ABSTRACT

Title of Thesis:	INVESTIGATING SEXUAL MATURITY IN MALE JONAH CRAB, *CANCER BOREALIS,* IN SOUTHERN NEW ENGLAND
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Fisheries management requires a thorough understanding of the processes involved in reproduction, including the ability to distinguish sexually mature individuals; understanding the physiological processes driving an individual to become sexually mature is equally as important. The Jonah Crab, *Cancer borealis,* contributes to a significant and growing male-driven fishery along the Atlantic coast of North America primarily from Newfoundland, Canada to Florida; however, little is known about their life history. This study focuses on investigating the relationship between morphometric and physiological maturity, and the size at which these life changes occur in the Southern New England stock. We also aim to understand better how the male sex differentiation hormone, insulin-like androgenic gland hormone, IAG, and its transcript levels relate to size in male *C. borealis*. The size at 50% sexual maturity (SM$_{50}$) in male *C. borealis* was estimated morphometrically to be at 105.9 mm carapace width (CW). This is larger than the estimate of 98.3 mm CW for Jonah crabs

in the Mid-Atlantic Bight, but lower than the estimate of 127.6 for *C. borealis* on the Scotian Shelf, consistent with a poleward gradient in size at maturity. Gonadosomatic index (GSI) differed significantly between CW size groups, maturity status, and season. Isolation of cDNA encoding *C. borealis* (*Cab-IAG* cDNA) was obtained from initial screening of AG transcriptome data and traditional cloning using 5', 3', Rapid Amplification of cDNA Ends (RACE). The partial IAG sequence obtained was 351 bps in length and 117 amino acids long; the putative amino acid sequence was structured similarly to other crustacean IAGs. Transcript levels were determined by qRT-PCR assays and compared to size and maturity status. The AGs originating from the ablated animals contain IAG transcripts at much higher levels than the intact animals using both transcriptome data and qRT-PCR assay. IAG transcript levels (copies/ug AG total RNA) of the animals with various CWs do show a significant difference, indicating IAG protein levels in the hemolymph could be a potential candidate of the physiological biomarkers for sexual maturity. Fisheries managers should use multiple measures of sexual maturity and consider factors including geographical distribution when establishing and assessing guidelines for this economically important species.

INVESTIGATING MORPHOMETRIC AND PHYSIOLOGICAL MATURITY ALONGSIDE INSULIN-LIKE ANDROGENIC GLAND (IAG) EXPRESSION AND SIZE IN MALE JONAH CRAB, *CANCER BOREALIS*, IN SOUTHERN NEW ENGLAND

by

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Preface

Chapter 2 of this thesis will be submitted for publication in the Journal of Crustacean Biology and is formatted under the guidelines specified for that journal.

Acknowledgements

Table of Contents

List of Tables

List of Figures

List of Abbreviations

AG   Androgenic gland

bp   Base pair

AGH   Androgenic gland hormone

ANOVA   Analysis of Variance

ANCOVA   Analysis of Covariance

cDNA   complimentary Deoxyribonucleic Acid

CW   Carapace width

GIH   Gonad-inhibiting hormone

GSI   Gonadosomatic index

GSP   Gene specific primer

gDNA   genomic Deoxyribonucleic

IAG   Insulin-like androgenic gland hormone

IMET   Institute of Marine Environmental Technology

MIH   Molt-inhibiting hormone

MTXO   Medulla terminalis

MTXO-SG   Medulla terminalis X-organ – sinus gland complex

NaK   Sodium, Potassium ATPase

qRT-PCR   quantitative Real Time Polymerase Chain Reaction

RNA   Ribonucleic Acid

RNAi   Ribonucleic acid interference

RSEM   RNA-Seq by Expectation-Maximization

SM50   Size at 50% maturity

VTG   Vitellogenin

Chapter 1: Introduction

Jonah crab, *Cancer borealis* is a data-poor species distributed along the Atlantic coast of North America as far north as Newfoundland and as far south as Florida (Haefner, 1977; Stehlik et al., 1991). Jonah crab are primarily found between 50 and 300 m at temperatures between 8 and 14 °C (Robichaud, 2000; Moriyasu et al., 2002). In the U.S., Jonah crab are landed as a bycatch of the American lobster trap fishery and have been for over 80 years. In the last 15-20 years, Jonah crab landings have increased significantly, due to the decline in Southern New England lobster (ASMFC, 2020). The majority of landings, over 95 %, occur in the New England region, specifically from Massachusetts and Rhode Island (ASMFC, 2020).

The Jonah crab fishery is managed under the Interstate Fishery Management Plan, which has set a 4.75-inch carapace length minimum size for Jonah crab being landed. Landings of undersized crabs and egg-bearing females are prohibited within state waters (ASMFC, 2020). Federal implementation of these regulations were established in December of 2019 (ASMFC, 2020). No broad range stock status assessment of the fishery currently exists in either state or federal waters. Minimal regional data is available from inshore state and federal water trawl surveys, however none of the surveys target Jonah crabs and therefore are not extremely useful in understanding Jonah crab stock or distribution (ASMFC, 2020).

Jonah Crab Life History

The presumptive size at sexual maturity is the guideline for most crustacean species harvested in US waters, including Atlantic snow crab, Tanner, Dungeness and King crab, which all support a male-driven industry (Pinfold, 2006; NPFMC 2011; PSMFC, 2014). Size at sexual maturity, in particular, can be utilized in establishing suitable minimum size limits for harvest. However, maturity amongst crustaceans can be separated by at least three different indices; morphometric, functional, and gonadal maturity. All three types of maturity must be present for an individual to be reproductively successful. These events do not always occur at the same life stage, resulting in a challenge in distinguishing between mature and immature individuals. Physiological maturity refers to the gonadal capability of producing viable and mature sperm; functional maturity is defined as the ability for an individual to partake in mating activities successfully, and morphological maturity is defined as the mean size at the onset of sexual maturity and/or appearance of secondary sex characteristics (Gerhart & Bert, 2008; Zhang et al., 2017).

Morphometric maturity indicates the relationship between allometric growth and the development of secondary sexual characteristics that are associated specifically with mature life stages in brachyurans (Hartnoll, 1978; Zhang et al., 2017). Secondary sex characteristics of brachyuran crabs, including the ratio of cheliped size to carapace size, can be used to determine the onset of sexual maturity (Hartnoll, 1974; Williner et al., 2014; Zhang et al., 2017). This method is heavily

relied upon in crustacean management because of its non-invasive and non-labor-intensive approach (Somerton, 1980; Paul, 1992; Williner et al., 2014; Zhang et al., 2017). Identifying this transition can provide one good indicator of size at maturity. Morphometric maturity is mainly relied upon in determining the size at which 50 % of the population is mature (=SM_{50}) and can in some species be a good indicator of gonadal maturity as well (Brown & Powell 1972; Goshima et al., 2000). However, SM_{50} does not always occur at the same size as other types of maturity (Goshima et al., 2000). A 2002 study off the Scotian shelf puts the morphometric maturity of *C. borealis* at 127.6 mm carapace width (CW), while gonadal maturity is estimated at 68.5 mm CW (Moriyasu et al., 2002).

The Jonah crab life cycle has not yet been fully defined; however, information has been gleaned from trawling events, stomach content analyses of predatory fish species, and SCUBA diver observations of behavior of the closely related species, *Cancer irroratus*. Females grow more slowly than males; it was estimated that after roughly 14 molts females would reach about 100 mm CW taking 8 years, and after 13-14 molts, males can reach 130 mm CW taking about 6-7 years (Williams, 1984; MCS Pre-Assessment Report, 2013). Pubertal molt comes earlier in the female life history, reaching the adult stage with a smaller body size and continuing to molt throughout their adult life. Larger females are observed to molt in December, and the majority of larger males from January to March (Reilly & Saila, 1978; MCS Pre-Assessment Report, 2013). Mating is believed to occur after the female has molted, brooding eggs for approximately 5-6 months until larvae are released into the water column (Elner et al., 1985; MCS Pre-Assessment Report, 2013). The spawning

3

season progresses from south to north, taking place from late winter to early spring in the Mid-Atlantic Bight, beginning in mid-July in Rhode Island, and August through September in Maine, (FOC, 2009). Understanding the life history of a species in all its aspects is critical for natural resource management to set sustainable regulations.

Sex Development in Malacostraca

Arthropods are an abundant and diverse phylum. Over 1.2 million species have been described, accounting for roughly 80 % of all known living animal species; they have adapted to and colonized practically all habitats on the planet (Ma et al., 2013). Similar to the widespread adaptation strategies exhibited within this phylum, a huge variety also exists within their life histories and reproductive strategies.

In general, sex determination in arthropods is determined genetically by factors on sex chromosomes. Sex determination in insects is triggered by a cascade of genes including a highly conserved (*doublesex*) gene at the downstream end, with less conserved primary signals and other genes upstream of the cascade (Wilkins, 1995; Beye et al., 2003; Verhulst et al., 2010; Beukeboom et al., 2012; Ma et al., 2013). Sex determination in crustaceans is heterogametic, however the mechanisms for which sex determination is determined within crustaceans is relatively unknown (Legrand et al, 1987; Ma et al., 2013). Unlike insects, some crustaceans show sexual plasticity facilitated by an endocrine process controlled by the androgenic gland (AG), and the androgenic gland hormone synthesized in the AG (Charniaux-Cotton, 1954; Katakura, 1961; Juchault & Legrand, 1964; Hasegawa & Katakura, 1981, Hasegawa & Katakura, 1985; Ventura et al., 2011; Ma et al., 2013). Malacostraca crustacea

4

contain the genetic information required to develop as both male and female; feminization relies on a feminizing gene, the product of which inhibits androgenic gland development and thus, the synthesis of androgenic gland hormone. For instance, the absence of an androgenic gland hormone results in female sex differentiation while the AGH allows developing male sex differentiation (Hasegawa et al., 1993).

The androgenic gland was first described in the blue crab, *Callinectes sapidus* as a tissue located sub-terminally to the vas deferens and attached to the posterior sperm duct (Cronin, 1947). After its discovery in other malacostracan species, including Tanner crab, *Chionoecetes bairdi* (Charniaux-Cotton, 1954; Charniaux-Cotton et al., 1966; Kon & Honma, 1970), AG manipulation experiments first defined its function in amphipod species, *Orchestia gammarella* (Legrand, 1955; Katakura, 1961; Juchault & Legrand, 1964). The product of the AG in amphipod species controls male sex differentiation, developing secondary sexual characters and sperm (Charniaux-Cotton et al., 1954). Further, in AG-ablated males, testes function ceased and the replacement of spermatogenesis by oogenesis was observed (Charniaux-Cotton, 1954; 1957). Moreover, in females, AG implantations caused vitellogenesis inhibition, and a lack of female-specific external sex character regeneration. After evidence supporting the role of the AG in male reproductive development that was understood in isopods and amphipods, morphological and physiological effects of AG became of prime focus in decapods (Juchault et al., 1978; Nagamine & Knight, 1987; Nagamine et al., 1980; Hasagawa et al., 1987; Suzuki & Yamasaki, 1998; Cui et al., 2005).

