AN ANALYSIS OF ION TRANSPORT PEPTIDE AND RELATED TRANSCRIPTS IN DESERT AND MIGRATORY LOCUSTS

by

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We accept this thesis as conforming to the required standard

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ABSTRACT

Maintenance of a homeostatic internal environment is necessary for all organisms to function. For the desert locust, a stable osmotic and ionic internal environment is especially critical when faced with widely fluctuating water and ion availability. The locust hindgut is the primary site of ion and fluid reabsorption. This reabsorptive process is regulated to carefully balance the internal environment. Although the transport mechanisms have been described thoroughly, the control of these processes is not well understood. Ion Transport Peptide (ITP) isolated from Schistocerca gregaria corpora cardiaca (CC) stimulates Na\(^+\), K\(^+\), Cl\(^-\) and water reabsorption in the locust hindgut. Both synthetic and expressed forms of ITP mimic the activity of S. gregaria CC in a biological assay. The ITP peptide and the related transcript ITP-L are the first insect members identified of a large crustacean hormone family.

The purpose of this study is to examine two questions regarding ITP:
1. Is ITP restricted to the brain and CC as suggested by bioassay results?
2. Do other insects have ITP or ITP-like peptides as suggested by bioassay results?

Using reverse transcription polymerase chain reaction (RT-PCR) I found ITP transcripts in the brain, corpora cardiaca and unexpectedly in the rectum. Using the same method, I found ITP is expressed during embryogenesis as early as day 9, and localized to the heads of day 12 eggs. ITP-L RNA is observed in all tissues tested and the temporal distribution of ITP-L is equally ubiquitous, as transcripts are demonstrated within \textit{in utero} eggs through to day 14. Western blot analysis shows an ITP peptide of about 7.6 kD in CC homogenates. Possible post-translational differences are suggested at the C-terminal end of the ITP peptide between S. gregaria and ITP of synthetic origin and ITP from Locusta migratoria CC. RT-PCR analysis demonstrates alternate 5' sequences for both ITP and ITP-L and that there are possibly two more ITP-like transcripts in the brain. Finally, I report the sequence of \textit{L. migratoria} ITP and ITP-L cloned from brain total RNA which shows high (>90%) identity relative to S. gregaria.
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<th>Description</th>
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<tr>
<td>+</td>
<td>positive charge</td>
</tr>
<tr>
<td>5-HT</td>
<td>5-hydroxytryptamine or serotonin</td>
</tr>
<tr>
<td>µL</td>
<td>microliter</td>
</tr>
<tr>
<td>A</td>
<td>deoxyadenosine</td>
</tr>
<tr>
<td>aa</td>
<td>amino acid</td>
</tr>
<tr>
<td>Ab</td>
<td>antibody</td>
</tr>
<tr>
<td>AKH</td>
<td>adipokinetic hormones</td>
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<tr>
<td>APS</td>
<td>ammonium persulfate</td>
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<tr>
<td>ATP</td>
<td>adenosine 5’ triphosphate</td>
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<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>BCM</td>
<td>Baylor College of Medicine</td>
</tr>
<tr>
<td>C-1</td>
<td>C-terminal antibody (ITP specific)</td>
</tr>
<tr>
<td>C-2</td>
<td>C-terminal antibody (ITP-L specific)</td>
</tr>
<tr>
<td>C</td>
<td>deoxycytidine</td>
</tr>
<tr>
<td>CC</td>
<td>corpus cardiaca</td>
</tr>
<tr>
<td>cDNA</td>
<td>complimentary DNA</td>
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<tr>
<td>CHH</td>
<td>crustacean hyperglycaemic hormones</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>CRF</td>
<td>corticotropin releasing factor</td>
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<tr>
<td>CTSN</td>
<td>chloride transport stimulating hormone</td>
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<tr>
<td>Da</td>
<td>daltons</td>
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<tr>
<td>DDT</td>
<td>1,1’-(2,2,2-trichloroethylidene)bis[4-chlorobenzene]; 1,1,1-trichloro-2,2-bis(p-chlorophenyl)ethane</td>
</tr>
<tr>
<td>DH</td>
<td>diuretic hormone</td>
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<td>DNA</td>
<td>deoxyribonucleic acid</td>
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dNTP - deoxynucleotide triphosphates
DP - diuretic peptide
DMSO - dimethyl sulfoxide
DTT - dithiothreitol
EDTA - ethylenediaminetetraacetic acid
EGTA - ethylene glycol-bis(β-aminoethyl ether) N, N, N’, N’-tetraacetic acid
EtBr - ethidium bromide
EtOH - ethanol
G - deoxyguanosine
HPLC - high pressure (performance) liquid chromatography
I_{sc} - short-circuited current
ITP - ion transport peptide
ITP-L - ion transport peptide-long
kD - kilodaltons
KLH - keyhole limpet hemocyanin
mg - milligrams
min. - minute(s)
mM - millimolar
MMLV - moloney murine leukaemia virus
MIH - molt-inhibiting hormone
mya - million years ago
N-1 - N-terminal antibody (recognizes both ITP and ITP-L)
NAPS - U.B.C. Nucleic Acid/Protein Service (unit)
NCC1 - nervus corporis cardiaci 1 (physical extension between PI and the CC)
NCC2 - nervus corporis cardiaci 2 (physical extension between PL and the CC)
NE - no product expected
Nps - neuroparsins
nvr - not very reactive
ORF - open reading frame
P^{32} - phosphorus-32
PAGE - polyacrylamide gel electrophoresis
PBS - phosphate buffered saline
pBs - plasmid Bluescript
PCR - polymerase chain reaction
PI-NSC - pars intercerebralis - neurosecretory cells
PL-NSC - pars lateralis - neurosecretory cells
pmoles - picomoles
RNA - ribonucleic acid
RT - reverse transcriptase
RT-PCR - reverse transcriptase - polymerase chain reaction
rxn. - reaction
sc - side chain
SDS - sodium dodecyl sulfate
Sf9 - \textit{Spodoptera frugiperda} cell culture
SOG - subesophageal ganglia
T - deoxythymidine
TAE - tris-acetate and EDTA buffer
TBE - tris-borate and EDTA buffer
TBS - tris buffered saline
TBS-T - tris buffered saline with tween-20
Temed - N,N,N',N'-tetra-methyl-ethylenediamine
T_m - melting temperature
<table>
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<tr>
<td>TNT</td>
<td>tween-20 and nonidet P-40 whole cell PCR buffer</td>
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<tr>
<td>VGF</td>
<td>ventral ganglia factor</td>
</tr>
<tr>
<td>VIH</td>
<td>vitellogenesis-inhibiting factor</td>
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INTRODUCTION

Rationale

Biological Control Strategies for Insects

The desert locust (*Schistocerca gregaria*), the migratory locust (*Locusta migratoria*) and related orthopterans exact a substantial toll on crops worldwide. *S. gregaria* plagues are a threat to crops and grazing lands in Africa, the Middle East and Southwestern Asia (Bennett 1993). Locust plagues occur sporadically and can persist for several years depending on rainfall, swarm movements and control efforts. From 1985 to 1995 international development agencies spent $383 million (US) on emergency control efforts in response to two major desert locust swarms (Joffe 1995).

Insecticides are the most popular method of pest control today, despite their obvious hazard to humans, either through direct exposure or secondarily via the environment. They can accumulate in soil, water and the produce itself. The use of insecticides is often not very effective. As with many insect species that are the target of chemical insecticide control, resistant strains are selected. An example of this, is the Colorado potato beetle (*Leptinotarsa decemlineata*) which has rapidly developed resistance to almost all known chemicals available for control (Ioannidis et al., 1991 and Rahardja and Whalon 1994). Insecticides often act to destroy non-target organisms, such as pollinators, natural enemies of the pests and soil arthropods. These non-specific actions and selection for resistance can lead to a secondary pest outbreak, thus causing more damage in the long run.

The benefits of pest control strategies are often outweighed by the accompanying economic and environmental costs of the control effort. The 'environmental costs' associated with the spraying of insecticides stems from the fact that most synthetic insecticides are broad spectrum nerve poisons. This means that they not only act on the nervous system of the pest species, but also on useful insects. Agriculture Manitoba (1991) grasshopper control guidelines suggest special precautions because insecticides used to control grasshoppers are also poisonous to honeybees. Another inconvenience of synthetic insecticides is their activity in mammals.
Chemical insecticides used to control locusts can be organized into four groups by their active ingredients 1) organochlorides, 2) organophosphates, 3) carbamates and 4) synthetic pyrethroids (Joffe 1995). Organochlorines, such as DDT and chlordane, are highly toxic to mammals. Because of their chemical stability and a low solubility in water, they accumulate in body fat (Gullan and Cranston 1994). The most common insecticides used for control of locust infestations are organophosphates, such as malathion and fenitrothion (Bennett 1993). Organophosphates are also highly toxic to mammals, but fortunately are less stable and are not stored in fat tissues (Gullan and Cranston 1994). Carbamates have characteristics similar to the organophosphates, while synthetic pyrethroids are fast acting, broad-spectrum insecticides with varying levels of persistence and toxicity (Joffe 1995).

The lack of specificity and the tendency to accumulate in the environment has prompted the development of pest-specific biological control strategies which employ entomopathogenic micro-organisms, plant toxins and hormone analogs, to name a few (Lomer and Prior, 1992 and Krall and Wilps, 1994). These strategies attempt to provide viable alternatives to chemical insecticides that are species specific and do not involve products that persist in the environment. For these reasons they are often termed 'environmentally friendly'.

There are currently over one hundred different insect neuropeptides isolated, 56 of which are found in S. gregaria and L. migratoria (reviewed by Schoofs et al., 1997). The use of hormone analogs or insect neuropeptides as control agents is reviewed by Masler et al. (1993), who promote this strategy for three primary reasons. Neuropeptides control major life processes (potentially disruptive), they act at very low levels (pico/femtomole range) and they can be subjected to 'analysis and manipulation' via molecular techniques. I would also consider insect neuropeptide control strategies more resistant to detoxification processes, because they are or they mimic essential native peptides. Price (1991) considers metabolic detoxification the primary mechanism of resistance in insects and identifies three major reaction types (Hydrolytic cleavage, cytochrome P-450-mediated and glutathione-mediated reactions). These individual detoxification
mechanisms often confer multiple resistance to toxin families and generally act non-specifically to make insecticides more hydrophilic. Insects have likely developed detoxification mechanisms in response to continuous exposure to harmful xenobiotics in their natural environment. The engineering of hormone analogs and neuropeptides for insect control may represent a substantial challenge to insect adaptive mechanisms.

Characteristics of the locust anti-diuretic peptide (ITP), make it an excellent candidate for the development of control strategies. The peptide has been cloned, sequenced and expressed (Meredith et al., 1996). An alternate antagonistic form (ITP-L) has been identified (Ring et al., 1997) and a sensitive bioassay system has been perfected (Hanrahan and Phillips 1982, Irvine et al., 1988). Thus far no ITP homologues have been found outside Orthoptera, but they likely exist. Any divergence between ITPs could be exploited to target specific 'pest' species. The use of an anti-diuretic peptide in control strategies is especially significant to insects. Insects are particularly susceptible to dehydration, because of their high surface to volume ratio (Lehmberg et al., 1993). For the desert locust, maintaining a relatively constant internal environment, both osmotic and ionic, is critical to survival in an environment where the availability of water and ions can fluctuate widely. Blocking the ITP receptor will lead to severe diuresis and dehydration in locusts. Moreover, locust ITP may act as an antagonist on ITP-like receptors of other insect orders. ITP-related neuropeptides have not been observed in vertebrates, whereas many other insect peptide hormones have considerable sequence homology to vertebrate peptide hormones (reviewed by DeLoof and Schoofs, 1990).

The preceding facts are a strong argument in favor of using ITP to develop biologically friendly control strategies that might eliminate some of the problems associated with traditional chemical pesticides. In the following sections I provide some background on the locust excretory and osmoregulatory systems where ITP acts. In Figure 1a a model of basic hindgut activities is presented. Figure 1b shows cell morphology and specific physiological processes. Figure 1c presents a model for the neuroendocrine regulation of the hindgut. These serve to demonstrate the importance of ITP in regulation of the locust hindgut and as a prime target for biological control strategies.
Background on the Excretory Process and its Control

Gut Anatomy
Locusts feed primarily on solid plant matter. This necessitates a straight gut with strong musculature and a cuticular lining to protect from abrasion. The nutrient levels in solid plant matter are relatively low; therefore, the intake of large amounts of food is necessary. The rate of water loss via the excretory system increases greatly after feeding (Coast, 1996).

