ABSTRACT

Title of Document INFLUENCE OF PREY DENSITY AND DIETARY SUPPLEMENTATION ON THE GROWTH AND DEVELOPLMENT OF THE BLUE CRAB, *Callinectes sapidus*

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The early developmental stages of arthropods often vary and appear to be influenced by dietary conditions. It is hypothesized that food availability and dietary components may affect the number and length of developmental stages of the blue crab, *Callinectes sapidus,* specifically reared in aquaculture. This hypothesis was examined with *C. sapidus* 1) larvae and 2) juveniles. 1) During the zoeal period, development from stage 1 to megalopae was monitored under a full factorial experiment with treatments: high and low prey density coupled with and without poly-β-hydroxybutyrate (PHB) supplementations. Our data showed that prey density influences variation in the zoeal development of *C. sapidus* by reducing the number of stages from 7-8 to 5-8 via stage skipping. Additionally,

PHB coupled with a high prey density caused increasing instances of stage skipping. 2) During the juvenile period, the growth was monitored for three molting events (57-165 days) under the following treatments: 0, 5, 10, and 20% chitin supplemented diets and squid control. Our data showed that chitin supplementation did not affect the growth of the juvenile *C. sapidus* (molt increment or interval).

INFLUENCE OF PREY DENSITY AND DIETARY SUPPLEMENTATION ON THE GROWT AND DEVELOPMENT OF THE BLUE CRAB, *CALLINECTES SAPIDUS*

By

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

AK	Arginine Kinase
ANOVA	Analysis of Variance
ANCOVA	Analysis of Covariance
ARC	Aquaculture Research Center
ASW	Artificial Salt Water
CBSAC	Chesapeake Bay Stock Assessment Committee
cDNA	complimentary Deoxyribonucleic Acid
CHH	Crustacean Hyperglycemic Hormone
CPRP	CHH Precursor-Related Peptide
DEPC	Diethylpyrocarbonate
DNA	Deoxyribonucleic Acid
EcR	Ecdysteroid Receptor
IMET	Institute of Marine Environmental Technology
MEGA	Molecular Evolutionary Genetics Analysis
MIH	Molt-Inhibiting Hormone
PHB	Polyhydroxybutrate
RNA	Ribonucleic Acid
RP-HPLC	Reversed-Phase High-{erformance Liquid Chromatography
qRT-PCR	quantitative Real Time Polymerase Chain Reaction
RXR	Retinoid X Receptor

CHAPTER 1 INTRODUCTION

The Chesapeake Bay blue crab, *Callinectes sapidus,* is a commercially (Miller et al., 2011; Pelton & Goldsborough, 2008), environmentally (Hines, 2007), and culturally important species. The blue crab commercial fishery for the Chesapeake Bay first began in the mid-nineteenth century (Kennedy et al., 2007). The blue crab is socially important to the Chesapeake Bay region because it is the embodiment of summer traditions and a symbol of identity for the Chesapeake region. Commercially, blue crabs are one of the most harvested species of the Bay. The mean commercial landing for blue crabs in the Chesapeake Bay is 65.7 million pounds a year from 1990-2016 (CBSAC, 2016). Furthermore, the blue crab is considered a keystone species as they are an important link in the Chesapeake Bay food web acting as a very dominant, opportunistic benthic predator and scavenger (Hines, 2007).

According to the 2016 Chesapeake Bay Blue Crab Advisory Report, the blue crab population is not depleted and overfishing is not occurring although overfishing has been a problem in the past. However, in the twelve years between 1994-2008, the population of crabs dropped to two-thirds the previous numbers in the early 1990s. Possible factors contributing to the population depletion were: a continuously low abundance of males, sperm limitation, sponge crab discards, and an increasing overwintering mortality for all crabs (CBSAC, 2015, 2016). Sperm limitation has been studied and viewed not as critical but the degree of which these factors contribute to the current stock is uncertain. The population remains very variable due to many overlying causes (CBSAC, 2016);

the Chesapeake Bay Blue Crab Advisory Report released in 2017 declared that the overall population decreased by almost 18% from 2016-2017 (CBSAC, 2017). Most concerning, the number of juvenile blue crabs decreased by 54%. Therefore, a deeper understanding of the early development of blue crab will assist management practices in the wild and future culture technologies within hatcheries.

1.1 Blue Crab Life History

The geographical range of the blue crab has been documented to stretch from Argentina to Nova Scotia (Norse, 1977; Williams, 1974) with a large population residing in the Chesapeake Bay (Williams, 1974). Blue crabs have a complex life cycle, which involves specific seasonal and geographical breeding and spawning patterns (Hines, 2007, 2003; Jivoff et al., 2007). Female blue crabs in the Chesapeake Bay migrate toward higher salinity areas to produce brood after mating and completing pubertal terminal molt. After release from a female, zoeae are moved via currents along the Atlantic continental shelf where they develop to megalopae (Churchill, 1919; Epifanio, 2007; Sulkin et al., 1980).

Consuming primarily zooplankton, blue crab zoeae require about 30-50 days to reach their post larval stage, the megalopa, under optimal culture conditions (Costlow & Bookhout, 1959). The blue crab requires at most eight zoeal stages to reach the megalopal stage with the majority of zoeae requiring seven stages. Using a microscope, zoeal stages can be determined by the setation of the maxillule and maxilla as well as the formation of pleopod buds on the telson (Costlow & Bookhout, 1959). With each increasing stage, there is a

progressive change in the number of setae on the maxilla and maxillule; except for the fourth and fifth zoeal stage, for which the number of setae on the maxillule remains the same. Pleopod buds first appear at the sixth zoea stage and progressively lengthen in subsequent stages. Succeeding the zoeal stage is the post larval or megalopal stage, which is distinguishable due to the appearance of claws (Costlow & Bookhout, 1959).

Following metamorphosis into the megalopal stage, the crabs enter the estuary via tides and migrate into brackish water to settle and complete their life cycle (Churchill, 1919; Kenneth & Thoman, 1984; Lipcius et al., 2007). Blue crabs remain megalopae for about 7-9 days and molt to the first juvenile instar under optimal conditions (Costlow & Bookhout, 1959). Blue crabs take 12-18 months to reach maturity in the Chesapeake Bay (Van Engel, 1958); however, this is largely temperature dependent and may vary depending on the timing of spawning (Lippson, 1973).

Blue crabs molt to grow; this process is repeated from hatching through adulthood. As the crab grows, the time between molting events, i.e., molt interval increases, like other crustaceans (Chippindale & Palmer, 1993; Klein-Breteler, 1975; Millikin & Williams, 1984; Vidal, 1980; Brylawski & Miller, 2006). The molt cycle includes several different stages: intermolt, premolt, ecdysis and postmolt. During the intermolt stage (stage C) the crab is feeding and storing nutrients in its tissues, hence increasing its tissue mass (Drach & Tchernigovtzeff, 1967). During the premolt stage (stage D), the crab is preparing to molt by separating the old exoskeleton from the newly formed exoskeleton derived from

accumulated reserves and the old exoskeleton. Ecdysis (stage E) is the actual molting process (shedding the old exoskeleton) followed by the postmolt stage (stage A & B) in which the crab is hardening its shell.

The crustacean hyperglycemic hormone family (CHH) members are responsible for many essential physiological pathways including carbohydrate metabolism and molting in decapods (Chung et al., 2010; Webster et al., 2012). Neuropeptides associated with CHH are: CHH1, CHH2, and the CPRP (crustacean hyperglycemic hormone precursor-related peptide) (Fig 1). CPRP is known as a circulating hormone, while the exact function of CPRP is clearly not yet defined to date and it doesn't have same function as CHH (Wilcockson et al., 2002). The minor isoform of CHH, CHH1, is almost identical to CHH2 except that the N-terminal residue is a glutamine rather than pyroglutamate (Chung and Webster, 1996).

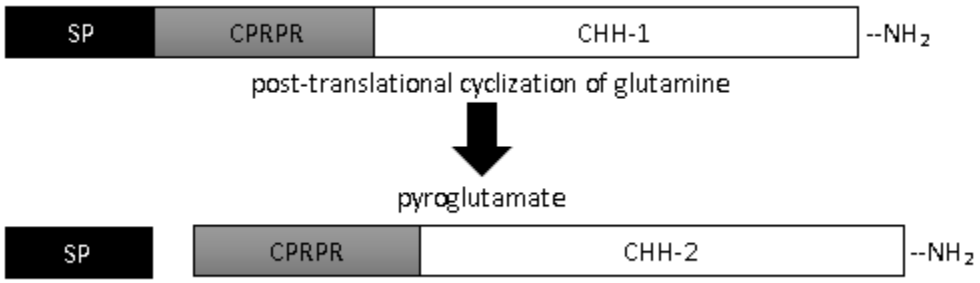


Figure 1. Schematic diagram of the neuropeptides (CPRP, CHH1 and CHH2) and the signal peptide (SP) associated with the crustacean hyperglycemic hormone (CHH).

Molting in crustaceans is regulated by numerous hormones and neurohormones but is under direct control by ecdysteroids (Chang et al., 1993).

Ecdysteroids, produced and secreted by the Y-organ, are suppressed during the intermolt by the molt-inhibiting hormone (MIH)/ CHH produced in the X-organ–sinus gland complex located in the eyestalk ganglia (Hopkins, 1982; Keller & O'Conner, 1982; Chang et al., 1990; Techa & Chung, 2015). During the premolt stage, ecdysteroids are no longer inhibited by MIH/CHH allowing molting to commence (Webster et al., 2012). During late premolt and during ecdysis, levels of CHH, from the foregut and hindgut, increase in the hemolymph. This increase in CHH levels regulate water and ion uptake allowing for successful molting and the subsequent increase in size during postmolt (Chung et al., 1999).

Ecdysteroids regulate ecdysone-response genes by binding to the ecdysteroid receptor (EcR), which then heterodimerizes with retinoid X receptor (RXR) (Durica & Hopkins, 1996; Zou & Fingerman, 1999). The EcR/ RXR heterodimer complex is translocated to nucleus and binds to DNA response elements of the transcription of early and late ecdysone responsive genes which regulate molting process. In *C. sapidus*, four different isoforms of EcRs and RXRs are found in all types of tissues (Techa & Chung, 2012).

Overall, molting is ultimately controlled by hormones and neurohormones (Costlow, 1963; Freeman & Costlow, 1980; Webster, 2015); however, the process can be influenced by environmental factors such as temperature and nutrition (McConaugha, 1985; Hartnoll, 2001; Hartnoll, 2015) and manipulated by chemical cues (Forward, et.al, 1996; O'Connor & Judge, 1997). It is presumed that a certain reserve of energy must be accumulated before molting can occur (Adelung, 1971). In crustaceans,growth rate is reduced when nutrition is below

the optimal level (Hartnoll, 1982), a reduction resulting from the combination of an extended intermolt period and a reduced molt increment even seen in zoeal development (Chittleborough, 1975; Minagawa & Murano, 1993). The development of later life stages is contingent on what occurs during zoeal development (Strathmann, 1985); which is why a deeper knowledge of zoeal biology is essential to understanding *C. sapidus* species.

