Differential Gene Expression in the American Lobster, *Homarus americanus*

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Abstract of Dissertation

An overarching goal in the study of neurobiology is to translate the capacity to perform an action to its underlying cellular and molecular mechanisms. As a result, nervous systems involved in central pattern generation have become well studied for the control of behavior. Amongst the most important model organisms for this field are the decapod crustaceans *Cancer borealis* (Jonah crab) and *Homarus americanus* (American lobster). Studies of these systems have defined our knowledge of the general principles of the organization and modulation of neural circuits, as well as garnered considerable clarity on crustacean physiology and behavior, including perception of sensory stimuli. However, this field has been stalled in its ability to use the full range of molecular technologies available. Both *Homarus americanus* and *Cancer borealis* currently lack a published genome or transcriptome, which limits the genetic resources available for use of approaches like qPCR, RNAi or CRISPR genome editing, and high-throughput sequencing (DNA-Seq, RNA-Seq, CHIP-Seq). My dissertation work established a *de novo* assembled transcriptome for *Homarus americanus* and defined differential gene expression across central nervous system and muscular tissues using RNA-Seq. This work also confirms the peptide proctolin as a circulating neuromodulator, concluding two decades of speculation and identifying its role in transcriptional regulation in nervous system tissues. The results of this work uncover links between proctolin and innate immunity, as well as thermoreception. We also identify possible candidate genes for thermoreception in homologs of thermosensory TRP A channels, and explore the behavioral component of thermoreception by establishing behavioral thermoregulation in *C. borealis*. Finally, transcriptome-wide profiles of the rhythmic pattern generating ganglia in *Homarus americanus* reveal candidates for neuronal genes that drive the production of motor output in these systems. Together, these studies offer important behavioral and genetic information into the field of crustacean neurobiology and lay the groundwork for an array of future studies ranging from characterization of thermoreception in nervous systems to the use of synthetic biology to construct neural networks.
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Glossary of Terms

**Anatomy**
AG – abdominal ganglia
CG – cardiac ganglion
CNS – central nervous system
COG – commissural ganglia
CPG – central pattern generator
PO – pericardial organ
SG – supraesophageal ganglion
STNS - stomatogastric nervous system
STG – stomatogastric ganglion

**Neuromodulators**
5H,T - serotonin
AST - allostatin
CCK – cholecystokinin
DA – dopamine
NO – nitric oxide
RPCH - red pigment concentrating hormone
Chapter 1: Introduction

Sections
A. Background
B. The model organisms Cancer borealis and Homarus americanus
C. Central pattern generators and anatomy of the lobster
D. Central pattern generators and the role of neuromodulators
E. The neuromodulator Proctolin
F. Central pattern generators and temperature
G. Transcriptomics using the RNA-Seq approach
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I. Experimental aims

Background

Even the simplest behaviors are governed by neuronal networks[1, 2]. Neuronal networks underlying behavior are highly adaptable and in some cases influenced by many integrated factors [3-6]. Neurons, the building blocks of neural circuits, can take on a number of identities [7, 8]. These physiological types are determined by the relative proportion of different ion channels present, and can often be altered by changes in metabotropic inputs [8-15]. Thus, the synthesis of proteins that comprise ion channels, metabotropic inputs (such as neuromodulators), and their receptors is integral to the neuronal conductance changes that govern a network and its motor output. Moreover, our understanding of the role of gene expression in response to synaptic and modulatory inputs is quite limited [5, 8, 12-17]. The overarching goal of this dissertation work is to examine the expression of protein-coding genes involved in distinguishing neural networks, and evaluate whether and how neuromodulators influence the expression of these genes.

In this research, I use transcriptome-wide mRNA sequencing to characterize gene expression patterns of CNS tissue containing central pattern generators, the neural networks that control rhythmic motor patterns, in the model Homarus americanus (the American lobster). I also examine the effect of a particular neuromodulator, proctolin, on gene expression in these neural networks. The majority of this research utilizes Illumina RNA sequencing (RNA-Seq) technology to determine molecular identities of these networks; the history and method of this approach is outlined in this section.
Additionally, as a behavioral complement to gene expression work in central pattern generation, I use temperature preference assays to establish behavioral thermoregulation in *Cancer borealis*, a model organism for central pattern generation.

*The model organisms* Cancer borealis and Homarus americanus

The Jonah crab (*Cancer borealis*) and the American lobster (*Homarus americanus*) are decapod crustaceans that inhabit the east coast of North America. The Jonah crab is known to inhabit waters along the Atlantic coast from Newfoundland to Florida, ranging in depth and temperature from 0 to 750 m and 2 to 24 °C [18, 19]. The American lobster is found from Newfoundland to North Carolina, and generally inhabits waters between 5 and 20 °C though it can tolerate temperatures between -1 to 35 °C [20]. Both organisms are economically valuable in the fishing industries of Canada and the northeast United States [21, 22]. For this purpose, physiological information on these species is of interest; however, these species are also well-studied models for central pattern generation for reasons including their reasonably low cost and easy maintenance, and the relative simplicity (tens or hundreds, rather than thousands of neurons) and the accessibility of their nervous system compared to vertebrate counterparts [23-25].

*Central pattern generators and anatomy of the lobster*

Central pattern generators are neural networks that produce repetitive actions such as the beating of the heart or gait patterns in walking or running [26, 27]. They are defined by their ability to produce rhythmic motor patterns even in the absence of sensory feedback. The continuous, measurable physical output of these networks allows for straightforward comparisons between actual behavior and network activity, such as the rhythmic chewing and grinding of the stomach controlled by the stomatogastric nervous system [28]. Moreover, these networks perform complex motor tasks in a coordinated, rhythmic
fashion while still adapting the locomotion pattern to many factors, such as goals of the individual or environmental perturbation [29, 30].

The central nervous system (CNS) of decapod crustaceans, like *H. americanus* and *C. borealis*, is decentralized, meaning all information processing and movement coordination is not localized to a brain and spinal cord—it can be coordinated in a combination of several distributed neuronal epicenters or “ganglia”. For example, information from primary sensory afferents, such as antennal chemoreceptors involved in olfaction, is sent to and processed in the olfactory lobes of the “brain” (the supraesophageal ganglia and commissural ganglia) before being passed along to downstream processes, such as the CPGs of the thoracic and abdominal ganglia (TG) [31]. Information can also bypass the brain, as in the case of rhythmic escape swimming. In this behavior, a stimuli (i.e. touch) activates lateral giant fibers in the tail and evokes a rapid subsequent swimming behavior coordinated by the subesophageal and abdominal ganglia (AG) [32, 33]. Such examples of sensory information are not necessary for the production of rhythmic motor patterns; rhythmic motor patterns can occur in isolated CPGs (the output is termed “fictive motor programs”). However, in other preparations (i.e. *in vivo*) the CPG can integrate sensory information to initiate, modify, or terminate movements [34].

In *H. americanus*, several central pattern generators control the locomotion of an organ, body segment, appendage, or sets of appendages (Figure 1). The abdominal ganglia (AG) and cardiac ganglia (CG) are the main focus of this dissertation research, though an accumulation of knowledge about CPGs also stems from the thoracic ganglia (TG) and stomatogastric nervous system (STNS). These systems will thus be described briefly as well.

The ventral nerve cord extends from the brain along the ventral side of the body and includes the commissural ganglia that wrap around the esophagus, the subesophageal ganglia, and five TG. TG control several behaviors including eating and grooming [20]. They also coordinate the motoneurons in rhythmic walking behaviors [35, 36]. The
stomatogastric nervous system (STNS) is located on the dorsal side of the foregut in the ophthalmic artery and controls digestive activities of the foregut [23]. The rhythmic movements of the gastric mill and pylorus are generated by two central pattern generators, resident in the stomatogastric ganglion (STG), which is composed of ~30 identifiable cells. These central pattern generators control the rapid pyloric rhythm (filtering) [37] and slower gastric mill rhythm (grinding) [38]. The circuit dynamics of this central pattern generator are extensively described [23, 39], and the fictive motor patterns generated by an isolated stomatogastric nervous system closely resemble those recorded in vivo [28, 40-44].

The neurogenic cardiac system is located on the dorsal side of the main body cavity of the lobster and controls the rhythmic pumping of the heart and distributes hemolymph (or ‘blood’) along the circulatory system [45-47]. This system includes the continually active cardiac ganglion (CG), motor neuron connections, heart, and a neurosecretory pericardial organ that releases neuromodulators both onto the cardiac ganglion and into the hemolymph for distribution to other networks [27, 48]. The abdominal network plays a role in posture, locomotion, ventilation, and rhythmic escape swimming [49-58]. It is part of the ventral nerve cord that extends from the thoracic ganglia along the ventral side of the tail. This network is composed of six abdominal ganglia, one for each tail segment, that control the swimmeret muscles that control the fin-like swimmerets located on the ventral side of abdomen used in positioning and large flexor–extensor muscles used for rhythmic escape swimming [49-51]. The sixth and final ganglia contains sensory and motor neurons to the telson, uropods, and anal musculature [59, 60].

Central pattern generators and the role of neuromodulators

Recent work has suggested neuromodulators as a dynamic mechanism for the fine-tuning of nervous activity through regulation of membrane channel gene expression, producing long-term changes in excitability [61-64]. A primary interest of my research was to establish how circulating hormones might affect nervous system activity over the long term through control of the number and type of membrane channels. By manipulating the
hemolymph levels of the neuromodulator proctolin and performing RNA-Seq on tissues containing CPG nervous systems, I address whether and how this modulator exerts long-term modulation over nervous systems through changes in gene expression.

First, it is important to understand how the numerous substances present in nervous systems affect the activity of these circuits, namely neurotransmitters, neuromodulators and neurohormones. Neurotransmitters are released from the presynaptic terminals of neurons and diffuse across the synaptic cleft to affect a post-synaptic neuron (or potentially two neurons), muscle cell, or other target cell [65]. In the stomatogastric system, the two most common neurotransmitters are glutamate and acetylcholine [66]. Neuromodulators are typically released from an extrinsic neuron and have the ability to modify more than one neuron or target cell with the appropriate receptor [67]. Neuromodulators typically act via G protein pathways to activate second messenger cascades that phosphorylate particular channels (among other mechanisms), thus modifying the ionic conductance or synaptic releases of target neurons [68]. The time course of alterations to the target neurons and their synapses depends on the continued presence of the neuromodulator and the dephosphorylation rates of affected channels [3, 39, 66, 69-76]. Dozens of neuromodulators have been identified in the stomatogastric system, including proctolin, dopamine, octopamine, GABA, serotonin, cholecystokinin (CCK), red pigment concentrating hormone (RPCH), etc. [6, 24, 66, 69] Some neuromodulators can also influence changes to the activity of a nervous system as circulating neurohormones in the bloodstream, released from neurosecretory organs [42, 76, 77].

In decapod crustaceans, dozens of neurohormones are directly released into the hemolymph from the pericardial organ (PO), a neurosecretory structure located in the pericardial cavity [48, 78]. Other release sources can include the sinus glands, eyestalks, thoracic ganglia, and command neurons [39, 79]. Identified targets for the released neurohormones include the cardiac ganglion, and the commissural ganglia and stomatogastric ganglion (STG) of the stomatogastric nervous system (STNS) [80, 81].
The neuromodulator proctolin was selected for this study for a number of factors. Acute effects of proctolin on nervous system activity are established and detailed below. Moreover, this neuromodulator has been isolated in the pericardial organ via mass spectroscopy and immunocytological techniques, indicating its role is not purely at a synaptic level, but system-wide as well [82-86].

Previous investigation on the effects of neuromodulators in crustacean CPGs primarily concentrated on bath applications of neuromodulators to isolated nervous systems or, in a few instances, semi-intact preparations [4, 6, 41, 43, 44, 66, 70, 71, 74, 77, 86-95]. Some behavioral changes (aggression, molting, etc.) due to systemic changes to neurohormone levels have also been characterized [96-100]. Moreover, Turrigiano and Selverston have established connections between hemolymph peptide content and stomatogastric activity in live lobsters [42]. This work has shown that levels of cholecystokinin (CCK)-like peptides in the hemolymph will initiate feeding behavior in vivo [42]. CCK-like immunoreactivity has been established in the STG and also in the neurosecretory pericardial organ and thoracic segmental nerves [95, 101].

Another study examining the interplay between hemolymph content and CPG activity postulated a mechanism for dictating increases in pyloric and gastric activity: partial pressure of oxygen (PO$_2$) levels in the hemolymph [92]. Rezer and Moulins also speculated on the STG sensing cues from the hemolymph via its position in the ophthalmic artery as a result of their in vivo studies [44]. In this work, the primary input nerve (the ‘stn’) was severed in vivo but STG activity remained, whereas severing the stn in vitro diminishes all STG activity. Researchers isolated the hemolymph to be responsible for this maintenance of STG rhythmic activity regardless of neuromodulatory inputs from the main input nerve [44]. These studies support that hemolymph content (either gaseous or hormonal) dictates the behavior of the STNS both in vivo and in vitro, and that in vivo activity closely resembles that seen in vitro. As a whole, these studies provide historical context and add justification to this proposed strategy of altering hemolymph hormone content while assaying expression changes the nervous system.
In the case of this dissertation work, the response investigated is transcription of mRNA. Thus, identifying a time course reasonable for ascertaining changes in mRNA transcription is an important background of the methodology. Recent studies have determined ion channel turnover rates, measured in half lives, in the range of 12-72 hrs for voltage-gated Na\(^+\), K\(^+\) and Ca\(^{2+}\) channels and AMPA receptors [61, 102-104]. One study suggested some K\(^+\) channel proteins can degrade or be removed from the membrane quickly (some within 30 minutes, reportedly), but require 8+ hour timeframe for increases in transcription [105]. In summary, establishing an exact minimum exposure length for assessing transcription differences is problematic since turnover and transcription rates are specific to channel and cell type. However, these studies support a basic timeframe of days, rather than minutes, for the modulation of neuronal excitability through gene expression and thus I use the outer range of this temporal framework (24-72 hrs) as the basis for a 3-day manipulation of hormone levels through injection of exogenous neurotransmitters.

In rodent models, long term exposure of hormones induces changes to cell membrane channel expression, specifically the expression of voltage-gated, shal-family potassium channels involved in A-type potassium ion channels and the cardiac transient outward potassium current (I\(_{to1}\)) [15, 106]. The regulatory effects of neurohormones on gene expression in the nervous systems of *H. americanus* are, at this time, only postulated.

Recent work has provided evidence that hormones, such as the red pigment concentrating hormone and dopamine, stabilize the pyloric rhythm and motor network output [16, 77]. Dopamine operates both via wired transmission (classical fast synaptic transmission) and volume transmission (hormonal release and modulator diffusion) in the stomatogastric nervous system, and has different phasic and tonic effects [10, 16, 17]. Expression of DA receptors seems to be specific and localized in the STG. LP neurons exclusively express type 1 DA receptors (D1R), and PD neurons express type 2 DA receptors (D2R) [8, 10, 16, 17]. Rodgers et al established that tonic nanomolar concentrations of DA cause a persistent increase and decrease in I\(_A\) \(G_{\text{max}}\) in LP and PD neurons, respectively. Through translation-inhibition experiments, they have shown evidence for a translation-dependent
mechanism driving these changes to $I_A$ (transient potassium current). These studies failed to show what changes may occur due to expression of D1R and D2R transcripts, likely due to the short duration of their DA exposure (1hr). Studies indicate a wide range of time frames for $K^+$ channel turnover, but measurable increases to transcription within an 8+ hour window [14, 104, 105, 107]. These changes in transcription are necessary to detect expression differences amongst samples using RT-PCR, microarray, or RNA-Seq.

*The neuromodulator Proctolin*

Proctolin is an invertebrate neuropeptide. It was first discovered in the hindgut of the American cockroach *Periplaneta americana*, by Starratt and Brown in 1975, and was the first invertebrate neuropeptide to be sequenced. After dissection of 125 kg of cockroaches, enough proctolin was collected to characterize this compound [108, 109].

Proctolin is a pentapeptide with the amino acid sequence H-Arg-Tyr-Leu-Pro-Thr-OH. It was originally of interest for its myotropic responses, or the ability to initiate contraction of the hindgut muscles in the cockroach at a threshold concentrations of $10^{-9}$ M [108, 109]. Since this discovery, the pharmacological action of proctolin has been investigated across numerous species of insects and crustaceans. Overall, proctolin produces an excitatory effect on target tissues, though responses are widespread across species and systems. In insects, proctolin increases the frequency and amplitude of heart beating, stimulates contraction of the fore- and hindgut, and increases the frequency and amplitude of contractions of the oviduct (for review, see Orchard 1989). In crustaceans, proctolin modulates neuromuscular connections and potentiation by grading the muscle tension resulting from a neural impulse [110, 111]. It can also activate or enhance the contraction of the heart [112, 113], enhance the contraction and work output of ventilatory muscle [114], and induce activity in the swimmeret system at a threshold concentration of $10^{-8}$ M and also increase burst duration of power-stroke motor neurons [115, 116]. In the stomatogastric nervous system, proctolin increases the frequency of
gastric mill and pyloric rhythms, and will generate gastric mill rhythms in quiescent systems [40, 41, 73, 117, 118].

As a result of the sequencing of the *D. melanogaster* genome, the receptor for proctolin (CG6986) was identified and functionally validated in 2003 [119]. Proctolin binds to a G-protein coupled receptor and activates a second messenger system involving IP$_3$ and/or cAMP. In this system, it is believed that the ligand (proctolin) activates a trimeric G protein that then activates phospholipase C (PLC), which cleaves PIP$_2$ to DAG and inositol 1,4,5-trisphosphate (IP$_3$). IP$_3$ binds to calcium channels on the endoplasmic reticulum and releases Ca$^{++}$ into the cytosol initiating cellular responses, such as increasing the amplitude of muscle contraction [120, 121]. Proctolin can act through adenylate cyclase to elevate levels of cAMP, which in turn increases calcium entry into the cytosol and initiates cellular responses [122].