Insects guts are usually divided up into three distinct functional regions. The foregut is responsible for ingestion, grinding, storage and transport of food to the midgut. Figure 1(a, b and c) shows the hindgut and the posterior portion of the midgut in cartoon form. In the midgut digestive enzymes are secreted and nutrients are solubilized for absorption (figure 1a). The Malpighian tubules are long thin blind-ended outgrowths of the anterior hindgut, that secrete solutes and water from the haemolymph relatively indiscriminately to form the primary urine. Material remaining from the midgut lumen and primary urine from the Malpighian tubules empties into the hindgut. The hindgut selectively reabsorbs water, salts, metabolites and other valuable molecules prior to elimination (figure 1a). The Phillips laboratory has described most of the major transport processes in the locust hindgut (as reviewed by Phillips and Audsley 1995 and Phillips et al., 1986). The variability of Malpighian tubule and hindgut contents versus the relative stability of haemolymph composition (reviewed in Phillips et al., 1986), indicates an essential role for the hindgut epithelia in maintaining an ionically and osmotically stable haemolymph.

Morphology/Physiology of the Locust Hindgut
The entire enteron is lined by a single epithelial cell layer and is surrounded by a basement membrane and a variably developed muscle layer. The structural differences between the ileal and the rectal epithelia reflect their different physiological roles. The locust ileum is about a third as thick as the locust rectum (Irvine et al., 1988). Of particular interest is
Figure 1a. Basic overview of the structures and functions of the locust gut in a longitudinal section, with arrows indicating direction of movement.
the presence of elaborate intercellular sinuses and channels in the rectum that are absent in the ileum, as shown in figure 1b. These rectal channels are believed to facilitate ion recycling (Gupta et al., 1980) which is essential for extracting water to create strongly hyperosmotic excreta in the rectum. The ileal epithelium is otherwise structurally very similar to the rectal epithelium and consequently has many common functions.

The ileum absorbs water and ions without any major changes in ionic or osmotic pressure. The rectum on the other hand is responsible for concentrating lumen contents to create strongly hyperosmotic and dry excreta prior to elimination during times of dehydration and starvation. The proposed model for reabsorption in the rectum, shown in figure 1b (rectal pad cell), suggests that active ion and proline reabsorption helps generate large ionic and osmotic gradients and due to solute recycling within the complex lateral spaces in the rectal epithelia a hypoosmotic absorbate can be produced (Phillips et al., 1986 and Wall, 1971).

The locust hindgut is primarily responsible for two physiological processes, excretion (removal of the waste products of metabolism) and osmoregulation (maintenance of a favorable body fluid volume and composition). Locusts carry out a delicate balancing act, to regulate these two processes. The excretion of nitrogenous wastes and osmoregulation (including pH balance) depends on the conservation of Na\(^+\), K\(^+\), Cl\(^-\) and water. Figure 1b shows the primary transport processes of the Malpighian tubules, the ileum and the rectum. The Malpighian tubules actively transport K\(^+\), urate, proline and some classes of toxic organic compounds into the lumen, but most other blood solutes diffuse into the tubule lumen. The resulting primary urine is isosmotic to the hemolymph, high in KCl and low in Na\(^+\). Water flows passively following the osmotic concentration difference created by KCl secretion. Some 80-95\% of Na\(^+\), K\(^+\), Cl\(^-\) and water secreted by the tubules is eventually reabsorbed by the hindgut depending on the state of hydration (reviewed by Phillips 1981; Phillips et al. 1986).
Figure 1b. Model of the locust hindgut showing specific solute and water movements and expanded views of the locust ileal and rectal pad cells (modified from Gupta et al., 1980). Note the complex channels and sinuses between rectal pad cells which allow for proline and ion recycling.
The ileum and rectum have very similar ion transport processes dominated by Cl⁻ transport and accompanying passive absorption of K⁺. The ileum is the major site of Na⁺ reabsorption and nitrogen excretion in the form of ammonium. The source of this ammonia is ammoiagenesis in the epithelium and it serves to assist in regulation of whole body pH by trapping H⁺ ions (Phillips et al., 1994). There is also an apical proton pump that actively secretes H⁺ into the ileal lumen and drives bicarbonate readsoption.

Structure of the Neuroendocrine/Neurosecretory System

Histological studies of *S. gregaria* and other orthopteroid insects has identified specific cells in the brain whose neurons extend in bundles to a remote neurosecretory site (Highnam 1961 and Virant-Doberlet et al., 1994). One example is the pars intercerabralis (PI), a collection of cells in the anterior medial brain that extend primarily to the storage lobe of the corpora cardiaca (CC) via the nervus corporis cardiaci 1 (NCC1). Another example is the pars lateralis (PL), a group of neurosecretory cells that are located anterior and laterally in the brain that also extend to the storage lobe of the CC via the NCC2. Figure 1c shows a simplified representation of various neurosecretory cells and their projections into the CC storage lobe. Neuropeptides are often associated with carrier proteins that escort them to the storage lobe and they may also be involved in processing and/or sorting of the neuropeptides prior to release. Oudejans et al. (1996) present evidence that locust adipokinetic hormones (AKHs) are transported in a carrier-independent manner within the hemolymph after release from the CC, despite being highly hydrophobic.

Insect Diuretic Factors

Diuretic factors isolated from insects fall into three categories: the corticotrophin-releasing factor related diuretic peptides (CRF related-DPs); insect kinin neuropeptides and the non-peptide 5-hydroxytryptamine (5-HT) or serotonin (reviewed by Spring 1990 and Coast 1996). Diuretic factors act to increase Malpighian tubule secretions by stimulating ion transport (reviewed by Nicolson 1993 and Beyenbach 1995).
Neuropeptides stored in neurohemal organs are released into the haemolymph upon the appropriate stimulus. An example of this is the triggering of stretch receptors in the abdomen of *Rhodnius prolixus*, in response to a blood meal, causing the release of diuretic hormone (Maddrell, 1964). Maddrell and Gee (1974) have shown release of diuretic factors from the CC can be accomplished by electrical stimulation of the NCC or CC.

A CRF-related diuretic peptide was first described by Kataoka et al. (1989) in *Manduca sexta* (*Manduca*-DP) as a 41 amino acid (aa), C-terminally amidated peptide. Blackburn et al. (1991) isolated a second smaller *Manduca sexta* DP which is a 30 aa peptide (*Manduca*-DP II). CRF-related DP's have now been isolated from *Acheta domesticus* (Kay et al., 1991a), *Locusta migratoria* (Kay et al., 1991b and Lehmberg et al, 1991) and *Periplaneta americana* (Kay et al., 1992). Other CRF-related DP's have been found in house flies, mosquitoes and mealworms (Clottens et al., 1994, Furuya et al., 1995 and reviewed by Coast 1996). Genomic and cDNA sequences are reported by Digan et al. (1992) for *Manduca sexta* diuretic hormone (*Mas*-DH). Patel et al. (1994) and Thompson et al. (1995) use immunocytochemistry to localize *Locusta*-DP in the central nervous system (CNS) and hemolymph of *L. migratoria*. Patel et al. (1995) present evidence for the hormonal activity of *Locusta*-DP by using antibodies to specifically block diuretic peptide activity in vivo. Audsley et al. (1997a) show a 5-fold increase in hemolymph DH postfeeding and report >5 pmoles of DH in CC, ~ 1 pmoles in the brain and 25-200 fmoles in thoracic and abdominal ganglia in 14 day old *L. migratoria* (Audsley et al., 1997b). A diuretic hormone receptor from *Manduca sexta* has been characterized and cloned (Reagan et al., 1993, Reagan 1994 and Reagan 1995). Also, Reagan (1996) reports the cloning of diuretic hormone receptor from *Acheta domesticus*.

Locustakinin shows colocalization (Thompson et al., 1994) and synergistic action with *Locusta*-DP (Coast 1996). However achetakinin is not synergistic with *Acheta*-DP, and kinins alone in general show 30-75% of the activity of a CRF-related DP from the same insect (Coast 1996).
Inhibitory Neuromodulator

CTSH
NPS
ITP

Anti-Diuretic Hormone

Diuretic Hormone

- CRF-DH
- Kinnins
- Serotonin

Osmosensitive cells in the Hemolymph could trigger the release of Anti-Diuretic Hormone from the CC.

Stimulation of gut stretch receptors could in turn activate the release of Diuretic Hormone from the CC.

Figure 1c. A simplified model of diuretic control in the locust hindgut, highlighting diuretic and antidiuretic factors isolated to date. In the left upper corner, the locust brain and associated structures are represented. The regions marked PI-NSC and PL-NSC represent the pars intercerebralis and the pars lateralis - neurosecretory cells respectively.
Nijhout (1994), suggests that the transport activities of the Malpighian tubules and the hindgut must be independently regulated (as shown in figure 1c) to keep the haemolymph ionically and osmotically stable when the organism is exposed to wide ranges of water and ions. Indeed, *Locusta-DV* and kinin have no action on ileal and rectal Cl⁻ and water reabsorption (Meredith and Coast, unpublished observation). The candidates for antidiuretic factors are not as well characterized as their diuretic counterparts.

**Insect Anti-Diuretic Factors**

There are three neuropeptides which are reported to show anti-diuretic activity. Chloride transport stimulating hormone (CTSH) was partially purified from CC of *S. gregaria* and stimulates rectal Cl⁻ reabsorption (Phillips et al., 1980 and Proux et al. 1984). Fournier and Girardie (1988) report a factor, Neuroparsins (Nps), which stimulates water reabsorption in the *L. migratoria* hindgut. Neuroparsins shows no effect on the short-circuit current or fluid transport in *S. gregaria* hindgut bioassays (Jeffs, 1993). Ion Transport Peptide (ITP) isolated from *S. gregaria* CC (Audsley et al. 1992a), which stimulates Cl⁻, K⁺, Na⁺ and fluid reabsorption, while also inhibiting H⁺ secretion across locust ilea (Audsley et al. 1992b) is the subject of this thesis. The sequence obtained by Audsley et al. (1992a) was used to identify an ITP brain cDNA clone and an Ion Transport Peptide long form (ITP-L) ileal cDNA clone (sequences shown in figures 2 and 3). Both clones have been expressed in a baculovirus system (Meredith et al., 1996) and ITP has been chemically synthesized by King et al. (in preparation) with three sulfur bridges and terminal amidation as predicted by Meredith et al. (1996). The transcript forms represented in figure 2 may not represent all of the possible transcripts and, to date, no genomic sequence for ITP or ITP-L has been recovered. Genomic sequence might indicate other ITP-like transcript forms, but the use of genomic DNA for investigations in locusts is hampered by genome size and large introns.
In this thesis I will address two main hypotheses. The first hypothesis is that ITP RNA and protein are restricted to brain and NCC (i.e. tissues that stimulate the hindgut bioassay). This hypothesis leads me to ask the questions:

**What is the distribution of ITP RNA and protein within the locust *S. gregaria*, and are there other ITP-like sequences or peptides found in *S. gregaria*?**

The second hypothesis is based on bioassays with crude CC extracts from other locusts (*L. migratoria*) and related orthopteroids (grasshopper, cricket and cockroach), which stimulate *S. gregaria* hindgut bioassays. Based on bioassays, other orthopterans are expected to have anti-diuretic stimulants with amino acid sequences similar to that of *S. gregaria*. From this hypothesis I ask the question:

**Do related orthopterans or other insects have ITP-like anti-diuretic factors?**

In this thesis I will demonstrate the distribution of ITP and ITP-L RNA in *S. gregaria* tissues and egg stages. I also show the distribution of ITP-like peptides in *S. gregaria* tissues. Finally, I use reverse transcription polymerase chain reaction (RT-PCR) and *S. gregaria* specific primers to clone ITP from *L. migratoria* brain RNA.
MATERIALS AND METHODS

Animals
Desert locust (*Schistocerca gregaria*) adults, 2-3 weeks past their final molt, were reared at 28 °C and 55% relative humidity, with 12 hours of light and 12 hours of darkness. Their diet consisted of lettuce and a mixture of dried grass powder, milk powder and bran. *L. migratoria* adults were raised under the same conditions as *S. gregaria*. *S. gregaria* eggs at various stages of development were collected and frozen in liquid nitrogen at the times specified. Eggs were dissected from adult females (in utero) and were excavated from the sand at 6, 9, 11, 12 and 14 days after being laid into the egg jars. Heads of 12 day embryos were obtained by removing the posterior 2/3 of the egg. The posterior 2/3 of these eggs were saved for 12 day thorax and abdomen preparations.