2. Variation in zoeal development

The life cycle of a species is predetermined by genetics; however, the development of crustaceans and insects show plasticity or variability in the earlier life stages. Traditionally, the duration and morphology of each crustacean zoeal stage was thought to be constant and any variation observed was believed to be anomalies resulting from rearing conditions (Gurney, 1942). However, many species display plasticity in zoeal development, which may be a natural adaptive mechanism to suboptimal conditions and/or competition of resources. Crustacean species such as *Pleuroncodes planipes* (Boyd & Johnson, 1963)*, Naushonia crangonoides* (Goy & Provenzano, 1978), *Petrolisthes elongates*, *P. novaezelandiae* (Greenwood, 1965)*, Porcellana longicornis, P. platycheles* (Lebour, 1943)*, Eualus occultus, E. pusiolous* (Pike & Williamson, 1961)*,* and *Upogebia deltaura* (Webb, 1919) show plasticity in zoeal development when wild caught and produced in laboratory conditions.

Plasticity in zoeal development is evident where molt interval and number of molts required to reach the megalopal stage vary per individual. This may result from individual genetic differences inherited from parents, which may

predispose zoeae to developmental plasticity. In insects, genetics has been attributed to variation in zoeal stages between the offspring of different individuals from the same population and in zoeae from different populations (Jones et al., 1981; Mira & Raubenheimer, 2002; Morita & Tojo, 1985; Telfer & Hassall, 1999).

Variation is common both between batches and within batches of crustaceans. *Clibanarius vittatus* batches from the same season but different years show variation in zoeal development (Lang & Young, 1977). High variation in zoeal development, specifically size, molting frequency, stage morphology, and survival, among batches originating from different females is evident in *Crangon sp.,* (Criales & Anger, 1986). Variation in the number of zoeal stages of a species due to local condition adaptations impacts its geographical distribution (Criales & Anger, 1986).

Variation also occurs within the same batch and may result from differences in conditions in the egg mass itself as seen in the *Hyas araneus* (Kunisch & Anger, 1984). Observations show that zoeae produced in one hatching event can vary developmentally from another hatching event. *P. planipes, H. americanus* and *Macrobrachium idea* show variation within a single batch (Boyd & Johnson, 1964; Pandian, 1970; Pandian & Katre, 1972). Zoeae from the same batch can differ in size, larval duration, and mortality (Kunisch & Anger, 1984). Within *C. sapidus,* genetic diversity is notably high in adults as well as larval populations (Feng et al., 2017; Plough, 2017), which may account for

developmental variation in zoeae when reared in the same conditions (Costlow, 1965).

Gender, population density, photoperiod, diet (quality and quantity), season (temperature, humidity and rainfall), genetics (maternal effects and inheritance) (Esperk et al., 2007), and chemical stimuli (Forward et al., 1996; Sui et al., 2014) influence variation in arthropods specifically the number of larval stages. However, environmental factors such as temperature and food availability appear to be a critical factor affecting development. Higher temperatures can affect zoeal development by reducing the number of stages from 5 to 4 as seen in *Galathea rostrate* (Gore, 1979) and *Palaemonetes varians* (Oliphant et al., 2013) or by increasing the number of stages from 5 to 5-13 as seen in *Pleuroncodes planipes* (Boyd & Johnson, 1964). Lower temperatures increase the number of stages from 5 to 6 as seen in *Eriocheir sinensis* (Anger, 1991) and from 5 to 5-7 in *P. varians* (Oliphant et al., 2013). The frequency of molting in *Crangon sp.,* is controlled mainly by temperature (Criales & Anger, 1986) compared to other environmental conditions. Extra zoeal stages are added when zoeae experience suboptimal conditions during development. For example, more zoeal stages occur in *Palaemonetes sp.,* and *Crangon sp.,* at stressfully high temperatures (Criales & Anger, 1986; Oliphant et al., 2013).

Food availability and dietary conditions such as low prey density and poor prey quality cause zoeal variation for crustaceans even at optimal temperatures (Broad, 1957; Costlow & Bookhout, 1959; Porter, 1960; Sandoz & Rogers, 1944; Templeman, 1936b). At 6 to18°C, the wrong types of prey tend to increase the

number of zoeal stages and of morphological forms in *Crangon sp.,* (Criales & Anger, 1986). At 25 to 27°C, low prey availability slows the growth of *P. pugio* and *P. vulgaris* (Broad, 1957a), and *C. sapidus* at 20 to 29°C (Sandoz & Rogers, 1944) by prolonging zoeal duration through lengthening intermolt period, hence reducing the molt frequency. Interestingly, the lack of prey extends zoeal duration by adding stage(s) from 3 to 3-4 in *Homarus americanus* (Templeman, 1936a), from 7 to 7-8 in *C. sapidus* (Costlow & Bookhout, 1959), from 5 to 5-6 in *Menipe mercenaria* (Porter, 1960), from 5 to 5-6 in *Scylla paramamosain* (Zeng et al., 2004) and from 7 to 7-13 in *P. pugio* and *P. vulgaris* (Broad, 1957b). On the other hand, optimal dietary conditions may result in the reduction of zoeal development either through fewer larval stages or shorter intermolt periods. *Emerita talpoida* reared in laboratory conditions was shown to skip the stage prior to the megalopal stage under optimal conditions (Rees, 1959).

Chemical stimuli have also shown to alter growth in many organisms. Poly-β-hydroxybutyrate (PHB) produced naturally by terrestrial and aquatic bacteria, enhanced the development rate of the *E. sinensis* (Sui et al., 2014), when supplemented to the diet. Other chemical substances found in the natural environment have shown to induce molting by shortening the length of the megalopal stage and has been attributed to organic substrates, vegetation, biofilms, conspecifics and similar species, and the presence of prey (Forward et al., 2001, O'Connor & Judge, 1997).

Developmental plasticity has been observed between the zoeae reared in the laboratory and those sampled from the wild (Boyd & Johnson, 1964; Costlow

& Bookhout, 1959; Goy & Provenzano, 1978; Lebour, 1943; Rothlisberg, 1980; Webb, 1919), suggesting variation may occur naturally, possibly due to genetics in addition to environmental conditions or laboratory/hatchery conditions. It is possible that there are other mitigating factors that have not been identified, but the developmental path an individual completes is greatly influenced by external factors like temperature and diet.

The life cycle of *C. sapidus* includes a relatively long larval development period (Church, 1911). The number of zoeal stages varies from eight stages in cultured and wild conditions (Costlow & Bookhout,1959; McConaugha et al., 1983) to six stages when females are induced to produce eggs during the winter (Sulkin et al., 1976). However, little is known about which zoeal stages are maintained or skipped.

3. Dietary conditions

In the Aquaculture Research Center (ARC) at the Institute of Marine and Environmental Technology (IMET, Baltimore, MD), hatchery technology has been developed to close the life cycle of *C. sapidus* by rearing blue crabs from embryo to adulthood in recirculating aquaculture systems (Zmora et al., 2005). Adult females are collected from the Chesapeake Bay every year and produce zoeae for the following year's spawning season (Bembe et al., 2017).

In our hatchery, blue crab zoeae are fed with a live food including algae, rotifers, and *Artemia* nauplii. The zoeal stages 1-4 consume a high density of rotifers consisting of about 50 rotifers/ml. At zoeal stage 3-4, *Artemia* nauplii are

introduced into the diet in addition to the rotifers until the megalopal stage. At the megaploal stage, only *Artemia* nauplii are fed. *Artemia* nauplii feeding continues once the crabs reach juveniles for three weeks (during ~3 juvenile stages); at this time fishmeal pellets (1-2 mm) are introduced to the crabs using a weight based ration. The *Artemia* nauplii are then replaced with shredded squid at the 4th juvenile stage. The juveniles are fed pellets of increasing size and shredded squid until use (Zmora et al., 2005).

When rearing animals in aquaculture settings, there is a tendency to overfeed (Millikin et al., 1980; Winget et al., 1976) for maximum productivity. Furthermore, dietary supplements are common practice in aquaculture to enhance growth and survival (Bricknell & Dalmo, 2005; Edun & Akinrotimi, 2011; Ringo et al., 2014; Yousefian & Amiri, 2009). Natural substances that are used as dietary supplements are:PHB (De Schryver et al., 2010; Najdegerami et al., 2012; Situmorang et al., 2013; Sui et al., 2014, 2013; Thai et al., 2014; Van Hung et al., 2015) and chitin (Cha et al., 2008; Esteban et al., 2001; Gopalakannan & Arul, 2006; Powell & Rowley, 2007; Shiau & Yu, 1998).

1.4 Poly-β-hydroxybutyrate (PHB)

PHB is a carbon and energy storage compound accumulated by many bacteria strains (Suriyamongkol et al., 2007). It is believed that PHB, once ingested, is degraded into β-hydroxybutyrate monomers and oligomers by bacteria during gastrointestinal digestion, and these compounds positively influence a species health (Yu et al., 2005). It has been shown that *Artemia* nauplii and rotifers, the primary live feed for larviculture, can ingest PHB (Defoirdt

et al., 2007b; Sui et al., 2014) and pass on the beneficial properties of PHB to zoeae, making PHB an attractive dietary supplement in aquaculture.

PHB acts as a microbial control agent combatting diseases and harmful bacteria (Defoirdt et al., 2007b). For many species raised in hatchery settings, PHB improves survival and/or growth (De Schryver et al., 2010; Defoirdt et al., 2007b; Laranja et al., 2014; Najdegerami et al., 2012; Van Hung et al., 2015) especially when species are challenged with pathogens (Defoirdt et al., 2007b; Laranja et al., 2014). The enrichment of live feed with PHB particles also shows immunity and growth benefits in crustacean species. PHB helped to protect *E. sinensis* from *Vibrio anguillarum* resulting in higher survival and better growth, especially when PHB was applied before pathogen contact (Sui et al., 2012). In *E. sinensis*, enhanced molting and weight gain were observed with increasing PHB concentration. An or the interval between molts was shortened when zoeae were fed PHB (Sui et al., 2014).

1.5 Chitin

Another dietary supplement used in aquaculture is chitin. Chitin, ($C_8H_{13}O_5N$), is the second most important natural polymer following cellulose and is synthesized for structural purposes such as cell walls and exoskeletons in many species including crustaceans. Crustacean exoskeletons contain about 15-40% chitin depending on the species (Kurita, 2006) and molt cycle stage (Kono et al., 1995; Zhang et al., 2014). Digestion of the old chitinous cuticle at the beginning of the molt cycle by proteinases and chitinolytic enzymes found in the

molting fluid (Samuels & Reynolds, 1993) allow about half of the organic content to be reabsorbed (Cameron & Wood, 1985).

Naturally, crustaceans consume some chitin through their prey. Stomach content analysis reveals that juvenile and adult blue crabs consume a variety of prey including bivalves, crustaceans, fish, plant matter/algae, polychaetes, and detritus (Hines, 2007; Reichmuth et al., 2009). Some crab species have the enzymatic capability of digesting chitin by producing protease and chitinase. Crustacean omnivores and carnivores such as *Leptograpsus variegatus* and *Nectocarcinus tuberculosus* contain high protease and chitinase activity in the stomach for digesting shells of invertebrates (Johnston & Freeman, 2005). In laboratory conditions, blue crabs regularly consume their own shed cuticle following ecdysis even as young as larvae (Sandoz & Rogers, 1944) possibly suggesting a beneficial dietary effect via reabsorption of chitin. It has been reported that the digestion of chitin has a significant nutritional contribution in shrimp (Clark et al., 1993).