5

Hormonal Regulation in Male Reproduction

The property of a factor secreted from the AG was suggested to be proteinaceous (King, 1964; Meusy, 1965; Malo & Juchault, 1970; Chaigneau & Juchault, 1979; Okuno et al., 1997). The first primary amino acid sequence and then cDNA encoding *AGH* was determined in *A. vulgare* (Martin et al., 1999; Okuno et al., 1999), both showing that AGH is structurally related to vertebrate insulins. The cDNA had an open reading frame (ORF) encoding a prepro*AGH* consisting of a signal peptide, B chain, C chain, and A chain (Okuno et al., 1999).

An AG specific insulin-like gene in decapods was first cloned in the crayfish *Cherax quadricarnatus* and termed insulin-like androgenic gland hormone (IAG) due to its structural similarity to vertebrate insulin (Manor et al., 2007). Since this discovery, insulin-like genes expressed in the AG were termed IAGs and have been cloned from nearly two dozen decapod species (Manor et al., 2007; Ventura et al., 2009; Chung et al., 2011; Li et al., 2012; Chung et al., 2014; Huang et al., 2014; Ventura et al., 2015; Lawrence et al., 2017). Decapod IAG deduced amino acid sequences reported thus far are presumptive preprohormones, like the isopod AGHs. The AGH ORF encodes a B and A chain separated by a C chain that is eventually cleaved out, producing a mature hormone comprising two chains A and B with inter-disulfide bridges forming between the two, and one intra-disulfide bridge within the A chain (Martin et al., 1999).

In some species, IAG also contains predicted N-glycosylation sites (Chung et al., 2011; Mareddy et al., 2011; Huang et al. 2014; Lawrence et al., 2017). N-

6

glycosylation occurs at the post-translational modification and can influence the tertiary structure of a protein or hormone, and is a feature in the isopod androgenic gland hormone, AGH as well (Okuno et al., 1999). The cDNAs encoding these peptide hormones otherwise show low homology; however, patterns are seen in the 3' and 5' untranslated regions regarding the length and putative regulatory site presence and frequency (Lawrence et al., 2017).

In decapods, IAG function is further defined using RNA interference (RNAi) to support earlier findings by AG manipulation studies. When IAG expression is reduced by RNAi in the red-claw crayfish, *C. quadricarinatus*, a reduction in sperm production and testicular degeneration was observed and in the prawn *Macrobrachium rosenbergii*, RNAi can be used to produce all-male progeny for aquaculture purposes (Rosen et al., 2010; Lezer et al., 2015). These and other RNAi experiments have further suggested that IAG is the isopod AGH, although functional evidence for the IAG peptide is currently lacking (Lugo, 2006; Ventura, 2009).

Crustacean sex differentiation is not solely controlled by IAG and is very likely part of a more comprehensive pathway involving upstream controls and downstream interactions. Produced and stored in the decapod specific medulla terminalis-X-organ-sinus gland complex (MTXO-SG) located in the eyestalk are regulatory neuropeptides that control physiological processes in both males and females (Keller et al., 1985; Kegel et al., 1989; Brady et al., 2012). The AG is thought to be negatively regulated via an endocrine axis between the XO-SG, AG, and in some reported cases the reproductive system. A variety of eyestalk ablation studies

have been done supporting this potential existence (Islas et al., 2015; Katayama et al., 2016; Padmanabhan & Raghavan, 2016; Vazquez-Guo et al., 2019).

Ample evidence supports the potential presence of an endocrine axis between the eyestalk ganglia and the AG. Eyestalk ablation studies showed increases in AG RNA synthesis (Brockenbrough Foulks & Hoffman, 1974) and induction of spermatogenesis within the testes (Demeusy, 1960; Payen et al., 1971). Specifically, in the blue crab, *C. sapidus*, bilateral eyestalk ablation caused increased *IAG* transcripts in the hypertrophised AG, but not hepatopancreas *IAG*. (Chung et al., 2011). Similar findings are reported in both blue swimmer crab *Portunus pelagicus* and prawn *M. rosenbergii* post-eyestalk ablation (Sroyraya et al., 2010; Phoungpetchara et al., 2011). Other species displayed dynamic changes in the reproductive system, as well as the AG. The result of bilateral eyestalk ablation in the adult male crayfish *C. quadricarinatus* resulted in hypertrophy of the AG and overexpression of AG polypeptides, one polypeptide in particular is suggested to phosphorylate a testicular polypeptide. Ablation affected not only the AG but caused a weight increase of the sperm duct due to spermatophore accumulation and resulted in empty spermatogenic lobules (Khalaila et al., 2002). Ablations appeared to induce precocious male gonad development in the prawn *M. nipponense* (Kim et al., 2002). AG hypertrophy and hyperactivity appears to be shared characteristic after eliminating the XO-SG complex regardless of species. This evidence is indicative of a type of control through factor(s) present within the eyestalk over the AG and in some cases, the reproductive system as well.

All peptide/protein hormones have corresponding receptors on their target tissues. The first insulin-like receptor identified in a decapod has given insight into the potential downstream interactions. A putative insulin-like receptor cloned from *M. rosenbergii* (Mr) suggested to interact with *Mr-IAG as the* long-term RNAi of *Mr-Insulin-like receptor* hypertrophied the AG, along with an increased production of *Mr-IAG*. The expression of this receptor was present in male AG, green gland, thoracic ganglia and head ganglia, as well as in female ovary and green gland (Sharabi et al., 2016). The expression of the receptor in non-reproductive tissues supports not only the potential complexity of an IAG pathway, but the possibility of a multifunctional role of IAG.

A study involving *M. nipponense* identified an IAG binding protein (IAGBP) as a player in IAG signaling, and investigated variations on the transcriptomic level via RNA interference. A reduction in *IAG* transcript levels is observed after injection of double-stranded *IAGPB* RNA. Silencing *IAG* reduced *IAGBP* transcription in tissues including AG, testis, muscle and hepatopancreas (Li et al., 2015). The relationship between IAG and IAGBP on the transcriptomic level suggests that IAG has a binding partner(s) that could play a signaling role (Li et al., 2015). Similar interactions have been reported in other species such as the Chinese mitten crab, *Eriocheir sinensis* (Song et al., 2018). Transcriptomes reveal a snapshot of what was actively being transcribed and what transcript levels are present. The transcriptome assembly of a lobster *Samariasus verreauxi* AG revealed that *IAG* was the predominant AG-specific transcript expressed. Other AG-related transcripts that are highly abundant included a membrane-anchored protein orthologue to *C.*

9

quadricarinatus; the expression levels are significantly highest in the AG but unlike in *C. quadricarinatus*, are present in other tissues including testis and ovary (Rosen et al., 2013a; Chandler et al., 2016). A group of highly expressed transcripts of interest coded for Alpha-2-macroglobulins, which are known to chaperone insulin and insulin-like factors (Borth, 1992; Chandler et al., 2016).

AG and Size: Sexual Maturation

Transcript levels of IAG varied with stage in Eastern Spiny Lobster, *Sagmariasus verreauxi*; immature males exhibited significantly lower abundances than mature males (Chandler et al., 2016). Transcript levels also varied by season in mud crab, *Scylla paramamosain*; IAG was elevated during mating season (Zhang et al., 2014). IAG hormone hemolymph levels have the potential to be used as a bio-indicator of a particular male life stage. However, there is a lack information regarding size and how it relates to IAG expression or sex characteristics that define male maturity.

It was hypothesized that IAG transcript levels would significantly differ between males who appear physiologically sexually mature and those that appear physiologically immature. Further, it was also hypothesized that certain maturity-related transcripts are present within the XO-SG complex, and that transcript levels in the AG of non-ablated males would be differently expressed in ablated males. To this end, two methods were used to isolate the cDNA sequence of *C.borealis* IAG from the AGL RNA-Seq analysis and molecular cloning. Then, it was further examined whether an endocrine axis exists between the XO-SG complex and the AG. Lastly,

differentially expressed genes were identified which were predicted to be involved in reproduction.

Project Objectives

The first study carried out focused on investigating the relationship between morphometric and physiological maturity of male Jonah crab, and the size at which these life changes occur. Gonadosomatic index (GSI) was calculated as the ratio of gonad weight to bodyweight and was related to carapace width, season and maturity status based on our observed SM_{50}. Spermatophore area was measured and the relationship to different secondary sexual characteristics was examined. It was then determined if the mean spermatophore size was affected by carapace width, size groups, or season.

The second part of this study involved isolating the male specific sex hormone, insulin-like androgenic gland (IAG), from the Jonah crab androgenic gland (AG). IAG cDNA was isolated using gene specific primers (GSPs) pulled from Jonah crab AG transcriptome and amplified using 5' and 3' Rapid Amplification of cDNA Ends (RACE). RT-qPCR assays were carried out to investigate if indeed *IAG* transcript levels relate to size and maturity status. Differential expression was determined between ablated and non-ablated males and differentially expressed genes of interest were pulled for further investigation and annotation. The IAG sequence was confirmed through a spatial distribution of *C. borealis IAG* expression in various tissues using an end-point reverse-transcriptase polymerase chain reaction (RT-PCR) assay.

Chapter 2: Morphometric and Physiological Maturity of Male Jonah Crab, *Cancer borealis*, in Southern New England

Introduction

Understanding the life history of a species is critical for natural resource management. In fisheries that target mature individuals, knowledge of size at sexual maturity is critical for establishing suitable minimum size limits (MSL) for harvest. Most crustacean species harvested in the US waters, including Atlantic Snow Crab *Chionoecetes opilio*, Tanner Crab *C. bairdi*, and Red King Crab *Paralithodes camtschaticus* all support male-driven fisheries that are managed based on the presumptive size at sexual maturity (Pinfold, 2006; NPFMC, 2011). Among crustaceans, however, sexual maturity can be defined by at least three different sets of criteria as morphometric, functional, or physiological (gonadal).

Indicators for all three definitions of maturity are based on the ability of an individual to be reproductively successful. These indicators, however, do not always occur at the same life stage, presenting a challenge in the distinction of mature and immature individuals. Physiological maturity in males refers to the gonadal capability of producing viable and mature sperm; functional maturity is defined as the ability for an individual to participate in mating activities successfully, and morphometric maturity depends on morphological changes throughout ontogeny (Gerhart & Bert, 2008; Zhang *et al.*, 2017).