RNA Extraction
*S. gregaria* and *L. migratoria* tissues were dissected and immediately frozen in liquid nitrogen. Extraction were performed on the day of dissection or material was stored at -80 °C until enough tissue was accumulated. There was no difference noted between tissues that were stored at -80 °C or used immediately, in terms of RNA yield or the quality of the RNA (i.e. degradation). Total RNA was extracted using one of two methods, the acid guanidinium thiocyanate-phenol-chloroform method of Chomczynski and Sacchi (1987) or using TRIzol (GIBCO, BRL). For TRIzol extractions, the manufacturer's protocol was followed, including an extra isolation step to remove proteins, fat, polysaccharides and extracellular material. Both extraction methods gave similar yields from 0.48 to 3.35 micrograms (μg) RNA / milligram (mg) tissue (wet weight). Even though TRIzol extraction is a modified Chomczynski and Sacchi protocol, the TRIzol method is simplified by premixed reagents and a coloured organic layer for easier phase separation.
Oligonucleotides

Synthesis of specific oligonucleotide sequences was accomplished with an automated DNA synthesizer (Applied Biosystems 380B-NAPS unit U.B.C.). Oligos were deprotected under standard conditions and purified with ammonia-butanol (Sawadogo and Van Dyke, 1991). Some primers were designed with restriction sites in flanking regions to simplify cloning (shown in bold on table 1). Oligos used as PCR primers were diluted in water to 20 picomoles (pmoles) /microliter (µl). See figures 2 and 3a for primer map, 5' primers A', A, B and C and 3' primers 3, 2 and 1. Random hexamers for reverse transcription were purchased from Pharmacia or GIBCO, BRL and diluted to 50 pmoles/µl.

Reverse Transcription - Polymerase Chain Reaction (RT-PCR)

Reverse transcription was performed on one µg of total RNA with 200 units of Superscript RNase H (GIBCO, BRL) which is a reverse transcriptase from the Moloney murine leukaemia virus (MMLV). The 20µl reaction (rxn.) volume contained buffer (50 millimolar (mM) Tris-HCl pH 8.3, 75 mM KCl and 3 mM MgCl₂) and 10 mM dithiothreitol (DTT), 0.5 mM of each deoxynucleoside triphosphate (dNTP), 20 units of cloned ribonuclease inhibitor (GIBCO, BRL) and 50 µM random hexamers. The mixture was incubated for 1 hour at 37 °C, followed by a 5 minutes (min.) incubation at 70 °C. The Polymerase Chain Reaction was conducted in a total volume of 100µl (20µl RT rxn. + 80 µl PCR mastermix). In addition to the 20µl RT reaction the 100µl volume contains PCR buffer (20 mM Tris-HCl pH 8.4 and 50 mM KCl), 6% DMSO, 2 mM MgCl₂, 0.1 mM of each dNTP, 20 µM of each primer (1 sense and 1 anti-sense) and 2.5 units of Taq (Thermus aquaticus YT1) DNA polymerase (GIBCO, BRL). The reaction was carried out on a Perkin Elmer/Cetus DNA thermal cycler (MODEL 480). The program of the cycler consisted of an initial denaturation of 10 minutes at 94 °C, followed by 30 cycles of 94 °C for 1.5 minutes (denature), 46-60 °C for 1 minute (anneal), and 72 °C for 1.5 minutes (extension) and after 30 cycles a final extension at 72 °C for 6 minutes. The annealing temperature was dependent on the specific primers used. The number of cycles and the annealing temperatures were determined by optimizing for expected products.
<table>
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<th>Primer</th>
<th>Primer Sequence</th>
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<th>$T_{m(b)}$</th>
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<tr>
<td>A</td>
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<td>60°C</td>
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<tr>
<td>B</td>
<td>CCACCCCGGATGCAACCAGAAGCAGCAG</td>
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<tr>
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<td></td>
<td>44°C</td>
<td>62°C</td>
</tr>
</tbody>
</table>

Table 1. List of primer sequences and corresponding melting temperatures ($T_m$). The $T_m$ values were calculated as follows $T_{m(a)} = 59.9 + 41 \times (% G + C) - 675/N$ and $T_{m(b)} = 2(A + T) + 4(G + C)$. **Bold** type indicates restriction sites and flanking sequence.
Figure 2. Scale map of the ITP brain cDNA clone (top) and the ITP-L ileum cDNA clone (bottom). Blocks A, A’, B and C represent the sense primers, and blocks 1, 2 and 3 represent the antisense primers. Solid bars indicate the ITP ORF sequence and the 121bp ITP-L insert is shown as a checkered bar. Asterisks (*) indicate stop codons. Diagonally striped bars represent sequences 5’ to the ORF sequences. Note the thick diagonally striped bar is upstream the ITP sequence while, the thin diagonally striped bar is upstream of the ITP-L sequence for these clones. White bars are sequence 3’ to the ITP ORF sequence.
Figure 3a. *S. gregaria* ITP and ITP-L primer sequence map shows an alignment of nucleic acid sequences (ITP-L above and ITP below). Primer sequences are indicated by arrows. Sense primers are A, A', B and C and anti-sense primers are 1, 2 and 3. The ITP-L insert sequence is indicated in **bold** type. Note that primer 1 is located within the insert sequence and is ITP-L specific. The putative ORF begins at position 1 below primer B. Note the alternate 5' sequences upstream from the ORF are associated with the A and A' primers. Asterisks (*) indicate stop codons and the solid vertical line indicates the start of the active peptide in ITP and the putative start in ITP-L.
Figure 3a.
DNA Electrophoresis

DNA products were fractionated on 2% agarose gels containing 0.05 μg/ml Ethidium Bromide, using a Biorad mini sub or a wide mini sub DNA Cell agarose gel electrophoresis apparatus. Gels were made with and submerged in Tris-borate buffer (TBE - 0.045M Tris-borate and 0.001M ethylene diaminetetraacetic acid disodium salt solution (EDTA)) for all agarose gels, with the exception of those used for electroelution. Gels used in electroelution and in the freeze-snap gel purifications were made with and run in Tris-acetate buffer (TAE - 0.04M Tris-acetate and 1 mM EDTA). The power source for electrophoresis was a Pharmacia LKB GPS 200/400 power pack and was run at 60-100V.

Preparation of Insert and Vector

Bands were extracted from agarose using a variety of methods:

1. Electroelution of DNA (modified method from McDonnell et al., 1977) from TAE gel slices was achieved by placing them in dialysis tubing in 1-2 mls TAE buffer (0.04M Tris-acetate and 0.001 EDTA) with both ends sealed. The tubing was then immersed in TAE buffer and electrophoresed at 80-100V for 2-3 hours or until the slices no longer fluoresced, current was reversed for 10 seconds and the solution within the tubing is pipetted into a clean micro-tube. DNA was extracted with butanol, the aqueous layer was precipitated with EtOH (100%) and the pellet was washed twice with 70% EtOH.

2. Freeze-Snap method of Qian and Wilkinson (1991), takes advantage of low melting point agarose (LMP agarose) gels in TAE buffer (0.04M Tris-acetate and 1 mM EDTA). Their protocol suggests a 30 minute electrophoresis at 20-50 V for DNA fragments less than 1 Kb, which is fine if only a single product is present per lane, but these conditions failed to separate the DNA products sufficiently when there was more than one band per lane. In these cases electrophoresis was carried out for one hour at 50 V.

3. Qiaex II gel purification kit (Qiagen, manufacturers protocol)

4. NucleoTrap gel purification kit (Macherey-Nagel, manufacturers protocol)

Both the Qiaex II and Nucleotrap gel purification kits use silica particles to bind DNA fragments for separation from the agarose gel.
PCR fragments that were cloned had restriction sites designed into the flanking ends of the primer sequences. Both the vector, pBluescript KS+ (Stratagene), and the bands were digested with the appropriate enzyme(s), separated on agarose gels and again purified from the gel. When fragments had the same restriction sites at both ends, the vector was digested and then dephosphorylated using 2 units of calf intestine alkaline phosphatase (Boehringer Mannheim) in a 100 μl reaction volume. The reaction mixture contained 50 mM Tris-HCl, 0.1 mM EDTA pH 8.5 and was incubated at 37 °C for 1 hour. To stop the reaction, 1/10 volume of 200 mM ethylene glycol-bis(β-amino ethyl ether)-N,N,N′N′'-tetraacetic acid (EGTA) was added and then the mixture was incubated at 65 °C for 10 minutes. The vector(pBS KS+) was then gel purified and ready for ligation. Fragments with non-complementary ends were not dephosphorylated, but were gel purified after digestion.

**Ligation and Transformation**

The ligation reaction had an insert-to-vector ratio of 3:1 in a reaction volume of 20 μl and uses 0.5 units of T4 DNA Ligase (GIBCO, BRL). The reaction mixture contained 50 mM Tris-HCl pH 7.6, 10 mM MgCl₂, 1 mM ATP, 1 mM DTT and 5% polyethylene glycol-8000. The ligation mixture was then incubated at 23-26 °C for 1-4 hours. Fifty μl aliquots of subcloning efficiency DH5-alpha competent cells (GIBCO, BRL) were transformed with 5 μl of ligation mix using the manufacturers suggested protocol. Standard Blue/White selection and ampicillin resistance were used to initially screen for positives. White colonies were PCR screened to confirm the presence of an insert (see below).
PCR Screening
White colonies were picked and grown on a masterplate. Cells were then picked from the masterplate and transferred to a 0.5 ml eppendorf tube containing 3 µl of 10X TNT buffer (0.5% tween-20, 0.5% nonidet P-40, 10 mM tris pH 8.0 and 500 mM KCl) and frozen until screening. PCR was conducted on the cell aliquots under the same conditions as mentioned above with two exceptions: 2 mM MgCl₂ and TNT buffer were used instead of the buffer provided by the manufacturer. Insert specific or vector specific primers were used for PCR screening. Positives from the PCR screen were grown up in liquid culture and plasmid purified (QIAGEN plasmid purification kit) and an aliquot was digested to ensure the appropriate sized fragment was in the cloning site of pBS. Plasmids that contained the appropriate sized insert were further purified on dialysis discs (Millipore) afloat on a milli-Q H₂O bath, prior to sequencing.

DNA Sequencing
Sequencing was accomplished using the ABI AmpliTaq (FS Taq) dye terminator cycle sequencing system and a 373 DNA automated sequencer. For double-stranded plasmid DNA, 500 ng of template was required. For sequencing of PCR product directly, 90 ng of template was required. In both cases, 3.2 pmoles of primer was used in a 20 µl reaction volume (U.B.C. Nucleic Acid/Protein Service unit-NAPS). Cycle sequencing extension products were purified of unincorporated terminators using Centri-Sep columns (Princeton Separations, Inc). All editing and alignment of sequence was done using MacVector 4.1 and Assembly-Lign 1.1.5 (Kodak) and sequence databank searches were conducted using the BCM Launcher (Baylor College of Medicine).
Tissue Homogenates
Dissected and frozen tissues were homogenized in 1X phosphate buffered saline (PBS pH 7.3) with a ground glass homogenizer, centrifuged to pellet insoluble matter (20 min. at 12,000g) and the supernatant removed and stored at -20 °C. The total sample volume was 25 μl (10% DTT and 12.5 μl 2X load buffer (50 mM Tris HCl, 100 mM DTT, 2% SDS, 0.1% bromphenol blue and 10% glycerol)). Samples were boiled for 5-10 minutes just prior to loading on gels. Samples were compared to baculovirus expressed ITP and ITP-L (Meredith et al., 1996) and chemically synthesized ITP (King et al., in preparation).

SDS Polyacrylamide Gel Electrophoresis
Proteins were fractionated on polyacrylamide gels under denaturing conditions. The resolving gel was 18% polyacrylamide (18% total monomer concentration (%T) / 2.67% crosslinking monomer concentration; bis-acrylamide (%C)) with 0.375 M Tris (pH 8.8), 0.1% SDS, 0.1% APS and 0.5% Temed. The stacking gel was 3.8% polyacrylamide (30%T / 2.67%C) with 0.12 M Tris (pH 6.8), 0.1% SDS, 0.05% APS and 0.1% Temed. The running buffer used was 1.92M Glycine, 0.25M Tris (pH ~8.5) and 34.6 mM SDS. Gels were run on a Tyler vertical gel apparatus at 30V and 4-16 mA for 12-14 hrs. The gel dimensions were 121 X 169 X 0.75 mm.