Chitin and its derivative, chitosan, are used in an array of applications due to its beneficial properties including food applications for human and animal consumption (Agulló et al., 2003; Shahidi et al., 1999), the medical field (Madhumathi et al., 2010; Park & Kim, 2010; Singla & Chawla, 2001), agriculture (Burrows et al., 2007; El Hadrami et al., 2010; Hirano et al., 1990), cosmetics (Morganti & Morganti, 2008), wastewater treatment (Aly et al., 1997), and chemical industries (Kurita, 2006).

More recently, chitin and chitosan have been applied to aquaculture. The addition of chitosan has been observed to stimulate the innate immune system in both fish and crustaceans (Esteban et al., 2001; Fang et al., 2015; Gopalakannan & Arul, 2006; Lin et al., 2012; Powell & Rowley, 2007). *Cyprinus carpio* showed better survival when challenged with *Aeromonas hydrophila* infection with a chitosan and chitin supplemented diet (Gopalakannan & Arul, 2006). Additionally, reductions in the abundance of two different *Vibrio* sp., were observed when chitosan was administered to oysters as a post-harvest process (Fang et al., 2015). Flounder fed moist pellets with chitosan also showed an increase in positive immune responses as well as a reduction in the chemical oxygen demand and suspended solids in the water quality (Cha et al., 2008).

However, species vary in their growth responses when provided a chitin supplement. The addition of chitin (2 or 5%) to the diet of *Penaeus monodon* enhanced feed efficiency, protein efficiency ratio and growth (Shiau & Yu, 1998). Yet, dietary supplementation of chitin and chitosan to the diet of tilapia significantly depressed growth (Shiau & Yu, 1999), whereas it did not affect flounder growth (Cha et al., 2008). The beneficial effects of chitin to crustaceans also depends on chitin levels; increasing chitin to 10% severely reduced the growth of the *P. monodon* and significantly depressed protein and lipid digestibility of the diet (Shiau & Yu, 1998). *C. sapidus* fed 20% chitin also showed a significantly lower final wet weight and longer molt interval (Allman et al., 2017).

It has been suggested that chitin is the major energy source for chitin synthesis because the reabsorption of the old cuticle provides enough material

for new chitin synthesis and energy for molting in *Orconectes limosus* (Gwinn & Stevenson, 1973). It was also found that supplementing iso-energetic diets with chitin at 0-16% did not significantly affect growth or feed conversion of juvenile *P. monodon;* it did significantly decrease the level of chitinase activity with increasing levels of dietary chitin (Fox, 1993).

There is also evidence that the eyestalks are also involved in the hormonal control of chitin (Knowles & Carlisle, 1959). CHH, synthesized in the X-organ-sinus-gland complex in the eyestalk, is known as a pleiotropic hormone and plays an important role in carbohydrate metabolism of crustaceans (Chung et al., 2010; Webster et al, 2012; Vinagre & Chung, 2016; Nagi et al., 2011). As stated earlier, it is unknown whether dietary chitin may modulate the levels of CHH in the eyestalk ganglia and chitinase expression in hepatopancreas of the animals at the intermolt stage.

1.6 Project Objectives

A series of experiments were carried out to better understand the early development of *C. sapidus*, specifically how food availability and dietary supplementation may be contributing factors in growth. The first experiment involved supplementing live prey (rotifers and *Artemia nauplii)* with PHB comparing the effects of prey density and PHB supplementation on zoeal duration, the number of zoeal stages and survival at room temperature (21-23°C). Newly-hatched *C. sapidus* larvae were reared to megalopae in 24-well plates under the following treatments: high and low prey density; and high and low prey density with poly-β-hydroxybutyrate (PHB) supplementation (50 and 100

mg/l). We predicted that a high prey density supplemented diet would decrease larval duration and the number of stages as well as improve survival. A high prey density treatment was modified from the standard Aquaculture Research Center (ARC, Baltimore, MD) regime and a minimal density was established.

A second experiment involved formulating diets supplemented with chitin and compared the effects of chitin supplementation on juvenile size, molt interval and changes in metabolism at constant temperature (21°C -23 °C). Juvenile *C. sapidus* were reared in individual compartments in a recirculating system under the following treatments: squid control; and chitin supplementation (0, 5, 10, and 20% of a wet weight of formulated diet). We hypothesized that a chitin supplement might enhance juvenile growth possibly by decreasing molt interval. To examine if chitin-enhanced growth was modulated by CHH and carbohydrate metabolism, eyestalk ganglia and hepatopancreas tissues were analyzed to determine CHH and chitinase expressions, together with CHH neuropeptide levels in the sinus gland. Additionally, hepatopancreas and hemolymph samples were assayed for measuring protein and carbohydrate levels (glucose, trehalose, and glycogen).

CHAPTER 2: EFFECTS OF PREY DENSITIES AND PHB SUPPLEMENTATION ON THE EARLY DEVELOPMENT OF THE BLUE CRAB, *Callinectes sapidus*

1. Introduction

Although genetically determined, the early life stages of arthropods, including crustaceans and insects, exhibit some degree of plasticity. Many species of decapod crustaceans have shown plasticity in larval development when reared both in the wild and in laboratory conditions via the addition or omission of zoeal stage(s). Examples include the anomuran crabs *Pleuroncodes planipes* (Stimpson, 1860) (Boyd & Johnson, 1963), *Petrolisthes elongatus* (H. Milne Edwards, 1837), and *P. novaezelandiae* (Filhol, 1885) (Greenwood, 1965), *Porcellana longicornis* (Linnaeus, 1767) and *P. platycheles* (Pennant, 1777) (Lebour, 1943), the thalassiniid shrimp *Naushonia crangonoides* (Kingsley, 1897) (Goy & Provenzano, 1978), the caridean shrimps *Eualus occultus* (Lebour, 1936) and *E. pusiolous* (Krøyer, 1841) (Pike & Williamson, 1961), and the upogebid *Upogebia deltaura* (Leach, 1816) (Webb, 1919).

Molting during the early life cycle, including in the larval stages, is also affected by external factors such as temperature and dietary conditions (Hartnoll, 2001, 2015). Food availability and dietary conditions such as low prey density and poor prey quality cause larval variation for crustaceans even at optimal temperatures (Broad, 1957b; Costlow & Bookhout,1959; D'Urban et al., 2014; Gimenez & Anger, 2005; Porter, 1960; Sandoz & Rogers, 1944; Templeman, 1936b). At 25 °C to 27 °C, low prey availability slowed growth in the palaemonid

shrimps *Palaemonetes pugio* (Holthuis, 1949) and *P. vulgaris* (Say, 1818), with the suppression of molt frequency and rate of development (Broad, 1957a), and in *C. sapidus* Rathbun, 1896 at 20 °C to 29 °C (Sandoz & Rogers, 1944) by prolonging zoeal development through lengthening intermolt period, hence reducing frequency of molting. Growth, measured by dry weight, survival, and development rates, was significantly decreased in the brachyuran crab *Panopeus herbstii* (H. Milne Edwards, 1834) when prey abundance was low (Welch & Epifanio, 1955).

Limited access to prey noticeably slowed development after the third zoeal stage in other decapods such as *Homarus gammarus* (Linnaeus, 1758) and *Necora puber* (Linnaeus, 1767) (D'Urban et al., 2014). Duration of the first zoeal stage was generally more prolonged in the brachyuran crab *Neohelice granulata* (Dana, 1851) when access to prey was limited (Gimenez & Anger, 2005). Lack of prey extends duration of zoeal development of many crustaceans by adding stage(s) from three to four in *Homarus americanus* (H. Milne Edwards, 1837) (Templeman, 1936b), seven to seven or eight in *C. sapidus* (Costlow & Bookhout, 1959), five to five or six in *Menippe mercenaria* (Say, 1818) (Porter, 1960), five to five or six in *Scylla paramamosain* (Estampador, 1950) (Zeng *et al.,* 20142004), and seven to seven to 13 in *Palaemonetes pugio* and *P. vulgaris* (Broad, 1957a). Survival to post-zoeae was also increasingly affected by a continuous prey limitation in *N*. *granulata* (Gimenez & Anger, 2005).

Polyhydroxybutyrate (PHB), although produced naturally by terrestrial and aquatic bacteria as a carbon energy-storage compound (Suriyamongkol et al.,

2007), shortened molt interval when provided as a supplement to the zoeae of the brachyuran *Eriocheir sinensis* (H. Milne Edwards, 1853) (Sui et al., 2014). It has been shown that *Artemia* nauplii and rotifers, the primary food staples for larviculture, can ingest PHB particles (Defoirdt et al., 2007; Sui et al., 2014) and pass on the beneficial properties of PHB to zoeae, making PHB an attractive dietary supplement in aquaculture. PHB acts as a microbial-control agent, combatting diseases and harmful bacteria (Defoirdt et al., 2007). PHB improves survival and/or growth in many crustaceans raised in hatchery settings (De Schryver et al., 2010; Defoirdt et al., 2007; Laranja et al., 2014; Najdegerami et al., 2012; Van Hung et al., 2015), especially when species are challenged with pathogens (Defoirdt et al., 2007; Laranja et al., 2014). In *Eriocheir sinensis,* PHB also showed increased survival when applied before pathogen contact (Sui et al., 2012).

The number of the zoeal stages of *C. sapidus* varies. Eight stages at most are found in cultured and wild conditions (Costlow & Bookhout,1959; McConaugha et al., 1983); six with zoeae produced during the winter (Sulkin *et al.,* 1976). Regardless of variation in the number of stages, some stages such as the first and eighth are always included in their development. In general, the minimal number of zoeal stages occurs at conditions optimal for survival, whereas extra stages result from suboptimal conditions during development (Anger, 2001; 2006; Anger et al., 2015; Gore, 1985; Sandifer & Smith, 1979). It is still unknown, however, which of the zoeal stages of *C. sapidus* are maintained or skipped.

We examined the influence of dietary conditions (prey availability and PHB supplementation) on the larval development of *C. sapidus,* specifically on the number and duration of zoeal stages, survival, and daily food consumption. The zoeae were produced by broodstocks kept in a blue crab hatchery (IMET, Baltimore, MD) and were used to determine the obligatory zoeal stages.

2. Materials and Methods

2.1 Collection of specimens

Primaparous adult female *C. sapidus* were collected in the mid-upper Chesapeake Bay during the falls of 2013 to 2015 (Supplementary material Fig. S1). The water temperature and salinity were measured as 16 ± 2 °C and 10 ppt, respectively. Females were transported to the blue crab hatchery in the Aquaculture Research Center (ARC), Institute of Marine and Environmental Technology (IMET), Baltimore, MD, USA, and placed individually in tanks (14.6 cm w × 29.4 cm l × 19.8 cm h) containing water at a salinity of 15 ppt for the screening of pathogens, a reo-like virus, CsRV1 (Bowers et al., 2010; Flowers et al, 2015) and the parasitic dinoflagellate *Hematodinium* spp. (Nagle et al., 2009). Crabs free of these diseases, about a total of 60 females from 2013 to 2015, were kept as broodstock at 21 ºC to 23 ºC in recirculating artificial seawater (ASW) at a salinity of 30 ppt at an 8:16 light/dark photoperiod condition (Bembe et al, 2017).