In brachyurans, morphometric maturity is indicated by a change in allometric growth and is related to the development of secondary sexual characteristics that are

associated explicitly with mature life stages (Hartnoll, 1978; Zhang *et al.*, 2017). Secondary sex characteristics of brachyuran crabs, including changes in the shape of chelipeds, can be used to determine the onset of sexual maturity (Hartnoll, 1974, Williner *et al.*, 2014, Zhang *et al.*, 2017). This method is widely used to manage crustacean fisheries because of its non-invasive and non-labor-intensive approach (Somerton, 1980; Paul, 1992; Williner *et al.*, 2014; Zhang *et al.*, 2017).

In decapod crustaceans, the transition from juvenile to adult occurs at the pubertal molt and is often distinguished by significant changes in the allometric growth rate of certain body structures (Hartnoll, 1978; Bueno, 2009). In male crabs, secondary sexual characters may include differentiation of the chelipeds. For species in the family Cancridae, it is typical for mating to take place between a recently molted, soft-shelled female and a hard-shelled male (Edwards, 1966; Hartnoll, 1969).

Identifying the size at which a transition in allometry occurs can provide one indicator of size at maturity. Morphometric maturity is typically defined as the size at which 50% of the population is mature (SM_{50}) based on the allometric relationship of two body parts (Ennis et al., 1990; Goshima et al., 2000). Morphometric maturity may coincide with gonadal maturity (Brown & Powell, 1972); however, the SM_{50} does not always occur at the same size as other types of maturity (Goshima et al., 2000; Moriyasu et al., 2002; Tallack, 2007).

The Jonah crab, *C. borealis*, is distributed between Newfoundland, Canada and Florida (Haefner, 1977), to depths >750 meters, and is primarily found between 50 and 300 meters, and temperatures of 8 – 14 °C (Robichaud, 2000; Moriyasu et al.,

2002). Caught initially as a bycatch species along with the American lobster, *Homarus americanus*, *C. borealis* has since become a highly desirable and increasingly valued species. Landings (primarily male crabs >120.65 mm CW) have steadily increased by 650% since early 2000 (ASMFC, 2020). Informed management policies are required for sustainable management. However, the Jonah crab is a data-poor species due to a lack of information on life history, reproduction, age, growth, maturity, or stock status (ASMFC, 2015). The size at 50% morphometric maturity of male *C. borealis* in the Scotian shelf has been estimated at 128 mm CW while gonadal maturity is estimated to occur at 68.5 mm CW (Moriyasu et al., 2002). In contrast, the SM_{50} for male *C. borealis* in the Mid-Atlantic Bight is estimated at the lower value of 98.3 mm CW (Olsen & Stevens, 2020).

This study aimed to investigate the allometric growth and physiological development of *C. borealis* from the North Atlantic region of Southern New England, where the greatest harvest of crabs occurs as bycatch in the American lobster fishery. To this end, the size at maturity (SM_{50}) of male crabs was estimated, and the sperm contents were measured to see if they were related to body size.

Materials and Methods

Animals

Male specimens of *C. borealis* were collected inshore in federally managed waters between Martha's Vineyard and Block Island, Rhode Island (40°52'N to 41°16'N and 70°20'W to 71°60'W). Crabs were collected by commercial fishers using modified (ventless) lobster pots re-designed to catch and keep small crabs, with

a mesh size of' ¾″. Samples included crabs of all sizes and were representative of what was landed each trip, over two years from September 2018 – November 2019. Crabs were collected during four seasons, defined as Winter (Dec – Feb); Spring (Mar. – May); Summer (June – Aug.); and, Autumn (Sept. – Nov.) (Thongda et al., 2015).

Crabs were shipped overnight to the Institute of Marine and Environmental Technology (IMET, Baltimore MD, USA), and acclimated for 1 to 2 weeks in a dark, cold room. Aquarium tanks (57 – 76 liters) holding approximately 38 liters of ~30 – 32 ppt artificial seawater housed 6 to10 individuals at a time at 10°C with constant aeration. Crabs were measured to the nearest 0.1 mm using a Mitutoyo electronic caliper. Carapace width (CW) was recorded by measuring the distance between notches anterior to both lateral spines. Chela length (ChL) was measured from the distal tip of the propodus to the ventral proximal condyle (Paul & Paul, 1996).

SM50

A set of R codes called "Crab Maturity" based on Somerton (1980) and developed by B. Stevens was used to estimate an SM_{50} for the male population in which the samples were collected. The code operates in three main steps. In the first step, the independent (carapace width) and dependent (chela length) variables were selected and log-transformed, and a single regression line through all the data was calculated. In the second step, the user selected a range of values for the lower and upper boundaries encompassing the unknown maturity region of the data, assuming

points to the left of the lower bound are immature and points to the right of the upper bound are mature.

Two separate regression lines were then estimated: one line for an immature cluster of crabs and a second line for a mature cluster of crabs. Each point that fell within the unknown maturity group was then assigned to either the immature or mature cluster based on the closest corresponding line, and the regression lines were recalculated. This process was repeated until no points changed lines, which usually required 4 – 10 iterations. The selection of the best boundary points was achieved by iteratively varying the boundaries in 5 mm increments and identifying the closest boundaries that produced the highest combined F-value for the two regression lines. The pooled residual sum of squares for the two-line model was then compared to the RSS for the single regression line to determine the best model for the data. In the third and final step, logistic regression of assigned maturity vs. CW was conducted using a generalized linear model (GLM) with a binomial link. The SM_{50} was defined as the inflection point of the curve, where 50% of crabs were classified as mature, and was calculated from the regression coefficients as

$$SM_{50} = B_0 \times B_1^{-1}$$

The standard error of the maturity estimate was calculated via bootstrapping, by resampling the data (with replacement) and recalculating the SM_{50} 1000 times. The confidence interval (CI) around the estimate was calculated as SE*1.96.

Gonadosomatic Index (GSI) and Sperm Content

The male reproductive system of decapod crustaceans includes a pair of testes, vas deferens, and ejaculatory ducts. A testis, vas deferens, and ejaculatory duct were removed from one side of each of 58 male crabs and weighed as 'gonad' to the nearest 0.1 g. The total gonad weight/animal was multiplied by two. Gonadosomatic index (GSI) was then calculated by the following expression (Anderson & Gutreuter, 1983):

$$GSI = \frac{\text{Gonad wet weight}}{\text{Total body wet weight}} \times 100$$

Spermatophore surface area (μm^2) was measured for 17 males across a wide range of sizes (110.08 –143.2 mm CW). All individuals were collected during the fall of 2019. The samples were prepared by first taking two separate distal vas deferens smears and diluting them seven times with crustacean saline (Webster, 1998). Approximately 2 – 3 drops of each spermatophore solution/animal was then transferred to two separate slides, and two photographs were taken in different areas of each slide (100× magnification with an Echo Revolve microscope, version 12.0). Every spermatophore within full view of the image frame and greater than 1000 μm^2 was measured and recorded. Spermatophores that were partially out of view were disregarded. Spermatophores smaller than 1000 μm^2 were counted but not measured, and were not included in the analysis, because they were too small to measure accurately.

Physiological Data and Statistical Analyses

Prior to analysis, the normality of GSI was tested using a q-q plot and Shapiro test, and both indicated that GSI was normally distributed (Shapiro W = 0.983, P = 0.555). Analysis of covariance (ANCOVA) was conducted to determine the relationship between GSI and CW, season, and maturity status based on our estimated SM_{50} (N = 58).

The relationship between spermatophore area and carapace width was analyzed with regression models, after eliminating the smallest crab (65.1 mm CW); all remaining crabs were in size range of 110–150 mm CW ($N = 17$). A simple regression model (Model R: Y = a + bX) was created by regressing Area (Y) on CW (X) using the gls command from the nlme package in R. A repeated measures model (Model RM) was created by regressing Area on CW, with observations repeated within each crab, i.e., crab (ID) was treated as a random variable: Y = a + BX | ID, using the lme command. Finally, a null version of model RM (Model 0: Y = a) was created with only the intercept and no slope. All three models were compared using the ANOVA command in R, and the model with the lowest AIC value was selected as the best model. All analyses were conducted using R version 3.6.1 (R Core Team, 2019).