Tris Tricine Polyacrylamide Gel Electrophoresis
For improved separation of small peptides, tris tricine polyacrylamide gels were used (Schägger and von Jagow 1987 and Klafki et al., 1996). The gel has the same dimensions as above. The separating gel was 16.5% polyacrylamide (16.5% Total monomer concentration (%T) / 2.4% Crosslinking monomer concentration-bis-acrylamide (%C)) containing 1.0 M Tris (pH 8.45), 0.1% SDS, 0.1% APS, 0.06% Temed and 10.4% glycerol. The spacer gel, consisting of 16.5% polyacrylamide (16.5%T / 1.0%C) containing 1.0 M Tris (pH 8.45), 0.1% SDS, 0.1% APS, 0.06% Temed, was poured to 20 mm above the separating gel. Stacking gel was 3.96% polyacrylamide (3.96%T/0.24%C) containing 0.744 M Tris (pH 8.45), 0.074% SDS, 0.1% APS, 0.1%
Temed and was poured to 10 mm below the bottom of the comb. The anode running buffer was 0.2M Tris at pH 8.9 and the cathode buffer contained 0.1M Tris, 0.1M Tricine and 0.1% SDS (pH 8.25). Gels were run on a vertical gel apparatus (Tyler) at 40V and 4-12 mA for 24 hrs.

Semi-dry Transfer of bands on Polyacrylamide Gels

Gels were blotted to TRANS-BLOT transfer medium (0.2μm nitrocellulose membrane; BIORAD). Membrane and Whatman (3 mm) paper were presoaked in Towbin transfer buffer (25 mM Tris, 192 mM Glycine and 20% Methanol at pH ~8.3) before transfer. The gel was then quickly wetted in transfer buffer and placed on the membrane and Whatman paper, which were both on the transfer apparatus (BIORAD Trans-Blot SD Semi-Dry Transfer Cell). More Whatman paper was soaked in transfer buffer and placed on the gel (i.e. transfer apparatus bottom plate \ Whatman \ Whatman \ nitrocellulose membrane \ gel \ Whatman \ Whatman \ transfer apparatus top plate). Gels were transferred for 1 hour at 10-12 V and 250-350 mA, air dried, wrapped in plastic-wrap and foil, then stored at 4 °C until probed.

Antibody Production

Figure 3b shows an 87 bp N-terminal boxed labeled N-l, encoding amino acids 57-85 of ITP. This DNA sequence was cloned into the pGex-2T vector (Pharmacia) following the methods outlined in Ring et al. (1997). A second peptide that matches amino acids 115-122 of ITP sequence (shown in figure 3b as the boxed sequence labeled C-1) and a third peptide, amino acids 120-134 in the ITP-L insert sequence (shown in figure 3b as the boxed sequence labeled C-2), were both synthesized with a CGG for coupling at the N-terminus, and coupled to Keyhole limpet hemocyanin (KLH) according to the method of Ziltener et al. (1987) as described in Ring et al. (1997). Polyclonal antibodies were raised by injecting 1 mg of fusion protein or synthetic peptide emulsified in Freund's complete adjuvant subcutaneously into New Zealand white rabbits. Rabbits were boosted with 0.5 mg protein emulsified in Freund's incomplete adjuvant at 6 and 10 weeks. Blood was taken 2 weeks following injection.
**Figure 3b.** *S. gregaria* ITP and ITP-L amino acid sequence map. ITP-L amino acid sequence shown above and ITP below. Boxed amino acids indicate peptides used as antigens for the production of antibodies labeled N-1, C-1 and C-2. Note that the isoleucine at position 60 (vertical arrow) in N-1 is replaced with a methionine in the pGEX expression product and the synthetic peptides C-1 and C-2 have a CGG at their C-terminal ends for coupling to KLH. Also note that C-1 is ITP specific and C-2 is ITP-L specific. The solid vertical line indicates a dibasic cleavage site.
Western Blotting Protocol (ECL western blotting protocol - Amersham)

Nitrocellulose membranes were blocked 12-16 hrs. at 4 °C in 0.1% Tween 20, 5% milkpowder in 1X TBS (Tris buffered saline pH 7.5). The blot was then washed at room temperature once for 15 min. and twice for 5 min. each in TBS-T (1X TBS with 0.1% Tween 20) with agitation. The blot was then incubated with the primary antibody (Ab) at a 1/10 000 dilution in 0.5% milkpowder, 0.1% Tween 20 in 1X TBS for 3 hours with shaking. The blot was then washed in TBS-T twice quickly, once for 15 min. and twice for 5 min. with shaking. The secondary antibody (anti-rabbit horseradish peroxidase linked- Amersham) was applied to the blot, also at a dilution of 1/10 000 in 0.5% milkpowder, 0.1% Tween 20 in 1X TBS, for one hour with shaking. The blot was then washed twice quickly, once for 15 min. and twice for 5 min. each in TBS-T with shaking. Then the blot was washed twice more with 1X TBS for 5 min. each at room temperature with shaking. The blot was quickly placed on Whatman paper to remove excess liquid and then incubated in a 1:1 mixture of ECL reagents (Amersham) for 1 min. at room temperature with shaking. The excess liquid was removed with Whatman paper and the blot was then wrapped in plastic-wrap and exposed (from 30 seconds to 30 minutes depending on signal strength) to Kodak X-OMAT RP XRP-1 film.
RESULTS

Tissue Distribution of ITP and ITP-L RNA in *S. gregaria*

To determine if the ITP transcript is restricted to neural tissues (brain, CC and ganglia) and to determine if the distribution of the ITP-L transcript is tissue specific, RNA was extracted, reverse transcribed and then amplified by PCR with *S. gregaria* sequence specific primers. Table 2 shows the product sizes (calculated to include flanking and restriction site sequences of primers) expected in RT-PCR reactions based on the original brain ITP and ileum ITP-L transcript clone sequences. Six primers were used, four sense (A, A', B and C) and two anti-sense (1 and 2). Based on the sequence of the two cloned transcripts, primer A and 1 are ITP-L specific and primer A' is ITP specific. The original two transcripts ITP and ITP-L were cloned from the brain and ileum respectively and primers were all derived from this sequence (see table 1 and figure 3a).

As a positive control to test the primers, the RNA, and the RT-PCR conditions, brain and ileum RNA were used for RT-PCR amplification as shown in figures 4a and 4b. The products of this amplification are expected to correspond to the product sizes given in table 2. For example, the A'2 primer set must yield a 432 bp fragment from brain RNA (Figure 4a) and the A2 primer set must produce a 711 bp fragment from ileal RNA (Figure 4b). Similarly, I would expect the brain RNA to yield 416 bp and 251 bp fragments with the B2 and C2 primers respectively, and the ileum RNA to produce a 537 bp and a 372 bp fragments with the B2 and C2 primers respectively. Any products other than the above mentioned (and as shown in table 2) are not predicted by the ITP and ITP-L transcript sequences originally cloned and may represent new transcripts. As a negative control for genomic DNA contamination, RNA was PCR amplified without being reverse transcribed first (results not shown).

Figure 4a shows RT-PCR products obtained when C2, B2 and A’2 primer sets are used to amplify from brain and ileal total RNA. When the C2 primer set is used in RT-PCR of ileal RNA, a band of about 370 bp is generated as predicted by the ITP-L ileal clone sequence. When the same primers are used to amplify brain RNA three bands are
Table 2. Expected RT-PCR product sizes based on ITP and ITP-L transcript clone sequences. Sizes are given in base pairs and NE indicates that no product is expected.

<table>
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<th></th>
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<th>A'2</th>
<th>A'1</th>
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Figure 4. Agarose gels of RT-PCR products of the B2, C2, A'2 primer sets in a) and the A2 primer set in b). *S. gregaria* brain and ileum total RNA is used in a) and brain ileum and rectum total RNA is used in b). Arrow heads indicate estimated fragment sizes in base pairs.
generated: a band of about 250 bp consistent with ITP and predicted by transcript clone sequence; a band of about 370 bp consistent with ITP-L; and a band of ~420 bp which was not predicted by existing transcript clones. A similar result is found when the B2 primer set is used in RT-PCR of ileal and brain RNA. The ileal RNA gives a band of about 540 bp which is predicted by the ITP-L sequence. Brain RNA again produces three bands: a predicted band at about 420 bp consistent with ITP sequence; a band at about 540 bp consistent with ITP-L sequence; and an unexpected band at ~580 bp not predicted by existing sequence. These data are the first evidence for an ITP-L transcript in the brain. The size difference between the B2 unpredicted band and the ITP-L band is ~50 bp and the size difference between the B2 unpredicted band and the ITP band is ~170 bp. The same approximate size differences are seen between the C2 unpredicted band and its corresponding ITP-L and ITP bands. This observation suggests that the unpredicted fragment at ~420 bp in the brain C2 lane represents the same transcript as the unpredicted fragment at ~590 bp in the brain B2 lane (Figure 4a). The transcript is shown in figure 7a as unpredicted form 1.

As predicted, the A'2 primer set shows a fragment at ~430 bp in the brain RNA lane. Unexpectedly, the A'2 primers show a fragment at ~570 bp in both the brain and ileum RNA lanes. This fragment may correspond to another new transcript, shown in figure 7a as unpredicted form 2. This product could represent an ITP-L fragment that has the A' sequence upstream of the ITP-L ORF. If this were the case, the fragment size expected would be 553 bp.

As shown in Figure 4b, the A2 primer set was used to amplify brain, ileum and rectum RNA. The A2 primer set produced two bands with brain RNA, one at about 590 bp and one at ~710 bp. A 711 bp band is expected based on the ileum clone sequence of ITP-L. The band at ~590 bp is not expected based on the brain cDNA clone sequence and is ~120 bp smaller than the ITP-L fragment (ITP-L insert is 121 bp), suggesting that the fragment at ~590 bp may represent the ITP ORF downstream of the ITP-L 5' sequence (containing the A primer) as represented by transcript form 2 in figure 7a. The ileal and rectal A2 lanes also have the predicted band, consistent with ITP-L at ~710 bp. Note
that the rectum RNA A2 lane only has the 710 bp fragment. This result is important because when the A'2, B2 and C2 primers are used with rectum RNA, fragments consistent with ITP are produced (as shown in Figures 5a and 7b).

Figure 4 is a good positive control for all the primers with the exception of the ITP-L specific primer 1. Table 2 predicts a B1 fragment size of 394 bp. Any RNA sample that produces a B2 fragment of 537 bp corresponding to ITP-L, must also produce a B1 fragment of 394 bp. To test the specificity of primer 1 and to confirm that the B2 fragment at ~540 bp represents an ITP-L transcript, Figures 5a, b and c compare the products of the B2 and B1 primer sets in several tissues. Figure 5a shows RT-PCR products of the B2 and B1 primer sets for brain, ileum and rectum total RNA. All three tissues show B2 and B1 bands consistent with ITP-L at ~540 bp for B2 and at ~390 bp for B1. This confirms the presence of an ITP-L transcript in the brain as suggested in figure 4. The 420 bp fragment in the brain is consistent with ITP. An unexpected result is the presence of a band at ~420 bp in the rectum B2 lane, suggesting that ITP transcripts are not limited to the brain and CC. This is inconsistent with the A2 primer pair results in figure 4a, which show no ITP transcript in the rectum lane, but an ITP sized fragment is found in rectum when the A'2 primer set is used (data not shown). In Figure 5b the same primer sets (B2 and B1) are used to amplify corpora cardiaca total RNA. The B2 lane shows two fragments, one at ~420 bp consistent with an ITP transcript and one at ~540 bp consistent with an ITP-L transcript. The B1 lane shows a band at ~390 bp, which is expected for ITP-L. Figure 5c shows PCR products of the same primer set used to amplify ventral ganglia total RNA. The results are consistent with only ITP-L, a B2 band at ~540 bp and a B1 band at ~390 bp. Ganglia do show the ITP-L transcript, but do not show the ITP transcript.