Females were examined for extrusion of eggs. Biopsies of the embryos were taken to determine the exact developmental stages of the embryos (Chung & Webster, 2004). The females carrying embryos at an imminent hatching stage,

i.e. one or two days prior to hatching, were transferred from broodstock tanks to a hatching container holding ~50 liters of ASW at a salinity of 30 ppt (Bembe et al., 2017). All the zoeae used were produced from May to September from 2014 to 2016.

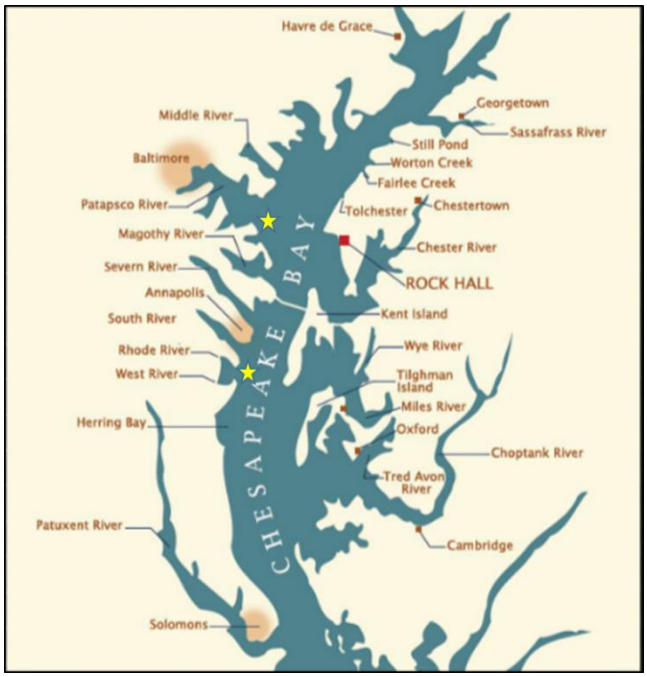


Figure 1. Primaparous adult female, *C. sapidus* were collected in the mid-upper Chesapeake Bay in the falls of 2013-2016 as indicated by yellow stars. http://www.havencharters.com/cruise.php

2.2 Raising of the larvae

Upon hatching, the zoeae that displayed strong positive phototaxis by swimming toward a beam that was produced by a high-intensity light (110 lumens), were collected for the experiments (Moresino & Helbling, 2010). These zoeae were first kept in a 1 l beaker holding ASW at a salinity of 30 ppt with mild aeration for 1–2 h to let zoeae settle. The experiment was carried out at constant temperature (21 ºC to 23 °C). On the day the zoeae hatched, counting as day 0,

each zoea was placed individually into a well containing 1 ml ASW previously filtered through a 0.22 µm membrane at a salinity of 30 ppt in 24-well plates (maximal volume ~3 ml volume/ well). The zoeae were fed daily around 11:00 to 12:00 according to the treatment (Table 1) until megalopae under a 12:12 light/dark photoperiod. Water was exchanged every other day by gently transferring zoeae to a new well plate containing the membrane filtered, fresh ASW at a salinity of 30 ppt.

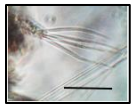
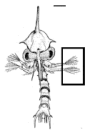
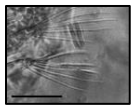
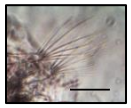
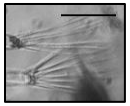
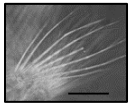
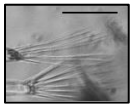
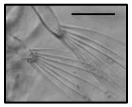
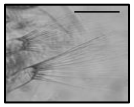
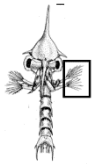
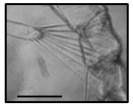
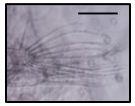
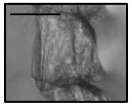
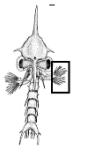
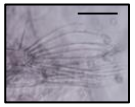
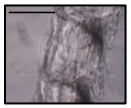
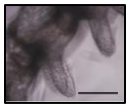
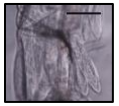
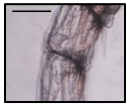
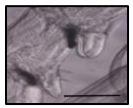
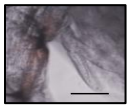
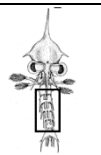
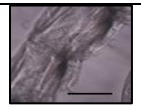
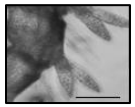
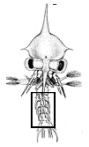
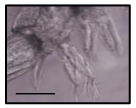
Table 1. Density of prey during *C. sapidus* zoeal development from hatching to megalopae

	Zoea 1-3	**Zoea 4-6**	**Zoea 6-Megalopa**	
	Rotifers/ml	**Rotifers ml + Fresh *Artemia*/ml**	**Fresh *Artemia*/ml**	**No. of batches**
High prey density	~50	20 ± 5; 7±1	15±1	6
PHB (mg/l) 0 50 100				3
Low prey density	~20	10 ± 4; 4±1	7±1	6
PHB (mg/l) 0 50 100				3

Every morning between 8:00 and 10:00, the zoeae were 1 examined for mortality and molting under a dissection microscope (magnification 20×) using the criteria of Costlow & Bookhout (1959). After each molt, the exuviae and/or zoeae were carefully removed using a 1 ml plastic Pasteur pipette and placed on a slide glass. Photographs of the setae of the zoeae at stages one through five

and pleopod buds of the zoeae at stages six through eight were taken using a AmScope MT camera under a compound microscope (100× magnification). The stages of all zoeae, except from stages three to five only in some cases, were identified. If a stage could not be distinguished due to the condition of the molt or the movement of zoeae, it was recorded as undetermined (Table 2).

Table 2. Drawings of eight zoeal stages of *Callinectes sapidus* and corresponding morphological differences used to determine stages (obtained from Costlow & Bookhout, 1959) with zoeal image (at magnification 100 ×): stage after hatching (A), stages after first molting event (B,C, D, E), stages after second molting event (F,G, H), stages after third molting event (I, J, K), stages after fourth molting event (L, M, N, O), stages after fifth molting event (P, Q, R), stages after sixth molting event (S, T), stage after seventh molting event (U). Image scale bar = 100 μm.

Stage description	Images of each zoeal stage and percentage of stages observed: given as % ± SE; *N* = zoeae studied
After hatching: 1st and 2nd maxillipeds have 4 swimming setae 	A  Features: 1st & 2nd maxillipeds with 4 setae: 100 ± 0.0; 136
After 1st molting event: 1st and 2nd maxillipeds have 6 swimming setae 	B  Features: 1st & 2nd maxillipeds with 5 setae: 5.3 ± 2.9; 8 C  Features: 1st & 2nd maxillipeds with 6 setae: 71.9 ± 5.0; 105 D  Features: 1st & 2nd maxillipeds with 7 setae: 19.9 ± 1.7; 28 E  Features: 1st & 2nd maxillipeds with 8 setae: 2.9 ± 2.9; 3
After 2nd molting event: 1st and 2nd maxillipeds have 8 swimming setae 	F  Features: 1st & 2nd maxillipeds with 7 setae: 11.7 ± 4.4; 15 G  Features: 1st & 2nd maxillipeds with 8 setae: 60.4 ± 3.7; 76 H  Features: 1st & 2nd maxillipeds with 9 setae: 16.7 ± 2.6; 20 Stage undetermined:11.2 ± 4.8; 13
After 3rd molting event: 1st and 2nd maxillipeds have 9 swimming setae 	I  Features: 1st and 2nd maxillipeds with 9 setae: 52.3 ± 8.2; 58 J  Features: 1st & 2nd max-illipeds with 9 & 10 setae: 9.9 ± 1.6; 10 K  Features: pleopod buds on segments 2-6 abdomen: 10.9 ± 4.7; 10 Stage undetermined: 27.0 ± 3.0; 28
After 4th molting event: 1st maxiliped has 9 and 2nd maxiliped has 11 swimming setae 	L  Features: 1st & 2nd max-illipeds with 9 & 10 setae: 16.0 ± 6.9; 14 M  Features: pleopod buds on segments 2-6 abdomen: 30.7 ± 3.0; n=31 N  Features: pleopod buds extend in length: 24.2 ± 7.0; 26 O  Features: pleopod buds project beyond border of abdomen: 12.5 ± 5.5; 13 Stage undetermined: 16.7 ± 5.1; 15
After 5th molting event: Pleopod buds appear on segments 2-6 of abdomen 	P  Features: pleopod buds on segments 2-6 abdomen:: 38.7 ± 13.3; 39 O  Features: pleopod buds extend in length: 25.5 ± 4.2; 23 R  Features: pleopod buds project beyond border of abdomen: 35.8 ± 4.2; 33
After 6th molting event: pleopod buds extend in length along abdomen 	S  Features: pleopod buds extend in length: 23.0 ± 5.6; 12 T  Features: pleopod buds project beyond border of abdomen: 77.0 ± 5.5; 42
After 7th molting event: Pleopod buds project beyond border of abdomen 	U  Features: pleopod buds project beyond border of abdomen: 100 ± 0.0; 12

2.3 Prey density experiments

Rotifers and *Artemia* nauplii were cultured in ARC following Zmora et al. (2005). Rotifers were fed the marine-microalga *Nannochloropsis* sp., (Nanno 3600™, strain CCMP525, Reed Mariculture, Campbell, CA) and baker's instant yeast (Fleischmann, Memphis, TN) *ad libitum* (Zmora et al., 2005). Artemia cysts (Artemia International, Fairview, TX) were rehydrated and decapsulated prior to hatching. Live *Artemia* nauplii (~18–22 h after hatching) were used for experiments.

The daily feeding regime for zoeal development was used based on a preliminary food consumption study that was modified from Sulkin (1978) and Zmora et al., (2005). The zoeae (stages 1 to 3) received only rotifers for the first 14 days, both rotifers and *Artemia* were delivered from 15–25 days (stages 4 to 6), and only *Artemia* from day 26 until the end of the experiment (stages 6 to megalopae). For the experiment using a high density of zoeae, 50 rotifers/ml and 15 *Artemia* nauplii/ml were provided based on the time prey remained in the gut of a zoea of the brachyuran crab *Scylla serrata* (Forskål, 1775) (Serrano, 2012). For the low prey density experiment, preliminary data showed that a minimum number of ~20 rotifers/ml in a 24-well plate was sufficient for promoting molting because there were always uneaten rotifers left in the wells. Similarly, seven *Artemia* nauplii/ml (~50% of high density treatment) was sufficient to promote growth in zoeae. Daily food consumption was monitored by subtracting the number of prey left after 24 h in the well from the initial number of rotifers and/or

Artemia delivered. In total, we used 596 zoeae from six batches produced by six different females over three years.

2.4 PHB supplementation experiments

Zoeae were exposed to high and low prey densities (Table 1). Rotifers and *Artemia* nauplii were enriched with 50 and 100 mg/l of PHB for ~18–22 h, as reported by Sui et al. (2014).