Results

SM50

A total of 273 male *C. borealis* were caught between September 2018 and November 2019 off the coast of Rhode Island. The lower and upper bounds of carapace width selected for crabs of unknown maturity status were log (80) and log (130), respectively (Fig. 1A). Crabs within the unknown range were assigned to the closest fitting regression line (Fig. 1B). The pooled RSS for the two-lined model was significantly less than the single regression line (F = 84.1, $P < 0.001$). The generalized linear model produced an SM_{50} of 105.9 ± 5.45 mm CW (mean $\pm$ CI) for male *C. borealis* (Fig. 1C).
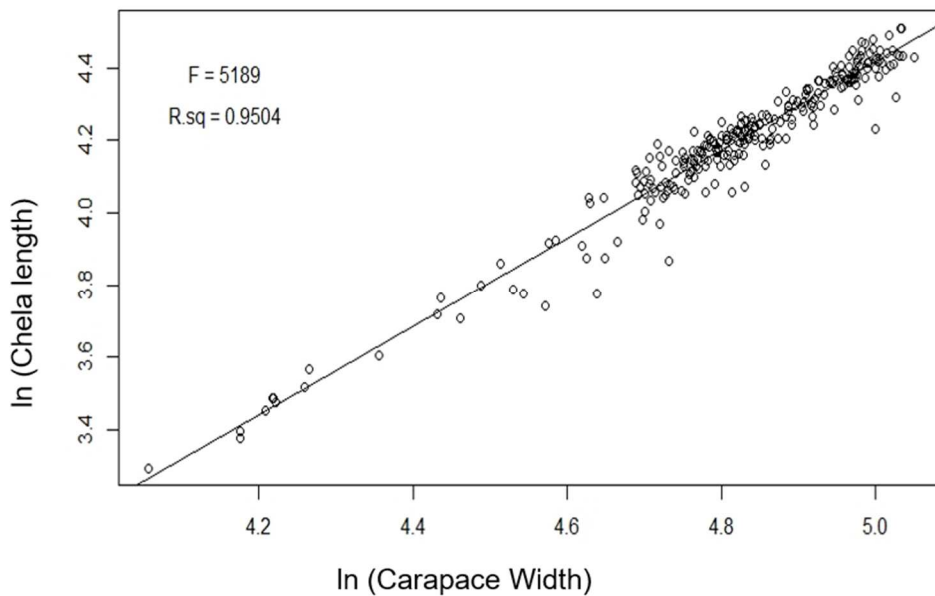
Figure 1A. Relationship between natural log-transformed chela length to carapace width of male Jonah crabs (*C. borealis*) (*n* = 273).
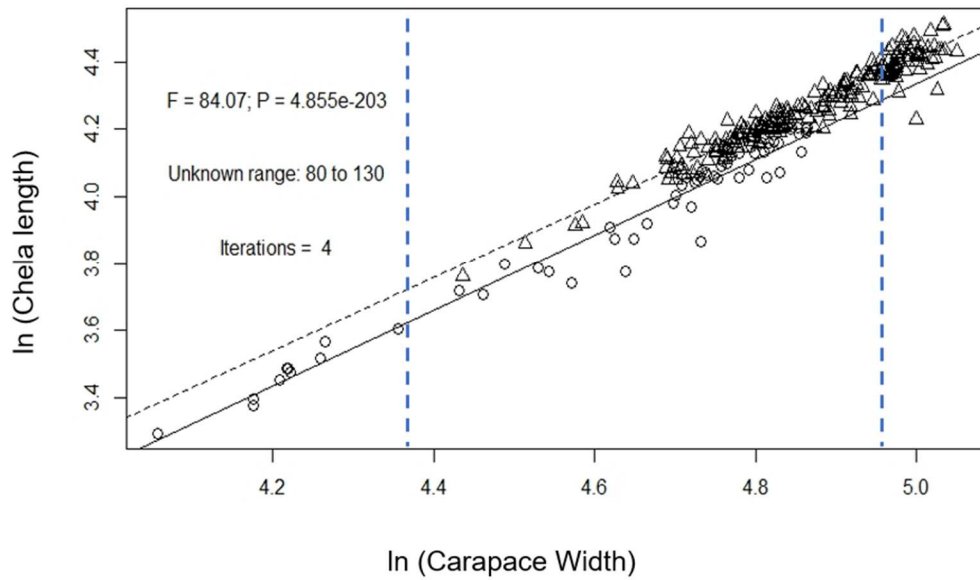
Figure 1B. Relationship of natural log chela length and natural log carapace width of male Jonah crab (*C. borealis*) for mature and immature regression lines (n = 273).
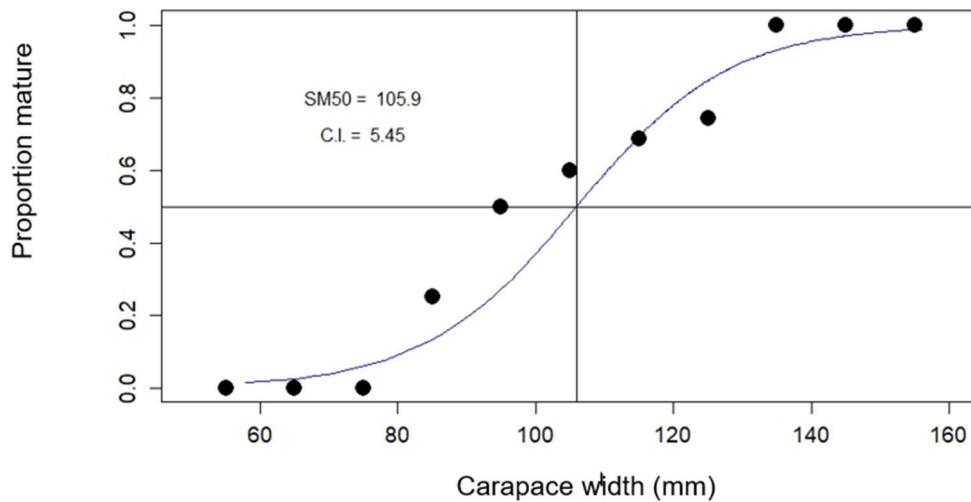
Figure 1C. Logistic regression of proportion maturity to carapace width. SM_{50} for male Jonah crab (*C. borealis*) is estimated at the point of inflection of the curve to be 105.9 ± 5.45 mm CW (n = 273)

Gonadosomatic Index and Sperm Content

Gonadosomatic index decreased significantly with CW at a rate of $0.025 \pm 0.01\%$ per mm ($P = 0.024$, Fig. 2) but increased significantly with maturity, by an average of $1.52 \pm 0.51\%$ ($P = 0.004$) (Table 1 & Fig. 3). The rate of GSI decrease with the size is amplified during the summer months by $-0.87 \pm 0.29\%$ per mm ($P = 0.004$) (Table 1 & Fig. 3). Spring and winter had no significant effect on the relationship of GSI with CW ($P < 0.05$) (Table 1 & Fig. 3).
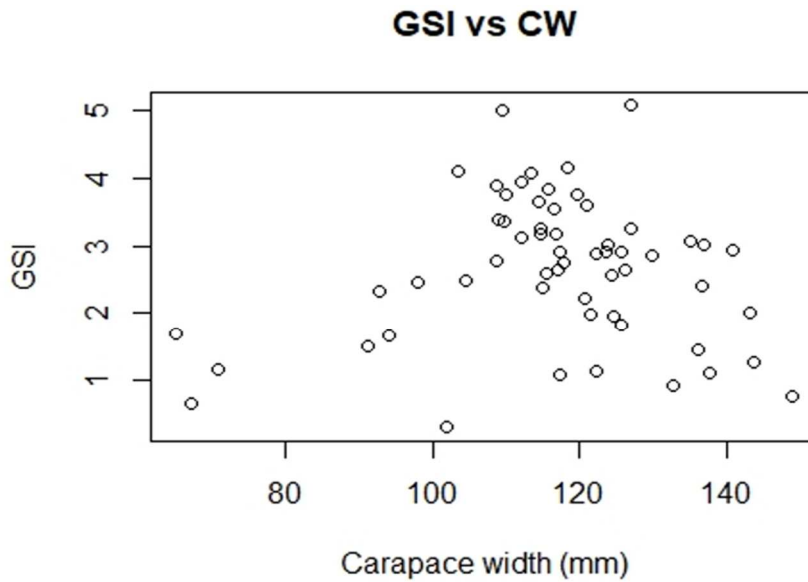
Figure 2. Plot of gonadosomatic index against carapace width (mm).
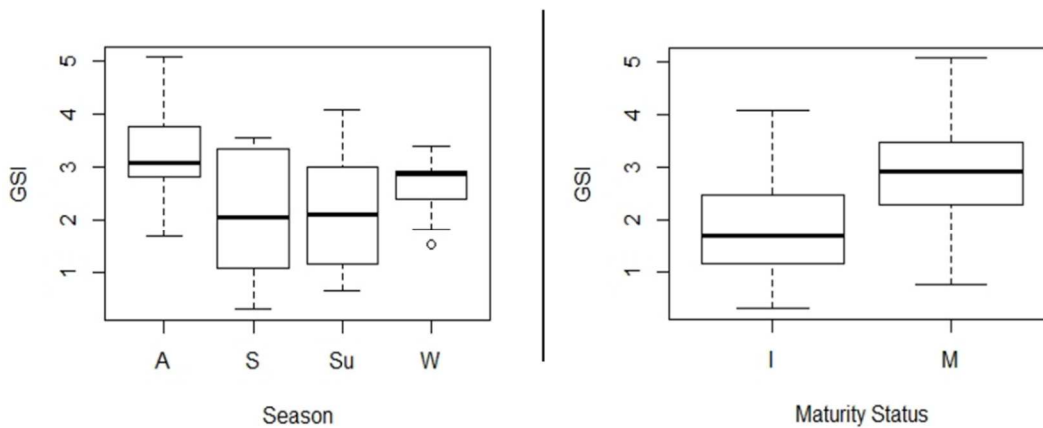
Figure 3. Boxplot of male *C. borealis* (n=58) gonadosomatic index across season (A = autumn, S = spring, Su = summer and W = winter). Boxplot of male *C. borealis* (n=58) gonadosomatic in males larger than the estimated SM_{50} (M = mature) and males smaller than the estimated SM_{50} (I = immature).

Table 1

Season	Intercept	Std. Error	t value	Pr(>|t|)
Estimate	4.82422	1.01536	4.751	1.63e-05***
Size	-0.02515	0.01084	-2.320	0.02430*
Maturity (M)	1.51968	0.50758	2.994	0.00421**
Spring	-0.84528	0.44513	-1.899	0.06313
Summer	-0.87400	0.28825	-3.032	0.00378**
Winter	-0.61795	0.35806	-1.726	0.09032

An ANCOVA was used to examine the effect of season, size and maturity status on GSI for male *C. borealis* (N = 58). Statistical significance was accepted at $P < 0.05$. Maturity group "M" is composed of males whose CW (mm) < SM_{50}. Autumn is not represented with the other season as it is included in the Estimate value.

Spermatophores present in the distal vas deferens of the males varied in size. Analysis with linear mixed-effects models, however, showed that CW had no significant effect on the size of spermatophores. The best model, with the lowest AIC value, was Model 0, i.e., the null model with repeated measures. The AIC value for Model 0 was 36.8 less than that for Model R, and 2 points less than Model RM, indicating that it differed from the latter only due to having one less variable (3 df vs. 4). Thus, the best predictor of spermatophore size was the mean value of 3998 ± 333 μm^2 (mean $\pm$ SE, $N = 17$).

Discussion

The present study aims to understand better the relationship between male body size and morphometric and physiological indicators of maturity, including GSI and spermatophore size.

A disconnect between the size at onset of morphometric, gonadal, and functional maturity has been reported in many brachyuran species of decapod crustaceans (Brown & Powell, 1972; Paul, 1992; Goshima et al., 2000). Even within a species, a disconnect can exist between different types of maturity; in female *C. pagurus*, morphometric and functional maturity appear to occur at different sizes making it hard to predict one based on the other, but in *C. pagurus* males, functional and morphometric maturity occurs at the same size (Ungfors, 2007). Functional maturity has been assessed for male crabs by observing precopulatory guarding behavior in the wild and in laboratory settings (Brown & Powell 1972; Paul, 1992; Stevens et al., 1993; Goshima et al. 2000). However, this can be challenging to

23

observe for species that reside at ocean depths, or which cannot be reared in laboratory settings. Due to these challenges and disconnects, examining multiple indicators of maturity can allow for a more complete understanding of sexual maturation.

Temperature and temperature-photoperiod interactions have been suggested as the factors affecting the geographical variation in size and onset of maturity and for latitudinal clines in metabolic rates, size, and growth (Giese, 1959; Kinne, 1970; Annala et al., 1980; Jones & Simons, 1983; Stearns, 1976). These could lead to slower growth and delayed maturity at higher latitudes. Latitudinal effects on size at maturity of decapod crustaceans, in particular, seem to be inconsistent because some species, including *C. irroratus and Carcinus maenas* show latitudinal clines, but others do not (Hines, 1989; Bakke et al., 2018).