Starratt and Steele, members of the team who first discovered proctolin, identified and characterized proctolin-degrading peptidases in 1984 [123-125]. These peptidases have a high affinity for proctolin and can act in quick time courses; injected exogenous proctolin reportedly has a half life of just four minutes [124, 125]. One such proctolin-degrading peptidase, dipeptidyl aminopeptidase III (GH01916), was purified, characterized and sequenced by Claire Mazzocco in 2003, again with the help of the *D. melanogaster* genome [126, 127]. The *D. melanogaster* gene (CG7105, Proct) encoding a precursor protein to proctolin was published the next year [128].

Distribution and localization of proctolin in nervous system tissues has been extensively studied. Immunoreactivity and mass spectrometry have identified proctolin and/or proctolin receptors in the stomatogastric, abdominal, thoracic and supraesophogaeal ganglia, as well as the neurosecretory pericardial organ [85, 88, 117, 129, 130]. It is due to the localization of proctolin to the neurosecretory pericardial organ that proctolin is suspected to act as a circulating hormone [129, 131, 132]. However, to date, proctolin has not been identified in the hemolymph of any crustacean.
Identifying proctolin levels in the bloodstream is technically challenging given the quick action of the endogenous peptidases, and the low concentrations of proctolin. To date, hemal proctolin levels have only been quantified in the American cockroach *Periplaneta americana* and the Madeira cockroach *Leucophaea maderae* [131, 133]. In these organisms, proctolin was observed at 100-200 pM (1-2 x 10^{-10} M) and 9 nM (9 x 10^{-9} M), respectively. It is important to note the value for the Madeira cockroach is based on reported values of 0.29 pmol/mg of protein in the hemolymph and an estimated 20 mg/ml hemal protein concentration [134]. Also, hemal proctolin levels were higher in females (~200 pM) compared to males (~100 pM) for the American cockroach[131].

At present, our knowledge of proctolin is lacking in the area of its function as a circulating neuromodulator, or neurohormone. Our working knowledge of proctolin stands to benefit from an accurate quantification of hemal proctolin levels and insight into the causal patterns for fluctuation. Additionally, areas such as hormonal control of transcriptional regulation have yet to be explored.

*Central pattern generators and temperature*

The role of temperature on central pattern generators has been of particular interest for a number of reasons. As poikilotherms, crustaceans are unable to internally regulate their body temperature. The individual must either use physiological mechanisms to compensate for resultant changes, or behaviorally thermoregulate to improved temperature conditions [135, 136]. These challenges are vital given the widespread effects of temperature on body operation and the impact of temperature changes on potential survivorship [137].

Previous research has characterized the effect of temperature on physiological mechanisms including heart rate, respiration, internal pH, walking speed, righting ability, and nervous system activity in crustaceans [138-151]. These studies included particularly interesting evidence for the role of acclimation in withstanding temperature extremes.
In the case of nervous system activity in the STNS, previous thermal history dictates the animal’s ability to withstand temperature conditions. In other words, warm acclimated (19°C) Jonah crabs are able to maintain normal nervous system function at higher temperature than cold acclimated (7°C) crabs [152]. Moreover, across these thermal ranges, the phase relationship between the three neurons activity in the pacemaker core of the pyloric rhythm (PD, LP, PY) remains remarkably constant [142]. The frequency of bursting increases with increased temperature, but the timing between involved cells is invariable [142]. These results indicate that nervous system function is remarkably stable over large temperature ranges (>15°C), but has limitations in its ability to compensate and these limits change based on acclimation temperature. These studies provoked the question of whether the Jonah crab exhibits decision-making behaviors in regards to temperature. Thus, one goal of my dissertation research attempts to elucidate this juxtaposition between flexibility and confines of nervous system temperature compensation by adding a behavioral component. This research addresses whether this species behaviorally thermoregulates and, if so, what is the “preferred temperature”?

Transcriptomics using the RNA-Seq approach

The majority of the research outlined in this dissertation utilizes RNA sequencing to answer questions on differential gene expression. In order to grasp the capabilities and limitations of this technology, it is important to understand the development of this assay.

The transcriptome is defined as the complete set of transcripts (RNA molecules) and their quantity in a cell or organism at a particular time in development or in a physiological condition [153]. Transcriptomics, or the study of transcriptomes and their functions, is a burgeoning field with applications ranging from molecular evolution to understanding how expression of genes contributes to complex disease [154, 155]. At present, next generation RNA sequencing (RNA-Seq), sometimes known as deep sequencing or whole transcriptome shotgun sequencing, is a commonly adopted approach for transcriptomic studies [156].
Prior to RNA-Seq, analysis of gene expression was mainly conducted using microarray or real-time or quantitative PCR (RT-PCR or qPCR). Microarray is a hybridization-based approached involving incubating cDNA to custom or commercially available oligo microarrays [157]. This approach can be used to determine and quantify transcripts, and can distinguish between alternatively spliced isoforms [158]. Its limitations include background noise at low levels due cross-hybridization and reduced range at high levels due to saturation of signals. Furthermore, microarray has an inherent dependency on knowledge of the organism’s genome. This dependency makes studies of non-genetic models, where there is no published genome, a challenge (for review, see Wang et al 2009).

RT-PCR is the fluorescence-based quantification of RNA using reverse-transcription polymerase chain reaction. RT-PCR is a robust technology capable of high sensitivity and high throughput; it often used in validation studies of RNA-Seq results, but has its own pitfalls as well [159]. First, it relies on amplification of RNA targets, which amplifies error [160]. Primer and probes must be validated, and exponential amplification compared to housekeeping genes known to be unaffected by experimental treatment (identifying those controls can be an additional challenge)[160]. Finally, and arguably the most important limitation, is that RT-PCR requires a priori selection of target genes and knowledge of the sequence of those targets.

Sequence-based technologies provide an alternative to these limitations, namely selection of target sequences. Sequence-based technologies began with Sanger sequencing, developed by Frederick Sanger and published in 1977 [161]. Sanger sequencing is a chain-termination method of determining sequence involving the incorporation of fluorescently-labeled, strand-terminating nucleotides during PCR, the separation of strand lengths through gel electrophoresis, and the imaging of terminating labels. Sanger sequencing provided a method for uncovering the sequence of genes and, eventually, whole genomes. It wasn’t until 30 years later that the first massively parallel sequencing method was released, developed by 454 Life Sciences and George Church [162-164]. This method binds single DNA molecules to beads and amplifies them via emulsion
PCR. These beads are then immobilized and undergo automated cycles of sequencing by impermanent ligation of fluorescently labeled tags and four-color imaging.

Pyrosequencing was groundbreaking in its speed and cost—the cost per base was a fraction (~1/9th) of Sanger sequencing [162]. Today, three major ‘next generation sequencing’ platforms dominate the market: Roche 454 by Roche Applied Sciences, Illumina HiSeq/MiSeq by Illumina, and SOLiD by Applied Biosystems.

Illumina adheres DNA molecular to flow cells, uses bridge PCR to form clusters of clonal copies, and then employs reversible terminators with fluorescent tags and imaging software to determine nucleotide sequence [166]. A con to Illumina sequencing, compared to Roche 454, is that it has shorter read sequences (~35bp compared to 200+bp); a pro to Illumina, compared to both Roche 454 and SOLiD, is increased performance in stretches of sequence with repeated nucleotides [164].

The ability to translate sequencing data into useable gene expression analysis was spearheaded by Ali Mortazavi and Brian Williams in Barbara Wold’s lab in 2008 [156]. These researchers developed “RNA-Seq”, a way to map transcripts to a transcriptome to quantify the presence and prevalence of a particular gene or transcript. This approach uses the ‘RPKM’ strategy, which uses counts of reads normalized to a 1 kb section of a transcript. In this way, the researcher is able to compare the expression of one gene to another using a comparison of coverage over defined space. This approach was quickly improved upon by the creation of ‘edgeR’ by Robinson and McCarthy and ‘DESeq’ by Anders and Huber [165, 166]. Both of these packages were developed for use in R/Bioconductor. EdgeR was developed using an overdispersed Poisson model to control for biological and technical variability [166]. DESeq, and later DESeq2, detect differential expression of genes based on negative binomial distribution, using data-driven estimates for variance and mean to allow for differential gene expression analysis with limited replicates (as is typically seen in these types of studies)[165, 167]. The data-driven estimates for variance and mean are an improvement over the approach of edgeR, which estimates dispersion using a common estimate across all genes.
The results of DESeq (or DESeq2) allow the interpretation of whether gene expression changes across a particular condition, and by how much, based on log2fold change from the control condition to the experimental condition. This information can then be further examined by breakdown of the gene products into biological pathways, such as KEGG analysis, or the associated biological process, cellular component or molecular function using gene ontology (GO) analysis [168, 169].

*Molecular approaches utilized in crustacean CPGs*

Given the initial reasons for interest in crustacean central pattern generators as a model system, a large amount of our present knowledge of these systems is based on electrophysiological approaches [23]. However, other imaging, computational, immunohistochemical, and molecular approaches have also been applied, though in the case of the molecular basis of CPG activity our knowledge remains quite limited. Some successful mRNA expression work has been conducted in the stomatogastric, most notably by Baro et al and Schulz et al [11, 170-172]. These researchers have employed RT-PCR approaches to gauge the difference in mRNA expression of K+ channels in the different neurons of the stomatogastric gangion. Schulz et al used pairwise correlations among ion channel (*shal* I_A, *shab* I_Kd, *shaw* I_Kd, *BKKCa*, *IH* I_H, and *para* I_Na) expression levels in identified neurons and reported signature expression levels in each cell. This work has established that relative concentrations of membrane channel mRNA differ in the pacemaker cells of the stomatogastric nervous systems [11]. Baro et al similarly detected levels and position of *shal* and *shaker* genes in identified neurons of the stomatogastric using RT-PCR and immunocytology [170-173]. Recently, an expansion of this work investigated neuromodulators as a mechanism for determining correlated levels of channel expression and conductance levels in motorneurons of the STG. This work identified co-regulation of ion channel expression in cell-specific manner and revealed removal of neuromodulation can strength, diminish or alter these correlations [174, 175].

However, these RT-PCR experiments have been limited to pre-selected genes, and mostly have focused on K+ channel genes in the *shaker*-family. Furthermore, changes to
these mRNA levels due to perturbations to the system, such as hormonal increases, have yet to be described. These gaps in our current understanding of the system can benefit from a transcriptome-wide approach to gene expression changes via RNA-Seq. Using Illumina RNA-Seq, we will be able to detect upregulation or downregulation of all annotated genes, not just a pre-selected group based on *a priori* knowledge.

**Experimental Aims**

*Aim 1: Behavioral thermoregulation in Cancer borealis, and effects of acclimation on temperature preference*

This study addresses the thermoregulatory behavior of the Jonah crab (*Cancer borealis*), a model organism for central pattern generators and examines the hypothesis that *C. borealis* navigates to particular temperatures to optimize its physiological state. Using acclimation to temperatures within the range of those normally inhabited in summer months (11 °C, 14.5 °C, 20 °C) and then conducting behavioral assays in a thermal gradient tank, this experiment characterized the thermotactic responses of the Jonah crab.

*Aim 2: Homarus americanus Transcriptome, Differential Gene Expression in Tissue Types*

This study establishes a molecular framework for understanding central pattern generation in the model organism, *Homarus americanus* (American lobster). These experiments first establish a transcriptome for *H. americanus*, for which there is no published genome or transcriptome to date, and then characterize gene expression patterns of CNS tissue containing central pattern generators. This study tests the hypothesis that sets of genes involved in neuronal differentiation are differentially expressed across tissue types to establish the identity of those systems.
Aim 3: Identification and quantification of hemal Proctolin, and its ability to regulate gene expression in the nervous system of Homarus americanus

Though proctolin is a well-studied neuromodulator in regards to its acute effects on nervous system activity, its role as a ‘hormone’ or circulating neuromodulator is only speculated. These experiments address two hypotheses: (1) Proctolin is present in the hemolymph as a circulating neuromodulator, and (2) Hemal proctolin regulates gene expression in central pattern generating nervous systems.
References


Figure 1.1. Anatomical diagram of the American lobster, *Homarus americanus*. STG – stomatogastric ganglion. PO – pericardial organ. COG – commissural ganglia. SG – supraesophogeal ganglion, also referred to as the brain. Adapted from Marder and Bucher 2007.
Chapter 2: Behavioral thermoregulation in *Cancer borealis*,
and effects of acclimation on temperature preference

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Temperature preference and acclimation in the Jonah Crab, *Cancer borealis*, pg 7-13, ©2014,
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Abstract

Temperature indisputably affects the physiology of poikilotherms. The role of temperature and its impacts on crustacean heart rate, internal pH and nervous system activity, have been characterized. Whether a species acts to seek or avoid particular temperatures to optimize a particular physiological character is less well understood. This study addresses the thermoregulatory behavior of the Jonah crab (*Cancer borealis*), a decapod crustacean that is economically valuable as an emerging fishery and also of importance as both a key species in intertidal and subtidal communities and a model organism for rhythmic pattern generating nervous systems. Using acclimation to temperatures within the range of those normally inhabited in summer months (11°C, 14.5°C, 20°C) and then conducting behavioral assays in a thermal gradient tank, this experiment characterized the thermotactic responses of the Jonah crab. Results indicate *Cancer borealis* will behaviorally thermoregulate and adjust the direction and magnitude of its movements towards a preferred temperature range. Moreover, the preferred temperature ranges of this species are significantly impacted by previous acclimation temperature.

**Key words:** *Cancer borealis*; crustacean; behavioral thermoregulation; temperature preference; acclimation; temperature gradient

1. Introduction

As poikilotherms, crustaceans are unable to regulate their body temperature internally and must rely on physiological or behavioral adaptations to maintain body function despite fluctuations in ambient temperature. The effects of temperature changes on crustacean physiology are widespread, including modifying acid-base balance [1], heart rate and cardiac output [2, 3], neuronal conductance properties, and the effects of neuromodulators [4-7]. Temperature also has demonstrable effects on crustacean behavior, including righting ability, walking speed, and activity levels [8-12].
The Jonah crab, *Cancer borealis* (Stimpson, 1859), is a decapod crustacean able to persist in a wide range of temperatures. This species is known to inhabit waters along the Atlantic coast from Newfoundland to Florida, ranging in depth and temperature from 0-750 m and 2-24°C [13, 14]. In Northern areas, there is a seasonal migration towards shallower waters from the spring to fall, followed by an emigration to deeper waters for winter months [15]. In New England and Canada, adult Jonah crabs of larger size (>11cm carapace width) are generally observed in benthic habitats of 50-300 m depths where the temperature ranges from 6-16°C [13]. However, smaller Jonah crabs are frequently found in the shallow subtidal and intertidal areas where temperatures can exceed 26°C in summer months [16-18].

Understanding thermoregulatory behavior of *C. borealis* is of general interest as this species is not only a model organism for understanding nervous system activity, but also an economically valuable fishery and important species in benthic communities. The stomatogastric nervous system, which controls the digestive activities of *C. borealis*, has a long history as a model for studying central pattern generators, neuromodulation, synaptic plasticity, and the connectivity of neural circuits [19-22]. Information on temperature preference and acclimation abilities will add an important component to our knowledge of this animal and its nervous system activity.

This species is also of increasing economic importance as part of the fisheries industry in Canada and the northeast United States. Limited research has been conducted on the influence of temperature on this species and the driving factors behind its seasonal migration patterns, despite the fact that these behaviors substantially affect fishing efforts. Previously considered a by-catch of commercial lobster traps, the Jonah crab is steadily increasing in yearly landings and price as stocks of other crustaceans, such as the red king crab and Dungeness crab, have been depleted [15, 23, 24]. Moreover, *C. borealis* is an important diet component of benthic and avian predators such as the wolfish *Anarhichas lupus* [25] and gulls *Larus marinus* and *L. argentatus* [26]. Its presence as a predator has been attributed to modifying the distribution and behavior of
the intertidal snail *Littorina littorea* [27] and green sea urchins (*Strongylocentrotus droebachiensis*) [28]. Investigating the preferred and avoided temperatures of this species, as well as its ability to acclimate to warming ocean waters, will be informative to our understanding of benthic population dynamics as well as our efforts in sustaining this species as an active fishery.

2. Materials and Methods

2.1 General

Adult *Cancer borealis* were purchased from commercial fisherman (Commercial Lobster Co., Boston, MA, USA), where they had been held in running seawater tanks around 5.6°C until time of sale. Crabs were then held in ambient running seawater tanks at the Northeastern University Marine Science Center; local ambient ranged from 12-21°C during the period of experimentation (July-November, 2012). No less than 14 days prior to experimentation, crabs were randomly assigned one of three treatment groups (11°C, 14.5°C, and 20°C) and moved to designated tanks for temperature acclimation.

Crabs were fed a diet of live mussels (*Mytilus edulis*) weekly; individuals were not fed 24 hours prior to experimentation. Mean crab size was 430±22 g or 14±1 cm CW, and due to this size constraint all individuals were male [29]. Previous work does not indicate abundance differences between male and female Jonah crabs based on temperature strata, but did note an absence of females at temperatures below 8°C [13].

2.2 Temperature Preference Assays

We designed and constructed a temperature gradient tank similar to those used in comparable experiments on eels [30], sea lamprey [31], and lobsters [32] (Fig. 2.1). Hot and cold seawater were circulated at opposite ends of a rectangular tank to establish a thermal gradient. An experimental chamber 284 cm long and 24 cm wide was partitioned off with plastic grating to prevent ‘corner-seeking’ (positive thigmotaxis) by the crabs,
which may affect their thermoregulatory behavior. A floor-mounted bubbler line ran the length of the experimental chamber and provided even aeration to eliminate vertical thermal stratification of the water column (water depth, 10-13 cm) and to maintain water quality at near-saturated oxygen levels. Location markers were mounted on the experimental chamber to ensure consistency in position readings. Preliminary trials without a thermal gradient resulted in an even distribution of individuals along the length of the tank, suggesting no bias for any particular region.