Rotifers were also fed a marine microalga *Nannochloropsis* sp., and Baker's yeast *ad libitum* (Zmora et al., 2005). *Artemia* nauplii were collected 18–22 h after hatching. There were no visual differences in appearance or behavior in the rotifers or *Artemia* nauplii fed with or without PHB. In total, 864 zoeae were used from the batches produced by three different females over two years.

2.5 Statistical Analysis

A power analysis was performed to determine the minimal number of batches to decrease batch to batch variation. Power analysis showed that to detect a difference of 0.5 with power of 0.80, at least six batches are needed. As a result, a paired *t*-test was performed to determine statistical significance in zoeal development (survival, number of stages, duration and prey consumption) between high and low prey density ($P < 0.05$). A $\chi 2$ test was performed to determine if there was a relationship between the number of stages and the following: density and duration of zoeal development. Additionally, an ANCOVA was performed to determine test the main and interaction effects of density and the number of stages controlling for the effects of duration of zoeal development.

Only three batches were used for the PHB supplementation experiment with each prey density so a randomized block ANOVA analysis was performed ($P < 0.05$) to determine significance of differences in zoeal development (number of stages and larval duration) between three concentrations of PHB supplementation at 0, 50 and 100 mg/l. A $\chi 2$ test was performed to determine if there was a relationship between the number of stages and PHB supplementation with a high and low prey density. Additionally, an ANCOVA was performed to determine test the main and interaction effects of PHB supplementation and the number of stages controlling for the effects of duration of zoeal development.

3. Results

3.1 Effect of prey density on larval development without supplementation

The density of prey (rotifers and/or *Artemia*) affected survival of zoeae (Fig. 2A). Prey density affected survival (Fig. 2A). The survival rate of zoeae kept under a high prey density was 64.0 ± 4.3% (391 zoeae surviving), which was significantly higher ($P < 0.001$, Student's paired *t*-test) than 38.2 ± 5.1% with a low prey density (205 zoeae surviving).

The zoeae with a high prey density (391 zoeae surviving) showed greater variation in the number of stages from five to eight: stages: five (10.6 ± 2.9%), six (42.4 ± 5.2%), seven (35.6 ± 3.3%), and eight (11.4 ± 2.8%) (Fig. 3). The majority of the zoeae reached the megalopal stage e after progressing through six or seven stages. Zoeae with a low prey density (205 zoeae surviving) progressed through six to eight stages: six (17.4 ± 3.4%), seven (56.5 ± 2.6%), and eight

(26.0 ± 4.0%). The majority of the zoeae reached the megalopal stage after progressing through seven or eight stages (Fig. 3). The number of stages required to reach the megalopal stage was significantly associated with the density ($P < 0.001$, $\chi 2$ test). On average, the zoeae with a high density of prey reached the megalopal stage in 6.4 ± 0.04 stages, compared to a low density, 7.2 ± 0.1 stages which was statistically different according to One-way ANOVA.

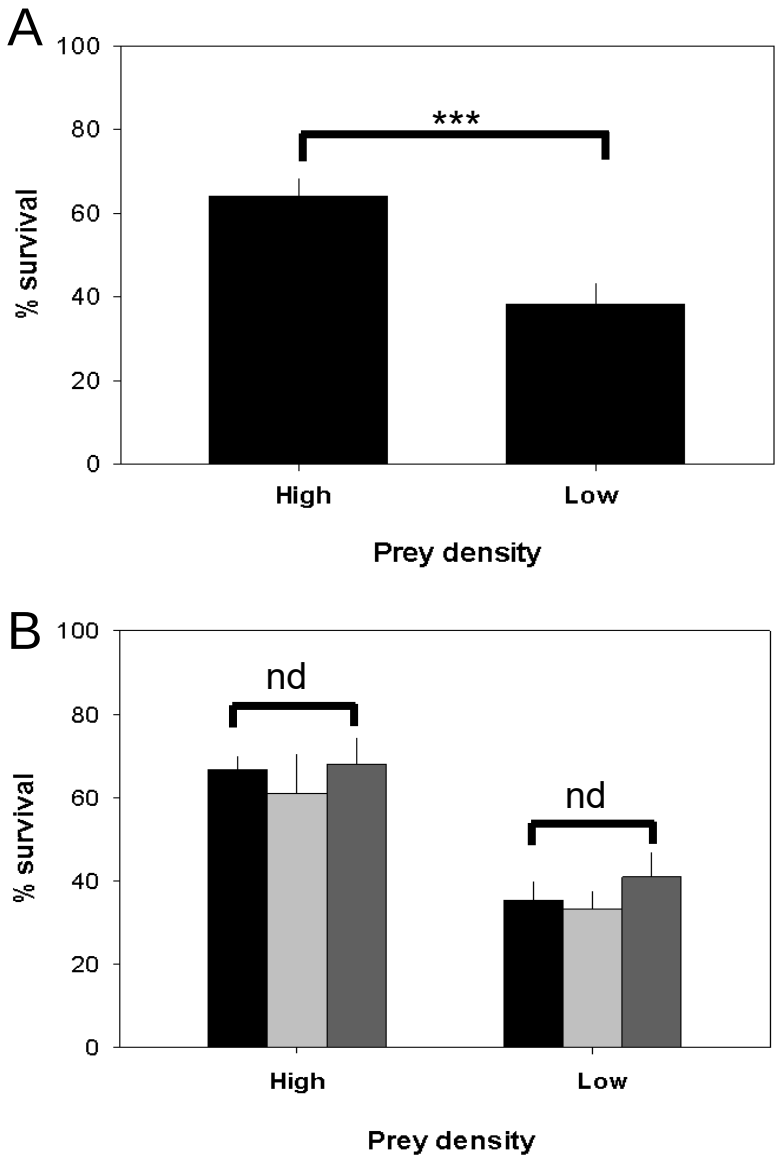


Figure 2. Survival (%) of the zoeae of *C. sapidus* (mean ± SE) fed a high (391 zoeae survived) and a low (*2*05 zoeae survived) density of prey (rotifers and/or *Artemia*) from hatching to megalopa. Zoeae obtained from six batches produced by six different females over three years. '***' significant difference at $P < 0.001$ (paired *t*-test) between the high and low prey density (A). Survival (%) of the zoeae under a high and low prey density from hatching to megalopae with 0 (black bars), 50 (light gray bars), and 100 (dark gray bars) mg/l PHB supplementation. Zoeae obtained from three batches produced by different females over two years. nd=no difference (randomized block ANOVA) (B)

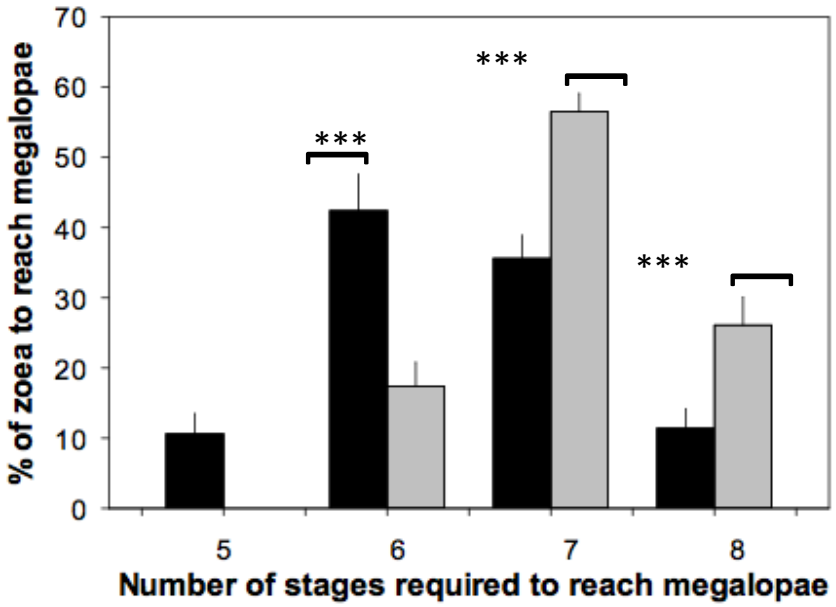


Figure 3. Number of stages (5, 6, 7, or 8) that zoeae of *C. sapidus* (mean ± SE) required to reach the megalopal stage e fed a high (black bars; 391 zoeae survived) and a low (gray bars; 205 zoeae survived) density of prey (rotifers and/or *Artemia*) from hatching to megalopae. Zoeae obtained from six batches produced by six different females over three years. '***' significant difference at $P < 0.001$ (paired *t*-test).

Prey density also influenced the duration of zoeal development (Fig. 4). With a high prey density (391 zoeae surviving), the duration was shorter than in a low prey density. The duration of zoeal development under a high prey density was 40.3 ± 0.5 days (five stages), 42.7 ± 0.3 days (six stages), 46.4 ± 0.4 days (seven stages), and 48.8 ± 1.2 days (eight stages). With a low prey density (205 zoeae surviving), the duration of zoeal development was 59.0 ± 0.7 days (six stages), 60.9 ± 0.5 days (seven stages), and 65.0 ± 0.7 days (eight stages). The duration of zoeal development was significantly associated with the number of stages (5, 6, 7 and 8) both with a high ($P < 0.001$, $\chi 2$ test) and low prey densities ($P < 0.005$, $\chi 2$ test). An ANCOVA was conducted to test the effect of density on the number of stages while accounting for the duration of zoeal development. The result indicates there is a change in the association between stage and duration between the two treatments ($P < 0.001$, ANCOVA).

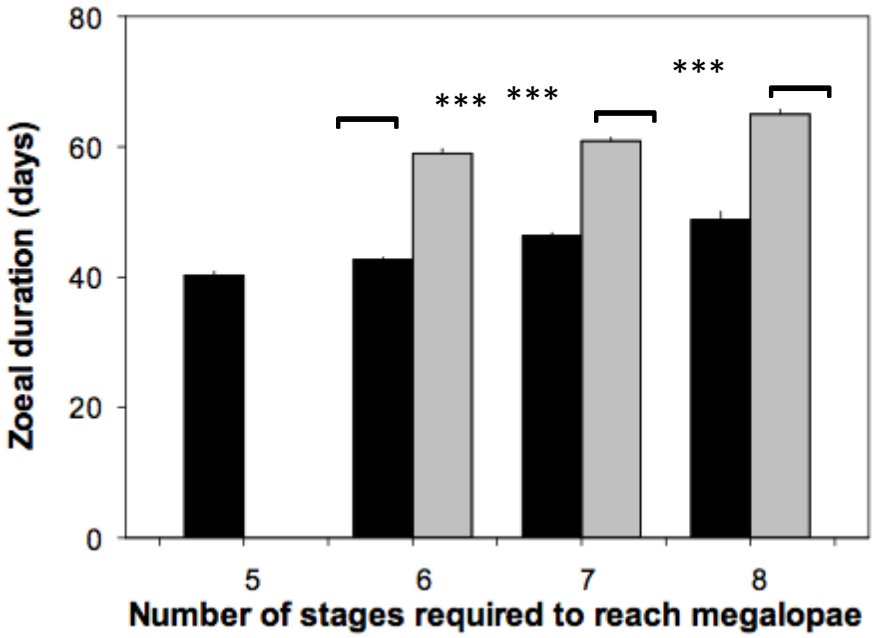


Figure 4. Duration (days) of the zoeae of *C. sapidus* zoeae (mean ± SE) from hatching to megalopae fed a high (black bars; 391 zoeae survived) and a low (gray bars; 205 zoeae survived) density of prey (rotifers and/or *Artemia*). Zoeae obtained from six batches produced by different females over three years. '***' significant difference at $P < 0.001$ (paired *t*-test).