Many terrestrial species exhibit increasing body mass with latitude (or decreasing temperature), a phenomenon known as Bergmann's or James's Rule (Blackburn et al., 1999), whereas others, particularly arthropods, exhibit the inverse relationship of decreasing body mass with latitude (or increasing with temperature) (Blanckenhorn & Demont, 2004). An inverse James's cline in female sexual maturity, i.e. positive correlation with temperature, has been reported for Snow Crabs *C. opilio* in Alaska (Somerton, 1981) and eastern Canada (Sainte-Marie & Gilbert, 1998), Red, Blue, and Golden King Crabs (*Paralithodes camtschaticus*, *P. platypus*, and *Lithodes aequispinus*, respectively) (Webb, 2014), Red Deep-sea Crab *Chaceon quinquedens*

(Martínez-Rivera et al., 2020), and Blue Crabs *Callinectes sapidus* in Texas (Fisher, 1999).

Olsen and Stevens (2020) suggested that the SM_{50} for *C. borealis* exhibits a pattern consistent with James' cline. Our estimate of morphometric size at maturity (105.9 mm CW) for male *C. borealis* at 40.5°N in Southern New England is 7 mm greater than the estimate of 98.3 mm CW made at 38.5°N in the Mid-Atlantic Bight (Olsen & Stevens, 2020), but 22 mm less than the estimate of 127.6 estimated at 45°N on the Scotian Shelf (Moriyasu et al., 2002). The difference between these estimates is proportional to the difference in latitude between these three sites. These values indicate a poleward trend of increasing size at maturity with latitude, consistent with James's cline, and should be considered when setting appropriate size limits for harvesting.

The spawning season for *C. borealis* is also thought to progress latitudinally, from south to north along the eastern coast of the U.S. In the Mid-Atlantic Bight, spawning is reported to take place from late winter to early spring; in Rhode Island spawning is observed beginning in mid-July, and off the eastern portion of George's Bank, MA, spawning is reported to take place in late summer from August through September (FOC, 2009; JCMSCR, 2013). The current estimates of GSI in Rhode Island were consistent with observations in previous reports. The proportional size of the male gonads is greatest immediately before mating and deposition of spermatophores into females. Ovigerous females are observed from March to June in

southern New England and believed to bear eggs for 5 to 6 months, which implies that mating season probably occurs between October and January (JCMSCR, 2013).

In sexually dimorphic crustaceans, the male reproductive system includes paired and symmetric testes, vas deferens, and ejaculatory ducts. Male crustaceans seem to have two types of testicular structures: lobular and tubular (Nagao & Munehara, 2003). *Cancer* males have lobulated testes where spermatogenesis occurs. The spermatozoa enter vas deferens, forming spermatophores, which give rise to white color in mature males.

The GSI values have commonly been used as a reproductive indicator in female crustaceans since there is a positive relationship between GSI and ovarian growth (Thongda et al., 2015). Females at maturity, especially ovigerous animals, tend to show higher GSI values than males (Parker et al., 2018).

The present study suggests that the GSI: size relationship is significantly lower during the summer, consistent with previous observations of mating season in this region and indicating that a post-mating period occurs during the summer for *C. borealis*. The GSI: size relationship varied little between Autumn, Winter, and Spring. This is because the majority of crabs in our sample were relatively large. A greater or more even size distribution of crabs might have produced a different result.

It is hypothesized that larger, or mature males would have a larger average GSI compared to smaller or immature crabs. The current study shows a significant decrease in the relationship between GSI and size, but GSI is statistically greater in

males considered to be mature based on our established SM_{50}. Further, with high GSI values, greater content of the gonads is expected due to more and/or larger spermatophores. The size of males did not have a significant effect on the mean spermatophore surface area. These results might have differed if we could have measured and analyzed spermatophores smaller than 1000 μm^2. Spermatophore presence in the vas deferens is often a good indicator of sexual maturity in male crabs; however, it does not always warrant that mating can successfully occur (Watson, 1970). It needs further investigating to assess reproductive potential if there is sperm/spermatophore viability relative to the male size and if there is an annual or biannual testicular maturation cycle in the species.

Acknowledgements

Chapter 3: Chapter 3: Isolation of insulin-like androgenic gland hormone (IAG) and *IAG* expression effect of size in Jonah crab, *Cancer borealis*

Introduction

The Jonah crab is an economically important yet data-poor species whose fishery has continued to grow steadily since the start of the 21st century (Jonah Crab Fishery, 2014). They are primarily landed in the Southern New England region but can be found from Newfoundland, Canada to Florida, US (Haefner, 1977). Jonah crab typically thrive between 8 and 14 °C and although typically found between 50 and 300 meters, they can exceed depths of 750 meters (Robichaud, 2000; ASMFC, 2020; Moriyasu et al., 2002). It is important to understand the Jonah crab life history in order to manage them in a sustainable manor. One aspect of this is the size at sexual maturity. The Jonah crab is currently managed under the Interstate Fishery Management Plan, which has set the minimum size for Jonah crabs to be landed at 120.65 mm carapace length (ASMFC, 2020).

The androgenic gland (AG) was first described in the blue crab, *Callinectes sapidus* in 1947 (Cronin, 1947). Since then, the AG has been identified across malacostracan species, and shown repeatedly to be heavily involved in male sexual development of both primary and secondary sex characteristics (Charniaux-Cotton, 1954; Legrand, 1955; Charniaux-Cotton et al., 1957; Kon & Honma, 1970; Juchault, 1978; Hasagawa et al., 1987; Suzuki & Yamasaki, 1998 & Cui et al., 2005). The first amino acid sequence for an AG-secreted factor suggested to be responsible for male

sexual development was described in *Armadillidium vulgare* and deemed androgenic gland hormone (AGH) (Martin et al., 1999; Okuno et al., 1999). AGH had insulin-like organization and following this, the discovery of insulin-like genes expressed in the AG have been deemed insulin-like androgenic gland hormones (IAGs), and have been cloned from many commercially important decapods (Manor et al., 2007; Okuno et al., 2009; Ventura et al., 2009; Chung et al., 2011; Li et al., 2012; Chung et al., 2014; Huang et al., 2014)

Insulin-like androgenic gland hormone is thought to be regulated by an endocrine axis between the AG and the decapod specific medulla terminalis-X-organ-sinus gland complex (MTXO-SG) located in the eyestalk. The neuropeptides produced MTXO-SG have been known to control physiological processes in both males and females including molting, reproduction, and metabolism (Keller et al., 1985; Kegel et al., 1989; Brady et al., 2012). Ample evidence supports the potential presence of an endocrine axis between the eyestalk ganglia and the AG. Eyestalk ablation studies have shown increases in AG RNA synthesis (Brockenbrough Foulks & Hoffman, 1974) and induction of spermatogenesis within the testes (Demeusy, 1960; Payen et al., 1971). Specifically, bilateral eyestalk ablation caused increased *IAG* transcripts in the hypertrophized AG in several species including the Blue crab, *C. sapidus*, Blue swimmer crab, *Portunus pelagicus*, and Freshwater giant prawn, *M. rosenbergii* (Sroyraya et al., 2010; Chung et al., 2011; Phoungpetchara et al., 2011).

The role of MTXO-neuropeptides on AG activity is also reflected in transcriptomic analysis. The AG transcriptome of the Eastern spiny lobster,

29

Sagmariasus verreauxi revealed IAG as the most highly expressed AG-specific

factor, and IAG expression as stage-dependent with lower expression in immature

males. Insulin-like androgenic gland hormone appeared to be eyestalk-regulated, with

expression 6-times higher in hypertrophied glands (Chandler et al., 2016). Bilateral

eyestalk ablation in *M. nipponense* (Mn) resulted in a 7.9 fold increase in *Mn-IAG*

expression. More specifically, the neurohormones partially responsible for regulating

Mn-IAG were identified via RNAi silencing of *Mn-GIH* and *Mn-MIH* which caused

significant increase in *Mn-IAG* expression (Li et al., 2015). In the Mud crab, *Scylla*

paramamosain, IAG is observed to be downregulated by CFSH (Liu et al., 2018).

The objectives of this study were to understand better how *IAG* expression

relates to size in male *C. borealis*, and to demonstrate that *Cab-IAG* is in part

regulated by the XO-SG complex. To this end, AGs were excised from ablated and

non-ablated males, RNA were isolated and submitted for RNA-seq analysis, and the

data were used to generate transcriptomes. Isolation of *Cab-IAG* cDNA was achieved

using gene-specific primers (GSPs) based on sequences from the Jonah crab AG

transcriptome. qRT-PCR assays were carried out to investigate whether Cab-*IAG*

transcript levels relate to size and maturity status. Differential expression was then

determined between ablated and non-ablated males and highly expressed genes were

annotated. The partial IAG sequence obtained was 351 base pairs (bps) in length and

117 amino acids long; the putative amino acid sequence was structured similarly to

other crustacean IAGs. Low sequence homology to other crustacean IAGs, however,

was observed. The transcriptomic analysis of AG revealed higher transcript

abundance in ablated males, and qRT-PCR revealed highest expression levels in ablated males, with no effect from size variation.

Materials and Methods

Animal Collection and Handling

Male specimens of *C. borealis* were collected from the Mid-Atlantic Bight off the coast of Bethany Beach, Delaware and West Ocean City, Maryland within the general boundaries of 37.7249 – 38.71028 °N; 74.34333 – 74.97732 °W and waters between Martha's Vineyard and Block Island, Rhode Island between the boundaries of 40°52'N – 41°16'N and 70°20'W – 71°60'W. Collection took place from September, 2018 – November, 2019, and males ranged in size from 91.3 – 143.72 mm CW. Crabs were captured predominantly as bycatch in lobster and black sea bass traps off commercial fishing vessels. The crabs were packed in coolers with ice packs and either driven on the same day, or shipped to the Institute of Marine and Environmental Technology (IMET, Baltimore, MD, USA). Crabs were immediately transferred to holding tanks in a dark, climate-controlled cold room at 10°C in approximately 30 ppt artificial seawater. Crabs were acclimated for no less than 5 days, and chilled on ice prior to dissection. All tissue samples collected were immediately placed on dry ice and kept at -80°C until further processing.

RNA Sample Preparation

Androgenic glands (AGs) were isolated from six males; three eyestalk-ablated males and three non-ablated of approximately the same size. Eyestalk-ablated males had eyes removed 14 days prior to AG dissection. Total RNA was isolated using TRIzol reagent according to the manufacturer's protocol. Equal quantities of total RNA from ablated and non-ablated samples were pooled separately for RNA-Seq.

