVAEAIRLLQELETKHAQH
Ang NPF  GTFTQRLLVALMIFALIADLSTLVAARPQDASVAAAIRYLQELETKHAQH
Ref NPF  NFHFWLLVLGCALIFVPSIVPSVWAKPSDPEQLADTLKYLEELDRFYSQV
Lom NPF  QSRPLALLVVAALVAAAVLVAAAEAQQADGEGLADALKYLQELDRYYSQV
Peh NPF  MQIQSVVCIAASLLVLSCTLQTSTAETDQRKSMAEVLQILQNLDKYYTQA
Dm  NPF  MRCILVACVALALLAAGCRVEASNSRPPRKNTMADAYKFLQDLDTYYGDR

Cup NPF  ARPRFGKR
Aea NPF  ARPRFGKR
Ang NPF  ARPRFGKR
Ref NPF  ARPRFGKR
Lom NPF  ARPRFGKR
Peh NPF  ARPRFGKR
Dm  NPF  ARVRFGKR
```

Figure A.4: Trimmed NPF ORF sequences for cladograms (GCG). Inclusion of *Bom* NPF and *Apm* NPF sequences provided insufficient data after trimming and were omitted for cladogram analysis.

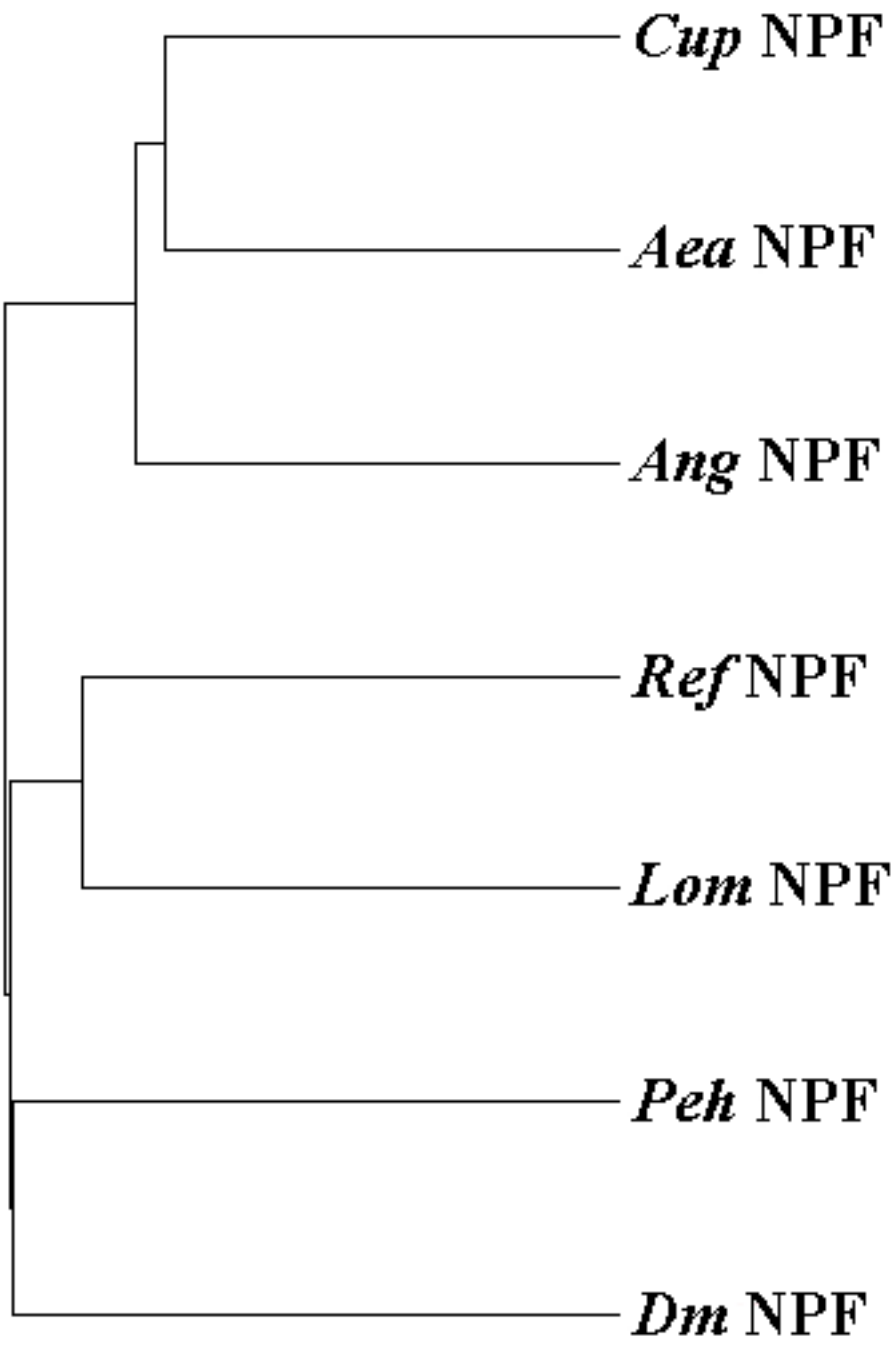


Figure A.5: Cladogram of insect NPF ORF sequences. ORFs were aligned with Clustal X and trimmed (Fig. A.4). The dendrogram was constructed by Jukes-Cantor distance analysis of the sequences within GCG.