The consumption of rotifers by the zoeal stages 1 to 6 was similar under high (11.6 ± 0.3; 26 zoeae studied) and low prey densities (10.9 ± 0.9; 20 zoeae studied). The consumption of *Artemia* was significantly higher ($P < 0.001$, Student's *t*-test) with a high prey density (6.6 ± 0.4;30 zoeae studied) compared to a low prey density (4.0 ± 0.9; 23 zoeae studied) from stages 4 to megalopae.

3.2 Effect of a high prey density on zoeal development with PHB supplementation

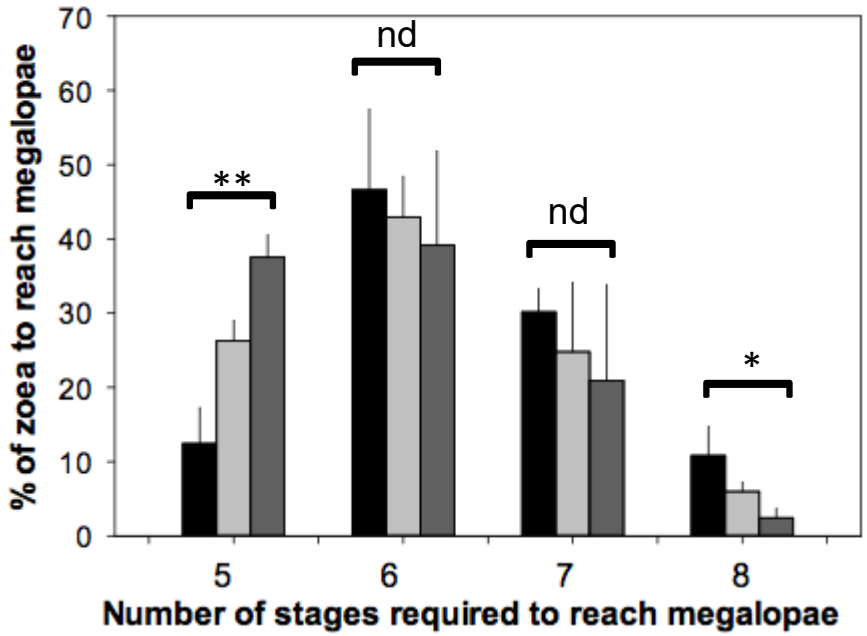
PHB supplementation did not significantly affect survival (258 zoeae survived) (Fig. 2B). The survival with 50 mg/l and 100 mg/l supplementation was 61.1 ± 9.3% and 68.1 ± 6.1%, respectively, the values of which were similar to 66.7 ± 3.2% without supplementation.

The zoeae still reached the megalopal stage after progressing through five, six, seven or eight stages with 50 mg/l and 100 mg/l PHB supplementations (Fig. 5A). With 50 mg/l supplementation, the zoeae reached the megalopal stage in five stages (26.3 ± 2%), six stages (42.9 ± 2 9.5%), seven stages (24.8 ± 9.4%), and eight stages (6.0 ± 1.2%). With 100 mg/l supplementation, the zoeae reached the megalopal stage in five stages (37.5 ± 3.0%), six stages (39.2 ± 4 12.7%), seven stages (20.9 ± 12.9%), and eight stages (2.4 ± 1.2%). Zoeae

reached the megalopal stage after five stages (12.5 ± 4.8%), six stages (46.6 ± 10.8%), seven stages (30.2 ± 3.1%), and eight stages (10.4 ± 3.9%) without supplementation.

The majority of zoeae reached the megalopal stage after progressing through six or seven stages with 50 mg/l of PHB and without PHB supplementation. The majority of zoeae reached the megalopal stage after progressing through five or six stages with 100 mg/l of PHB. The number of stages required to reach the megalopal stage was significantly associated with the PHB supplementation ($P < 0.001$, $\chi 2$ test). According to a randomized block ANOVA followed by post hoc Schaffe test, number of stages required to reach the megalopal stage is statistically different with and without PHB supplementation for five and eight stages with a high prey density (Fig 5A). On average, the zoeae with a 50 mg/l and 100 mg/l reached the megalopal stage in 6.3 ± 0.14 stages and 5.7 ± 0.09 stages, respectively, which is statistically different from 6.5 ± 0.09 stages with 0 mg/l according to One-way ANOVA.

A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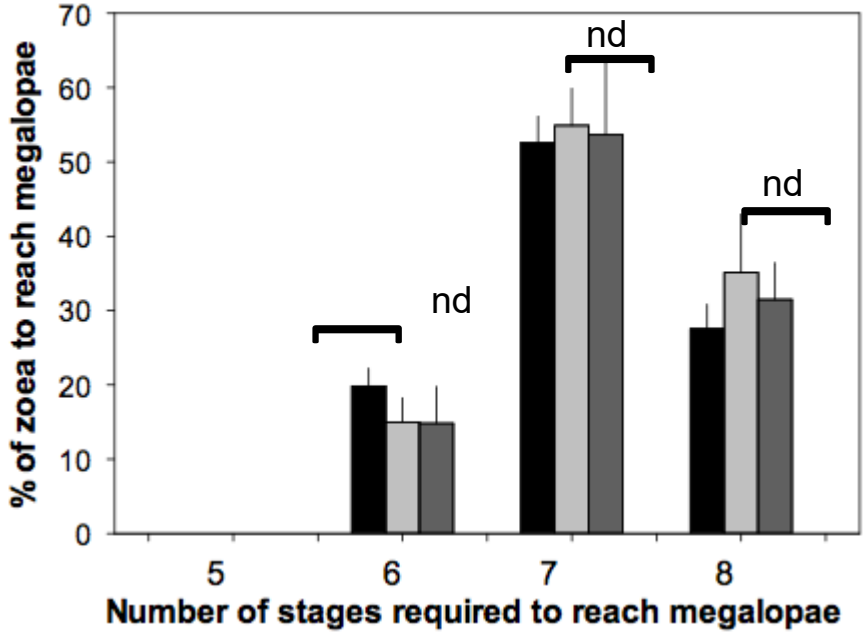
Number of stages required to reach megalopae

B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Number of stages required to reach megalopae

Figure 5. Number of stages (5, 6, 7, or 8) that zoeae of *C. sapidus* (mean ± SE) required to reach the megalopal stage fed a high prey density (258 zoeae survived) (A) and a low prey density (150 zoeae survived) (B) supplemented with 0 (black bars), 50 (light gray bars), and 100 (dark gray bars) mg/l PHB. Zoeae obtained from three batches produced by different females over two years. nd = no difference. '*' indicate significant differences ($P < 0.05$). '**' significant difference at $P < 0.01$ (randomized block ANOVA).

PHB supplementation had no effect on the duration of zoeal development (Fig. 6A) according to randomized block ANOVA analysis. With 50 mg/l supplementation, the duration of zoeal development was 35.5 ± 1.4 days (five stages), 41.9 ± 0.8 days (six stages), 42.2 ± 1.2 days (seven stages), and 49.2 ± 3.6 days (eight stages). With 100 mg/l supplementation, the duration of zoeal development was 39.9 ± 0.7 days (five stages), 37.7 ± 0.7 days (six stages), 43.1 ± 1.3 days (seven stages), and 48.5 ± 1.5 days (eight stages). The duration of zoeal development was 38.9 ± 0.6 days (five stages), 40.9 ± 0.7 days (six stages), 47.3 ± 0.8 days (seven stages), and 57.0 ± 1.8 days (eight stages) without supplementation. An ANCOVA was conducted to test the effect of PHB supplementation on the number of stages while accounting for the duration of zoeal development. The result indicates there is a no change in the association between stage and duration between the three supplementation concentrations even though it appears the 0 mg/l treatments had a higher duration of zoeal development with seven and eight stages.

Rotifer consumption by the zoeal stages 1 to 6 was similar with or without PHB supplementation. With 50 mg/l (27 zoeae studied) and 100 mg/l

supplementations (28 zoeae studied), rotifer consumption was 11.8 ± 0.2 and 11.8 ± 0.3, respectively, compared to 11.6 ± 0.3 (26 zoeae studied) without supplementation. The consumption of *Artemia* by the zoeal stages 4 to megalopae was also similar with or without supplementation. With 50 mg/l (29 zoeae studied) and 100 mg/l supplementations (32 zoeae studied), *Artemia* consumption was 6.5 ± 0.4 and 6.6 ± 0.4, respectively, compared to those without supplementation: 6.6 ± 0.4 (30 zoeae studied).

3.3 Effect of a low prey density on zoeal development with PHB supplementation

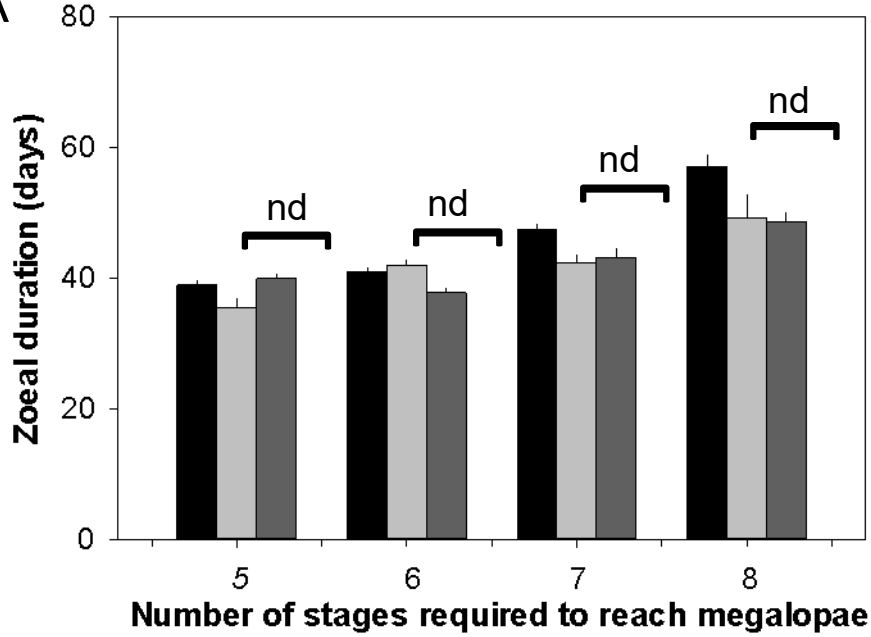
PHB supplementation did not affect survival (150 zoeae surviving) (Fig. 2B). Survival with 50 mg/l and 100 mg/l supplementations was 33.3 ± 4.2% and 40.9 ± 5.9%, respectively, values that were similar to 35.4 ± 4.3% without supplementation.