Transcriptome Data Analysis

Paired-end Illumina sequences were first trimmed using Trimmomatic with a sliding window trimming procedure (10 bp window trimming when mean Phred quality is below 20) and requiring a minimum final trimmed length of 75 bp. A quality assessment (% Q30) of trimmed data was performed using seqtk. A Phred score of 30 indicating a base-calling probability of error of 1 in 1000 was applied. Trinity (v2.8.6) was then used to assemble a single transcriptome of both data sets combined (AGs of ablated and non-ablated males). Statistics on the assembly are reported in Table 2. The completeness of both clustered and unclustered assemblies generated by Trinity were evaluated using Benchmarking Universal Single-Copy Orthologs (BUSCO) analysis. Transcriptomes were compared against the arthropoda_odb9 lineage dataset using the dependencies augustus-3.3.1 (E-value threshold of 1e-05). BUSCO ran the search with 1066 total BUSCO groups, resulting in a 93.6% completeness score.

A blasted diamond annotation was performed to identify transcripts that were potential IAG hits. The potential candidates were filtered through using a few criteria:

(i) long sequence hits (> 100 bp), (ii) high % identity and (iii) low E-values. The top ten selected DNA sequences were translated to candidate protein sequences and aligned to reference IAG sequences using BLAST to confirm their identity (adjustments were made here to ensure the frames were all reading from forward to reverse). The selected sequences were aligned using Muscle and additional gene-specific primers were developed based on the most conserved regions based on this alignment.

Transcript quantification analysis was achieved via RSEM and is broken down into two main steps. First, a set of reference transcript sequences are generated and second, RNA-Seq reads are aligned to that set of reference transcripts producing alignments that are then used for abundance estimation. The top ten most highly expressed transcripts from eyestalk ablated and non-ablated AGs were identified using BlastX. The transcript abundance generated by RSEM of *CabIAG* was also examined in eyestalk-ablated and non-ablated male *C. borealis*.
bowtie2 v2.1.0.

Cloning of IAG and Tissue Distribution

Total RNA was isolated from the AGs and other tissues (state) using QIAzol lysis reagent (Qiagen, Santa Ana, CA, USA), and following the manufacturer's protocol. Total RNA was quantified using a NanoDrop Lite Spectrophotometer (Thermo Scientific, Waltham, MA, USA). A two-step PCR method was employed as previously reported (Chung et al., 2011 & Lawrence et al., 2017). Gene-specific primers (Table 3) were generated based on transcripts pulled from our AG

transcriptome, and that most closely aligned to other crustacean IAGs. Primers *CabIAG*-5R2 and *CabIAG*-3F1 were used to obtain an initial sequence, under the following conditions: 94 °C for 2.5 min; annealing temperatures decreasing 2 °C/cycle from 57 °C to 53 °C for 9 cycles; followed by 27 cycles at 94 °C for 30 s; 58 °C for 30 s; 68 °C for 1.5 min; and the final extension at 68 °C for 7 min. One microliter of the initial TD-PCR product served as the template for the nested PCR and was amplified using nested universal primer (BD Biosciences) and *CabIAG*-5R2 and *CabIAG*-3F3 were used to amplify a partial sequence. Bands with the expected size of ~450–550 bp were excised for DNA extraction (Qiagen), followed by subsequent cloning and sequencing as previously described (Chung et al., 2011).

For the tissue distribution, total RNA was extracted as described above from adult male and female tissues including eyestalk ganglia, thoracic ganglia complex, hemocytes, brain, muscle, hepatopancreas, testis/ovary, androgenic gland/ spermatheca. Approximately 1-2 μg of total RNA of each tissue sample was subjected to the first strand cDNA synthesis using the PrimeScript™ Reverse transcriptase reagent kit with a gDNA eraser (TaKaRa). The spatial distribution of CabIAG transcripts was established using an end-point PCR assay in which cDNA from each tissue (12.5 ng of total RNA equivalent) was amplified with *CabIAG-F1* and *CabIAG-R4* (Table 3) under the following PCR conditions: 94°C for 2.5 min, followed by 30 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min, and the final extension at 72°C for 5 min. Na, K-ATPase (CabNaK-ATPase) was used as a reference gene, and amplified in the same cDNA samples using CabNaK-ATPase-QF and CabNaK-ATPase-QR primers listed in Table 3.

cDNA Sequencing Analysis

The sequence was translated using ExPasy translate (https://web.expasy.org/translate/) and the open reading frame was confirmed using ORF finder. The presence of a partial signal peptide was identified utilizing Signal P (http://www.cbs.dtu.dk/services/SignalP/). A neighbor-joining tree of a partial *CabIAG* and all other full-length IAG and AGH sequences deposited in GenBank to date was constructed using ClustalW2 (https://www.ebi.ac.uk/Tools/msa/clustalw2/).

qPCR analysis of IAG Transcripts and Statistical Analysis

Total RNA was extracted as described above, and cDNA synthesis was carried out using the PrimeScript™ Reverse transcriptase reagent kit with a gDNA eraser (TaKaRa). The *CabIAG* RNA was measured via qPCR analysis using the primer set *CabIAG-3F1* and *CabIAG-5R4* to amplify the partial *CabIAG* sequence of 351 bps. This product was subcloned into a pGEMT vector and the plasmid DNA served as standard. qRT-PCR data is presented as the mean ± SE copies/ug total RNA. The seasonal data (N = 30) were analyzed using a one-way ANOVA (Rstudio v3.5.2). Statistical significance was accepted at $P < 0.05$. Size and expression were analyzed using a paired two sample t-test and the statistical significance was accepted at $P < 0.05$ (N = 30). All other data was analyzed using Welch two sample t-test. Statistical significance was accepted at $P < 0.05$ (N = 54).

Results

Transcriptome Analysis of AGs

The *de novo* assembly generated 67,206,181 Illumina reads producing a total number of 123,421 contigs, or overlapping DNA sequences (Trinity version 2.8.6); half of these (N50) contigs were at least 1,956 base pairs in length. The % GC content was 43.37. The assembly generated 84,041 Trinity genes.

Table 2**.** Summary statistics for the *de novo* assembly of *C. borealis* androgenic gland. Statistics are based on all contigs.

Statistical Summary	Number of base pairs
Contig N50	1,956
Median contig length	443
Average contig	965.57
Total assembled bases	119,172,099
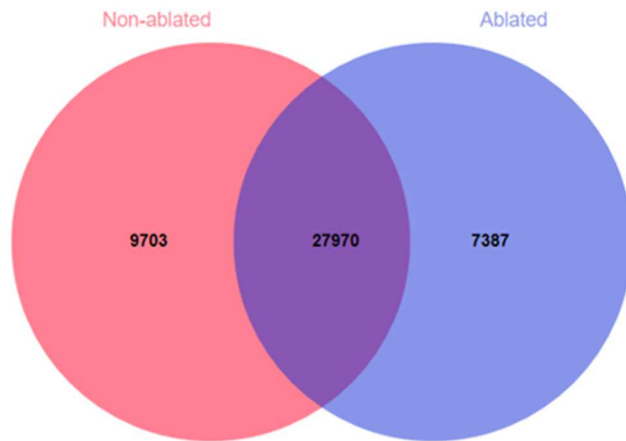
Figure 4. Differential expression analysis of Trinity derived genes in ablated and non-ablated male *C. borealis* androgenic glands using jvenn.toulouse.inra.fr.

Differential Expression Analyses

The de novo assembly of androgenic glands from non-ablated and ablated male *C. borealis* show 27,970 trinity genes in common, 9,703 trinity genes that are specific to AG of non-ablated males and 7,387 trinity genes that are specific to ablated males (Fig. 4).

Table 3. Gene-specific primer (GSP) sequences used for the isolation of the partial-length *CabIAG* cDNA from the androgenic gland of male *C. borealis*. Primers used for qRT-PCR are represented with a star, and primers used for control gene *CabNaK-ATPase* expression are labeled as such.

Primer Name	Primer Sequences (5' → 3')
Cab-3F1 *	TTCACCATAAAACTCTTATTTCAGGTACTGCTG
Cab-3F2	TCTTACACTACCAAGTCTACCTTCCATCCCCGC
Cab-3F3	GGGCTGACGCTGACACCAATG
Cab-3F4	TGCGACCTACAAACTGACCT
Cab-5R1	ACAATACTCCGATACCTCCTG
Cab-5R4 *	ACACACGTAGGACATGGATCTCTG
Cab-NaK-ATPase (forward)	TGGGTCTTGTAATCTTGTGTCTCTAGG
Cab-NaK-ATPase (reverse)	ACACACGTAGGACATGGATCTCTG