Based on the original ITP and ITP-L transcript clones, the A' primer sequence is ITP specific, while the 1 primer sequence is ITP-L specific as shown in figures 2 and 3a. Thus, I would expect no product if the A'1 primer set were used in an RT-PCR amplification. In figure 4a the A'2 primer set produces an unpredicted band estimated at ~570 bp. A 553 bp fragment is expected if the ITP-L ORF were downstream of the A'
Figure 5. Agarose gels of RT-PCR products a) shows *S. gregaria* rectum, ileum and brain total RNA b) compares corpora cardiaca total RNA and c) compares ganglia total RNA for the B1 and B2 primer sets. Arrow heads indicate estimated fragment sizes in base pairs.
sequence. To test if the A' sequence is associated with the ITP-L specific sequence, I used the A'1 primer set to PCR amplify brain and ileum cDNA in figure 6. If the A' sequence is upstream of an ITP-L like transcript then I expect a fragment of 410 bp with the A'1 primer set. Figure 6 shows RT-PCR products of the A'2 and A'1 primer sets for brain and ileal total RNA. The brain and ileum again both show a fragment at ~570 bp for A'2 primer set and the brain A'2 lane also has a band at ~432 bp consistent with ITP, as previously reported in figure 4a. The A'1 lanes both have a band at ~410 bp consistent with an ITP-L like transcript. The fragment estimated at ~570 bp is within the margin of error and could represent the ITP-L transcript. A 553 bp fragment is expected if the A' sequence lies upstream of the ITP-L ORF, suggesting that the A' sequence may be upstream of the ITP-L ORF in some transcripts.

In figure 7a, six transcript forms suggested by RT-PCR products are shown in cartoon format. Transcript forms 1' and 3' represent the transcripts originally cloned from brain and ileum cDNA respectively. Of the six possible transcripts the first four are predicted by sequence and the last two are not. Transcript forms are referred to as ITP if they contain the ITP ORF sequence (black bar) and ITP-L if they contain the checkered bar. The crosshatched bar of transcript form 5 represents a sequence that is ~50 bp larger than the ITP-L ORF and ~170 bp larger than the ITP ORF, but appears to maintain a fragment comparable to the B-C region of ITP and ITP-L (shown as a solid black bar). Transcript form 6 (gray bar) possibly represents the unpredicted band at ~570 bp generated with the A'2 primer set. The A' sequence of forms 1',4 and 6 was originally believed to be ITP specific, but has been shown here to be associated with the ITP-L ORF also. The A primer (forms 2 and 3) is also found associated with both the ITP and ITP-L ORF's.

Figure 7b summarizes the RT-PCR results for *S. gregaria* tissues (brain, ileum, rectum, corpora cardiaca, ganglia, Malpighian tubules and flight muscle). This figure summarizes data for tissues that are not otherwise represented in the results section. Of the tissues assayed, ITP transcript form 2 is found only in the brain, while form 1 is only found in the brain, corpora cardiaca and rectum. Transcript form 3 (ITP-L) is found ubiquitously in all tissues tested.
Figure 6. Agarose gels of RT-PCR products of *S. gregaria* ileum and brain total RNA amplified using the A'1 and A'2 primer sets. Arrow heads indicate estimated fragment sizes in base pairs. The faster migrating brain A’2 fragment is estimated at about 430bp and the A’1 brain and ileum fragments are estimated at about 410bp.
Figure 7. a) Summary of predicted ITP and ITP-L like RNA transcript forms in cartoon format (drawn to scale). Solid black bars represent ITP ORF sequence and the checkered bars represent the 121 bp ITP-L insert sequence. The diagonally stripped bars represent 5' sequences, with the thick diagonal lined bars representing the sequence associated with the A' primer sequence and the thin diagonal lined bars representing the 5' sequence associated with the A primer sequence. The hatched bar represents unknown transcript sequence, predicted by the C2 fragment at about 420 bp and the B2 fragment at about 580 bp. Transcript form 5 is referred to as Unpredicted 1. The solid gray bar of transcript form 6 is another unknown transcript not predicted by existing sequence, and represents the A'2 fragment at about 570 bp. This transcript form is referred to as Unpredicted 2. The small empty boxes labeled A, A', B, C, 1 and 2 represent primers, with the sense primers above the sequence bars and the anti-sense primers below. If a primer is shown on a transcript form cartoon this indicates that it has been used to amplify a product of a size predicted by known sequence. Transcript forms 1' and 3' represent the original cDNA clones from the brain and ileum respectively. Transcript forms 2, 4, 5 and 6 have not been previously shown. 1' and 2 are ITP transcripts, 3' and 4 are ITP-L transcripts and 5 and 6 are completely unpredicted and their presence is speculative.
Transcripts

Figure 7a.
**Figure 7b.** Summary of the tissue distribution of the transcript forms shown in a. Transcript cartoons marked with a bullet (•) represent originally cloned forms. Those transcripts marked with an asterisk (*) represent forms cloned and sequenced by me. Tissues are indicated along the top. Continued on the following page.
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Figure 7b. cont.
Transcript form 4 represents an area bordered by the primers A' and 1. When these primers were used only on brain and ileum RNA, both tissues show this form to be present. The fragment estimated at ~570 bp (form 6) may actually represent the 553 bp fragment expected if the A' sequence were upstream to the ITP-L ORF and therefore represent the same transcript as transcript form 4. Forms 4 and 6 are both present in the brain and ileum. Form 5 (crosshatched bar) is the most interesting of the unpredicted forms, because it shows fragments consistent with internal priming as represented by the C2 (~420 bp) and B2 (~580 bp) fragments (figure 4a). Transcript form 5 is only found in brain tissue.

**Sequences of *Schistocerca gregaria* RT-PCR Clones**

Despite being able to generate RT-PCR products of predicted sizes, I was not completely confident that those products represented ITP and ITP-L transcripts. To confirm the reliability of the RT-PCR assay, I cloned and sequenced several of the RT-PCR products. The A2 products (~710 bp and ~590 bp) of RT-PCR from total brain RNA and B2 product (~540 bp) from ileum total RNA were cloned and sequenced (as shown in figure 7b). The sequence of the A2 711 bp brain clone is identical to the original ileal clone sequence and is the first evidence that ITP-L transcripts are found in the brain. The 590 bp A2 brain clone sequence was identical to the original brain ITP sequence except for two changes. The 590 bp brain clone does not contain the same 5' sequence as the original clone. The 5' sequence is however exactly identical to the 5' sequence of the original ileal clone (no A' sequence). The other change noted was the absence of a start codon; both clones sequenced had an ACG instead of an ATG. Other than the missing start codon the sequence was identical to the ITP ORF. This result may only represent a Taq polymerase error prior to cloning or may actually be ACG and represent a transcript with a larger ORF. Direct sequencing of the PCR product will be needed to decide between these two options. The B2 ITP-L product (540 bp) from ileum total RNA was also cloned and sequenced. The sequence is consistent with ITP-L sequence previously reported by Meredith et al. (1996). Neither of the unpredicted putative transcript forms have been cloned and sequenced to date.
Temporal Distribution of ITP and ITP-L RNA in *S. gregaria* Egg Stages

Bioassay results (Jeff Chen unpublished undergraduate thesis, 1994) reveal the appearance of an ileal stimulant at day 12 of embryogenesis. Could this be due to the appearance of ITP at this time? To determine when the ITP and ITP-L transcripts can be detected during embryogenesis, staged *S. gregaria* egg RNA was extracted and reverse transcribed, then PCR amplified with the same sequence primers used for tissue RT-PCR (table 1). Based on physiological activity, I would expect that an ITP-like transcript is present by the day twelve egg stage. The A’2 primer set should show an ITP fragment of 432 bp, while the B2 primer set should give a 416 bp ITP fragment. I can not make any predictions about ITP-L transcripts, because this peptide has not yet been assigned a physiological role in adult locusts, let alone during embryogenesis.

Total RNA extracts from various developmental stages were used to determine when the A’ (5’ sequence) ITP RNA is first present during embryogenesis. As shown in figure 8a, egg stage RNA was amplified using RT-PCR and the A’2 primer set. A fragment consistent with ITP band at ~430 bp is present as early as the day 9 egg stage and is absent from the day 12 abdomen and thorax RNA. The appearance of the ITP transcript was expected on or before day twelve. An unpredicted band at ~570 bp is present continuously from day 9 to day 14 and is not restricted to the head or abdomen and thorax.

To determine the temporal distribution of the ITP and ITP-L transcripts during development, staged RNA was PCR-amplified with the B2 primer set. Figure 8b shows a result similar to figure 8a (A’2 primer set), with a fragment consistent with ITP of ~420 bp seen in 12 day head RNA and 14 day whole egg RNA lanes. When the A’2 primer set was used in figure 8a, the ITP like transcript was observed as early as day 9, but was also specific to the day 12 heads. The fragment consistent with ITP-L (~540 bp) is present in utero and in every other stage tested in figure 8b. The unpredicted band at ~580 bp is present as early as day 9 and is also restricted to the heads of twelve day embryos.
Figure 8. Agarose gels of RT-PCR products from total RNA extracts of various staged S. gregaria eggs. a) A'2 primer set is used in amplification and in b) the B2 primer set is used. Note the day 12 eggs are divided into heads and abdomen/thorax RNA extracts. Arrow heads indicate estimated fragment sizes in base pairs.
Figure 9 summarizes the RT-PCR products that are produced from total RNA extracts of *S. gregaria* eggs. This figure also represents data not shown elsewhere in the thesis. The ITP-L product (3') is present at all stages tested, while the product which corresponds to A'(5' sequence) ITP (1') is found as early as day 9 embryos, is restricted to the head of the embryo in day 12 RNA and is found in day 14 embryos. The second ITP (2) transcript with the A 5' sequence, does not appear before day 12 and is also restricted to the heads of the day twelve eggs. The appearance of an ITP transcript at about day 9 roughly corresponds with physiological activity of embryo stages given that ITP synthesis might be delayed after its mRNA appears (Jeff Chen unpublished undergraduate thesis, 1994). Jeff Chen found that physiological activity in the thorax and abdomen of day twelve eggs, but this may be attributed to other active factors, like the 37 000 Da stimulant in the ventral ganglia (Bilgen, 1994). The two unpredicted transcript forms are present as early as the day 9 egg RNA. Unpredicted form 1 is restricted to the head of day 12 eggs similar to the ITP transcripts, while unpredicted form 2 is not.
Figure 9. Summary of developmental distribution of ITP and ITP-L like transcripts. The left hand column shows transcript forms previously described in figure 7a. Numbering system and shading is the same as in 7a. Egg stages are indicated along the top of the figure. Arrows show stages of development when the transcripts are present.
Western Blots of Tissue Homogenates

To determine peptide distribution, Western blots of tissue homogenates and synthetic ITP were done and probed with antibodies (Ab's) to ITP/ITP-L (N-1), ITP only (C-1) and ITP-L only (C-2) as shown in Figure 3b. Before examining the distribution of ITP and ITP-L peptide in tissue homogenates, I demonstrated the specificity of the antibodies used. To demonstrate Ab specificity, I compare Western blots of synthetic ITP and Bacculovirus/Sf9 expressed ITP and ITP-L probed with corresponding pre-immune blots. Figure 10 shows control Western blots of synthetic ITP and baculovirus expression products (ITP and ITP-L) detected with the N-terminal antibody (N-1) in Figure 10a, the C-terminal ITP specific antibody (C-1) in Figure 10b, the C-terminal ITP-L specific antibody (C-2) in Figure 10c. The N-1, C-1 and C-2 blots are compared to the pre-immune sera from the rabbits used to produce each of the antibodies. Antibody N-1 is made to N-terminal sequence shared by both ITP and ITP-L. To demonstrate the specificity of the N-1 Ab, in figure 10a N-1 Western blots of synthetic ITP and Sf9 expressed ITP and ITP-L show bands with relative mobilities of ~7.6, ~9.8 and ~9.8-10.0 kD respectively. The corresponding pre-immune blot shows no bands in the 12.0-7.0 kD range for all the tissue homogenates tested. There are low relative mobility bands in all the lanes at about 37 kD. The ileum homogenate lanes for *S. gregaria* and *L. migratoria* and the *L. migratoria* rectum lane all show greater mobility bands in the 13-37kD range. These slow relative mobility pre-immune bands will be referred to later in figure 11 when tissue homogenates are probed with the N-1 antibody.