For temperature readings, thermistor probes (Digikey, Thief River Falls, MN, USA) were mounted at equidistant locations along the experimental gradient and read into a data file at 10 sec intervals via Arduino Mega 2560 Board (Arduino, http://arduino.cc). At the experimental temperatures used, the thermistors are accurate ±0.5°C with ~2.5 sec response time. The thermistors were calibrated and repeatedly retested using both traditional and digital thermometers. Thermometers were also used to confirm consistent temperatures along the depth of the water column and across the width of the experimental chamber. The position of the crab, based on the midline/center of symmetry, in relation to thermistors was used to calculate the immediate surrounding temperature of the individuals during the trial.

The mean temperature range spanned 16°C from the hot to cold end of the chamber. For each trial, temperatures were available at ranges greater and less than the acclimation temperature (on average, 7.5-23.5°C for 11°C crabs, 10-28°C for 14.5°C crabs, and 11.5-27°C for 20°C crabs). Determination of the preferred temperature was achieved by the ‘acute’ method described by Reynolds and Casterlin [33], whereby animals were placed in the gradient and repeated measures of their location were taken over a short duration of time. In this experiment, observations occurred every 5 minutes for one hour, with the first 40 minutes constituting the ‘investigatory period’ and the mean immediate surrounding temperature of the last 20 minutes constituting the ‘preferred temperature’ [34, 35]. Crabs were introduced to the experimental chamber at randomly selected central locations along the length of the temperature gradient. The temperature at which an individual was placed in the tank is considered the ‘introductory temperature’. Due to
experimental variability in the temperature gradients, a small number of individuals (7 of 46) were added at selected central entry points to ensure a range of introductory temperatures above and below the acclimation temperature of each group. There was no significant difference in introductory temperatures between groups ($p>0.05$). Due to possible influence from conspecifics, each trial contained a single individual and water was changed between trials. To avoid handling effects, crab weight and sex were measured after the trial. One crab died during a trial and is not included in results. Data were analyzed with linear regression, ANCOVA, One-Way ANOVA and Tukey post-hoc comparisons using JMP software 5.0.1a (SAS Institute, Inc., Cary, NC, USA) and R 2.15 (R Development Core Team, 2012). All values presented are ± the standard error of the mean (s.e.m.).

2.3 Retrials

A limited number of individuals from each group were randomly selected for a re-trial (11°C n=4, 14.5°C n=4, 20°C n=3, total n=11). After a second period of acclimation to their previous acclimation temperature (re-acclimation period ranged from 4-38 days, with an average of 18±4 days), individuals were placed into the temperature gradient tank for another temperature preference assay conducted in the same manner as previously described. The aim of this experiment was to assess how reliably an individual maintains its temperature preference. Data were analyzed with One-Sample T-test and Repeated Measures ANOVA using JMP software 5.0.1a (SAS Institute, Inc., Cary, NC, USA) and R 2.15 (R Development Core Team, 2012). Beyond this subset of individuals, all crabs were used in only one trial.

3. Results

3.1 Effects of a Temperature Gradient

Two groups of 14.5°C acclimated crabs were introduced to the experimental chamber. The control individuals were introduced to the chamber without a thermal gradient
(ambient seawater temperature, ~17°C). The experimental group had a temperature gradient from 10-28°C. Both groups displayed an initial investigatory period with large changes in tank position, followed by an overall decrease in positional change and movement (Fig. 2.2). In general, control crabs exhibited larger positional changes initially, compared to experimental crabs, and then displayed little movement for the last fifty minutes. Experimental crabs initially moved smaller distances, and continued to make small adjustments to their position over time.

3.2 Movement Along the Temperature Gradient

As the experimental crabs moved around the gradient tank over the time period of their trial, their movements began narrowing the range of their immediate surrounding temperature (Fig. 2.3). After forty minutes, movements resulted in less than a 1°C change in immediate surrounding temperature, and thus a mean immediate surrounding temperature for this time period (the final 20 minutes of the trial) serves as a reliable indicator for the acute preferred temperature.

The temperatures at which the individuals were introduced into the gradient tank were not significantly different between the acclimation groups ($p>0.05$). However, an analysis of covariance on the change in immediate surrounding temperature of the individuals between their introduction to the tank and their final preferred temperatures indicates that the introductory temperature did have a significant impact on the direction and magnitude of the crab’s movement along the gradient ($R^2=0.33$, $F_{(5,40)}= 4.01$, $p<0.01$). The interaction between acclimation group and introductory temperature is not significant and indicates that this response was consistent across all acclimation groups (Table 2.1). For each of the acclimation groups, individuals placed at higher introductory temperatures moved to cooler temperatures and individuals placed at the lower introductory temperatures moved to warmer temperatures (Fig. 2.4).

3.3 Preferred Temperatures
As individuals navigated the temperature gradient, the observed movements demonstrated an exploration of the temperature gradient followed by continued dwelling at a preferred temperature. An analysis of covariance indicates that acclimation temperature had a significant effect on the preferred temperature ($R^2=0.32$, $F_{(5,40)}=3.78$, \(p<0.01\)) of the individuals (Table 2.2).

A lack of significant interaction between acclimation and introductory temperature indicates that the effect of introductory temperature was consistent amongst acclimation groups. A comparison of effect sizes reveals that acclimation temperature had a stronger effect on preferred temperature than the temperature at which the individual was introduced into the tank.

A one-way ANOVA supported that there were significant differences in preferred temperatures between the different acclimation groups ($F_{(2,43)} = 4.54$, $p<0.05$) (Fig. 2.5). Interestingly, for each group the preferred temperature was higher than the acclimation temperature. Preferred temperatures for the 11°C, 14.5°C, and 20°C crabs were 16.3±1.1°C, 17.3±1.3°C and 21.3±1.3°C, respectively. Moreover, post-hoc tests using the Tukey-Kramer method revealed a significant difference in preferred temperature between the 11°C crabs and 20°C crabs ($p<0.05$).

### 3.4 Retrials

No individual directed its movement to the same temperature in both the initial trial and retry; however, the mean preferred temperature for each acclimation group was unchanging between trials. A repeated-measured ANOVA revealed a significant difference in preferred temperature between acclimation groups $F_{(2,8)}=7.491$, $p<0.05$, but not between trials $F_{(1,8)}=0.006$, ns. It can be concluded that a general temperature preference is exhibited by individuals acclimated to different temperatures, but that a given individual does not return to a precise preferred temperature.
4. Discussion

4.1 Behavior in a Temperature Gradient

Results show that *Cancer borealis* are thermotactic, meaning they move towards or away from temperature stimuli, based on the behavioral differences between the individuals exposed to a temperature gradient and those exposed to an environment with homogenous temperature. The movements observed in the tank during the investigatory period were similar to those described as ‘casting behavior’. Casting behavior describes a zig-zagging flight of insects searching a wind tunnel for pheromone cues [37]. A similar behavior is described in blue crabs (*Callinectes sapidus*). This species remains mostly inactive when food cues are absent and, when present, demonstrates increased activity and incidence of changing angular headings to locate food odor sources [38]. These behaviors, as with that observed by the crabs upon introduction to a thermal gradient, allows the organism to survey its surrounding environment for chemosensory or thermal cues. All crabs exposed to a thermal gradient displayed some initial back-and-forth exploration along the gradient. For crabs exposed to a homogenous temperature environment, this behavior was observed in only 50% of individuals, and in all groups this movement decreased over time. It is also important to highlight this initial casting behavior spanned shorter distances in crabs exposed to a temperature gradient than those in homogenous water temperature, suggesting an aversion or negative thermotaxis from more extreme temperatures of the gradient.

It is generally believed that crustaceans display minimum motor activity while in their preferred temperature [10, 12, 39]. After the investigatory period in the temperature gradient, the reduced number of moving crabs and the short distances moved by the active crabs is indicative of the crabs reaching their preferred temperature range. The small movements continued by those crabs that remained active resulted in little change to their immediate surrounding temperature (<1°C).

4.2 Introductory Temperatures and Thermotaxis
Varying the introductory temperatures to those above and below the acclimation temperature, rather than placing in the gradient tank at its acclimation temperature, highlighted the thermotactic ability of this animal. While *C. borealis* does perform an initial exploratory ‘casting’ or ‘shuttling’ behavior, it is evident that it exhibits an overall propensity to orient away from introductory temperatures too warm or too cold. When given a range of temperatures, an individual modifies the direction and magnitude of its total movement to navigate to a preferred temperature range. This behavioral trend was consistent amongst all acclimation groups; however, the preferred temperature was significantly different based on the previous thermal history.

### 4.3 Temperature Preference and Acclimation Ability

Results show that acclimation temperature plays an important role in the preferred temperature of an individual. Crabs acclimated to 11ºC had a significantly different preferred temperature than those acclimated to 20ºC. This study demonstrates a positive linear relationship between acclimation temperature and preferred temperature in *C. borealis*. This relationship has been established in many species such as bluegill fish [33], the octopus *Octopus maya* [40], and the crayfish *Astacus astacus* [41]. However, not all crustacean species follow this trend [39]. The crayfish *Procambarus clarkii*, for instance, does not demonstrate a change in temperature preference amongst differentially acclimated individuals [42].

Common practice for obtaining a ‘final temperature preferendum’ via the acute method is to map the intersection of the median preferred temperatures and a line of equality [43, 44]. This line represents the point at which acclimation and preferred temperatures are equal. Previous research supports that the final temperature preferendum is the same whether established through this described ‘acute’ method or via the ‘gravitational method’, which allows animals to move within a temperature gradient for 2 or more days [33]. The acclimation groups in this study preferred temperatures higher than their acclimation temperature so a 45º line does not intersect directly; however, the acute
method equality line intersects the 95% confidence interval of the median (M=15.4°C) for the 14.5°C acclimation group. Thus the closest projected final temperature preferendum for the species would be 15.4°C.

Temperature preferences determined in this study are congruent with those performed on the American lobster *Homarus americanus*, a species sharing the same habitat as *C. borealis*. Reynolds and Casterlin determined the final temperature preferendum for *H. americanus* at 16°C [45]. In two recent studies, lobsters roughly acclimated to 15.5°C displayed a preferred temperature of 16.5°C and 15.7°C with a final temperature preferendum of 15.9°C [Crossin et al., 32, 46].

Our observations and results corroborate that crustaceans have keen perception of their surrounding temperature. Cardiac assays on the lobster have established the organism’s ability to sense environmental temperature changes to the resolution of 0.5°C [47]. The neural mechanism for thermoreception and initiation of thermotaxis is still largely unknown, and would greatly add to our understanding of how this species senses temperature changes in its environment and amends its physiological responses and behavior accordingly. Previous research in the semi-terrestrial fiddler crab *Uca pugilator* identified the claw as a candidate for temperature sensing and thermoregulation [48]. A study in the spiny lobster *Panulirus japonicus* ties nerves in pericardial organ, a neurohormonal secretory organ, to temperature stimulation [49]. Other studies on crustaceans have suggested changes to internal pH, ventilation rates, and heart rate as mechanisms for thermoreception and thermoregulation [50, 51]. Nevertheless, the widespread physiological effects of temperature on these parameters make it difficult to establish whether these mechanisms are simply a result of changing ambient temperature or if they are involved in the signaling pathway to induce thermotaxis.

An interesting aspect of this investigation is the role of acclimation on temperature preference in the context of previous assays on cardiac performance and nervous system activity. Recent work suggests acclimation of *Homarus americanus* to warmer (20°C) temperatures increases the upper limit of thermal tolerance for cardiac performance and
increases survivability at high temperatures (30°C) [Camacho et al., 2]. Moreover, studies on the stomatogastric nervous system of *Cancer borealis* indicate that warm acclimated (19°C) crabs can maintain normal pyloric rhythm function at higher temperatures than crabs acclimated to cool temperatures [52]. With consideration to these studies, warm acclimated decapod crustaceans have a heightened ability to withstand temperatures toward their upper extremes and, as our results indicate, also show a preference towards warmer temperatures. Future work could reveal whether the fundamental processes behind the compensatory abilities of the cardiac and nervous systems also play a factor in modifying the temperature preferences based on acclimation as well, and should specifically address the mechanisms underlying acclimation abilities. This information may also be beneficial in understanding the deleterious effects of acclimation to warmer temperatures, as we observed that *C. borealis* is acclimating to temperatures within the range of thermal stress for *H. americanus* [53, 54]. Prolonged exposure to these temperatures (>20°C) affects lobster immunocompetence and tolerance to changing salinity and oxygen levels [53, 55, 56].

The capacity for *C. borealis* to acclimate and prefer higher temperatures may also have important repercussions in the migratory patterns and catchability of this species. Temperature has been indicated in triggering the migration of the spiny lobster *Panulirus argus* [12, 57]. Moreover, studies focused on the American lobster *Homarus americanus*, which is often targeted by those hauling *C. borealis* as well, have linked increasing ocean temperatures and catch [12, 58, 59]. And on a local scale, researchers found the highest catch per unit effort at its preferred temperature [46]. Given the preferred temperature for the lobster differs by less than 1°C from that which we determined for *C. borealis* (16°C and 15.4°C, respectively), this species may follow the same trend. Our results demonstrate that *Cancer borealis* uses its thermotactic ability to behaviorally thermoregulate. *C. borealis* will adjust the direction and magnitude of its movements to navigate towards a preferred temperature range, and this preferred temperature range is modified by its thermal history. As water temperatures rise, migratory patterns of *C. borealis* and the upper limits of its acclimation ability will be of continued interest in maintaining this species as an active fishery.
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References


59. Drinkwater, K., M. Tremblay, and M. Comeau, *The influence of wind and temperature on the catch rate of the American lobster (Homarus americanus)*
Figure 2.1. Temperature gradient tank and example of typical thermal gradient along the length of the tank. Mean crab size is 14±1 cm CW.
Figure 2.2. Comparison of the movement of individuals throughout a 60 minute trial in a tank with no thermal gradient (control) and a tank with a thermal gradient. Movement is reported by the number of centimeters an individual moved since the previous observation. Values are group means ± s.e.m.
Figure 2.3. Average change in immediate surrounding temperature as a result of movement along a temperature gradient decreases over time. ΔT indicates the difference in immediate surrounding temperature between the first and last minutes of the time period. Values are group means ± s.e.m for each 20 minute time period of the 60 minute trial.
Overall movement along temperature gradient indicates that individuals from all acclimation groups will move away from extreme high and low temperatures. Movement along temperature gradient represented as difference between the introductory temperature and final preferred temperature ($\Delta T$), plotted against the introductory temperature. Introductory temperature is defined as the temperature at which the individual was placed in the temperature gradient tank. Preferred temperature is defined as the mean surrounding temperature of the individual during the final 20 minutes of the trial.
Figure 2.5. Acclimation temperature significantly impacts the preferred temperature of *Cancer borealis*. Groups not connected by a horizontal line are significantly different ($p<0.05$).
Table 2.1. Analysis of covariance demonstrates that the introductory temperature significantly affects the change in surrounding temperature produced by an individual’s movement along the temperature gradient. The change in temperature is calculated as the difference between the introductory temperature and preferred temperature. The response of individuals to the temperature gradient was consistent across all acclimation groups, and was not significantly affected by the acclimation temperature assigned to the individuals ($p < 0.05$). *** indicates $p$ value < 0.001

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Table 2.2. Analysis of covariance demonstrates that both the introductory temperature and the acclimation temperature have a significant effect on the preferred temperature of *Cancer borealis* during the trials. The interaction between acclimation temperature and introductory temperature is not significant. An effect size test indicates acclimation temperature has a stronger effect on preferred temperature than the introductory temperature. * indicates $p$ value < 0.05

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Chapter 3: Transcriptome-wide profiles of nervous system tissues in the lobster, *Homarus americanus*

*In review at BMC Genomics. Formatting of abstract and methods reflect those required for publication in BMC Genomics.*
Abstract

Background:
The American lobster, *Homarus americanus*, is an important species as an economically valuable fishery, a key member in marine ecosystems, and a well-studied model for central pattern generation, the neural networks that control rhythmic motor patterns. Despite multi-faceted scientific interest in this species, currently our genetic resources for the lobster are limited. In this study, we *de novo* assemble a transcriptome for *Homarus americanus* using central nervous system (CNS), muscle, and hybrid neurosecretory tissues and compare gene expression across these tissue types. In particular, we focus our analysis on genes relevant to central pattern generation and the identity of the neurons in a neural network, which is defined by combinations of genes distinguishing the neuronal behavior and phenotype, including ion channels, neurotransmitters, neuromodulators, receptors, and other gene products.

Results: This study used RNA-Seq to investigate transcriptional differences across neural and non-neural tissues from the American lobster, *Homarus americanus*. Using samples from the central nervous system (brain, abdominal ganglia), abdominal muscle, and heart (cardiac ganglia, pericardial organs, muscle), we *de novo* assembled the lobster transcriptome. We use RNA-Seq to characterize gene expression patterns of CNS, muscle, and hybrid neurosecretory tissues. We also compare control tissues with those challenged with the neuropeptide proctolin *in vivo*. Our analysis concentrates on genes characteristic of central pattern generators and terminal selector genes associated with neurons in other systems. Our transcriptome generated 34,813 transcripts with known protein annotations. Of these, 5,000-10,000 of annotated transcripts were significantly differentially expressed (DE) across tissue types. We found 369 transcripts for ion channels and identified receptors and/or proteins for over 18 different neurotransmitters and neuromodulators. Results indicated tissue-specific expression of select neuromodulator (allostatin, myomodulin, octopamine, nitric oxide) and neurotransmitter (glutamate, acetylcholine) pathways. We also identify differential expression of ion channel families, including kainite family glutamate receptors, inward-rectifying K⁺
(IRK) channels, and transient receptor potential (TRP) A family channels, across central pattern generating tissues.

**Conclusions:** Our transcriptome-wide profiles of the rhythmic pattern generating abdominal and cardiac nervous systems in *Homarus americanus* reveal candidates for neuronal features that drive the production of motor output in these systems.