cDNA Sequence Analyses & Tissue Distribution

Figure 5. The partial-length cDNA and deduced amino acid sequence of *CabIAG* isolated from the androgenic gland of male *C. borealis*. The partial predicted signal peptide is italicized. Two predicted cleavage sites (R-X-X-R) are boxed and four of the six insulin-like peptide conserved cysteine residues are circled. The number of nucleotides is displayed on the left and amino acids are numbered on the right.
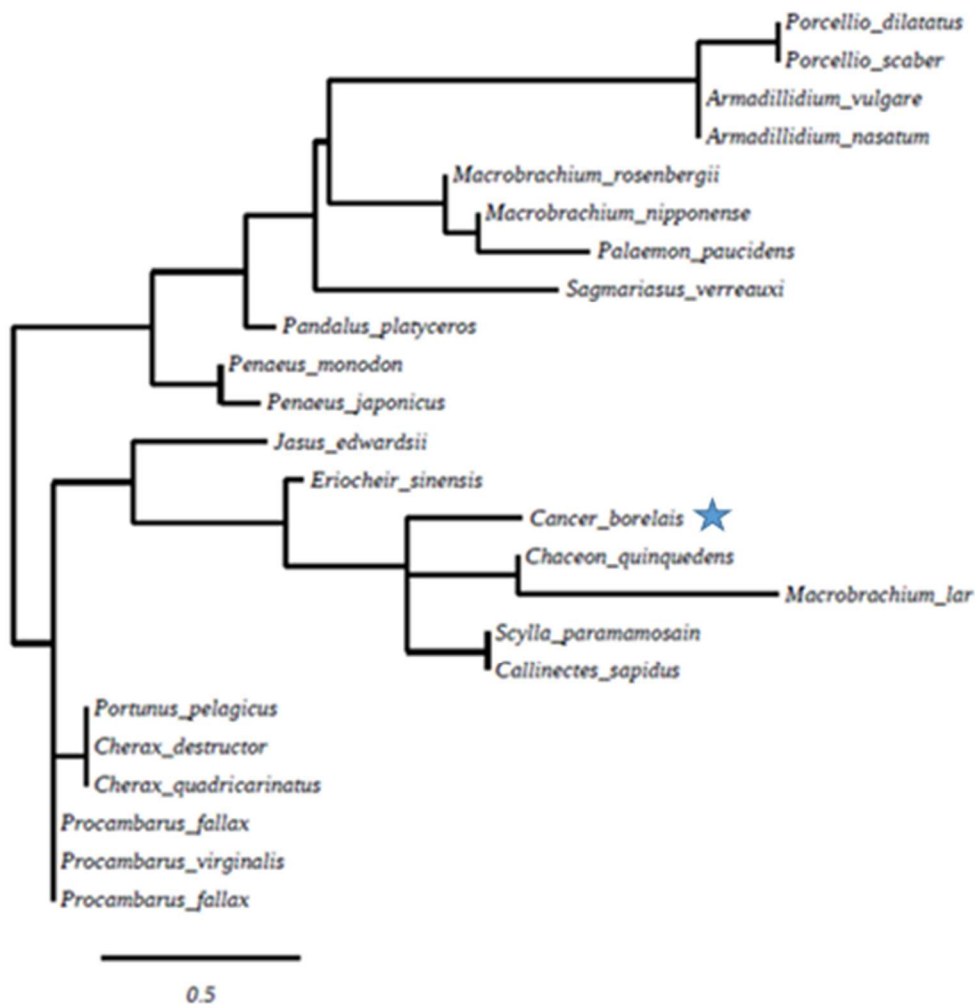
Figure 6. Maximum likelihood tree (Phylogeny.fr) of the open reading frame sequence of crustacean insulin-like androgenic gland hormones (IAGs) and androgenic gland hormone (AGHs) obtained from without distance corrections using clustalo Version 1.2.4. *Cancer borealis* is represented with a blue star; *Portunus pelagicus*, ADK46885.1; *Cherax quadricarinatus,* ABH07705.1; *Cherax destructor, ACD91988.1*; *Sagmariasus verreauxi*, AHY99679.1; *Macrobrachium nipponense*, AGB56976.1; *Macrobrachium rosenbergii*, ACJ38227.1; *Eriocheir sinensis*, QAT96396.1; *Procambarus virginalis*, AUL52689.1; *Procambarus fallax*, AUL52688.1; *Chaceon quinquedens*, ASA45642.1; *Scylla paramamosain*, AFY09905.1; *Jasus edwardsii*, AIM55892.1; *Penaeus monodon*, ADA67878.1; *Callinectes sapidus*, AEI72263.1; *Penaeus japonicus*, BAK20460.1; *Palaemon paucidens*, BAJ84108.1; *Procambarus fallax*, ASM94213.1; *Macrobrachium lar*, BAJ78349.1; *Pandalus platyceros*, ASM94212.1; *Armadillidium vulgare*, BAA86893.1; *Armadillidium nasatum*, KAB7497038.1; *Porcellio dilatatus*, BAC57013.1; *Porcellio scaber*, BAC57012.1. The scale bar indicates the number of amino acid sequence substitutions per site. Distance represents neighbor-adjoining bootstrap values.
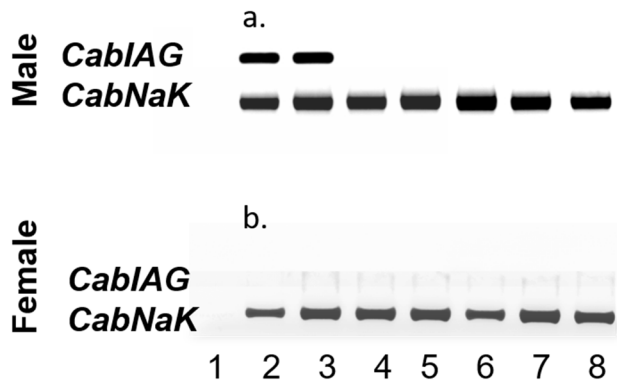
Figure 7. Spatial expression analysis of *CabIAG* in adult (a) male (b) female tissues. Male: 1: no template control; 2: AG; 3: AG from eyestalk-ablated individual; 4: eyestalk ganglia; 5: thoracic ganglia complex; 6: brain; 7: hemocytes; 8: testis. Female: 1: no template control; 2: spermatheca; 3: eyestalk ganglia complex; 4: thoracic ganglia complex; 5: muscle; 6: hemocytes; 7: hepatopancreas; 8: ovary. Na, K-ATPase (*CabNaK-ATPase*) was used as a reference gene for amplification.

The partial-length cDNA of *CabIAG* containing 351 nucleotides was isolated from the AG of adult male *C. borealis* using primers generated from the AG transcriptome in the previous step (Fig. 5). The deduced amino acid sequence encodes a partial open reading frame (ORF) with 117 amino acids. A neighbor-joining tree was generated using crustaceans AGH and IAG ORFs including the partial-length CabIAG. The partial *CabIAG* most closely aligns with *C. quinquedens* and *C. sapidus* and clustering with other crab species (Fig. 6). The lobster, crayfish, isopod, and prawn/shrimp species also cluster with each other as well. The only anomaly is that of *P. pelagicus*, which clusters more closely with the lobsters even though it belongs to the family Portunidae.

Control gene *CabNaK-ATPase-ATPase* was amplified in all tissues of male and female. The only tissue expressing *CabIAG* is the AG, which show similar expression patterns in both eyestalk-ablated and non-ablated AGs (Fig. 7).
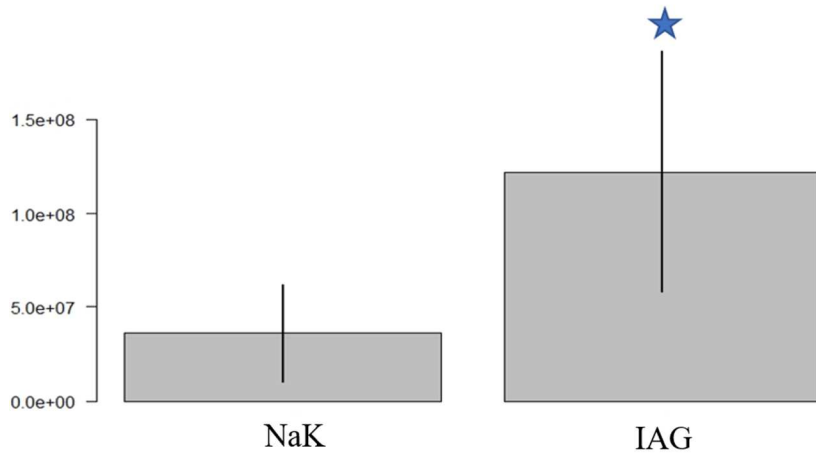
Figure 8. Quantitative real-time PCR (qRT-PCR) expression profile of the *CabIAG* and control gene *CabNaK-ATPase* in the androgenic gland of male *C. borealis*. Statistical significance is represented by a star, and was accepted at P < 0.05.

 Figure 9. Quantitative real-time PCR (qRT-PCR) expression profile of the *CabIAG* in the androgenic glands of eyestalk-ablated and non-ablated individuals. Statistical significance is represented by a star and was accepted at P < 0.05.

Statistical Analysis

There was a significant difference due to size (carapace width) on expression (t = 3.807, p = 0.0007). The control gene *CabNAK-ATPASE* 4.4 x 10^6 ± 9.92 x 10^5 showed little variance compared to that of *CabIAG* (1.26 x 10^8 ± 3.30 x 10^7) with significantly lower expression (t = 3.67, p = 0.001) (Fig. 8). The expression of *CabIAG* in AGs of ablated males (3.60 x 10^8 ± 1.28x 10^7) was significantly greater than that of non-ablated males (1.22 x 10^8 ± 3.21 x 10^7, p = 7.503e-08) (Fig. 9). There was no significant seasonal effect on expression of *CabIAG* (Anova, F = 1.866, p = 0.16, Fig. 10).
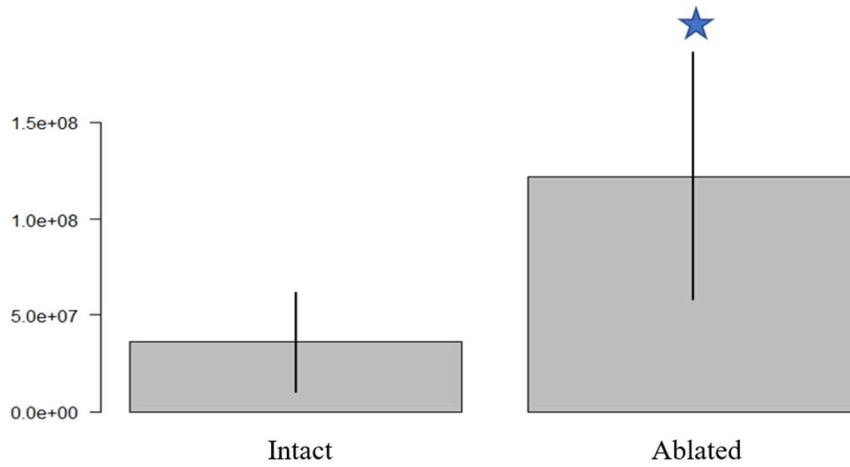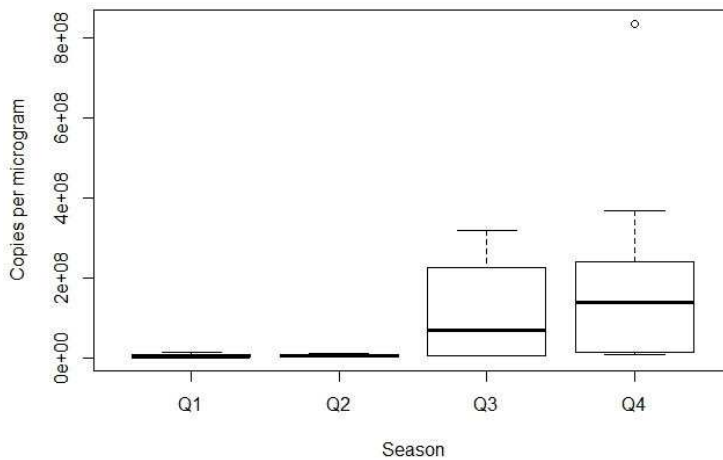

Figure 10. Quantitative real-time PCR (qRT-PCR) expression profile of the *CabIAG* in the androgenic gland in individuals during different seasons: Q1: winter; Q2: spring; Q3: summer; Q4: fall.