The zoeae still reached the megalopal stage after progressing through six, seven or to eight stages with 50 mg/l and 100 mg/l PHB supplementations (Fig. 5B). With 50 mg/l supplementation, the zoeae reached the megalopal stage in six stages (14.9 ± 4.9%), seven stages (54.9 ± 4.9%), and eight stages (35.1 ± 7.8%). The zoeae reached the megalopal stage in six stages with 100 mg/l supplementation (14.8 ± 3.2%), seven stages (53.7 ± 9.8%), and eight stages (31.5 ± 4.9%). Zoeae reached the megalopal stage in six stages (19.8 ± 2.3%), seven stages (52.6 ± 3.4%), and eight stages (27.5 ± 3.2%) without supplementation. Number of stages required to reach the megalopal stage is not statistically different with and without PHB supplementation for five, six, seven or eight stages with a low prey density according to randomized block ANOVA (Fig

5B). The majority of zoeae reached the megalopal stage after progressing through seven or eight stages. On average, the zoeae with a 50 mg/l and 100 mg/l reached the megalopal stage in 7.3 ± 0.12 stages and 7.2 ± 0.01 stages, respectively, similar 7.1 ± 0.05 stages with 0 mg/l according to One-way ANOVA.

PHB supplementation also had no effect on the duration of zoeal development according to randomized block ANOVA (Fig. 6B). The duration of zoeal development was 60.7 ± 1.7 days (six stages), 61.2 ± 0.6 days (seven stages), and 64.3 ± 0.8 days (eight stages) with 50 mg/l supplementation; the duration was 61.4 ± 1.9 days (six stages), 62.5 ± 0.9 days (seven stages), and 64.3 ± 0.8 days (eight stages) with 100 mg/l supplementation. The duration of development was 59.2 ± 1.0 days (six stages), 62.7 ± 0.6 days (seven stages), and 65.3 ± 0.7 days (eight stages) without supplementation.

A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Zoeal duration (days)
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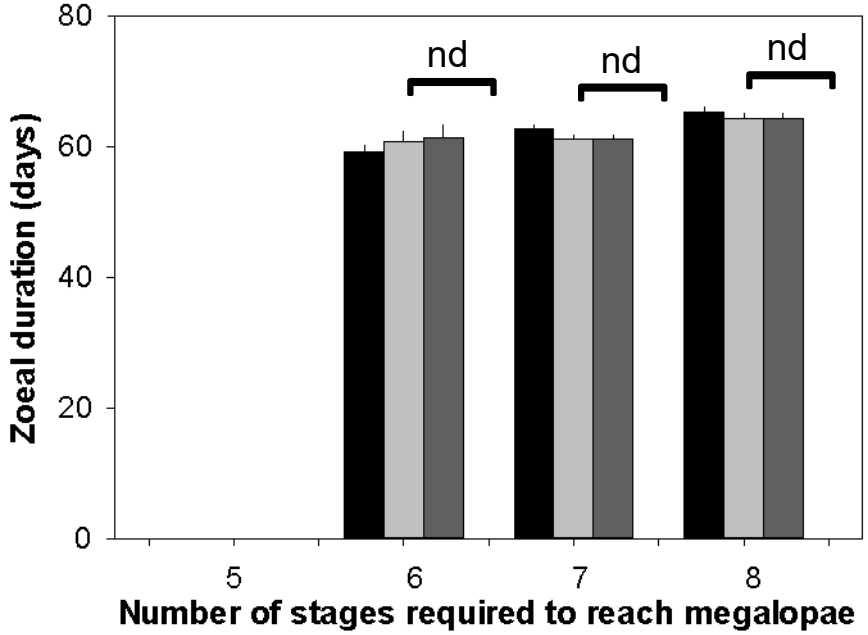
Number of stages required to reach megalopae

B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Zoeal duration (days)
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Number of stages required to reach megalopae

Figure 6. Duration (days) of the zoeae of *C. sapidus* (mean ± SE) from hatching to megalopa fed a high prey density (258 zoeae survived) (A) and a low prey density (150 zoeae survived) (B) supplemented with 0 (black bars), 50 (light gray bars), and 100 (dark gray bars) mg/l PHB. Zoeae obtained from thee batches produced by different females over two years. nd=no difference.

Rotifer consumption of zoeal stages 1 to 6 was similar 1 with or without PHB supplementation. With 50 mg/l (21 zoeae studied) and 100 mg/l supplementations (21 zoeae studied), rotifer consumption was 10.8 ± 0.4 and 10.7 ± 0.4, respectively, compared to those without supplementation, 10.9 ± 0.4 (20 zoeae studied). The consumption of *Artemia* of the zoeae stages 4 to megalopae was also similar with or without supplementation. With 50 mg/l (22 zoeae studied) and 100 mg/l supplementations (20 zoeae studied), *Artemia* consumption was 4.1 ± 0.4 and 4.0 ± 0.1, respectively, compared to those without supplementation, 4.0 ± 0.9 (23 zoeae studied).

4. Discussion

4.1 Effect of prey density on zoeal development

High prey density yielded better survival even if food consumption was similar. On average, 12–15 rotifers/day were consumed regardless of prey density during earlier zoeal development (stages 1 to 6). When considering survival between high and low densities, a low prey density of rotifers in early development (stages 1 to 6) is suboptimal due to the higher energy cost for foraging. It will be of interest measuring the energy cost of foraging of the zoeae under different prey densities. During later zoeal development (stages 4 to

megalopae), food consumption was greater at higher prey density compared to a low prey density. The nutritional status of the zoeae during development under conditions of high and low densities remains to be studied specifically lipid levels. Declining zoeal survival is associated with decreasing lipid accumulation (Torres et al., 2002).

There is a relationship among prey density, larval duration, and the number of zoeal stages. Zoeae raised under a high density of prey spent 6.4 ± 0.04 stages, lower than those under a low density, 7.2 ± 0.1 stages. As the number of zoeal stages required to reach the megalopal stage decreased, duration of zoeal development was reduced albeit slightly. *Callinectes sapidus* usually has seven to eight zoeal stages (Costlow & Bookhout, 1959).

The number of stages typically tends to increase under suboptimal conditions in insects except in Orthoptera and Coleoptera (Esperk et al*.,* 2007). Although we did not measure the energy cost for foraging, zoeae with a low prey density could have a higher energy cost, thus taking a longer time to molt. In such case, molting is often delayed as reported by Gore (1985). The mechanism by which zoeae fed a high prey density display frequent stage(s) skipping remains to be studied. It is likely that a high prey density could stimulate skipping of stages in zoeae under favorable environmental conditions.

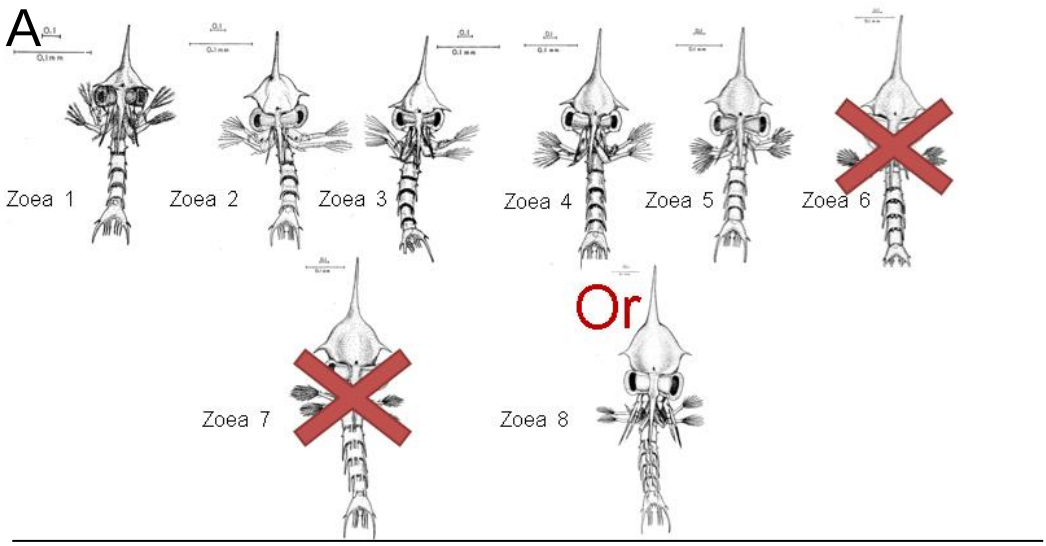
4.2 Stage skipping

The stage skipping observed in our study is not unusual. Stage skipping is described when a zoeae eliminates a stage in development (Costlow, 1965). Developmental plasticity in early larval development regardless of food source

could be an inherent feature of many crustacean species. Crustaceans show developmental plasticity in terms of the number of zoeal stages whether in the wild, as in the case of shrimps (*Palaemonetes* spp*., N. crangonoides,* and *Euaus* spp*.*)*,* anomuran crabs (species of *Petrolisthes* and *Porcellana*) and the upogebiid *U. deltaura* (Boyd & Johnson, 1963; Goy & Provenzano, 1978; Greenwood, 1965; Lebour, 1943; Pike & Williamson, 1961; Webb, 1919) as well as those reared in the laboratory as in shrimps (*Palaemonetes spp.*), brachyuran crabs (*E. sinensis, M. mercenaria,* and *C. sapidus*), and the lobster (*H. americanus*) (Anger, 1991; Boyd & Johnson, 1963; Broad, 1957a; Costlow, 1965; Gore, 1979; Oliphant et al., 2013; Porter, 1960; Sandoz & Rogers, 1944; Templeman, 1936a).

Moreover, the occurrence of additional zoeal stages under poor diet in terms of quality and quantity, has been observed in brachyuran crabs *S. paramamosain* (Zeng et al*.,* 2004)*, M. mercenaria* (Porter, 1960), and *N. granulata* (Pestana & Ostrensky, 1955), shrimps *Crangon spp.* (Criales & Anger, 1986) and *Palaemonetes spp.* (Broad, 1957b)*,* and lobster *H. americanus* (Templeman, 1936b). Our results with the zoeae under a high prey density treatment showed higher incidence of stage skipping and/or combing than those with a low density. By combining stages, a molt shows a combination of morphological characteristics from two contiguous larval stages (Costlow & Bookhout, 1959) (Table 2B, D, F). Additionally, it was observed that consecutive stages were never skipped in the zoeae development.

Common patterns in development were also observed when zoeae skipped stages (Fig. 7). Most often when zoeae skipped one stage in development, either stage six or seven was skipped (Fig. 7A). Most often when zoeae skipped two stages in development, and stages two and three were combined and either stage five, six or seven was skipped (Fig. 7B). Most often when zoeae skipped three stages in development, stages two and three were combined and stage five and seven were skipped (Fig. 7B).