To show the specificity of the C-1 Ab, figure 10b compares the controls for the ITP specific Ab C-1. In the C-1 blot synthetic ITP lanes show 7.6 kD bands and the Sf9 expressed ITP (2µl) and ITP-L (4µl) lanes show bands of 9.8 and 19.0 kD relative mobilities respectively. The C-1 Ab does recognize ITP and does not recognize ITP-L, but probably reacts non-specifically with a 19.0 kD peptide. The corresponding C-1 pre-immune blot shows no bands for synthetic ITP, expressed ITP (2µl) and ITP-L (4µl) and *S. gregaria* and *L. migratoria* 2 CC homogenates. In figure 10c the specificity of the ITP-L specific Ab C-2 is demonstrated. The C-2 Western blot shows only one band in
Figure 10. Shown are control Tris Tricine PAGE Western blots for the 3 antibodies used, compared to their pre-immune serum. a) Antibody N-1 [1/2000] was used to probe a blot with 10 and 20 pmoles of synthetic ITP, Sf9 expressed ITP (2μl) and ITP-L (4μl). Note the pre-immune N-1 [1/500] blot shows several low relative mobility bands in all tissue homogenates and more bands in the 10.0-37.0 kD range for both ileum and the L. migratoria rectum homogenate lanes. b) Antibody C-1 [1/2000] is ITP specific and Western blots show lanes of 30, 20 and 10 pmoles synthetic ITP, Sf9 expressed ITP (2μl) and ITP-L (4μl). The corresponding C-1 [1/500] pre-immune blot has lanes with 2 CC from S. gregaria, 20 pmoles synthetic ITP, 2 CC from L. migratoria and Sf9 expressed ITP (2μl) and ITP-L (4μl). c) Antibody C-2 [1/2000] is the ITP-L specific Ab. Lanes were loaded with 20 pmoles of synthetic ITP and Sf9 expressed ITP (2μl) and ITP-L (4μl). The C-2 [1/500] pre-immune blot was loaded exactly the same as the C-2 Ab blot. Arrow heads indicate estimated band sizes in kD.
Figure 10.
the ITP-L (4μl) lane at a relative mobility of ~9.4 kD and no bands in the expressed ITP (2μl) and synthetic ITP (20 pmoles) lanes. The corresponding C-2 pre-immune blot also shows no bands. I can conclude from figure 10, that the N-1 polyclonal Ab does specifically recognize synthetic ITP and the Sf9 expressed forms of ITP and ITP-L. The pre-immune serum does not recognize any bands in the 7.0-12.0 kD range in a variety of S. gregaria and L. migratoria tissue homogenates. The C-1 ITP specific Ab does appear to be specific for the synthetic and expressed forms of ITP, while not recognizing expressed ITP-L. The C-1 pre-immune serum does not recognize any bands. Lastly, figure 10c demonstrates the specificity of the C-2 Ab for expressed ITP-L. C-2 does not recognize synthetic or expressed ITP and the pre-immune blot is completely clear.

The N-1 Ab is raised to a region that is common to both ITP and ITP-L sequences as shown in figure 3b. To determine the distribution of ITP and ITP-L peptides in locust tissues, I use the N-1 [1/10 000] Ab in Western blots of brain, rectum, ileum and corpora cardiaca tissue homogenates from S. gregaria and L. migratoria is presented in figure 11a. The tissue results are compared to synthetic ITP as a positive control. The synthetic ITP has a relative mobility similar to that of S. gregaria CC and L. migratoria CC. In Figure 11a, the synthetic peptide has a relative mobility of ~7.6 kD. The S. gregaria 2 corpora cardiaca lane has a similar band at ~7.6 kD, while the L. migratoria 2 corpora cardiaca lane shows a slightly lower mobility (~8.0 kD). In the L. migratoria brain, L. migratoria rectum and S. gregaria ileum lanes there are low relative mobility bands at about 27-28 kD. In the L. migratoria rectum, S. gregaria rectum and S. gregaria ileum lanes, there are also bands at ~13.5-13.7 kD. Bands at about 13 kD and in the 27-28 kD range are seen in the pre-immune blot in figure 10a, suggesting that the lower mobility bands found in figure 11 in locust hindgut and brain tissues do not represent ITP-like peptides. As predicted by the RT-PCR results, the CC of both locusts show ITP-like band. The RT-PCR results also show ITP transcripts in the brain and rectum (figure 5a and 7), but in both of those tissues ITP peptide is not detected. The negative result in the brain is likely because the concentration of ITP in the brain is low compared to the CC. The negative result in the rectum could be because the transcript is not always translated or as in the brain, it may be at a low concentration.
Figure 11. Tris Tricine PAGE Western blots using the N-1 Ab to probe two separate blots. Lanes are marked across the top of the blot and arrow heads indicate estimated band sizes in kD. Note the difference in relative mobilities of bands between the two blots. a) Shows synthetic ITP at about 7.6kD while in b) the same bands are at about 8.0-8.2 kD.
As a demonstration of the variability in relative mobilities and of the Ab sensitivity between two identical gels run under the same conditions, a second N-1 Western blot is presented in figure 11b. This Western blot shows 4 corpora cardiaca (gland equivalents) from both species, 2 CC (gland equivalents) for S. gregaria only and 10 and 20 pmoles of synthetic ITP. The results are almost identical to figure 11a, with two exceptions. The relative mobility of the synthetic peptide and S. gregaria CC bands are estimated at ~8.0-8.2 kD. The second exception is in the L. migratoria 4 CC lane, which again has a band with a relative mobility slightly lower than the synthetic ITP band, but this time also shows two more bands at relative mobilities of ~13 kD and ~14 kD, suggesting variation in Ab sensitivities. The comparison of these two blots demonstrates a variability of about 0.5 kD in the relative mobilities of the ITP bands between gels. Other possibilities include differences in extraction, or in biological variability between samples.

To determine which tissues express ITP, the C-1 [1/10 000] Ab was used to probe Western blots of various tissue homogenates from S. gregaria and L. migratoria. As shown in figure 12a, the ITP C-terminal specific Ab (C-1), made to amino acids 115-122 of ITP (see figure 3b), was used in a Western blot of L. migratoria 2 CC gland equivalents, synthetic ITP at 10, 20 and 30 pmoles and 2, 3, 4 and 5 CC gland equivalents from S. gregaria. The synthetic ITP gave a band as expected, at the relative mobility of ~7.6 kD. The L. migratoria lane has a band at the relative mobility of ~8.0 kD. The surprising result is that none of the S. gregaria CC lanes show any bands of any size.

Because tissue homogenates often give variable results and to be sure that the corpora cardiaca lanes contained ITP, I washed and reprobed the same membrane used in 12a with N-1 [1/10 000] antibody. As shown in figure 12b, the synthetic ITP and L. migratoria CC show bands of approximately the same sizes as in figure 12a. Most interestingly, the S. gregaria CC lanes all show a band at a relative mobility of ~7.6 kD. It can be concluded from figure 12, that the C-1 Ab does not recognize the ITP peptide found in S. gregaria CC, but does recognize ITP from L. migratoria CC and synthetic ITP. This specificity may indicate that the synthetic peptide and the ITP from L. migratoria CC
Figure 12. Tris Tricine PAGE Western blots using the C-1 Ab in a) and the same blot was washed and reprobed with Ab N-1 in b). The lanes are labelled across the top of the blot, with four doses (5-2) of CC from *S. gregaria*, three concentrations of synthetic peptide (30, 20 and 10 pmoles) and 2CC from *L. migratoria* were run. Note there are no bands for *S. gregaria* CC lanes in a) but there are bands in b). Also note that the *L. migratoria* CC lane shows a band in both blots. Arrow heads indicate estimated band sizes in kD.
are not processed the same way as the ITP peptide found in *S. gregaria* CC. This theory is supported by the fact that the C-1 Ab was raised to a recognize an 8 amino acids (see figure 3b).

Antibody C-2 is made to the last 15 C-terminal amino acids of ITP-L (see figure 3b). The C-2 region of ITP-L in *S. gregaria* is identical to the same region in *L. migratoria*. To determine which tissues express ITP-L, the C-2 [1/10 000] Ab was used to probe Western blots of various tissue homogenates from *S. gregaria* and *L. migratoria*. As shown in figure 13, the only band produced was at a relative mobility of ~9.4 kD in the baculovirus expressed ITP-L lane. No other bands were seen. Although the C-2 Ab recognizes the expressed form of ITP-L, no ITP-like peptide is detected in any of the tissue homogenates. This may be because the ITP-L peptide, if present may be in relatively low concentrations. The other possibility is that the ITP-L RNA is never translated or not translated all of the time.
Figure 13. Tris Tricine PAGE Western blot using the C-2 Ab. The lanes are labelled across the top of the blot, comparing synthetic ITP and expressed ITP and ITP-L to *L. migratoria* and *S. gregaria* tissue homogenates. Arrow head indicates estimated band size in kD.
Locusta migratoria Brain RNA Clones and Sequence

Western analysis strongly suggests that *L. migratoria* CC contain an ITP-like peptide with both the N-1 and C-1 sequence. To examine the possibility further total brain RNA was isolated and subjected to RT-PCR using *S. gregaria* sequence specific primers A2 and C3 (see figure 3a). The resulting bands were cloned and sequenced. Multiple independently obtained clones were sequenced in both directions. The amino acid and nucleic acid sequences are compared with *S. gregaria* sequences for ITP and ITP-L, as reported below.

To date the only peptides with sequence similarities to *S. gregaria* ITP and ITP-L were from a family of crustacean hyperglycaemic hormones (CHH) as reported by Meredith et al. (1996). I report here 2 more members of this family in insects. The comparison of amino acid and nucleic acid sequences from two related species can provide insight into conserved functional regions. Non-conserved regions may represent non-functional (spacer sequence) or functional regions that have become species specific and could explain activity differences between peptides. In this case bioassay results indicate *L. migratoria* and *S. gregaria* tissues exhibit similar activity (Macins et al. in preparation). To examine sequence similarities between *L. migratoria* and *S. gregaria* ITP and ITP-L sequence alignments are shown in figures 14 and 15.

In figure 14a, the putative active region of *L. migratoria* (starting SSFDI....) is identical to ITP amino acid sequence in *S. gregaria*. In figure 14b, the putative *L. migratoria* active peptide (starting SSFDI....) of ITP-L is identical to ITP-L amino acid sequence in *S. gregaria*, except for an E to D substitution at position 118. The overall amino acid sequence identity for ITP is 91.5%, between *L. migratoria* and *S. gregaria*. There are 2 amino acids omitted and 9 amino acid changes within the ITP ORF of *L. migratoria*. There is a similar result for ITP-L, which has 90.3% amino acid sequence identity with *S. gregaria* ITP-L, with 2 amino acids omitted and 11 amino acids changed. An interesting aspect of these homologues is that the amino acid changes are all for amino acids with similar physical properties and with one exception (noted above position 118 of ITP-L).
Figure 14. a) Comparison of *S. gregaria* (above) and *L. migratoria* (below) amino acid sequences for the ITP open reading frame (aa 1-130). Differences in *L. migratoria* sequence are indicated in **bold**. Start of the active peptide is indicated by a vertical line between amino acids 55 and 56.  

b) Comparison of *S. gregaria* (above) and *L. migratoria* (below) amino acid sequences for the ITP-L open reading frame (aa 1-134). Differences in *L. migratoria* sequence are indicated in **bold**. A putative dibasic cleavage site is indicated by a vertical line between amino acids 55 and 56.
all changes occur 5' to the dibasic cleavage site between positions 55 and 56 and within the predicted signal peptide sequence. Table 3 compares amino acid substitutions between *S. gregaria* and *L. migratoria*. All substitutions are for amino acids with similar characteristics. Table 3 also indicates the Dayhoff numbers for accepted amino acid substitutions (Dayhoff, 1978).

Figures 15a and 15b show the nucleic acid sequence comparisons of *L. migratoria* and *S. gregaria* ITP and ITP-L sequence. When *L. migratoria* and *S. gregaria* nucleic acid sequences are compared, there is a 91.9% identity for ITP and a 91.9% (93.2% to the ITP stop) identity for ITP-L. There are 26 nucleic acid changes and 6 deletions when comparing *L. migratoria* ITP sequence with *S. gregaria* ITP sequence. A similar result is found when comparing *L. migratoria* ITP-L sequence with *S. gregaria* ITP-L sequence, where there are 27 nucleic acid changes and 6 deletions. Figure 15c compares ITP and ITP-L nucleic acid sequences from *L. migratoria*. There is only a single bp change between the two sequences other than the 121 bp insert in ITP-L, suggesting that the two transcripts are not splice variants of the same genomic sequence and are transcribed from separate genomic sequences.