**Keywords:** transcriptome, RNA-Seq, gene expression, nervous system, central pattern generation, locomotion, neuronal differentiation, lobster, *Homarus americanus*

**Background**

An overarching goal in the study of neurobiology is to translate the capacity to perform an action to its underlying cellular and molecular mechanisms. As a result, nervous systems involved in central pattern generation have become a well-studied model for the control of behavior [1, 2]. Central pattern generators are neural networks that produce repetitive actions such as the beating of the heart or gait patterns in walking or running. They are defined by their ability to produce rhythmic motor patterns even in the absence of sensory feedback [3]. The continuous, measurable physical output of these networks allows for straightforward comparisons between actual behavior and network activity. Moreover, these networks perform complex motor tasks in a coordinated, rhythmic fashion while still adapting the behavior to environmental contingencies [3, 4]. The accessibility of these networks combined with this juxtaposition between precision and flexibility has sparked decades of research on the subject, especially in invertebrate models like the American lobster *Homarus americanus*, where these networks are relatively simple (tens or hundreds, rather than thousands, of neurons) compared to their vertebrate counterparts.

In this study, we utilize the American lobster for its role as a model organism in the study of neural networks, though it is also an economically valuable fishery and an important species in marine ecosystem dynamics. To perform this work, we *de novo* assemble a transcriptome for this species, which has no published genome or transcriptome to date.
This genetic resource will expand our ability to study this species in both a physiological and ecological context, as well as within the framework of neurobiology and central pattern generation.

In the lobster, perhaps the simplest central pattern generating network (CPG) is the cardiac system that controls the rhythmic pumping of the neurogenic heart and distributes hemolymph (or ‘blood’) throughout the body (Figure 1A). This system includes the continuously bursting cardiac ganglion (neural control center) and motor neuron connections embedded within the heart itself [5]. The cardiac system generates the heartbeat in the frequency range of 0.5-1.5 Hz [6]. Cardiac performance can be influenced by external factors (including temperature and temperature acclimation)[6-8], and by release of neuromodulators from the pericardial organ [9]. The pericardial organ is an important member of both the cardiac and endocrine systems [10]. It is a neurosecretory structure attached to the lateral walls of the pericardial cavity that releases neuromodulators through nerve trunks with dense assemblies of release terminals [5, 11]. These nerves release neuromodulators as hormones into the hemolymph for distribution to other networks and also innervate the heart to directly modulate the cardiac ganglion [12, 13].

Another well-studied model for central pattern generation in the lobster is the abdominal ganglion, which contains several CPG networks that coordinate locomotion and rhythmic escape swimming behaviors and also play a role in posture [14-16]. This system is composed of six abdominal ganglia, one for each abdominal segment, located in the ventral nerve cord. These ganglia control the swimmeret muscles that operate the fin-like swimmerets (located on the ventral side of abdomen) used in locomotion, righting, and ventilation, the slow extensor-flexor muscles used during backward walking, and the large flexor–extensor muscles used for rhythmic escape swimming [14-23]. The abdominal system controls both phasic and tonic muscle fibers and thus, by comparison, can operate in time domains slower and more rapid than the cardiac system [24, 25]. It can maintain ‘background activity’ in maintenance of posture and also generate rapid tail flips or swimmeret beating at frequencies up to 5 Hz [24, 26].
Characterizing the function and connectivity of these circuits led to a proposed control architecture for innate behavior: the command neuron, coordinating neuron, central pattern generator model. CPG networks are characterized by a particular ability to respond to external and internal variables (temperature, pH) while maintaining stable performance [1, 2, 7, 27, 28]. Study of these networks has also led to breakthroughs in our overall understanding of the role of neuromodulation in shaping the activity of neural circuits [29, 30]. Neuromodulators are signaling molecules that act in concert to modify the intrinsic firing properties of neurons, and can transform the functional connectivity of neural circuits and alter their output. Neural activity can be induced, modified, or terminated by input from multiple neuromodulators [30, 31]. Through extensive work, researchers have identified dozens of neuromodulators in crustacean decapods and characterized their ability to alter circuit dynamics in vitro and, in some cases, motor activity or behavior in vivo [29]. Neuromodulators can be released both intrinsically (from a cell within the circuit) and extrinsically (from another area of the nervous system, such as the neurosecretory pericardial organ)(Figure 1A). However, despite evidence of systemic release of neuromodulators, they are not ubiquitous within the nervous system—immunocytological work and, recently, a characterization of the H. americanus peptidome across tissue types suggests localized distribution of neuromodulators [32]. Exploring the full specificity of expressed neuromodulators and, particularly, their receptors across nervous system tissues is an important next step towards a more complete understanding of the complex interplay of neuromodulators in functional motor networks.

Accordingly, the activity of a neural network is not just conditional to modulatory inputs, but also to the response of the participating neurons to these inputs. The response is dictated by intrinsic properties of those neurons such as the number and kind of ion channels on the membrane [33]. Thus, recent focus has shifted to answering the question: what genetic constructs underlie the production of these stereotyped motor patterns? Current theoretical and molecular research demonstrates correlations between gene expression of different ion channel proteins are actively regulated to maintain robust neuronal output [34-40]. In the invertebrate CPGs the stomatogastric and cardiac ganglia,
Despite variable expression levels of a particular ion channel, there exist characteristic sets of correlated expression of these genes. The relationships between potassium channels (\textit{shal} \textsuperscript{1A}, \textit{shab} \textsuperscript{Kd}, \textit{shaw} \textsuperscript{Kd}, \textit{shaker} \textsuperscript{1A} and \textit{BKCa} \textsuperscript{IKCa}) and membrane conductances in identified cell types are particularly well described [34-36, 39]. These results support the idea that neuronal identity is not defined by the expression of unique genes, but by specific combinations of genes [41].

In this study, we characterize the transcriptional profiles of two types of nervous system tissues: motor, sensory, and command neuron tissue from the abdominal ganglia and supraesophageal ganglia (or ‘brain’), and hybrid neural/muscle tissue from the heart. We compare these central nervous system (CNS) and ‘hybrid’ heart tissues to muscle tissue and with each other to identify representative transcriptomic signatures of neural tissue types. Finally, we compare abdominal ganglia to heart tissue to discover transcriptome-wide differences between these two central pattern generating tissues.

We also explore the role of a circulating neuromodulator in the transcriptional regulation of ion channels by including a hormonal treatment with proctolin. Proctolin, an endogenous pentapeptide, was selected for this study, because its physiological role as an excitatory neuromodulator is well characterized [42]. Proctolin can increase the frequency of action potentials, increase the amplitude of muscle contraction, and initiate activity in quiescent systems [43-48]. It also may function at a system-wide hormonal level though, at present, its role as a hormone and its mode of action over longer time frames is not well understood [49, 50].

The experiments in this study were performed by sampling three types of tissue: muscle (abdominal muscle, \(n=3\)), neural (abdominal nerve cord, \(n=3\); supraesophageal ganglion, \(n=1\)), and neuromuscular hybrid (heart, \(n=3\)). Individuals used were treated with three daily exogenous proctolin injections to temporarily raise hemal concentrations of this neuromodulator \textit{in vivo} to \(10^{-6}\)M (treated, \(n=5\)) or injection of physiological saline.
The effects of increased hemal proctolin across treated and untreated tissues are limited and addressed briefly. All other analyses are conducted bioinformatically controlling for the effects of this treatment in our differential gene expression analysis, and reflect only changes in tissue type (Supplementary Table 1). For analyses of tissue type we include both central pattern generating abdominal ganglia and descending inputs (brain) to characterize the differences between these CNS tissues and muscle or heart tissues. However, for direct comparison of the abdominal and cardiac networks, differential gene expression analysis was re-run with only the abdominal ganglia and heart samples.

By employing a transcriptome-wide approach to investigate neural tissue types and examine two central pattern generating networks, we aim to distinguish combinations of factors involved in determining neuronal identity and function of these systems, including ion channels, neurotransmitters, neuromodulators, receptors, and other gene products.

Results and Discussion

De novo transcriptome assembly and annotation

To date, there remains no published genome for *Homarus americanus*. Thus, for this study, we *de novo* assembled and BLAST annotated a reference transcriptome for *H. americanus* in Trinity using 119.7 million reads from four tissue types (heart, abdominal muscle, abdominal nerve cord, brain) stemming from four lobsters. The assembly resulted in a transcriptome of 115,757 contigs, with an N50 of 1,289. The maximum and minimum contig lengths were 17,481 and 201 bp, respectively, with approximately 25% of the contigs exceeding 1,000 bp in length. tBLAST against NCBI and UniProtKB’s Swiss-Prot/TrEMBLE databases resulted in reliable protein annotations (e value < 10^-4) for 34,813 contigs, or approximately 30% of the assembled contigs. These 34,813 protein annotations contained 12,389 unique proteins, as determined by redundancies in Entrez Gene IDs.

Mapping Entrez Gene IDs to Gene Ontology (GO) annotations identified 11,383 GO
categories, including 7,161 gene products attributed to biological processes, 1,090 attributed to cellular components, and 3,132 attributed to molecular function. The GO terms attributed to the greatest number of genes, in descending order, were nucleus (GO:0005634), cytoplasm (GO:0005737), protein binding (GO:0005515), integral component of membrane (GO:0016021), membrane (GO:0016020), metal ion binding (GO:0046872), and plasma membrane (GO:0005886).

**Differences in expression between all tissue types and treatment groups**

Samples were visualized using a principal component analysis (PCA) and sample-to-sample distances as data quality assessments and also to visualize relative relatedness between samples (Figure 1B). PCA and sample-to-sample distances (Figure S1) indicate a closer relationship between transcripts within a given tissue type than between hormone treatment groups. PCA also closely clusters the two types of neural tissues represented, the supraesophogel (brain) and abdominal ganglia (hereafter together referred to as CNS nervous system or nerve tissues). In Figure S1, a simple representation of Euclidian distances demonstrates nervous system tissues are farther from muscle and heart tissues than muscle and heart tissues are from each other.

**Differential expression between treated and untreated tissues**

Three daily treatments elevating hemal levels of the neuropeptide proctolin resulted in significant differential expression (adj \( p < 0.05 \)) of 255 transcripts. Of these transcripts, 79 had reliable protein annotations (31%). Gene ontology (GO) enrichment analysis did not identify significantly overrepresented (SO) GO categories (adjusted \( p \) value < 0.05, BH method) in treated tissues. Of the annotated transcripts, 80% were upregulated and largely included proteins involved in immune and neural systems. Neural transcripts included innexin, proteins involved in the gap junctions between electrically connected neurons (adj \( p < 0.05 \), +3 log\(_{2}\)fold change), and transient receptor potential channels (TRPA1, TRP pyrexia) (adj \( p < 0.05 \), +3 log\(_{2}\)fold change). The greatest log\(_{2}\)fold change was observed in three transcripts annotating to anti-lipopolysaccharide factors (ALFs) (adj \( p < 0.001 \), +5 log\(_{2}\)fold change), peptides with potent anticoagulation and antimicrobial abilities.
ALFs have been identified in the lobster and other crustaceans as part of the innate immunity, a system that largely takes place in the hemocytes where antimicrobial compounds are synthesized and stored for release into the hemolymph [51-53]. These results indicate a relationship between hemal proctolin and increased synthesis of antimicrobial proteins.

The connection between another neuromodulator, octopamine, and immune responses is well documented in invertebrate systems [54]. Octopamine increases total hemocyte count and nodule formation, enhances inositol trisphosphate (IP$_3$) production in hemocytes, and increases phagocytosis [55-57]. The neuromodulator serotonin (5-Hydroxytryptamine) is also known to regulate phagocytosis, hemocyte production, and nodule formation [56-58]. The ability of octopamine to regulate immune responses is mediated by G-protein coupled receptors (GPCRs) and second messenger systems. The identified receptor for proctolin is also a GPCR, suggesting a similar mechanism by which proctolin may regulate immune response [59]. Here we see hemal proctolin levels upregulate the transcription of ALFs, as well as a stress activated protein kinase identified in another crustacean immune response [UniprotKB: G0ZJ53] (adj p < 0.001, +4 log$_2$ fold change) [60]. These results indicate a novel role for hemal proctolin in innate immunity pathways, and suggest this neuromodulator may be multi-faceted in its role as a signaling molecule and act on both neural and immune systems.

**Differential expression between heart and muscle tissues**

We found 15,046 transcripts with significant differential expression (adj p < 0.05) in heart tissues when compared to abdominal muscle tissues; of these differentially expressed (DE) transcripts, 32% had reliable protein annotations (Table 1). GO enrichment analysis indicated 11 SO categories in heart tissues. The SO categories included extracellular region (GO:0005576), myoblast fusion (GO:0007520), actin binding (GO:0003779), structural constituent of muscle (GO:0008307), calcium ion binding (GO:0005509), and response to heat (GO:0009408).

**Differential expression between central nervous system and muscle tissues**

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Results indicate 28,479 DE transcripts (adj \( p < 0.05 \)) in CNS tissues (abdominal and supraesophageal ganglia) compared to muscle tissues; 35% annotated and included 4,755 unique genes. These genes comprised 22 significantly overrepresented GO categories (adj \( p < 0.05 \)), including plasma membrane (GO:0005886), cell adhesion (GO:007155), extracellular region (GO:005576), axon guidance (GO:007411), synapse (GO:0045202), and neurotransmitter secretion (GO: 0007269).

**Differential expression between central nervous system and heart tissues**

We found 20,179 DE transcripts between nerve and heart tissues, including 7,388 with protein annotations (37% of all DE transcripts), 3,797 unique genes, and 38 significantly overrepresented GO categories (\( p<0.05 \)). Enriched GO categories included plasma membrane (GO:0005886), structural constituent of ribosome (GO:0003735), axon guidance (GO:0007411), extracellular matrix (GO: 0031012), and translation (GO: 0006412).

To address which genes were consistently DE in the neural and heart tissues compared to muscle tissues, we constructed a Venn diagram (Figure 2). Overlap of DE transcripts identified 6,771 transcripts differentially expressed across both contrasts and 2,069 DE across all three contrasts; these groups included many of the nervous system genes described in more detail below.

**Regulation of neurotransmitters and neuromodulators**

The nervous system of crustaceans is rich in neuromodulatory substances that act as extrinsic and intrinsic regulators of nervous system activity. To date, over a dozen of these neuroactive molecules have been identified [61]. Our goal was to assess the presence and relative expression of these known crustacean neurotransmitters and neuromodulators across neural and non-neural tissues. Proteins involved in signaling pathways were detected for 18 different neuroactive substances, including known crustacean neurotransmitters (acetylcholine, glutamate, serotonin, dopamine) and neuromodulators allostatin (AST), FLRFamide, cardioactive peptide (CCAP),
octopamine, orcokinin, dopamine, etc. These proteins included neuropeptides, receptors, and enzymes involved in neurotransmitter pathways (i.e. synthesis). As expected, most of these genes were upregulated in the nervous system tissues compared to muscle tissues (Table 2). However, some neuromodulators exhibited tissue-specific expression signatures for particular neuromodulator sequences or receptors (Figure 3). For instance, the neuropeptide myomodulin was DE and downregulated in nervous tissue compared to both muscle ($p < 0.05$, -7 log$_2$ fold change) and heart ($p <0.05$, -6 log$_2$ fold change) tissue, suggesting a localized role in neuromuscular signaling. There was also a bifurcation of regulation of neuronal nitric oxide synthase (NO) across nerve and muscle tissues, suggesting the proteins or protein isoforms involved in NO signaling differ across tissues. NO is of particular interest as its recently been shown to gate the polarity of endocannabinoid-modulation to shift the excitation-inhibition balance of a synapse and thus mediate long-term potentiation of rhythmic locomotor circuits [62-65]. All six transcripts for the carboxyl-terminal PDZ ligand of neuronal nitric oxide synthase protein (70729) were downregulated in nerve tissue compared to muscle, and three of the six were DE ($p<0.05$). Three nostrin (115677, 521834) transcripts were DE and downregulated (-5 log$_2$ fold change or greater) across the tissue types, but five other nostrin transcripts were DE and upregulated (+2 log$_2$ fold change or greater).

In comparing the heart tissue (which contains both muscle and the cardiac ganglion nervous system) with the other nervous tissues (abdominal chain and brain), there was a contrast in the expression of different octopamine receptors. Octopamine receptors beta 1-R, 2-R, and 3-R were upregulated in other nervous system tissues, compared to heart tissues ($p$ value, ns, +2 log$_2$ fold change or greater). In contrast, both octopamine receptors transcripts matching UniProtKB Q25188.1 of *Heliothis virescens* were downregulated (1 of 2 transcripts DE, $p$ value $< 0.05$, -2 log$_2$ fold change) and thus more highly expressed in cardiac tissue (Figure 3). Octopamine $\beta$-receptors are a group of receptors homologous to vertebrate $\beta$-adrenergic receptors [66]. These receptors respond to octopamine by increasing intracellular cAMP but display disparate pharmacological profiles, suggesting differences in sequences between these receptors results in varying functional roles in
signaling activities [66, 67]. Our data provide context for localization of expression and expression levels in *H. americanus*, and suggest differences in receptor sequences for octopamine (as well as other signaling molecules) may play a role in defining specificity in targets and effects of these molecules as a circulating neuromodulators.

**Transcription Factors for Neuronal Differentiation**

To identify candidates for transcription factors involved in the differentiation of our neuronal tissues, we performed a blast analysis for transcription factors identified in terminal selector programs, or pathways that that control the expression of identifying features of mature individual neuron types [33, 68-72]. We searched for 24 different gene sequences, all of which were found within our transcriptome (e-val < 10^{-4}) (Table S2). Of these sequences, we found several transcription factors with a high proportion (>40%) of transcripts DE and upregulated in our nervous system tissues, including *ceh-36*, an Otx-type homeobox gene involved in chemosensory neuron differentiation, *ttx-1*, an Otx-type transcription factor involved in thermosensory neuron identity, and *ets-5*, an ETS domain transcription factor involved in CO₂/O₂ sensory neurons [71].