Discussion

The organization and structure of CabIAG was similar to other insulin-like peptides, however when compared to other crustacean IAGs, low amino acid sequence similarity was observed. The CabIAG had predicted proteolytic cleavage motifs of R-X-X-R at the predicted C-terminal end of both the B chain and C chain). These sites are presumed to be involved in the removal of the C peptide and formation of the functional heterodimeric form consisting of the A chain and B chain (Mareddy et al., 2011). Proteolytic cleavage of the C peptide is a post-translational modification that affects activity of the peptide. (Okuno et al., 2002, Ma et al., 2013). Four out of the six cysteine residues conserved in insulin-like peptides and other decapod IAGs were present, further supporting the formation interaction between the A and B chains. Two inter-chains are thought to be formed between chains and one intra-chain disulfide bond is thought to form within the A chain and is consistent in other decapod species.

Tissue distribution results reveal *CabIAG* is expressed exclusively in the AG as expected and in common with most other crustaceans. It is important to note however that *CabIAG* expression differs from that of *C. sapidus*, *S. paramamosain* and *F. chinensis* where *IAG* is expressed in tissues in addition to the AG in males, and/or in the female tissues as well (Li et al., 2012; Chung et al., 2014). The expression of IAG showed similar patterns in both ablated and non-ablated AGs, however, we expected to see higher expression in the ablated male tissues. There was higher expression seen on the transcriptomic level when examining IAG

homologs/IAG of that showed high similarity to crustacean IAGs when using BLAST.

The level of *CabIAG transcripts* did differ by size of an individual and other factors seem to have an influence over expression. There was no significant effect of season on *CabIAG*, however there is a clear pattern in the expression that is similar to that of *C. borealis* predicted mating season (Elner et al., 1985; MCS Pre-Assessment Report, 2013). There is also a large influence of the eyestalk over *CabIAG*; a significant increase after 14 days post-ablation was observed. This is evidence for the existence of an endocrine access between the XO-SG and AG, and regulatory effects of the eyestalk on AG have been described in *C. sapidus* and *Cherax quadricarinatus* (Khalaila, et al., 2002; Chung et al., 2011).

Intact *C. sapidus* showed *IAG l*evels of $8.9 \pm 0.3 \times 10^7$ copies/μg total RNA whereas AK expression of intact animals is $3.9 \pm 0.5 \times 10^5$. The *IAG* expression was significantly elevated six fold $56.8 \pm 12.9 \times 10^7$ copies/μg total RNA 7 days post-ablation (Chung et al., 2011). Insulin-like androgenic gland hormone is involved in a more complex pathway that needs to be further explored to understand better the male sex differentiation pathway(s), and the factors that influence this. In the oriental river prawn, *Macrobrachium nippon*ense, 40 novel genes were identified meaning their expression levels were highest in the AG compared to other sex tissues (vas deferens, ovaries, testis). *Mn-IAG* expression in the AG was similar to that of 16 other genes that were candidates in potential sex developmental roles (Jin et al., 2013).

Expression levels of *CabIAG* were significantly higher than the control gene, *CabNaK-ATPase*, which is atypical of control gene expression. In intact *C. sapidus*, the expression of control gene *arginine kinase* was 3.9 ± 0.5 x 10^5, and more highly expressed than *IAG*. Expression of *IAG* in intact *C. borealis* was higher than other reported expression levels at 1.22 x $10^8 \pm 3.21$ x 10^7. The expression of IAG for *C. sapidus* even after reaching its peak level 7 days post-eyestalk ablation was still less than that of intact *C. borealis* $56.8 \pm 12.9 \pm 10^7$ copies/µg total RNA (Chung et al., 2011).

Conclusion

The current study aimed to isolate and confirm the presence of *CabIAG* in male *C. borealis* in order to investigate the effects as a function of carapace width. The partial-length CabIAG sequence shows low sequence identity with other crustacean IAGs, but shares structures and features conserved in other decapod IAGs. The presence of Cab*IAG* was observed only in the AG and in no other male or female tissues. Expression was measured in copies/µg and was significantly influenced by size, and should be further investigated to examine if expression levels are influenced solely by body size, or if more specifically, expression statistically differs in crabs above and below our estimated SM_{50} is. There was higher expression in eyestalk-ablated males, and was observed to have a possible seasonal affect although there were no significant differences in expression between seasons. We infer the potential existence of an endocrine axis between the XO-SG complex, and that *IAG* expression is regulated via this axis via factors which act on the AG. The *CabIAG* expression

patterns should be further investigated to include samples from all life stages of *C. borealis*, and include other sexual characteristics that IAG is known to influence including spermatogenesis.

Chapter 4: General Discussion and Future Direction

Potential Factors Influencing Sexual Maturity

Latitudinal clines are prevalent in the life cycle of mammals, fish, birds and arthropods, particularly affecting size, metabolic rates, growth and onset of maturity (Giese, 1959; Kinne, 1970; Stearns, 1976; Annala *et al*., 1980; Jones & Simons, 1983; Jonsson & Abée-Lund, 1993; Kojola & Laitala et al., 2001; Blanckenhorn & Demont, 2004; Gardner et al., 2009). It is clear that latitude should be considered in the management of species that inhabit a wide range of latitudes.Tthis study estimated the SM_{50} for male *C. borealis* to be 105.9 mm CW at a latitude of approximately 40.5°N. In comparison the SM_{50} of *C. borealis* was estimated to be 127.6 mm CW at 45°N (Moriyasu et al., 2002), and at the smaller value of 98.3 mm CW at 38.5°N (Olsen & Stevens, 2020). Latitude, which can affect temperature and temperature-photoperiod, appears to be a critical factor affecting the size at maturity of this species, and should therefore be considered in Jonah crab management policies. Latitude should also be considered during further investigation of *CabIAG* expression patterns at different life stages.

The SM_{50} was estimated utilizing a logistic curve model and although it is representative of our data, other models may also be appropriate. One limitation for this model was the size of the dataset; specifically the low number of small crabs which may have caused an upward bias in the maturity estimate.

Size and IAG

The expression of *CabIAG* was significantly influenced by size of males, and the trend should be investigated further to evaluate whether or not maturity is a factor in expression. A higher concentration of total RNA could be correlated with the larger AGs. Transcription levels are often presented as copies/ug total RNA. This is only valid when the size of experimental animals is statistically similar or the same. The data are presented as copies/ AG total RNA since the current study involves different sized animals.

Insulin-like androgenic gland hormone is known to be involved in development of primary and secondary characteristics including spermatogenesis (Lugo et al, 2006; Ventura et al, 2009; Rosen et al., 2010; Lezer et al., 2015). Therefore, reproductive activity and sex characteristic development depends on season, which is examined throughout both studies. Season had a significant influence on the GSI

Size of AG has been seen to undergo seasonal changes. In *S. paramamosain*, the AG increases in size and cell number reaching its peak during mating season (July-Sept.); the AG begins to degenerate in October and reaches its smallest size in December (Liu et al., 2018). Similar AG cycles or seasonal stages were seen in *Travancoriana schirnerae* (Devi & Smija, 2014). Not only should methods of quantifying *IAG* be examined, but perhaps defining AG stages/cycle to ensure future comparisons are being made amongst the same AG stage.

Future Work

The size of males did not have an effect on spermatophore surface area in our study.It was observed, however, that smaller males tended to have a larger number of smaller spermatophores that were too small to measure accurately, and therefore were excluded from the results. If all spermatophores (including those of <1000 μm) could be accurately measured and incorporated into the calculation of mean surface area size/individual, this could shift the mean of those smaller individuals potentially to a point of significant difference. Other measures of sperm content have also been established, including spermatophore viability in *Birgus latro* and *Hapalogaster dentata* (Sato & Goshima, 2006; Sato et al., 2008). It was observed that the number of sperm inside spermatophores tended to vary, however our sample size was not large enough to perform meaningful statistical analyses. Therefore, further investigation of the relationship between carapace width and the number of sperms per spermatophore could be interpreted as another means of measuring sperm content if a significance difference in size is established.

A number of regulatory neuropeptides are produced and stored in the XO-SG complex and regulate metabolism and molting (Loredo-Ranjel et al., 2017; Lv et al., 2017). A number of neuropeptides also play a role in regulating sex-related process including sexual differentiation and development (Skinner, 1985). The differential expressed gene analysis (Fig. 5) between the ablated and non-ablated AGs reveals a large pool of potential factors under the regulation of the XO-SG complex, some of which could be involved in the sex differentiation process. Aside from IAG and the

membrane-anchored AG-specific factor identified in *Cherax quadricariantus*, very few other genes involved in sex differentiation have yet to be characterized and/or have their roles confirmed (Rosen et al., 2013a). Many genes of potential involvement, however, have been identified to be involved in male sexual differentiation. A total of 47 sex-related gene families were identified in the AG of *Macrobrachium nipponense* and other genes have been identified as playing a role in sexual character developmental in *Eriocheir sinensis* (Jin et al., 2013; Fu et al., 2017). Future studies should investigate these candidate genes identified as sex-related in order to classify and confirm their functions and place within the sex differentiation and developmental pathways.

General Conclusions

The *de novo* transcriptomic assembly of the androgenic gland provided insight into *IAG*, the factors that influence this hormone and the potential for an endocrine access between AG and the XO-SG complex. It is evident that IAG is involved in a dynamic pathway and further investigation into the genes regulating IAG is required. This investigation should start with a transcriptomic analysis of the eye-stalk ganglia in immature and mature males to examine the differentially expressed genes, and identify genes in the potential regulation of *IAG* or other AG-expressed genes of interest.

The expression of *IAG* in male *Sagmariasus verreauxi* is stage-dependent, with low expression in immature males and significantly higher expression in mature males (Chandler et al., 2016). Similarly, in female *C. sapidus*, the female sex

precursor *VTG* can be used to identify the ovarian stage by. IAG hormone hemolymph levels could have a potential to serve as a bioindicator of sexually mature males, which could aid in management decisions of crustaceans by providing another or more precise means of detecting maturity (without sacrifice) within the poorly understood *C. borealis* life history. The *CabIAG* was affected by size in this study, and was determined to be affected by GSI via paired two sample t-test (t = 3.807, p = 0.000674). Since season affects GSI, it would be appropriate to do further investigation into the combined effects of GSI and season. Further steps should be taken to investigate size effects on *IAG* more thoroughly by increasing both the sample size and obtaining a greater/broader size range. More factors should be examined as our seasonal data was limited and no molt cycle data was collected.

When investigating sexual maturity in *C. borealis* including potential regulators of sex differentiation and primary and secondary sex characteristics, it is clear that these aspects are not synchronous in a size-dependent manner. Sexual maturity has been studied in crustaceans for decades, as a better understanding can bring advancements to aquaculture and species management. Maturity in *C. borealis* is clearly a dynamic process that has yet be fully understood. Multiple aspects of sexual maturity need to be investigated when considering the ability to successfully mate, and when evaluating or establishing size limits for management purposes.

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