```
Cup NPF   MASTSSSSRINNNRHAVRSSASSAFTQRLLIGLLVCTLVLDLSCLTEARP
Aea NPF                    MTFSTSSSFSRRALVALLVCTLLIDLSSFTDARP
Ang NPF                       MASGTFTQRLLVALMIFALIADLSTLVAARP

Cup NPF   QD.DPTSVAEAIRLLQELETKHAQHARPRFGKR.GYLQPASYGQDEQEVN
Aea NPF   QD.DPTSVAEAIRLLQELETKHAQHARPRFGKR.SYLNPAGYGQDEQEDD
Ang NPF   QDSDAASVAAAIRYLQELETKHAQHARPRFGKRGGYLNPAIFGQDEQEVD

Cup NPF   LYLNR
Aea NPF   WQDSTFTR
Ang NPF   WQDSTFSR
```

```
Ref NPF   MQNFHFWLLVLGCALIFVPSIVPSVWAKPSDP...EQLADTLKYLEELDR
Lom NPF   MSQSRPLALLVVAALVAAAVLVAAAEAQQADGNKLEGLADALKYLQELDR

Ref NPF   FYSQVARPRFGKRAELRPVTEQEAAPDD.SSDSLWRQFASRR
Lom NPF   YYSQVARPRFGKRAELRPDVVDDVIPEEMSADKFWRRFARRR
```

```
Peh NPF       MQIQSVVCIAASLLVLSCTLQTSTAETDQRK.MKSMAEVLQILQNL
Dm  NPF   MCQTMRCILVACVALALLAAGCRVEASNSRPPRKNDVNTMADAYKFLQDL

Peh NPF   DKYYTQAARPRFGKR.....................NYGGLDERLMVRNS
Dm  NPF   DTYYGDRARVRFGKRGSLMDILRNHEMDNINLGKNANNGG..........

Peh NPF   YQFLSVQAKQFDNDNKLITESGRGTV
Dm  NPF   ...................EFARGFNEEEIF
```

```
Apm NPF   MQSYSNTIYLTL.ILFIFGIMIVHGEPEPMARPTRPEIFTSPEELRRYID
Bom NPF2        MRLTLSAILLFAAILSCSAQAQYPRPRRPERFDTAEQISNYLK

Apm NPF   HVSDYYLLSGKARYGKRGNVLYSVPDVNYPWDTMKTVVENSQRSQQLKLE
Bom NPF2  ELQEYYSVHGRGRYGK................................

Apm NPF   KRKQKDSELLGEHETYGAKKETSRIDTRPCHVLDSIERYYDDVQ
Bom NPF2  ............................RQMHIADASVIFRESPFFEHSLN

Apm NPF
Bom NPF2  EDGLLKKFGYK
```

Figure A.6: Clustal X alignment and pileup of subgroups of NPF ORF amino acid sequences. ▌ Identical. ▐ Conserved.