We also blasted our transcriptome for the homeodomain transcription factor *Shox2*, which has been linked to excitatory interneurons in the rhythmic pattern generating kernel for spinal locomotion in mice [73]. The top hit (comp32264_c0_seq2) had a 99% sequence identity match to *Shox2* and was DE and upregulated in heart tissues compared to muscle tissue (adj *p* < 0.05, +6 log fold change) and downregulated in nerve tissues compared to heart tissues (adj *p* < 0.05, -7 log fold change), indicating this transcription factor is highly expressed in our cardiac network compared to the abdominal ganglia, brain, or muscle.

**Candidate genes for differentiation between abdominal and cardiac CPGs**

To identify divergent genetic signatures across the abdominal and cardiac central pattern generating networks, we conducted a separate gene expression analysis of just abdominal
nerve cord tissues (n=3) over heart tissues (n=3) and then examined transcripts involved in neuronal identity.

In summary, 14,955 transcripts were differentially expressed; 38% of transcripts had protein annotations. Gene ontology analysis identified 31 significantly enriched GO categories \((p < 0.05)\) (Figure 4). Many of these results, like the structural constituents of muscle (GO:0008307) and respiratory chain (GO:0070469), were downregulated in the abdominal nerve cord and are attributable to differences in nervous system tissue versus mixed muscle/nervous system tissue (Table 3). Thus, our analysis focused on GO categories and genes involved in neuronal differentiation with the goal of addressing the differences in the CPG nervous systems of the cardiac ganglion and abdominal ganglia.

Enriched GO category terminal bouton (GO:0043195) suggested a role for kainite family glutamate receptors (GluK) in differentiation of these CPGs. GluK2 receptors are DE and upregulated in abdominal ganglia (GluK2 \(p < 0.001, +6 \log_2\) fold change) whereas GluK3 and GluK5 are DE and downregulated (respectively, GluK3 \(p < 0.001, -10 \log_2\) fold change and GluK5 \(p < 0.05, -5 \log_2\) fold change). For more broadly relevant GO categories, such as plasma membrane and extracellular matrix, we employed previous techniques for identifying neuromodulators, as well as isolating ion channels and receptors (Figure 5,6). Overall, this analysis detected 369 transcripts annotating to membrane ion channels or receptors. Results identified downregulation of the kainite glutamate receptors (GluK3, GluK5) as well as specified downregulation of myomodulin A \((p < 0.001, -5 \log_2\) fold change) and octopamine receptors \((p < 0.05, -2 \log_2\) fold change). In contrast, cardioactive peptide (CCAP) is upregulated \((p < 0.001, +9 \log_2\) fold change) in the abdominal CPG. This result is particularly interesting given the nerve terminals in the heart tissues from the neurosecretory pericardial organ; the expression of CCAP was first discovered in the pericardial organs, hence the etymology \([74]\). Here, CCAP is indeed expressed in the heart tissues, but has higher expression in the abdominal system. Also upregulated in the abdominal system were two variants of allostatin annotating to allatostatin-cc (Nilaparvata lugens) \((p < 0.001, +7 \log_2\) fold change) and C-type preproallatostatin (Pandalopsis japonica) \((p < 0.001, +6 \log_2\) fold change) and the
neuromodulator red pigment containing hormone (RPCH) ($p < 0.05$, +4 log$_2$ fold change) (Figure 5). Several contigs of acetylcholine pathway proteins were also upregulated in the abdominal CPG including subunit alpha 6 ($p < 0.001$, +10 log$_2$ fold change) and acetylcholinesterase ($p < 0.001$, +7 log$_2$ fold change). Collectively, these results indicate CCAP, AST, RPCH, GluK2, and acetylcholine as strong candidates for the function of abdominal networks, and GluK3, Gluk5, myomodulin and octopamine as signatures of the cardiac network.

We observed all voltage gated L-type Ca$^{2+}$ channel subunits and the Ca$^{2+}$ channel subunit alpha-2/delta-1 are DE and upregulated in the abdominal ganglia (Table 4). We also observed that glutamate-gated Cl$^-$ channels (GLUCL) and calcium-activated Cl$^-$ channels (CLCA) were largely DE and upregulated, with log$_2$ fold changes of many transcripts greater than +5 (Figure 6). K$^+$ channels slo-1/slowpoke are also DE and upregulated. These large-conductance, big potassium or ‘BK’ channels are involved in calcium-dependent K+ currents ($I_{\text{KCa}}$), participate in repolarization of presynaptic termini, and are important to the timing of action potentials [75-77]. Interestingly, recent studies in *Drosophila melanogaster* with slowpoke knockout mutants and RNAi have identified timing deficiencies in rhythmic motor patterns and, particularly, a drastically decreased ability for slo mutants to initiate rhythmic flight activity [78, 79]. Future studies could explore whether the slo K+ channel is a key constituent in intermittently active, rather than continually active, rhythmic motor generating nervous systems.

Ion channels that were DE and downregulated in this comparison, thus displaying significantly increased expression in cardiac systems, included a predicted Cl$^-$ channel and two inward-rectifying K$^+$ (IRK). IRK channels are important for neuronal excitability and the timing of neuronal activity [80, 81]. Combined, these observations suggest families of channels are critical to the function and timing of motor pattern generating circuits including IRK channels for the heart and slo family BK channels and CLCA channels for the abdominal system. For a full list of differentially expressed ion channels, please refer to Table S3.
Heart tissue in response to heat

An unexpected result of this study was the identification of significant overrepresentation of thermosensory genes in heart tissue. Interestingly, in GO enrichment analysis of heart vs. muscle tissues the only significantly overrepresented biological process was ‘response to heat’ (GO:0009408), with over 50% of involved genes differentially expressed (18 of 32 genes in category, \( p < 0.05 \)).

As mentioned, central pattern generators are characterized by their ability to maintain robust output across changing internal and environmental factors [27, 82]. For poikilotherms, this ability is particularly important given there are no internal mechanisms for regulating temperature—an environmental factor with undeniable effects on all biological processes. The thermal behavior of *H. americanus* and other decapod crustaceans is well documented. They are able to respond to small changes in temperature (<0.5° C) and maintain rhythmic nervous system function over large temperature ranges [8, 82-84]. Moreover, these organisms are thermotactic and navigate toward preferred temperatures to optimize physiological function [85-87]. Our results may indicate candidate genes for this precise response to temperature in the cardiac and central nervous system tissues (Table 5). Of the 18 DE genes involved in heat response, two were DE and downregulated in all contrasts: heat shock 18kDa protein and protein Efl21. Additionally, TRP channel pyrexia, locomotion defects protein, and hippocampal cholinergic neurostimulating peptide, were DE and upregulated in both heart and nervous system tissues when compared to muscle tissues.

In particular, this analysis identified two transient receptor potential (TRP) channel genes, pyrexia and painless, that belong to TRPA family (Table 2). Full analysis of our transcriptome identified 5 subtypes of TRP channels including three types of TRPA family genes (TRPA1, pyrexia, and painless), TRP M2 and TRP M3. TRP channels are a diverse family of channels permeable to \( \text{Na}^+ \) and/or \( \text{Ca}^{2+} \) and involved in many varied types of sensory reception. Of the TRP channels identified in our transcriptome, research in other models establishes TRP M3 as an osmoreceptor, M2 is a possible oxidant stress sensor, and TRPA genes in temperature sensing [88]. The TRPA channels pyrexia and painless may be responsible for sensing different temperature ranges [89-92].
differential expression of these channels in our samples, the upregulation of pyrexia in heart tissues and the upregulation of painless in CNS tissue, suggests a possible mechanism by which these systems are able to efficiently sense and react to temperature changes.

Conclusions
Our study detected tissue-specific patterns of increased expression of neuromodulators in the heart (octopamine), muscle (myomodulin), and nervous system (RPCH, AST, tachykinin, FLRFamide), as well as tissue-specific variation in expression sequences for octopamine and NO. We identified several neuronal factors contributing to the identity of our abdominal and cardiac systems. For the abdominal network, we observed significantly increased expression of acetylcholine receptors, GluK2, voltage gated L-type Ca\(^{2+}\) channels, calcium-activated Cl\(^{-}\) channels, the K\(^{+}\) channel slo-1/slowpoke, and significantly increased expression of neuromodulators CCAP and AST. For the cardiac network, we observed increased expression of the neuromodulators myomodulin and octopamine, and the GluK3, Gluk5, and IRK channels. We identified a set of neuronal differentiation transcription factors in these systems, including ceh-36, ttx-1, ets-5 and Shox2. This research also elucidated a novel role for the neuromodulator proctolin in regulating immune responses, and identified an overrepresentation of response to heat in the heart tissues of lobster, and suggests a possible mechanism for thermoreception in this poikilotherm stemming from thermosensory TRPA channels in nervous system tissues.

Materials and Methods

Sample collection and preparation
Adult *Homarus americanus* were purchased from local commercial fisherman (F/V Jacqueline Bess, Nahant Fish and Lobster, Nahant, MA) and held in ambient running seawater tanks (10.29±0.01 °C) at the Northeastern University Marine Science Center. Lobsters were not fed prior to experimentation. Four different tissue types were collected from live *Homarus americanus*: (1) abdominal nerve cord \((n=3)\), (2) supraesophogeal
ganglion (the “brain”)(n=1), (3) heart and pericardial cavity with neurosecretory pericardial organ (n=3), (4) muscle tissue from abdominal muscles (n=3). Prior to dissection, individuals were subjected to once-daily hormone treatments of the proctolin (Arg-Tyr-Leu-Pro-Thr) (injection of proctolin in physiological saline) or control treatments (physiological saline injection) for a three-day period. Hormone treatments served to temporarily increase systemic proctolin concentration to 10⁻⁶ M, based on standard hemolymph/bodyweight calculations [93] and physiologically relevant concentration levels [13, 45, 47]. Efficacy of injections increasing systemic proctolin was confirmed by quantitative mass spectrometry. The period of treatment was selected based on time frames of ion channel turnover, a process that can take hours to days [94-98].

Samples were removed with forceps and surgical scissors, flash-frozen in TRI® Reagent with liquid nitrogen, and pulverized with RNase-DNase-free pestles (VWR, Radnor, PA). To prep samples for RNA-Seq on the Illumina platform, mRNA was separated from the extracted Total RNA with Dynabeads® Oligo(dT)₂₅ (Invitrogen Life Technologies, Grand Island, NY). cDNA libraries were constructed using the Vollmer laboratory protocol for non-genetic model organisms [99-101] and NEBNext® reagents for Illumina® (New England Biolabs, Ipswich, MA). Samples were multiplexed and sequenced (single-end, 109bp) on the Illumina HiSeq2000II platform at Tufts TUCF Genomics.

**Data processing**

RNA Sequencing on the Illumina HiSeq2000II platform yielded 145.3 million total reads, averaging 14.56 million reads per sample. Reads were trimmed and quality controlled to a Phred score of Q=30. Read contamination was resolved prior to assembly by aligning all raw reads, using Bowtie (version 0.12.7) and custom Perl scripts, against several indices and retaining only unmapped reads. These indices were constructed from the following inputs: (1) metazoan rRNA databases (NCBI), (2) viral, fungal, and bacterial genomes (NCBI), and (3) Illumina TruSeq adaptor sequences and PCR primers. Next, *de novo* assembly of the transcriptome was conducted in *Trinity* (version 8-14-2013), allowing for the detection of punitive alternative splice variants [102, 103]. Subsequently, contigs from our transcriptome were BLAST annotated against NCBI, UniProtKB’s
Swiss-Prot, and TrEMBLE protein databases; matches with an e-value below $10^{-4}$ were considered protein-coding genes. Reads were aligned using Bowtie and include all partial and multiple alignments. Bowtie alignments of our dataset against selected genes from major signaling pathways as well as housekeeping genes, revealed high efficiency in gene recovery compared to available genomes of closely related species. Blast output was parsed to the top hit for each contig, and NCBI gi numbers were converted to Entrez Gene IDs using ID mapping files from UniProt Knowledgebase (UniProtKB). All gene ID numbers provided are Entrez Gene IDs unless otherwise specified. Moreover, Entrez Gene IDs were mapped to gene ontology (GO) annotations using NCBI gene2go.

Differences in gene expression were analyzed from raw read counts using the R package DESeq2 1.4.5 [104] and merged with annotation files (for breakdown of analyses, see Table S1). Results from differential expression analysis were further analyzed for difference in expression of gene ontologies using the R package GOseq (version 1.16.2), which determines expression of gene ontology categories accounting for over-detection of differential expression for long or highly expressed transcripts [105]. As multiple contigs often mapped to the same protein annotation, enrichment analysis was conducted on contigs with greatest read coverage for each given Entrez Gene ID. GOseq was run using gene annotation length as bias data and our GO category mapping file. $P$-values for overrepresented GO categories were adjusted using the Benjamini and Hochberg (1995) method for false discovery rate control. GO enrichment was visualized in REVIGO using GOseq output [106].
References

1. Stein PSG: **Motor Systems, with specific references to the control of locomotion.** *Ann Rev Neurosci* 1978, **1**:61-81.


78. McKiernan EC: Effects of manipulating slowpoke calcium-dependent potassium channel expression on rhythmic locomotor activity in Drosophila larvae. *PeerJ* 2013, **1**:e57.


Figure 3.1. A. Schematic drawing of the lobster *Homarus americanus*, indicating tissue types collected for this study. Nervous system tissue samples include either abdominal ganglia (abdominal nerve cord) or the supraesophageal ganglion (brain). All heart tissue samples include the heart muscle with attached cardiac ganglion and neurosecretory pericardial organ. Muscle tissue samples are a section of the abdominal muscle. Adapted with permission from John Wiley and Sons, from Skiebe 1999.
Figure 3.1. B. Principal component analysis of transcripts across three tissue types (heart, muscle, and nervous system tissue) and two treatment levels (hormone treated and untreated). This 2D representation of the first two principal components of these 10 samples demonstrates a greater effect of tissue type than treatment, and also a greater effect of tissue type than variation across individuals. Component 1 explains 87.84% of the variance (SD=2.96); component 2 explains 4.08% of the variance (SD=0.64).
Figure 3.2. Venn diagram of differentially expressed transcripts by tissue comparison. Values indicate number of transcripts with significant differential expression ($p$ value < 0.05) across that contrast (HvM, heart vs. muscle tissues; NvM, nerve vs. muscle tissues; NvH, nerve vs. heart tissues).
Figure 3.3. Heatmap of select neuromodulators and receptors with tissue specific expression patterns. Relative to each other, red cells indicate high levels of expression and blue cells indicate levels of low expression. For nerve tissues, AG – abdominal ganglion, SG – suprasphageal ganglion.
**Figure 3.4.** Gene ontology analysis of abdominal nerve cord vs. heart tissue. Analysis is divided by category: **A.** Biological Process. **B.** Cellular Component. **C.** Molecular Function. Each panel includes only significantly enriched GO terms (adjusted $p$ value < 0.05) and depicts number of differentially expressed contigs ($p < 0.05$) that are upregulated (black bars, positive log2foldchange) or downregulated (gray bars, negative log2foldchange). For full list of GO terms with ID numbers, refer to Table 3.
Figure 3.5. Analysis of neurotransmitters, neuromodulators, and their receptors in the abdominal nervous system compared to the heart system. Red dots indicated significantly DE contigs (p < 0.05). Orange dots indicate DE contigs with log2fold change greater than 5 or less than -5.
Figure 3.6. Analysis of membrane channels in the abdominal nervous system compared to the heart system. Red dots indicated significantly DE contigs ($p < 0.05$) annotating to known membrane channels. Orange dots indicate DE contigs with log2fold change greater than 5 or less than -5.
Figure 3.S1. Heatmap display of sample-to-sample Euclidean distances between samples, calculated by regularized log transformation.
Figure 3. S2. Treemap of enriched biological process GO categories in heart tissues compared to muscle tissues. Box size indicates relative level of overrepresentation in heart tissues. The only significantly overrepresented biological process was response to heat (GO: 0009408), with over 50% of involved genes differentially expressed (18 of 32 genes in category, $p < 0.05$).
Table 3.1. Number of transcripts out of 115,757 total transcripts with significant differential expression (adj $\ p < 0.05$). Annotated transcripts depicts the number of differential expressed (DE) transcripts with a reliable protein annotation. Unique annotations indicate the number unique genes within the annotated DE transcripts, as determined by associated Entrez Gene ID numbers.

<table>
<thead>
<tr>
<th>Contrast</th>
<th>Differentially Expressed Transcripts</th>
<th>Annotated Transcripts</th>
<th>Unique Annotations</th>
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<tr>
<td>Nerve vs. Muscle</td>
<td>28,479</td>
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<tr>
<td>Nerve vs. Heart</td>
<td>20,179</td>
<td>7,388</td>
<td>3,797</td>
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</table>
Table 3.2. Expression of known crustacean neuromodulators and neurotransmitters in the *Homarus americanus* transcriptome. Transcripts manually curated into functional categories by their annotations. Count is the number of transcripts mapping to a function; DE indicates the number of transcripts differentially expressed in nervous system tissues compared to muscle tissues (adj $p < 0.05$); up and down signify the number of DE transcripts upregulated (positive log$_2$ fold change) or downregulated (negative log$_2$ fold change), respectively.

<table>
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<tr>
<th>Function</th>
<th>Count</th>
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<th>Up</th>
<th>Down</th>
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Table 3.3. Gene ontology analysis of abdominal nerve cord vs. heart tissue. Analysis includes only significantly enriched GO terms (adj p value < 0.05) and depicts the ‘count’ or number contigs mapping to genes in the GO category, number of DE contigs (adj p < 0.05), DE contigs upregulated (“up”, log₂ foldchange > 0) or downregulated (“down”, log₂ foldchange < 0) in the abdominal ganglia compared to the heart ganglia.

<table>
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<th>GO ID</th>
<th>GO Term</th>
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<td>GO:0008307</td>
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<td></td>
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103
Table 3.4. Membrane channels differentially expressed across central pattern generating nervous systems. Count is the number of transcripts annotating to a channel type; DE indicates the number of transcripts differentially expressed in abdominal compared to heart tissues (adj $p < 0.05$); up and down signify the number of DE transcripts upregulated (positive log$_2$ fold change) or downregulated (negative log$_2$ fold change), respectively.