**Clones from Acheta domestica, Periplaneta americana and Trichoplusia ni.**

To determine if other orthopterans or other insects have ITP-like transcripts, *S. gregaria* primers were used to amplify reverse transcribed RNA from various insect species. Total brain/head RNA from *A. domestica, P. americana* and *T. ni* was used in RT-PCR reactions. Crude CC extracts from *A. domestica* and *P. americana* show biological activity on the hindgut assay (Meredith et al., 1996), making them good candidates for having ITP. Low stringency conditions (low annealing temperatures and high magnesium levels) in PCR reactions were necessary to generate bands. However it is also likely that I amplified non-specifically. All resulting bands that were cloned and sequenced did not resemble ITP or ITP-L. Further investigation of the ORFs resulted in low identity matches with a wide range of protein domains.
<table>
<thead>
<tr>
<th>position of aa</th>
<th>S. gregaria amino acid</th>
<th>Common Characteristics</th>
<th>L. migratoria amino acid</th>
<th>Dayhoff (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>9</td>
<td>Glutamine(Q)</td>
<td>polar</td>
<td>(H)Histidine (+)</td>
<td>1.6 2.6</td>
</tr>
<tr>
<td>17</td>
<td>Proline(P)</td>
<td>aliphatic(sc) / small</td>
<td>(S)Serine (polar)</td>
<td>2.3 7.7</td>
</tr>
<tr>
<td>19</td>
<td>(+) Arginine(R)</td>
<td>polar</td>
<td>(Q)Glutamine</td>
<td>11 8.1</td>
</tr>
<tr>
<td>21 and 30</td>
<td>(aliphatic) Leucine(L)</td>
<td>hydrophobic / sc nvr</td>
<td>(F)Phenylalanine (aromatic)</td>
<td>12 2.4</td>
</tr>
<tr>
<td>34 and 39</td>
<td>(small/aliphatic) Valine(V)</td>
<td>hydrophobic</td>
<td>(L)Leucine</td>
<td>15 2.1</td>
</tr>
<tr>
<td>41</td>
<td>(hydrophobic) Threonine(T)</td>
<td>polar / small</td>
<td>(S)Serine</td>
<td>29 2.0</td>
</tr>
<tr>
<td>49</td>
<td>(non-hydrophobic) Proline(P)</td>
<td>small</td>
<td>(A)Alanine (hydrophobic)</td>
<td>29 9.5</td>
</tr>
<tr>
<td>ITP-L only</td>
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<td>polar</td>
<td>(R)Arginine (+)</td>
<td>8.1 1.1</td>
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<tr>
<td>ITP-L only</td>
<td>(small) Aspartic Acid(D)</td>
<td>charged / polar</td>
<td>(E)Glutamic Acid</td>
<td>4.0 3.9</td>
</tr>
</tbody>
</table>

Table 3. Comparison of S. gregaria amino acids with corresponding L. migratoria substitutions. The position of the amino acid substitutions are indicated in the left hand column (position 1 is the start codon as shown in figures 14a and b). The bracketed characteristics are specific to the adjacent amino acid relative to the substitute. Amino acid substitutions are found in both ITP and ITP-L except when indicated. Asterisk (*) indicates the amino acid falls within the putative active peptide. The Dayhoff numbers represent the frequency for an amino acid to change to another amino acid given as a percentage of the total changes found for that amino acid as calculated by Dayhoff (1978), with the arrow heads indicating the direction of substitution (i.e. at position 9 > indicates the change from Q to H and < indicates from H to Q). The abbreviations sc and nvr represent side chain and not very reactive respectively.
ATG CAC CAC CAG AAG CAG CAG CAG CAG CAG AAG CAG CAG GGA GAG GCT CCG TGC
ATG CAC CAC CAG AAG CAG CAC CAG AAG CAG CAG GGA GAG GCT TCA TGC

CGA CAT CTC CAG TGG CGG TTA TCA GGG GTC GTC TGC GTC TGC GTC GTA GCT
CGA CAT TTT CAG TGG CGG TTA TCA GGG GTC TTC TGC GTC CTC CTC GTA GCT

AGC CTC GTT TCC AGG GCG GCT TCC AGC CCG TTG GAT CCA CAC CAC CTT GCC AAA
AGC CTC CTG TCC TCA GCA GCA TCC AGC CCG CTG GAT GCA CAC CAC TTA GCC AAA

AGG TTC TTC TTC GAC ATC CAG TGT AAA GGA GTT TAC GAC AAG AGC ATC TTT GCA
AGG TTC TTC TTC GAC ATC CAG TGT AAA GGA GTC TAC GAC AAG AGC ATC TTC GCA

CGC CTA GAC CGC ATC TGC GAA GAT TGC TAC AAC CTA TTC CGC GAA CCT CAG CTC
CGC CTC GAC CGC ATC TGT GAA GAT TGC TAC AAC CTG TTC CGC GAA CCT CAG CTC

CAC TCT CTG TGC AGA TCT GAC TGT TCC AAG AGC CCA TAC TCC AAA GGT TGT CTT
CAC TCT CTA TGC AGA TCT GAC TGT TCC AAG AGC CCA TAC TCC AAA GGT TGT CTT

CTG GGG AAG AAG TAG* S. gregaria
CTG GGG AAG AAG TAG* L. migratoria

Figure 15a. Comparison of S. gregaria (above) and L. migratoria (below) ITP nucleic acid sequences. Differences in the L. migratoria sequence are in bold type. The asterisks (*) indicate the stop codons and the blank spaces indicate deletions. The solid vertical line indicates the beginning of the active peptide.
Figure 15b. Comparison of *S. gregaria*(above) and *L. migratoria*(below) ITP-L nucleic acid sequences. Differences in the *L. migratoria* sequence are in bold type. The asterisks (*) indicate the stop codons and the blank spaces indicate deletions. The solid vertical line indicates the beginning of the active peptide.
ATG CAC CAG AAG CAG CAC CAG AAG CAC CAC CAG GGA GAG GCT TCA TGC CAG CAT TTC CAG TGG CGG TTA TCA GGG GTC TTC TGC GTC CTC CTC GTA GCT CAC CAT TTC CAG TGG TCA GGG GTC TTC TGC GTC CTC CTC GTA GCT AGC CTT CTT TCC TCA GGG GCA TCC AGC CCG CTC GAT GCA CAC CAC TTA GCC AAA AGC CTT CTT TCC TCA GGG GCA TCC AGC CCG CTC GAT GCA CAC CAC TTA GCC AAA AGG TCC TTC TCC GAC ATC CAG TGT AAA GGA GTC TAC GAC AAG AGC ATC TTC GCA AGG TCC TTC TTC GAC ATC CAG TGT AAA GGA GTC TAC GAC AAG AGC ATC TTC GCA CGC CTC GAC CGC ATC TGT GAA GAT TGC TAC AAG CTG TTC CGC GAA CCT CAG CTC CGC CTC GAC CGC ATC TGT GAA GAT TGC TAC AAG CTG TTC CGC GAA CCT CAG CTC CAC TCT CTA TGC AG CAC TCT CTA TGC AGG AAA GAC TGT TTC ACA TCT GAC TAC TTC AAA GGA TGC ATC GAT GTT CTA CTT CTT CAA GAT GAC ATG GAA AAG ATA CAG TCT TGG ATA AAA CAA A TCT GAC TGT TTC AAG AGC CCA TAC A TCT GAC TGT TTC AAG AGC CCA TAC TTC AAA GGT TGT CTT CAG GCA CTA CTT CTG ATA GAT GAA GAA GAA AAA TTT AAC TTC AAA GGT TGT CTT CAG GCA CTA CTT CTG ATA GAT GAA GAA GAA AAA TTT AAC CAG ATG GTG GAA ATA CTG GGG AAG AAG TAG ITP CAG ATG GTG GAA ATA CTG GGG AAG AAG TAG ITP-L

**Figure 15c.** Comparison of *L. migratoria* ITP(above) and ITP-L(below) sequences with the sequence differences in **bold type.** A single base pair change in codon 8 (**A-G**) and the 121bp insert in ITP-L sequence are indicated with **bold type.** There are two codon spaces that indicate *S. gregaria* codons not found in *L. migratoria.*
DISCUSSION

The purpose of this study was to examine the distribution of ITP within *S. gregaria* tissues and in other insect species. Although some of my findings are consistent with our current model others are not and require further investigation. What follows is a discussion of these results and some possible explanations for all of the results.

**Tissue distribution of *S. gregaria* ITP and ITP-L transcripts**

The RT-PCR of *S. gregaria* tissues and egg stages directly addresses the question concerning RNA distribution. ITP RNA is present in the brain, corpora cardiaca and the rectum as shown by figure 7b. The brain shows the ITP ORF sequence associated with both 5' sequences (A and A'). The CC and rectal RNA show products consistent with the ITP ORF, but only with the A' 5' sequence. The presence of ITP RNA in the brain and CC is completely consistent with the model of neuropeptide production in the neurosecretory cells of the brain. The appearance of ITP in the rectum is completely unexpected. This ITP may be associated with nervous tissue that innervates the rectum and may be acting as a neuromodulator locally. This could be confirmed by whole mount staining with an antibody that recognizes native ITP, to see if staining is localized to nervous tissue or whether it is associated with the rectal epithelia.

ITP-L RNA is found in all tissues tested with the A2 primer set, the A2 products and the internal primer products (B2 and C2) are all consistent with ITP-L as indicated by figure 7b. The tissues tested include brain, ilea, recta, CC, ganglia, Malpighian tubules and flight muscle. When the A'1 primer set is used to amplify brain and ilea RNA, products consistent with ITP-L are also generated (RNA from other tissues not tested). This is an unexpected result for two reasons; the A' 5' sequence has not previously been associated with ITP-L like products and when A' is used with antisense primers further 3' of primer 1 (i.e. primer 2) a product consistent with ITP-L is not found (553 bp fragment expected). This suggests that the sequence that lies 3' to primer 1 (of the product shown in form 4 of figure 7) is different than the sequence of this same region in the A2 ITP-L band (form 3 figure 7). This may be due to an intron between primer 1 and 2 that is
sufficiently large to disrupt amplification with the A'2 primer set or this product represents a novel RNA species derived from an ITP-L like gene. Another possibility is that form 6 (Unpredicted 2), which is estimated to be at about 570 bp, is actually a 553 bp fragment and represents the expected product if the A' primer is upstream of the ITP-L ORF. Possible roles of ITP-L will be discussed later.

The unpredicted bands (1 and 2) were only found in the brain of adult animals and as early as day 9 in development. These bands have not been cloned or otherwise characterized. To determine if they are artifacts or genuine RNA transcripts with ITP/ITP-L like sequences, they should be cloned and sequenced. Otherwise they could be subjected to high stringency southerns or band purified to re-PCR the isolated band products as suggested by Zacharias et al. (1994). Unexpected band 1 shown in form 5 of figure 7a is particularly interesting because the region bordered by the primers B and C seems to be conserved. Until these bands are further characterized not much more can be said about them. However, I would not find it surprising that other ITP/ITP-L like molecules exist and function in the regulation of the hindgut. This is supported by Nässel (1996) who suggests that it may be possible to increase the complexity of a signaling system without increasing the number of anatomically defined synaptic contacts, but by simply increasing the number and complexity of signaling molecules. It is further supported by Audsley et al. (1992a), who observed a second peak of stimulatory activity (30% total) more hydrophobic than ITP during high pressure liquid chromatography (HPLC) purification.

**Temporal distribution of *S. gregaria* ITP and ITP-L transcripts**

As shown in figure 10, ITP-L RNA is present throughout development and is not limited to either the head or thorax and abdomen in 12 day eggs. ITP RNA appears in two forms, the ORF with the A 5' sequence and the ORF with the A' 5' sequence. The A' 5' sequence form appears as early as day 9 and is head specific at day 12. The A 5' sequence form only appears at day 12 and is also head specific. The appearance of ITP RNA in egg stages is completely consistent with physiological data. Homogenates of staged eggs show activity at day 12 (Chen, 1994). Chen did not however, find that physiological
activity was restricted to the head of day twelve eggs, but this may be attributed to other active factors found in the abdomen and thorax. One candidate for activity is the ventral ganglia factor (VGF) isolated by Bilgen (1994) that has an estimated molecular weight of 37 000 Da.