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<th>Type</th>
<th>Abbreviation</th>
<th>Annotation</th>
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Table 3.5. The significantly differentially expressed genes in heart tissues in the “response to heat” GO category (GO:0009408) (heart tissue vs. muscle tissue, adj \( p < 0.05 \)). This GO category is significantly overrepresented in heart tissues as compared to muscle tissues (adj \( p < 0.05 \)) and contains 18 DE genes out of 32 total annotated genes. Only DE genes with a log₂-fold change greater or less than 2 are displayed in this table.

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<td>3771872</td>
<td>Heat shock protein 67B2</td>
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<td>Translation initiation factor eIF-2B subunit alpha</td>
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Table 3.S1. Experimental design for represented differential gene expression analyses conducted in DESeq2.

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<tr>
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<td>Nerve vs. Muscle</td>
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<td>Heart vs. Muscle</td>
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<td>Tissue Type – Separate Nerve Tissues</td>
<td>~ Treatment + Tissue Type</td>
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<td>Abdominal Ganglia vs. Heart</td>
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Table 3. S2. Terminal selector genes differentially expressed in heart tissue compared to muscle tissue and central nervous system tissue (supraesophageal ganglion, abdominal ganglion) compared to muscle tissue. Count indicates the total number of transcripts annotating to proteins (e-val < $10^{-4}$) identified as transcription factors, co-factors, or other regulatory genes involved in differentiating the terminal identity of a neuron type. Columns denote the total number of DE transcripts (DE, $p < 0.05$), and number of upregulated (up) and downregulated (down) DE transcripts in contrasts: heart vs. muscle tissue and nervous system vs. muscle tissue.

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<th></th>
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<td>Otx-type homeodomain</td>
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<td>3</td>
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Table 3.S3. Differentially expressed membrane channels in the abdominal ganglia compared to the heart. Table includes only DE contigs with a log2fold changes greater than four or less than negative four.

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<tr>
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<td>Cl</td>
<td>6.3</td>
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<tr>
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Chapter 4: Identification and quantification of hemal Proctolin, and its ability to regulate gene expression in the nervous system of *Homarus americanus*
Introduction

Dynamic signaling is important for the nervous system activity. Signaling molecules can act in varied time frames and spatial scales, from the rapid and local action of neurotransmitter release across a synapse to the impact of hormones over longer time frames and systemic levels [1]. Neuromodulators are signaling molecules released by either intrinsic (i.e. a neuron in a neural network) or extrinsic inputs, and act to initiate, modify, or terminate the activity of a neural network [2]. The release of neuromodulators from extrinsic sources, for example neurosecretory release of a neuromodulator into the bloodstream, can blur the line between a neuromodulator and hormone [3]. And though we know many neuromodulators in invertebrate systems share duty as both neuromodulators and hormones [4], our knowledge of the role of these substances as circulating neuromodulators¹ is limited.

In this study we exploit the American lobster, *Homarus americanus*, as a model organism in the study of neuromodulation. Decades of research on the lobster (and other decapod crustaceans like the Jonah crab *Cancer borealis*, crayfish *Procambarus clarkii*, or spiny lobster *Panulirus interruptus*) has identified dozens of crustacean neuromodulators and characterized their ability to modulate the activity of neural circuits through bath applications *in vitro* and, occasionally, injections *in vivo* (for recent reviews on neuromodulation, see Harris-Warrick 2011 or Marder 2012)[5, 6]. Though the connection between hormones and regulation of gene expression is long established [7, 8], only recently have studies begun explore the role of neuromodulators in regulating transcription or translation-dependent mechanisms [9, 10].

In particular, a notable study by Temporal et al. identifies neuromodulators as a dynamic mechanism for the fine-tuning of nervous activity in a cell-specific manner through regulation of ion channel gene expression [11, 12]. Another work, by Rodgers et al.,

¹ For issues of clarity, we will continue to use the term neuromodulators or ‘circulating neuromodulators’ to refer to neuromodulators that are both released within a neural network and also released from neurosecretory structures to circulate in the bloodstream like hormones.
examines tonic expression of dopamine as a translation-dependent mechanism for maintenance of rhythmic motor output [9].

The focus of this study is to examine the role of proctolin in transcriptional regulation at time courses relevant to ion channel turnover. Proctolin is a pentapeptide (Arg-Tyr-Leu-Pro-Thr) with excitatory properties as neuromodulator acting on numerous nervous systems [13, 14]. The structure of proctolin is well conserved across invertebrate species, and its role in insects is well described [15]. The modulatory activity in decapod crustaceans includes increasing the amplitude of muscle contraction of the heart via excitation of the cardiac ganglia [16]. In the stomatogastric nervous system (STNS) in vitro, proctolin increases the frequency of the pyloric rhythm and the gastric mill rhythm, and will initiate the gastric mill rhythm in quiescent preparations [17-19]. In in vivo injection experiments, proctolin initiates rhythmic chewing and gastric mill activity [20]. In the abdominal system in vitro, proctolin initiates fictive locomotion in the motorneurons that control movement of the swimmerets [21, 22].

Proctolin has been identified throughout the nervous system tissues of Homarus americanus using immunohistochemistry and mass spectrometry approaches, including the stomatogastric ganglia (STG), subesophageal ganglia, thoracic ganglia (TG), circumesophageal ganglia (CEG), cardiac ganglia (CG), abdominal ganglia (AG, and pericardial organ (PO) [17, 23-26]. In the STG, proctolin has been quantified at 0.26 pmol/ganglion in proctolin-like immunoreactivity (n=8) [18]. Other ganglia (TG, AG, CG, supra- and sub-esophageal ganglia) range in proctolin-like immunoreactivity from 0.5-4 pmol/ganglion, with the highest concentration in the supra- and sub-esophageal ganglia [24]. In neurons within the TG, CEG, and AG proctolin coexists and possibly is co-transmitted with acetylcholine, serotonin and dopamine [25, 27]. In the PO the highest observed proctolin concentration is 18 pmol [24]. The PO is a neurosecretory structure in the pericardial cavity that releases neuromodulators both onto the cardiac ganglion and into the hemolymph [28]. Due to its localization to the PO, proctolin has long been suspected to act as a circulating neuromodulator [24, 25]. However, to date, proctolin has not been quantified in the hemolymph (‘blood’) of any decapod crustacean.
Threshold concentrations of proctolin required to elicit a neural response vary by species and system. In *Panulirus interruptus*, *Cancer borealis* and *Cancer irroratus*, bath-applied proctolin *in vitro*, has excitatory effects on the gastric mill and pyloric rhythms at a threshold concentration of $10^{-9}$-$10^{-8}$ M [18]. Proctolin stimulates activity in quiescent preparations and excites nervous system activity in a dose-dependent manner, with concentrations of $10^{-6}$ M stimulating decreased period, increased action potential/burst, and increased amplitude of membrane potential oscillations for sustained periods [18]. In the crayfish *Pacifastacus leniusculus*, proctolin excites vigorous swimmeret activity at a threshold concentration of $10^{-8}$ M and an EC$_{50}$ of $1.6 \times 10^{-6}$ M [21, 22]. In *Homarus americanus*, the measured threshold for excitatory action on the STNS *in vitro* was $10^{-8}$ M in one study [18] and $10^{-10}$ M in another [19]. For injections *in vivo*, chewing was initiated at a threshold hemal concentration of $\sim 10^{-9}$ M (1 ml of $1.5 \times 10^{-7}$ M), at an average latency of 53 seconds following injection [20]. Higher doses are required to elicit all modes of chewing. Injections of 1 ml of $1.5 \times 10^{-6}$ M proctolin triggered chewing in the squeeze mode and $1.5 \times 10^{-4}$ M initiated chewing in the cut-and-grind mode [20].

We first sought to isolate and quantify proctolin levels in the hemolymph using quantitative mass spectometry. In the tissue, proctolin has been measured at concentrations between $2.1$-$2.5 \times 10^{-5}$ M (TG, CEG, AG)[25]. We expected lower concentrations in the hemolymph, at levels near those estimated for EC$_{50}$ and threshold concentrations ($10^{-6}$-$10^{-10}$ M). Next we experimentally amplified proctolin levels to physiologically relevant levels ($10^{-6}$M) using three once-daily injections of exogenous proctolin *in vivo* and collection of nervous system and muscle tissues for RNA-Seq on the fourth day. This time frame was selected for its relevance in the regulation of ion channel turnover. Turnover and transcription rates are specific to channel and cell type, and thus can be challenging to quantify. However, previous studies have determined ion channel turnover rates, measured in ‘half lives’, in the range of 12-72 hrs for voltage-gated Na$^+$, K$^+$ and Ca$^{2+}$ channels and AMPA receptors [29-32]. One study suggested some K$^+$ channel proteins can degrade or be removed from the membrane quickly (some within 30

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$^2$ EC$_{50}$ is the concentration of a drug that gives half-maximal response.
minutes, reportedly), but require 8+ hour timeframe for three-fold increases in transcription [33]. These experiments selected a longer time frame within this window to observe transcription changes; our samples were collected 72 hr after first injection, 24 hr after last injection in a three-day period. Using these methodologies, the primary objective of this research is establishing whether proctolin is capable of functioning as a circulating neuromodulator, and examining a molecular snapshot of how this peptide regulates the activity of nervous systems over the long term through control of mRNA transcription.

**Materials and Methods**

*Animal collection*

Live American lobsters (*Homarus americanus*) were purchased from local lobstermen (F/V Jacqueline Bess, Swampscott, MA) and held in an ambient flow-through seawater tank at the Northeastern University Marine Science Center. Experiments were performed between December 2013 and January 2015 and included winter (December-February) and spring (May) seasons. Individuals were held at naturally occurring water temperatures and light cycles and starved for a two-week acclimation period before hemolymph or tissue collection.

*Quantification of proctolin in the hemolymph*

Proctolin labeled with a stable heavy isotope was used for quantification of the endogenous peptide and as an internal control added immediately to hemolymph samples [34]. For heavy labeled proctolin, a stable isotope-labeled arginine was substituted in the peptide (Arg-Tyr-Leu-Pro-Thr) for a mass shift to 659.37 MW; this peptide was custom-synthesized from ThermoFisher Scientific (Rockford, IL). Hemolymph was sampled by withdrawal using a sterile needle attached to a syringe. Samples were collected from the body cavity through the soft tissue below the first tail segment on the dorsal side. Hemolymph was immediately measured for volume and combined with equal amounts of acidified methanol (90:9:1 methanol, acetic acid, DI water)[23]. Heavy labeled proctolin was added and the samples were frozen (-80º) until processing. Extracted samples were
evaporated, desalted using C18 micro-columns, and analyzed via C18 nano-LC/MS on an Orbitrap Fusion mass spectrometer using a targeted SIM method centered around the heavy (659.37) and light (649.36) proctolin masses. Endogenous proctolin was calculated by comparing the ratio between the integrated areas of the heavy and light peaks (Figure 4.1).

Differential gene expression analysis across proctolin-treated and untreated individuals using RNA-Seq

Individuals were randomly divided into two treatment groups: proctolin-treated and untreated/control. These individuals received once-daily pericardial injections of proctolin (American Peptide Company, Vista, CA) in physiological saline (proctolin-treated) or physiological saline (control) for a three-day period. Hormone treatments served to temporarily increase systemic proctolin concentration in vivo to $10^{-6}$ M, based on standard hemolymph/bodyweight calculations [35]. Efficacy of injections increasing systemic proctolin was confirmed by quantitative mass spectrometry.

On day four (24 h after the final injection), tissues were collected from the live lobsters. Samples were removed with forceps and surgical scissors, flash-frozen in TRI® Reagent with liquid nitrogen, and pulvérized with RNase-DNase-free pestles (VWR, Radnor, PA). To prep samples for RNA-Seq on the Illumina platform, mRNA was separated from the extracted Total RNA with Dynabeads® Oligo(dT)25 (Invitrogen Life Technologies, Grand Island, NY). cDNA libraries were constructed using the Vollmer laboratory protocol for non-genetic model organisms and NEBNext® reagents for Illumina® (New England Biolabs, Ipswich, MA)[36-38]. Collected tissues included four tissue types: (1) abdominal nerve cord (n=3), (2) supraesophageal ganglion (the “brain”)(n=1), (3) heart and pericardial cavity with neurosecretory pericardial organ (n=3), (4) muscle tissue from abdominal muscles (n=3). These ten samples are comprised of tissues from four total individuals (two proctolin-treated, two control) (Supplementary Table 4.1). Samples were multiplexed and sequenced (single-end, 109bp) on the Illumina HiSeq2000II platform at Tufts TUCF Genomics (Boston, MA).
RNA Sequencing on the Illumina HiSeq2000II platform yielded 145.3 million total reads, averaging 14.56 million reads per sample. Reads were trimmed and quality controlled to a Phred score of Q=30. De novo assembly of the transcriptome was conducted in Trinity (version 8-14-2013), allowing for the detection of punitive alternative splice variants [39, 40]. Subsequently, contigs from our transcriptome were BLAST annotated against NCBI, UniProtKB’s Swiss-Prot, and TrEMBLE protein databases; matches with an e-value below $10^{-4}$ were considered protein-coding genes. Reads were aligned using Bowtie and include all partial and multiple alignments. Differences in gene expression across proctolin-treated (n=5) and untreated tissues (n=5) were analyzed from raw read counts using the R package DESeq2 1.4.5 [41, 42]. Analysis of differential gene expression across tissue type comparisons was also conducted using DESeq2, across contrasts using following tissue types: central nervous system tissues (abdominal ganglia, brain; n=4), heart (heart muscle with cardiac ganglia and PO; n=3), and muscle (abdominal muscle; n=3). We examined CNS vs. heart, CNS vs. muscle, and heart vs. muscle. For table of sample tissue type and treatment groups, see Supplementary Table 4.1.

Results

Detection and quantification of proctolin in the hemolymph

Using quantitative mass spectrometry, endogenous proctolin was detected in the hemolymph of *H. americanus* (Figure 4.1). Proctolin was detected at mean concentration of $1.54E-10 \pm 4.67E-11$ (S.E.M., n=19); the maximum observed concentration was $6.26E-10$ and the minimum observed concentration was $1.82E-13$ (Table 4.1).

Hemal proctolin across gender, body size and blood volume

Hemal proctolin levels within male and females populations were not normally distributed (Shapiro Wilk normality test, males: $W = 0.78$, *p* value < 0.05; females: $W = 0.69$, *p* value < 0.05) (Supplementary Figure 4.1). Thus, we conducted a Wilcoxon rank
sum test to identify whether the distribution of hemal proctolin levels are significantly different across gender. Median hemal proctolin levels across male and female populations were 1.87E-11 (n=9) and 3.19E-11 (n=10), respectively; the distributions in the two groups did not differ significantly (Wilcoxon rank sum, W = 43, p-value = 0.90) (Figure 4.2).

The volume of hemolymph in lobsters is conditional on molt cycle; for adult, intermolt lobsters, hemolymph volume is 30% of the total body weight [43]. To assess if proctolin concentration was related to the size or blood volume in individuals, we modeled this relationship with a linear regression and determined that body weight is not a strong predictor of hemal proctolin concentration (R² = 0.09) (Figure 4.3).

**Differential gene expression across proctolin treated and control tissues**

Differential gene expression analysis was conducted to analyze the effect of three daily injections of exogenous proctolin (10⁶M), compared to control treatments of sterile saline injections, on our neural and muscle tissues. Our proctolin treatment resulted in significant transcriptional changes in the expression of 255 transcripts (adjusted p value < 0.05, BH Method) (Supplemental Figure 4.1). Of these 255 significantly differentially expressed (DE) transcripts, 79 transcripts had reliable protein annotations (31%). Moreover, 80% of these annotated transcripts exhibited upregulated expression in our treated tissues. Gene ontology (GO) enrichment analysis did not identify significantly overrepresented GO categories (adjusted p value < 0.05, BH method) in treated tissues compared to control tissues.

Among our DE genes, we identified numerous transcripts involved in innate immunity and hematopoiesis, as well as genes involved in the regulation of the nervous system and response to temperature (Figure 4.4). We also identified four 3- to 4-log2fold upregulated transporters: a cationic amino acid transporter [UniprotKB: B5D5N9.1], a copper uptake protein [UniprotKB: Q5RAS6.1], and two protein-coupled folate transporters [UniprotKB: Q6DCX5.1 and Q7ZWG6.1]. Two proteins involved in transcription,
nuclear transcription factor Y subunit alpha [UniprotKB: P23708.2] and MDS1 and EVI1 complex locus protein EVI1-B [UniprotKB: B7ZRM8.1], were also upregulated. For a full list of DE genes, refer to Table 4.2.

The greatest log2fold change in response to increased hemal proctolin was observed in three transcripts annotating to anti-lipopolysaccharide factors (ALFs) [UniprotKB: C0KJQ4.2] (5+ log2fold upregulated) and an SAPK substrate protein [UniprotKB: AEL23050.1] (4+ log2fold upregulated). Both of these protein types have established connections to innate immunity in crustaceans [44, 45]. While it is possible the increased expression of these transcripts was due to the injections themselves, we do not believe this response is due to experimental procedures given that treated and control individuals were handled identically. The control individuals received sterile saline injections and treated individuals received the same procedure and saline solution, only with added exogenous synthetic proctolin.

In addition to ALFs and SAPK proteins, we observed upregulation of immune-related genes DNA repair protein REV1 [UniprotKB: Q4KWZ7.1] and C-type lectin domain family 4 member D [UniprotKB: Q8WXI8.2]. We also observed an upregulation of astakines [UniprotKB: Q56R10.1] (3-log2fold upregulated), cytokines involved in hematopoiesis and innate immunity in crustaceans [46].

Increased hemal proctolin levels also resulted in the upregulation of several genes involved in the regulation of nervous system activity, most notably innexin [UniprotKB: Q9VAS7.1], a protein involved in gap junctions between electrically-connected neurons, and four transcripts annotating to thermosensory transient receptor potential (TRP) channel genes in the A family: two TRPA1 [UniprotKB: Q18297.5] (3+ log2fold upregulated) and two TRP pyrexia [UniprotKB: Q9W0T5.2] (3+ log2fold upregulated). We also observed upregulation of Ankyrin-3 [UniprotKB: Q12955.3], a protein involved in the targeting of ion channels, and a nitric oxide synthase protein [UniprotKB: Q26240.1]. Nitric oxide pathways have been implicated in both neural and immune systems [47, 48]. Finally, we explored whether any of the DE TRP channels were DE across our sampled tissue types and identified one of the TRPA1 transcripts was
significantly upregulated in heart tissues compared to muscle tissues (adj \( p < 0.05, +3 \log_{2} \text{fold change} \)).

**Expression of proctolin receptors in nervous system tissues**

Another goal of this study was to characterize the expression of proctolin receptors in our nervous system, neurosecretory hybrid and muscular tissues. To perform this analysis, we performed a BLAST of the proctolin receptor sequence and known isoforms from *Drosophila melanogaster* and the putative proctolin receptor sequence from *Daphnia pulex* against our transcriptome. Using an e value threshold of 1E-10, this analysis identified five transcripts matching these sequences (51% median percent identity). The expression of these transcripts was not significantly different across proctolin treated and control tissues (ns, adj \( p > 0.05 \)). For tissue type comparisons, one of the transcripts was DE and upregulated in CNS vs. heart tissue (adj \( p < 0.001, +5 \log_{2} \text{fold change} \)) and CNS vs. muscle tissue (adj \( p < 0.001, +4 \log_{2} \text{fold change} \)). All other transcripts were not significantly different across any contrast, though some transcripts exhibit greater expression levels in particular tissue types relative to others (Figure 4.5).

It is not clear from this study which of these sequences might be a functional receptor for proctolin. All transcripts had significant matches (e value < 1E-4) to either the GPCR for FMRFamide [UniprotKB: Q9VZW5.1] or a putative rhodopsin-like GPCR [UniprotKB: XP_002426691.1] in our initial BLAST annotation of the *H. americanus* transcriptome. For multiple sequence alignment of our *H. americanus* transcripts, the FMRFamide and rhodopsin-like GPCRs, and proctolin GPCR isoforms, refer to Figure 4.6. The similarity between the putative proctolin receptor transcripts and FMRFamide receptors was unsurprising given that GPCRs can share common structural features and display high sequence identity within families (>35%) [49]. Phylogenetic analysis of neuropeptide receptors in *D. melanogaster* identifies FMRFamide and proctolin receptors as nearest neighbors, along with receptors for myosopressin [50]. In addition, these receptors may be promiscuous in their affinity to neuropeptides with FMRFamide treatment blocking the excitatory effects of proctolin [51].

**Co-expression of neuromodulators with proctolin in nervous system tissues**
To determine which neuromodulators and neuromodulator receptors are co-expressed with our differentially expressed (DE) putative proctolin receptor transcripts, we selected other neuromodulators that were also DE and upregulated across the same contrasts. Our DE putative proctolin receptor was upregulated in CNS vs. heart tissue (adj \( p < 0.001 \), +5 log2fold change) and CNS vs. muscle tissue (adj \( p < 0.001 \), +4 log2fold change). Of our transcripts annotating to known neuropeptides and neuromodulator receptors or synthesis/degradation pathways, we selected those transcripts that were also DE and upregulated in CNS vs. heart tissue and CNS vs. muscle tissue. We then restricted our analysis to those transcripts that had a greater than 40% ratio of DE transcripts to total transcripts by gene function (i.e. GABA transporter) (Supplementary Table 4.2).

This analysis revealed that the putative proctolin receptors were co-expressed with crustacean cardioactive peptide (CCAP), three types of allostatin, orcokinin, and two transcripts annotating to FMRF-amide like neuropeptides (Figure 4.7). Our putative proctolin receptor was also co-expressed with prohormone-1, RPCH, and two types of tachykinins. For a full list of DE transcripts, see Table 4.3.

**Discussion**

*Hemal proctolin across gender, body size and blood volume*

Using these techniques, we were successful in isolating and quantifying proctolin from the hemolymph of *Homarus americanus*. To our knowledge, only two studies have quantified hemal proctolin levels in invertebrate systems. One study identified 0.29 pmol/mg of protein in the hemolymph of the adult Madera cockroach, *Leucophaea maderae* (n=1) [52]. In cockroaches, this value reportedly converts to an estimated \( 10^{-9} \) M concentration [15]. If this ratio was accurate for *H. americanus*, it would convert to 1.29x\( 10^{-8} \) M based on the mean total protein content in *Homarus americanus* hemolymph (n=500; 4.45 ± 0.09 g dL\(^{-1}\) [53]. Another study, in the female locust *Locusta migratoria*, hemal proctolin varied between 107.3 ± 11.7 fmol/ml (n = 5) for virgin (non-egg-laying females) and 204.4 ± 15.4 fmol/ml (n = 6) for hemolymph egg-laying females
[54]. These concentrations (100-200x10^{-12} M) for *L. migratoria* are within the range we observed in *H. americanus* hemolymph; by contrast, the levels observed in *L. maderae* are much higher than *H. americanus*. In regards to differences across gender, we note that hemal proctolin concentrations were not significantly different across sex. Previous work in *L. migratoria* suggests proctolin may play a role in reproduction. Proctolin receptors are located on the oviduct membrane in *L. migratoria*, and proctolin stimulates oviduct contraction [55, 56]. The higher titres in *L. migratoria* egg-laying females compared to virgin females support this link [54]. However, our data report no differences between males and females and, consequently, do not support a direct connection between proctolin levels and gender in the lobster.

Additionally, hemal proctolin concentration and body weight were not correlated (Figure 4.2). Because body weight can be used to estimate blood volume, this result indicates there is not a relationship between proctolin concentration and blood volume.

Overall we infer no link between hemal proctolin levels and blood volume or gender. We propose, given the wide variation observed in proctolin concentration across individuals, that other factors may be stronger indicators of proctolin concentration. These factors could include both physiological conditions, as well as environmental constituents, and provide many possible pathways for future research.

**Novel role for proctolin in the innate immune system**

Along with quantifying the basal levels of hemal proctolin, we wanted to understand how fluctuations in these levels can regulate changes in transcription of mRNA. Increased hemal proctolin levels *in vivo* correlated with the differential expression of 79 annotated transcripts. The greatest upregulation in expression was observed in ALFs, which have been identified in the lobster and other crustaceans as part of the innate immune system [57, 58]. Marine crustaceans lack adaptive immune mechanisms, possessing only an innate immune system based on self/nonself recognition and cellular and humoral processes [59]. In this system, antimicrobial peptides and proteins play a large role in host defense [60]. ALFs are one such peptide; ALFs were first isolated in the hemocytes
of horseshoe crabs *Limulus polyphemus* and characterized for their potent antimicrobial abilities [61]. They have since been identified in a number of marine crustaceans, including *H. americanus* [58, 62, 63]. These compounds are synthesized and stored in the hemocytes for release into the hemolymph [44, 58, 64]. Here we see hemal proctolin levels upregulate the transcription of ALFs, as well as a stress activated protein kinase and other proteins important to crustacean immunity, implicating a novel role for hemal proctolin in innate immune pathways.

To our knowledge, the connection between proctolin and innate immune pathways has not been previously explored; however, the relationship between other neuromodulators, like octopamine and serotonin, and immune responses are well-documented in invertebrate systems [65]. Octopamine levels increase in the hemolymph upon an immune challenge [66]. Elevated octopamine levels increase total hemocyte counts, as well as increase nodule formation, enhance inositol trisphosphate (IP$_3$) production in hemocytes, and increase phagocytosis [67-69]. Serotonin (5-hydroxytryptamine) also regulates phagocytosis, hemocyte production, and nodule formation [68-70]. The ability of octopamine to regulate immune responses is mediated by G-protein coupled receptors (GPCRs) and second messenger systems [71-73]. The identified receptor for proctolin is also a GPCR, suggesting a similar mechanism by which proctolin may regulate immune response [74].

*Upregulation of thermosensory TRP channels link proctolin to thermoreception*

A surprising result was the 3-fold upregulation of two types of TRP channels, TRPA1 and TRP pyrexia, as a result of increased hemal proctolin. TRP channels are a superfamily of cation channels with a wide range of activation mechanisms spanning from external (light, sound, temperature) to internal (osmolarity) stimuli [75, 76]. Even a single channel can vary in its specificity and react to multiple stimuli. TRPA1 channels, for instance, can react to both chemical cues (cannabis, citonella) [77, 78] and mechanical stimuli [79]. There is also a wide body of evidence implicating TRPA channels in thermoreception, particularly in invertebrates [80].
The role of TRP channels as thermosensors is evolutionarily conserved across species [80]. In humans, TRPV, TRPM and, recently, TRPA channels have been linked to temperature sensing [81-84]. For poikilotherms, the fruit fly *D. melanogaster* is the predominant model for studies on TRP channels and has four TRPA channels: TRPA1, pyrexia (pyx), painless (pain), and waterwitch (Wtrw) [80, 85]. Of these, TRPA1, pyx and pain are involved in temperature sensing. TRPA1 is essential for warm avoidance 25-29º C or sensing of comfortable temperatures (18-24º C), but not cool avoidance [86-88]. Pyrexia and painless are involved in sensing of noxious warm temperatures. Painless is activated at temperatures greater than 42º C; pyrexia is activated at temperature ~ 40º C and is important for tolerance for noxious warm temperatures [89, 90].

The temperature activation ranges of TRPA channels in the lobster *Homarus americanus*, compared to the terrestrial fruit fly *D. melanogaster*, are of particular interest. The preferred temperature and critical thermal temperatures of *H. americanus* are lower than *D. melanogaster*. The preferred temperature is 16º C [91, 92] compared to 25º C [93]; the approximate critical thermal maxima (defined as the upper temperature at which normal locomotor activity is compromised) is 29º C [94] compared to 41º C [95]³. The activation ranges for pyrexia and painless determined in *D. melanogaster* exceed habitable temperatures for *H. americanus*. Thus, if these channels are thermosensors in *H. americanus*, it is likely that the temperature sensing ranges of these channels differ between the species. Additionally, the temperature responsiveness between isoforms of TRPA1 can vary [87]; here we see two potential isoforms in the transcriptome of *H. americanus* that were upregulated by proctolin, which may indicate disparate coverage in activation temperatures as well.

Furthermore, the connection between proctolin and transcriptional regulation of TRPA channels is particularly interesting given the interest in neuromodulators on reliable

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³ Critical thermal maxima (CT\textsubscript{max}) was determined by cardiac assay for *H. americanus* and knockdown temperature for *D. melanogaster*. It is important to note that procedural differences between methods can result in small variations in the determination of CT\textsubscript{max}.
circuit function. Neuromodulators can reconfigure neural circuits, altering firing frequency, phase dynamics, synaptic strength, and other parameters (for recent review, see Marder 2015) [96]. Neuromodulators can also differentially regulate expression of ion channels transcripts and conductance levels in a cell-specific manner [11, 12]. Our results indicate proctolin upregulates transcription of TRPA channels that play an important role in membrane potential and signal transduction. Examining the conditions upon which hemal proctolin increases, and thereby upregulates expression of TRPA, may be critical to understanding the compensatory mechanisms at play in maintaining reliable nervous system function. Finally, our results pose a possible explanation for the abundance of neuromodulators (and associated isoforms) identified in crustaceans with apparently redundant functional effects on nervous system activity: these molecules have disparate targets for gene expression changes. In other words, mechanistic changes resulting from release of one or the other neuromodulator under a particular condition may result in effects that cannot be observed through experimentation with these neuromodulators in vitro and over acute time periods. Future studies exploring the release of neuromodulators as signaling molecules in time frames relevant to transcriptional or translational regulation will improve our understanding the maintenance of reliable network function.

Localization of proctolin receptors and co-expression with other neuromodulators

Examining the expression of putative proctolin receptors identified probable downstream targets of hemal proctolin. Our data reveal expression of putative proctolin receptors in every tissue sampled: heart, abdominal muscle, abdominal ganglia and supraesophogeal ganglia (or ‘brain’). Our data also reveal expression of TRP channels in all tissues sampled, with one TRPA1 transcript DE in heart compared to muscle tissues. These expression patterns suggest all tissues may be targets of transcriptional regulation of TRPA channels by proctolin, with perhaps particularly increased expression of TRPA1 in the heart.

Of the five transcripts we isolated as putative proctolin receptors, one transcript exhibited
significant upregulation across particular tissue types: CNS vs. heart tissue and CNS vs. muscle tissue. We used this expression profile to select for similar expression patterns in receptors for other neuromodulators. This search identified tachykinin and dopamine as other signaling molecules that share the same targets as proctolin. We also identified a number of neuropeptides co-expressed with the DE putative proctolin receptor, including CCAP, allostatin (AST), orcokinin, FMRF-amide like neuropeptides, prohormone-1, and RPCH. These results suggest candidate neuropeptides with functional importance to the modulation of target tissues for proctolin.

Conclusions

This study identified proctolin in the hemolymph of the lobster *Homarus americanus* at a mean concentration of 1.54E-10 M. In the *H. americanus* transcriptome, putative proctolin receptor mRNA was identified and located in heart, abdominal muscle, abdominal ganglia, and brain tissues. Co-expressed receptors for other circulating neuromodulators (tachykinin, dopamine) were also localized across tissue type comparison, as well as expression of neuropeptides (CCAP, AST, FMRF, RPCH). RNA-Seq across proctolin-treated and untreated tissues revealed that increase of hemal proctolin in vivo resulted in the transcriptional regulation of several genes important to innate immunity in crustaceans. These results identify proctolin as another neuromodulator, alongside octopamine and serotonin, with links to regulation of immune response. We also observed that hemal proctolin upregulates the expression of TRPA family cation channels, which act as thermosensors in other systems, unveiling a potential interplay between proctolin and the thermoregulatory behavior of the lobster.

Acknowledgements

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Figure 4.1. Detection of endogenous proctolin in the hemolymph of *H. americanus*. Samples were processed on an Orbitrap Fusion mass spectrometer using a targeted SIM method centered around the heavy (659.37) and light (649.36) proctolin masses. Endogenous proctolin was calculated by comparing the ratio between the integrated areas of the heavy and light peaks.
Figure 4.2. Concentration (M) of proctolin in the hemolymph of wild caught *H. americanus* is not significantly different across females (F) and males (M).
Figure 4.3. Concentration (M) of proctolin in the hemolymph of wild caught *H. americanus* compared to body weight of the individual.

Figure 4.4. Significantly differentially expressed transcripts across proctolin treated and untreated tissues (adj $p < 0.05$). Red dots highlight transcripts annotating to anti-lipopolysaccharide factors (ALF), and TRP A channels TRPA1 and pyrexia (PYX).
Figure 4.5. Heatmap displaying expression levels of putative proctolin receptors across tissue type in *Homarus americanus*. Red boxes indicate greater expression levels compared to blue boxes. In column labels indicate: CNS – central nervous system, SG – supraesophageal ganglia (brain), AG – abdominal ganglia.
Figure 4.6. Rooted phylogenetic tree of ClustalW multiple sequence alignment. Contigs are from our *Homarus americanus* transcriptome are aligned against selected nucleotide sequences from NCBI. All sequences are from *Drosophila melanogaster* unless otherwise indicated. Sequences include: proctolin receptor variants A-E [AHN59339.1, AAX52478.1, AAX52477.1, AAN09130.1, AAF45980.2] and FMRFamide receptor [NM_001274418.2]. Sequences from other arthropods include: *Daphnia pulex* putative proctolin GPCR [GenBank: GL732542.1] and *Pediculus humanus corporis* rhodopsin-like GPCR [XM_002426646.1].
Figure 4.7. Heatmap displaying neuromodulator pathway transcripts co-expressed with our DE putative proctolin receptor (indicated with arrow). Red boxes indicate greater expression levels compared to blue boxes. In column labels indicate: CNS – central nervous system, SG – supraesophageal ganglia (brain), AG – abdominal ganglia.
Supplementary Figure 4.1. Histograms depicting the distribution of molar hemal proctolin concentration in male (M) and female (F) populations.

Supplementary Figure 4.2. Volcano plot of differential gene expression across proctolin treated and control tissues performed in DESeq2. Red dots indicate significantly differentially expressed transcripts (adj $p < 0.05$).
Table 4.1. Proctolin concentration (M) in hemolymph of wild caught Homarus americanus.

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Table 4.2. Significantly differentially expressed (adj $p < 0.05$) transcripts in proctolin treated tissues compared to control tissues.

<table>
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<th>adj $p$</th>
<th>Log2fold</th>
<th>Annotation</th>
<th>UniprotKB</th>
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<td>SAPK substrate protein 1-B</td>
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<td>Putative fed tick salivary protein 7 [Rhipicephalus pulchellus]</td>
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</table>
Table 4.3. Neuromodulator pathway transcripts co-expressed with our DE putative proctolin receptor, meaning transcripts are DE and upregulated in CNS vs. heart and CNS vs. muscle contrasts. Displayed transcripts only include those in functional categories with over 40% of transcripts DE across these contrasts. For full list of functions and transcript counts, see Supplementary Table 4.1.