If the two ITP 5' sequences represent pre and post splice forms, that implies that the A' 5' sequence is replaced with the A 5' sequence by the splicing event. If the two forms represent alternate splice variants of a yet unknown precursor nothing more can be concluded by this result. It is possible that the 5' sequence is responsible for localization of RNA or peptide and/or post-transcriptional/post-translational modifications of ITP which could affect its activity.

It would be interesting to investigate the significance of the appearance of the ITP transcript and the biological activity in the bioassay found at day 12. This is a time of active synapse formation in this species (C. S. Goodman, personal communication). The temporal distribution of the two unpredicted forms is also interesting. Unpredicted band 1 as shown in figure 10 has the same profile as A' ITP (transcript form 1). Unpredicted 1 appears at about day 9 and is head specific in the day 12 egg. Unpredicted 2 also appears at day 9, but is not head specific in the day 12 egg.

*S. gregaria* ITP and ITP-L clones

In figure 7b the RNA forms marked with an asterisk (*) indicate bands that I cloned and sequenced. The cloning and sequencing of ITP from brain and ITP-L from brain and ileum of *S. gregaria* gives sequence that agrees with earlier published results (Meredith et al., 1996) with one exception, the lack of an ATG in the ITP clones. This could represent a Taq error or an RT error. Alternatively the ITP start may be further 5' and the ITP propeptide may be larger than previously thought. Based on a single clone I find it difficult to draw any conclusions from this result. Sequences for ITP and ITP-L from brain and ileum confirm that ileal and brain bands actually represent RNA that codes for ITP and ITP-L and increases my confidence that RT-PCR products accurately represent ITP and ITP-L RNA in other tissue types and in the staged tissues of *S. gregaria*. 
It would be interesting to clone and sequence the unpredicted RNA bands. Alternatively the genomic sequence for ITP/ITP-L would provide clues to the authenticity of these bands.

**Western Blots of* S. gregaria* and* L. migratoria**

The Western blots of *S. gregaria* and *L. migratoria* tissues directly addresses the question concerning protein distribution of ITP. As expected, in figures 11a and 11b I see bands in both *S. gregaria* and *L. migratoria* CC lanes that are of a relative mobility very similar to synthetic ITP. The *L. migratoria* band consistently migrates slower than the *S. gregaria* CC band and the synthetic ITP band. This may be due to post translational modifications that occur in the migratory locust and not in the desert locust. All other tissues (brain, ilea and recta) show no ITP like bands. I did expect to see ITP like bands in the brain homogenate lanes, but because there are relatively few neurosecretory cells in the overall population of brain cells, it is not surprising that bands were not seen. The overall concentration of ITP is expected to be much less in brain than in the CC. Other bands are present in rectal and ileal lanes. The relative mobilities of these bands were very similar to bands seen in the pre-immune blots (Figure 10a). The size and the corresponding pre-immune bands suggests that the rectal and ileal bands (Figure 11a and 11b) do not represent ITP/ITP-L like peptides. In figure 11b, the *L. migratoria* CC lane has two bands of about 13 and 14 Kd relative mobilities that do not have corresponding bands in the pre-immune blots. These bands may represent ITP-like peptides, however there is no other evidence for such a claim (i.e. RT-PCR bands).

Possibly the most interesting result in the Western blots is found in figure 12, where in 12a the ITP specific antibody does not recognize any bands in the *S. gregaria* CC lanes. On the same blot bands of the appropriate relative mobilities are found in the synthetic ITP lane, the Sf9 expressed ITP lane and the *L. migratoria* CC lane. When the same blot was reprobed with the N-terminal (N-1) anti-body the *S. gregaria* lanes had bands of the expected mobilities as shown in figure 12b. This result strongly suggests that the primary ITP peptide in the *S. gregaria* CC is different than the ITP in *L. migratoria* CC and from
the synthetic ITP. The region that the ITP specific (C-1) antibody is made to, is only 8 amino acids in length (115-122) as shown in figure 3b. Considering that the lower limit for antibody detection is 6 amino acid residues (Harlow and Lane 1988), this 8 aa peptide may only present one epitope. There are two possibilities raised by this result. The first is there are post-translational modifications of the C-1 epitope that impair antibody binding. The second is that there are sequence changes in this 8 residue region that are sufficient to alter the epitope presented.

Figure 13 shows a blot probed with ITP-L specific antibodies. The only band present at about 9.4 kD is in the positive control lane (Sf9 expressed ITP-L). It is no surprise that the tissue homogenate lanes show no bands. Even if ITP-L is translated, it would probably be in low concentrations and thus not detected.

**Ab sensitivity**

A single CC shows between 10-20 pmoles of ITP, when compared to known amounts of synthetic ITP in Western blots. This result is consistent with physiological studies that show 0.5 CC able to generate a maximal response on hindgut bioassays. To get a similar bioassay response 10 pmoles of synthetic ITP are needed (Meredith personal communication). The detection limit of ITP with antibodies is about 5 pmoles. The CC is the most concentrated source of ITP, so I would expect that other tissues which are far less concentrated sources of ITP (i.e. brain) would be negative in Western blot assays. Concentrated homogenates of tissue do not show appropriate sized bands for these peptides in Western blots. In order to detect ITP or ITP-L it may be necessary to partially purify and concentrate the peptide from the tissue homogenate before electrophoresis.

**S. gregaria and L. migratoria ITP and ITP-L Sequence Comparisons**

When comparing ITP nucleic acid sequences of *S. gregaria* and *L. migratoria*, 91.9% of the sequence is identical. Also 91.9% is identical when *S. gregaria* and *L. migratoria* ITP-L nucleic acid sequences are compared. The same high level of conservation is found at the amino acid level. ITP sequence is 91.5% identical and ITP-L sequence is 90.3%
identical between *S. gregaria* and *L. migratoria*. All of the amino acid changes occur in
the first 150 bp of the ITP ORF, making all nucleic acid changes in the active peptide (8)
silent ones. Similarly, ITP-L has all changes in amino acid sequence before position 55
(i.e. in signal peptide sequence) except for a single conservative amino acid change (D to
E) at position 118. The active peptide of ITP is identical for both *S. gregaria* and *L.
migratoria*, which suggests ITP is a functional peptide. This same level of conservation
in ITP-L suggests an active role for it as well. The identity of the ITP sequences in these
two species agrees with the results of reciprocal bioassays of their CC on ileal *I*SC
(Macins et al., in preparation).

**ITP and ITP-L Amino Acid Substitutions and Peptide Comparisons**
The amino acid substitutions in *L. migratoria* all share characteristics in common with
their *S. gregaria* counterparts and have relatively high Dayhoff numbers for accepted
amino acid substitutions (as shown in Table 3). All but one of the substitutions are
within the propeptide region (amino acids 1-55) and yet the predicted signal peptide
cleavage site (between 44 and 45) is conserved in both species. It is suggested by the
clustering of changes in this region that the active peptide is more sensitive to any amino
acid changes than the propeptide region, no matter how conserved the changes are. Most
of the changes within the putative signal peptide region (residues 20-44), are conservative
and maintain the necessary charged, hydrophobic and polar regions as outlined by
Creighton (1993). The substitutions within the hydrophobic region (25-39) at residues
30, 34 and 39 share the common characteristic of hydrophobicity. The substitution at
position 41 (T-S) exchanges one polar residue for another and thus conserves the polar
region of the signal peptide (40-44). In summary five of the nine amino acid substitutions
in ITP are considered non-conserved by a BLAST alignment, but of these the L-F change
at residue 30 conserves the hydrophobic region of the signal peptide despite their other
differences.

Because no changes occur in the active peptide region’s amino acid sequence in ITP, the
differences in recognition by the C-1 Ab between *S. gregaria* CC and *L. migratoria* CC
may be due to species specific post-translational modifications in *S. gregaria* that do not
allow C-1 Ab binding. The C-1 peptide region contains several candidates for post-translational modification; the aspartic acid (D) at position 122 could undergo phosphorylation or hydroxylation, the lysine (K) at position 126 could also be phosphorylated and the asparagine (N) at position 128 could be hydroxylated (Creighton, 1993).

**ITP and ITP-L Peptides from other Insect Species**

Attempts at finding ITP or ITP-like peptides in other insects has a two fold purpose. The first is to determine regions of conserved sequence which can provide valuable clues about the functional regions of ITP. The second purpose is to eventually take the knowledge of the structure and function of ITP and its role in the osmoregulatory processes of pest insects and develop species specific methods of biological control. The cloning and sequencing of ITP and ITP-L in the related locust, *Locusta migratoria*, is a first step in identifying ITP homologues in Lepidopterans and other commercially important pests. The use of sequence specific and degenerate primers based on regions of similarity with crustacean hormones (CHH, MIH etc.) to amplify RNA isolated from the brains or heads of *Acheta domesticus, Periplaneta americana* and *T. ni* generated products of approximately the expected size, but when cloned and sequenced these products bore no resemblance to ITP or ITP-L. The stringency of the hybridizations was low to facilitate binding of the primers to supposedly non-identical sequences. This can account for the non-ITP sequences found. Due to the relative evolutionary closeness of *S. gregaria* and *L. migratoria*, it is not surprising that they share a common antidiuretic factor. The same is not likely to be true for more distantly related species (phylogenetically), whose CC's do not stimulate the locust ileal ISc assay.

Meredith et al., (1996) puts ITP and ITP-L into a larger family of crustacean peptides that includes various hyperglycemic hormones (CHH), molt-inhibiting hormones (MIH), vitilogenesis inhibiting hormone (VIH) and a gonad inhibiting hormone (GIH). Neither CHH nor MIH stimulate the locust ileal bioassay and ITP does not react on a CHH enzyme linked immunosorbent assay (ELISA) (E. S. Chang, personal communication). According to Kukalová-Peck (1991) the fossil record indicates that Crustacea were
present 600 million years ago (mya) and Orthoptera are found in the fossil record up to 300 mya. The differences in sequence between the crustaceans and insects have had at least 600 mya to develop and take on new functions.

Role for ITP
My results support the role of ITP as a neuropeptide that is produced in the brain and stored in the CC. ITP RNA found in the CC may be due to contamination from the neurosecretory cells of the brain, may represent RNA being transported to the CC for translation or may indicate a local source of ITP RNA in the CC. Based on the differences in recognition by the ITP specific anti-body, suggests that the ITP peptide in S. gregaria CC is different from the ITP in L. migratoria CC and the synthetic CC. As mentioned above this is likely due to postranslational modifications and not sequence differences, although it is possible that the ITP sequence we have does not represent the only ITP or ITP-like transcript.

Possible role for ITP-L
Based on the sequence similarity between ITP and ITP-L, where all of the changes in sequence are at the C-terminal end, and the sequence similarity between S. gregaria and L. migratoria ITP-L’s, it seems reasonable to assume that ITP-L does have some function within locusts. Ring et al. (1997) show an antagonistic effect between ITP and ITP-L in the hindgut bioassay, while ITP-L alone has no effect. This suggests that ITP-L may play a role in regulation of fluid and ion reabsorption in locusts. Even though the ITP and ITP-L C-terminal ends are 40% identical and 57% conserved (over 35 residues) and completely identical at the N-terminus, they show quite different activities, suggesting an important role for the C-terminal tail. It is possible that the N-terminal is able to bind the receptor, but the ITP C-terminal is needed for activation of second messengers. Thus the observed antagonistic action of ITP-L on the ITP stimulation could be explained by N-terminal binding to the ITP receptor without C-terminal activation. ITP may be released into the hemolymph to increase fluid and ion reabsorption, while ITP-L may be released locally to act as an inhibitor to fine tune the response. However, before assigning any role to ITP-L it should be shown that the peptide exists in vivo.
Conclusions
In this thesis I have demonstrated ITP transcripts in the brain, corpora cardiaca and unexpectedly in the rectum. The appearance of ITP during embryogenesis is as early as day 9, and localized to the heads of day 12 eggs. I have also shown an ITP-L distribution in all tissues tested and the temporal distribution of ITP-L is equally ubiquitous, because transcripts are demonstrated within in utero eggs through to day 14. ITP peptide is shown in CC homogenates and possible post translational differences are indicated in the C-1 peptide region, between ITP from S. gregaria and ITP from synthetic origin and ITP from L. migratoria CC. I have also demonstrated alternate 5' sequences for both ITP and ITP-L and shown that there are possibly at least two more ITP-like transcripts found in the brain. Finally, I report the sequence of L. migratoria ITP and ITP-L cloned from brain total RNA and show high (>90%) identity relative to S. gregaria.
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