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<th>Neurotransmitter</th>
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<th>CNS vs. muscle</th>
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<td>Prohormone-1</td>
<td>Red pigment-concentrating hormone</td>
<td>NA</td>
<td>7.63E-03</td>
<td>4.75</td>
</tr>
<tr>
<td>RPCH</td>
<td>Tachykinin-like peptides receptor 99D</td>
<td>43551</td>
<td>1.34E-02</td>
<td>2.46</td>
</tr>
<tr>
<td>Tachykinin</td>
<td>Tachykinin-like peptides receptor 99D</td>
<td>43551</td>
<td>3.54E-04</td>
<td>5.94</td>
</tr>
<tr>
<td>Tachykinin</td>
<td>Tachykinin-related peptide 5</td>
<td>41456</td>
<td>2.14E-02</td>
<td>4.20</td>
</tr>
</tbody>
</table>
**Supplementary Table 4.1.** Samples prepared for Illumina sequencing, depicting tissues and treatment groups. AG – abdominal ganglia. SG – supraesophageal ganglia (brain). CNS – central nervous system tissue.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Sample</th>
<th>Tissue Type</th>
<th>Individual</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>AG</td>
<td>CNS</td>
<td>B</td>
</tr>
<tr>
<td>Control</td>
<td>AG</td>
<td>CNS</td>
<td>A</td>
</tr>
<tr>
<td>Control</td>
<td>Heart</td>
<td>Heart</td>
<td>B</td>
</tr>
<tr>
<td>Control</td>
<td>Muscle</td>
<td>Muscle</td>
<td>B</td>
</tr>
<tr>
<td>Control</td>
<td>Muscle</td>
<td>Muscle</td>
<td>A</td>
</tr>
<tr>
<td>Proctolin</td>
<td>AG</td>
<td>CNS</td>
<td>C</td>
</tr>
<tr>
<td>Proctolin</td>
<td>Heart</td>
<td>Heart</td>
<td>C</td>
</tr>
<tr>
<td>Proctolin</td>
<td>Heart</td>
<td>Heart</td>
<td>D</td>
</tr>
<tr>
<td>Proctolin</td>
<td>Muscle</td>
<td>Muscle</td>
<td>C</td>
</tr>
<tr>
<td>Proctolin</td>
<td>SG</td>
<td>CNS</td>
<td>C</td>
</tr>
</tbody>
</table>

**Supplementary Table 4.2.** Transcripts co-expressed with proctolin receptor and presented in Figure 4.7 and Table 4.3. Count is number of transcripts with annotations belonging to each functional group. DE indicates the number of transcripts significantly differentially expressed (adj p <0.05) within that group. Only transcripts within functional groups with over 40% of transcripts DE are included in proctolin co-expression heatmap and table.

<table>
<thead>
<tr>
<th>Function</th>
<th>Count</th>
<th>DE</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCAP</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Conversion of dopamine to noradrenaline</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Catalyzes the conversion of GABA</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Brain peptide SYWKQCAFNAVSCF-amide</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>RPCH</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Tachykinin</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>ACH transporter</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>AST</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>GABA transporter</td>
<td>12</td>
<td>7</td>
</tr>
<tr>
<td>Hydrolysis of ACH</td>
<td>6</td>
<td>3</td>
</tr>
<tr>
<td>Dopamine receptor</td>
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<td>3</td>
</tr>
<tr>
<td>FMRFamide-related neuropeptide</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>Orcokinin</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>Tachykinin receptor</td>
<td>5</td>
<td>2</td>
</tr>
</tbody>
</table>
Chapter 5: Conclusions and recommendations

Sections
A. Recap of experimental aims and findings
B. Future directions in temperature and crustacean physiology
C. Future directions in molecular and synthetic biology

Recap of experimental aims and findings

Aim 1: Behavioral thermoregulation in Cancer borealis, and effects of acclimation on temperature preference

This study addressed the thermoregulatory behavior of the Jonah crab (Cancer borealis), an important species for its role as an economically valuable fishery and a well-studied model for nervous system activity. C. borealis is thermotactic; it behaviorally thermoregulates and adjusts the direction and magnitude of its movements towards an environmental affordance that permits maintenance of a preferred body temperature. Moreover, the preferred temperature ranges of this species are significantly impacted by previous acclimation temperature [1]. The significance of these findings is multifaceted. First, C. borealis actively makes taxic decisions based on temperature. Second, previous experience significantly impacts preferred temperature, suggesting a physiological state change based on acclimation that also impacts decision-making. Finally, in our studies, C. borealis prefers 15.4°C. This matches the optimal living temperature of the species.

Aim 2: Homarus americanus Transcriptome, Differential Gene Expression in Tissue Types

This study established a molecular framework for understanding central pattern generation in the model organism, Homarus americanus (American lobster) by de novo assembly of the H. americanus transcriptome and characterization of gene expression patterns across CNS, heart and muscle tissues. Alignment of the lobster transcriptome resulted in 115,757 contigs, with 30% annotating to known proteins (e value 1E-4). In a
principal component analysis, tissue type explained a greater proportion of the variation than proctolin-treatment or differences across individuals. Therefore, the greatest contributor to gene expression differences between samples was the tissue from which the sample originated. Overall, differences between tissue types accounted for significant differential gene expression of 13-25% of the transcriptome (adj $p < 0.05$).

Genes involved in ‘response to heat’ are overrepresented in heart tissue, suggesting a possible site and candidate genes (including TRP A channels and specific heat shock proteins) for thermoreception in this poikilotherm.

Analysis of DE genes indicated tissue-specific patterns of increased expression of neuromodulators in the heart (octopamine), muscle (myomodulin), and nervous system (RPCH, AST, tachykinin, FLRFamide), as well as tissue-specific variation in expression of octopamine and NO. This study also identified several neuronal factors contributing to the identity of our abdominal and cardiac systems. For the abdominal network, we observed significantly increased expression of acetylcholine receptors, calcium-activated Cl$^-$ channels, and K$^+$ channels $slo-1$/slowpoke, and significantly increased expression of neuromodulators CCAP and AST. For the cardiac network, we observed increased expression of the neuromodulators myomodulin and octopamine, and kainite family glutamate receptors and inward-rectifying K$^+$ (IRK) channels.

Overall, this study revealed genes underlying the differentiation of neural networks and their production of stereotyped motor patterns. It revealed dozens of candidate genes that may provide fruitful avenues for future research. Interestingly, one of our candidate genes, the calcium-activated K$^+$ channel slowpoke, has recently been validated for its critical importance to the generation of rhythmic locomotion. The role of slowpoke in myogenic central pattern generators was examined in flight activity of the fruit fly Drosophila melanogaster. This study demonstrated slowpoke knockout mutants have a drastically decreased ability to initiate flight, but fly normally if flight begins [2]. The implication that slowpoke is imperative to initiation, but not maintenance, of rhythmic motor activity corroborates our finding in H. americanus where this gene is upregulated in the
intermittently active abdominal system compared to the continually active cardiac system. Similar experiments in knockouts for kainite family glutamate receptors and IRK channels may provide important insight into the role of these membrane components in continually active rhythmic motor pattern generating networks.

Finally, identification of transcription factor proteins that act as terminal selector genes in the differentiation of neuronal phenotypes paves the way for studies of transcriptional regulation by promoter regions or epigenetics in this system. The American lobster is well studied because of the phenotypic accessibility of its neural circuits for electrophysiological and pharmacological inquiries. By assembling a transcriptome and identifying conserved genetic features, this work provides an important substrate for future experiments merging electrophysiological and molecular approaches.

**Aim 3: Identification and quantification of hemal Proctolin, and its ability to regulate gene expression in the nervous system of Homarus americanus**

Proctolin is a neuropeptide with well-described excitatory neuromodulatory properties; however, to date its presence as a circulating hormone and role in gene expression are unknown. We identified proctolin in the hemolymph of the lobster *Homarus americanus* at a mean concentration of $1.54 \times 10^{-10}$ M, and in the range of $6.26 \times 10^{-10}$ to $1.82 \times 10^{-13}$ M. These observed concentrations reach the threshold level for excitation of nervous system activity, but are lower than expected. In previous studies, the lowest threshold concentration for excitation of nervous system activity *in vivo* and *in vitro* is $10^{-10}$ M [3], with other calculations of threshold concentrations for varied motor patterns ranging from $10^{-9}$ to $10^{-4}$ M [4-7]. A possible explanation for the discordance between threshold and hemal concentrations could be the uptake and storage in nervous system tissues, for local release at higher concentrations. This explanation is corroborated by tissue levels previously determined on the order of $10^{-5}$ M [8]. Another non-exclusive explanation is the rapid release and degradation of proctolin at time points or conditions not observed in this study (n=19). An increase in sample size and inclusion of, rather than controlling for,
individuals with varied physiological backgrounds (e.g. diet, molt cycle) may both identify hemal levels closer to threshold concentrations and also elucidate mechanisms behind the variation we observed in hemal proctolin concentrations.

In the *H. americanus* transcriptome, putative proctolin receptor mRNA was identified and located in heart, abdominal muscle, abdominal ganglia, and brain tissues. RNA-Seq across proctolin-treated and untreated tissues revealed that increasing hemal proctolin *in vivo* resulted in the transcriptional regulation of several genes important to innate immunity in crustaceans. These results indicate proctolin as another neuromodulator, alongside octopamine and serotonin, with links to regulation of immune response and open up interesting possibilities regarding the interconnectedness of the nervous and immune systems. We also observed that hemal proctolin upregulates the expression of the TRPA family of cation channels, which act as known thermosensors in other systems, unveiling a potential interplay between proctolin and the thermal behavior of the lobster. Overall, this study identifies proctolin is a circulating neuromodulator or ‘neurohormone’—finally ending conjecture that began in the early 1980s—and defines a framework for its hemal concentration at $10^{-10}$ to $10^{-13}$ M. Proctolin’s role as a circulating modulator may be tied to transcriptional regulation in both the nervous and immune systems.

*Future Directions in Temperature and Crustacean Physiology*

As a whole, the experiments performed in this dissertation work unveiled interesting interactions between temperature and crustacean physiology and behavior. As poikilotherms, temperature indisputably plays an important role in the physiology and behavior of marine crustaceans like the American lobster and the Jonah crab. The Jonah crab inhabits waters along the Atlantic coast from Newfoundland to Florida, ranging temperature from 2 to 24°C [9, 10]. The American lobster is found from Newfoundland to North Carolina, and generally inhabit waters between 5 and 20°C though it can tolerate
temperatures between -1 to 35 °C [11]. Temperature impacts everything from growth and reproduction [11, 12] to cardiac and nervous system performance and thermal limits [13-16]. In particular, acclimation to warmer temperature allows the cardiac system to function at higher temperatures compared to individuals acclimated to cool temperatures [15, 16]. Likewise, in the STNS, the pyloric rhythm is able to maintain remarkable phase compensation across a wide range of temperatures; however, this upward limit of this range differs depending on thermal history. In cool acclimated crabs, nervous system activity “crashes” at temperatures that are permissive in warm acclimated crabs [13, 14]. Despite physiological interest in temperature effects, the question remained: do these animals “care” about temperature? Temperature preference assays are a straightforward way to address the nature of this question [17-20]. Using this approach, this dissertation work confirmed that C. borealis makes decisions based on temperature, and that these decisions are dependent upon previous acclimation temperatures [1]. Furthermore, subsequent experiments on transcriptomic profiles of tissue types in H. americanus identified possible mechanisms for thermoreception. To date, the mechanism for temperature sensing in decapod crustaceans (crabs, lobsters) is unknown, despite evidence that these animals can sense temperature changes of less than 0.5°C [21]. This temperature sensitivity was conducted via cardiac assay [21]; other work examines the claw [22] and the pericardial organs [23] as sites for thermoreception.

We found a significant overrepresentation of genes in the GO category “response to heat” (GO:0009408) (adj $p < 0.05$) in heart tissue vs. muscle tissue. Included in this category were several forms of heat shock proteins, and transient receptor potential (TRP) channels pyrexia and painless (Entrez Gene IDs: 38037, 37985). Overrepresentation of genes involved in temperature sensing in the heart tissue compels two non-exclusive interpretations. First, the heart could be the major organ for thermoreception in crustaceans. While this is possible, the antennae is a more likely candidate given its role in olfaction and mechanoreception, and the thermosensory capabilities of antennae in flying insects are well-documented [24, 25]. Second, thermoreception could be integral to the nervous system itself. In mammals, thermosensory TRPV channels are expressed in a wide range of tissues [26]. Because TRPA channels in invertebrates are homologs of
mammalian TRPV channels and play well documented roles in thermoreception, expression of these channels in the nervous systems we assayed (heart, abdominal ganglia, supraesophageal ganglia) suggests these central pattern generating nervous systems may receive temperature cues directly as well as from other sources (such as via the antennae). Finally, we identified proctolin-regulated TRPA channels, establishing a novel connection between this circulating neuromodulator and thermosensation. At present, the interplay between neuromodulators and temperature is of particular interest. The circulating neuromodulator serotonin changes the amplitude of inhibitory junction potentials in a temperature-dependent manner and shifts the temperature at which they change polarity by ~7 °C [27]. Proctolin stabilizes STNS rhythms at temperatures that would otherwise exceed thermal limits and result in a “crashing” of the STNS rhythm [28].

In unpublished work conducted as a part of this dissertation, I examined the fluctuation of hemal proctolin across seasons and temperatures using methods previous described in Chapter 4. Hemal proctolin was significantly different across trials conducted on wild-caught lobster populations (F/V Jacqueline Bess, Nahant, MA) from December 2013 to January 2015 (One-way ANOVA, F(3,5) = 7.36, p < 0.01). Post-hoc tests using the Tukey–Kramer method revealed a significant difference between the May 2014 trial and the December 2013, February 2014, and January 2015 trials (p < 0.05). As temperature varied between the trial conducted in May (13°C) and the winter months (2-7°C), I performed an experiment analyzing the effect of acclimation temperature on hemal proctolin. Wild-caught winter lobsters were collected (January 2015; F/V Jacqueline Bess, Nahant, MA) and subjected to 2-week temperature acclimation at 7°C and then 17°C. Hemolymph was collected after each acclimation period. There was no significant difference between cold acclimation treatment (M=1.60E-12, SD=1.05E-12) and warm acclimation treatment (M=3.21E-12, SD=2.7E-12) (Paired t-test, t(2) = -0.32, p = 0.78). These results indicate hemal proctolin in wild-caught populations can vary significantly across trials; this variation may be induced by changes in season but is not due to water temperature alone. Future experiments may elucidate whether light-dark cycles, water
temperature, and other seasonal factors interact to increase or decrease hemal proctolin concentrations in natural populations.

Collectively these experiments provide ample framework for future investigations into the interplay between proctolin, thermoreception, and temperature. First, methods development for quantifying proctolin in hemolymph would improve resolution in the fluctuation of peptide levels across time course trials. Specifically, development of a microdialysis system to systematically withdraw hemolymph samples without physical intervention over periods of activity vs. inactivity, and changes in acute temperature, acclimation temperature, or light cycle would improve our understanding of how proctolin fluctuates over acute and persistent time frames and conditions.

Next, investigating thermosensitive TRPA channels in central pattern generators will help us understand these networks and their ability to support robust rhythmic activity across wide thermal ranges. Future experiments should describe distribution of TRPA channels across both sensory systems (antennae, dactyl walking legs) and networks of command and motoneurons (circumesophogeal ganglia, STNS). Additional experiments should investigate the functional significance of these channels in response to temperature by conducting electrophysiological recordings on an isolated central pattern generating nervous system in vitro (i.e. STNS) while blocking TRPA channels and slowly ramping up temperature. These experiments address whether TRPA channels are necessary for phase compensation in response to increased temperature. They also explore whether temperature response in central pattern generators relies on thermoreception mechanisms present within these networks, rather than descending inputs from sensory appendages, and inform our overall understanding of how this network and this animal respond to temperature.

Future Directions in Molecular and Synthetic Biology

Assembling the lobster transcriptome, identifying candidates for differentiation of nervous system tissues from muscle tissues and between two types of nervous system
tissues (abdominal ganglia, heart), and describing how hormonal regulation can alter
gene expression in these tissues paves the way for future molecular studies and provides a
“toolkit” needed to build neural networks using synthetic biology.

The central pattern generating nervous systems of the decapod crustaceans, specifically
the stomatogastric nervous system, are one of the few model systems where connectivity
and network function can be rigorously examined at the level of cellular dynamics and
also translated into output behaviors [29]. The organisms most commonly studied for
these model systems include the crayfish Procambarus clarkii, the clawed lobsters
Homarus americanus and Homarus gammarus, the spiny lobsters Panulirus interruptus
or Panulirus argus, and the crabs Cancer borealis and Cancer irroratus. However, to
date, none of these organisms have a published genome. The closest published genome to
these crustaceans is the water flea Daphnia pulex [30]. To our knowledge, the closest
published transcriptomes are for a handful of crustaceans including the Eastern rock
lobster Sagmariasus verreauxi [31], the crayfish Procambarus clarkii [32], green crab
Carcinus maenas [33], the blue crab Callinectes sapidus [34], the Chinese mitten crab
Eriocheir sinensis [35], the green mud crab Scylla paramamosain [36], the swimming
crab Portunus trituberculatus [37], the banana shrimp Fenneropenaeus merguiensis [38],
and the amphipod Parhyale hawaiensis [39]. The assembly and publication of the H.
americanus transcriptome provides a genetic resource for new research on the biology of
the lobster, including designing of primers for qPCR/RT-PCR and identifying targets for
RNAi. Moreover, isolation of the neuropeptidome and differential expression of
neuromodulator, receptors, and other gene products involved in neuromodulation across
tissue types provides baseline information for electrophysiological or molecular studies
on co-expression of neuromodulators or constellation pharmacology [40].

Finally, these studies have ramifications in the emerging field of synthetic biology, the
engineering of biomolecular systems or cellular capabilities [41, 42]. In particular,
describing gene expression signatures across nervous system tissues and how
perturbations like chronic neuromodulation shape these genetic constructs lay the
groundwork for larger scale research programs using the principles of central pattern
generating nervous systems in the lobster to design neurons and functional neural networks using synthetic biology. Specifically, use of these technologies can progress into establishing genetic differences across neuronal phenotypes at a single cell level, and then testing these principles through the designing and creation of synthetic neural circuits. In other words, synthetic biology has developed an important means to “understand life by building it”, and defining the compulsory genes for creation of the neurons composing a neural circuit is the first step to pushing forward our understanding of these dynamic systems through this innovative approach [43].
References


Figure 5.1. **A.** Effects of season on hemal proctolin concentration in wild-caught lobster populations. **B.** Effects of 2-week temperature acclimation on hemal proctolin in winter lobsters, January 2015 (ns, $p > 0.05$).