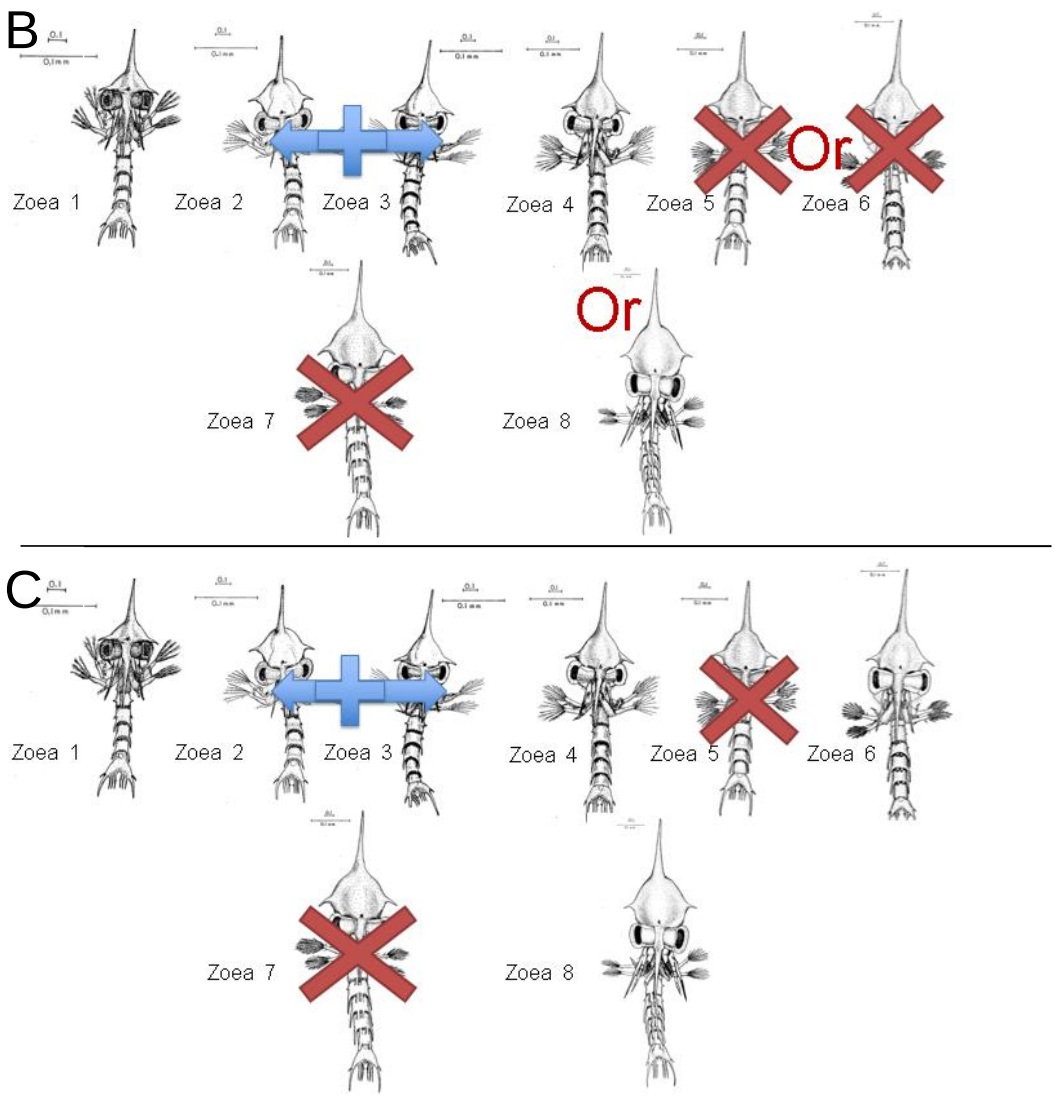


Figure 7. Frequently observed patterns of *C. sapidus* zoeal development when one (A), two (B), or three (C) stages skipped.

4.3 PHB supplementation

PHB had no effect on food consumption or survival of the zoeae of *C. sapidus* with a high or low prey density, from first zoeae to megalopae, in contrast to the brachyuran crab *E. sinensis* (Sui et al., 2014). PHB supplementation enhance survival rate of *E. sinensis* zoeae 14 days after hatching and onwards. The zoeae of this species progress through five to six

stages in one to two months, reaching the megalopal stage of approximately 3 mm in total length (Montu et al., 1996)., whereas *C. sapidus* zoeae progress through a maximum of eight stages in a similar period, reaching the megalopal stage of approximately 1 mm in total length (Costlow & Bookhout, 1959). It could be possible that a concentration of 100 mg/l was not be high enough to cause a significant effect in *C. sapidus* given its molting frequency.

PHB supplementation, coupled with a high prey density, increased the instances of stage skipping when compared to the absence of supplementation. Although food consumption was similar between 50 mg/l, 100 mg/l, and prey without supplementation, stage skipping could be related to energy reserves. PHB supplementation could provide an additional nutritional value to the zoeae. Although the exact nutritional value of PHB is unknown, PHB has been described as influencing digestive health (Nhan et al., 2010; Yu et al., 2005).

PHB coupled with a high prey density had no effect on the duration of zoeal development but did affect the number of stages. A change in the number of stages without influencing duration of development suggests that zoeae are skipping stages but remaining longer as in the previous or subsequent stages to compensate for skipped stages.

4.4 Changes in food source during zoeal development

Zoeae change their food source during development (McConaugha, 1985). The zoeae of *C. sapidus* are very small (~0.25–1 mm) (Churchill, 1919) and requires small-size prey, compared to those of many other species of

brachyuran crabs (0.5–1.5 mm) (Hines, 1986). In crustacean aquaculture, rotifers (70–350 μm) are often used as a suitable prey for early larval development as they provide sufficient nutrition for survival and development of the zoeae upon capture and consumption (McConaugha, 1985; Nghia et al., 2007; Ruscoe et al., 2004; Sulkin, 1978; Sulkin & Epifanio, 1975).

It appears that stage skipping during zoeal development is mostly driven by nutritional conditions. When the *C. sapidus* zoeae were fed rotifer alone (stage 1 to 3), survival was similar between a high and low prey density. Species with longer zoeal stages requires diet change during their development (Sulkin, 1978). When feeding *Artemia* (stage 4 to megalopae), survival is significantly higher with a high prey density than a low one. This can be explained because the zoeae under a low-density prey spend more effort to capture and consume the *Artemia* compared to the zoeae feeding at a high prey density.

When zoeae were fed rotifers alone (stages 1 to 3), instances of stage skipping and combining were observed. Stage combining did not occur when zoeae stages from 4 to 8 were fed *Artemia* but stage skipping was observed instead. Zoeae showed instances of stage skipping throughout development no matter the prey type. Stages that could not be distinguished due to degraded exuviae or the movement of zoeae was noted as undetermined (Table 2). The percent of undetermined stages, however, did not affect the overall data of stage skipping or the number of zoeal stages.

4.5 Ecological significance of plasticity during larval development

We propose that plasticity in zoeal development may provide an adaptive advantage and plays an important role in the survival of the zoeae. Variability in zoeal development could be an adaptive strategy under suboptimal (Sandifer & Smith, 1979). Lengthening zoeal duration may increase the vulnerability to other pelagic predators (Vaughn & Allen, 2010), whereas stage skipping may generate smaller individuals which may be a disadvantage when competing for resources. Future experiments should measure the size and weight of larvae/juveniles to determine if stage skipping changes size and/or weight of individuals. Further understanding is required on how selective pressures like prey availability may affect the duration of larval development, allowing to predict post larval metamorphosis and successful settlement in the wild.

5. Conclusion

Prey density influences variation in zoeal development of *C. sapidus* by increasing stage skipping, reducing the number of stage(s) from seven–eight to five–eight. The zoeae with a high density of prey reached the megalopal stage in 6.4 ± 0.04 stages, compared to a low density, 7.2 ± 0.1 stages. Under suboptimal conditions, zoeal survival is low and the number of stages required from hatching to megalopa is higher as well as longer zoeal duration. Additionally, a high prey density coupled with PHB supplementation causes increasing incidences of stage skipping. Our data show that prey density is a critical factor possibly affecting the nutritional status of zoeae, influencing the plasticity in the zoeal development of *C. sapidus* when the temperature is constant.

CHAPTER 3: EFFECT OF CHITIN SUPPLEMENTATION ON THE EARLY DEVELOPMENT OF THE BLUE CRAB, *Callinectes sapidus*

1. Introduction

Dietary supplementations such as natural substances, probiotics, prebiotics, immunostimulants and plant, algae and bacteria extracts as well as commercial products, have been used in aquaculture to improve growth and survival (Cook et al., 2003; Bricknell & Dalmo, 2005; Wang et al., 2006; Nakagawa et al., 2007; Nayak et al., 2007; Grisdale-helland et al., 2008; Rawling et al., 2009; Nhan et al., 2010; Ai et al., 2011; Gatesoupe, 1999; Watson et al., 2013; Sui et al., 2014; Ringo et al., 2014; Van Hung et al., 2015).

Chitin is the second most important natural polymer following cellulose and it is synthesized for structural purposes such as in cell walls of fungi and exoskeletons in many species including crustaceans. Crustacean exoskeletons contain about 15-40% chitin depending on the species (Kurita, 2006). Due to their beneficial properties, chitin and its derivative, chitosan, are used in an array of applications which include the medical field (Madhumathi et al., 2010; Park & Kim, 2010; Singla & Chawla, 2001), food applications (Agulló et al., 2003), agriculture (Burrows, 2007; El Hadrami et al., 2010; Hirano et al., 1990), cosmetics (Moranti & Morganti, 2008), wastewater treatment (Aly et al., 1997), and industrial chemicals (Kurita, 2006).

Chitin and chitosan have also been applied as a dietary supplement in aquaculture (Cha et al., 2008; Gopalakannan & Arul, 2006; Powell & Rowley,

2007; Shiau & Yu, 1998; Shiau & Yu, 1999). A 5% addition of chitin to the diet of *Penaeus monodon* enhanced feed and protein efficiency ratios and growth (Shiau & Yu, 1998). Additionally, a chitin-supplemented diet improved survival of adult male *Carcinus maenas* (Powell & Rowley, 2007). However, dietary supplementation of chitin to the diet of tilapia significantly depressed growth (Shiau & Yu, 1999), conversely, it had no significant effect on flounder or common carp (Cha et al., 2008; Gopalakannan & Arul, 2006) suggesting the beneficial growth effects of chitin and its derivatives are specific to crustaceans.

Additionally, chitin may provide an innate benefit to crustacean growth based on observations that crabs consume chitin through prey naturally (Hines, 2007; Reichmuth et al., 2009) as well as from their shed cuticle following ecdysis. Many crab species have the enzymatic capability of digesting chitin by producing protease and chitinase. Omnivores and carnivores such as *Leptograpsus variegatus* and *Nectocarcinus tuberculosus* contain high protease and chitinase activity in the stomach for digesting shells of invertebrates (Johnston & Freeman, 2005). Furthermore, it has been reported that the digestion of chitin has a significant nutritional contribution in shrimp (Clark et al., 1993).

The crustacean hyperglycemic hormone (CHH) is responsible for many essential physiological pathways including carbohydrate metabolism and molting in decapods (Chung et al., 2010; Webster et al., 2012). As a pleiotropic hormone, CHH has multiple target tissues including the hepatopancreas (Katayama & Chung, 2009; Kummer & Keller, 1993; Chung & Webster, 2006) which is the major metabolic site and the largest tissue in decapod crustaceans. When

crustaceans experience stressful conditions, CHH is released by the sinus gland to the hemolymph (Chung & Webster 2005; Chung et al. 2010; Webster et al. 2012). The increase in CHH levels is followed by hyperglycemia possibly via glycogenolysis in muscle and hepatopancreas (Chung et al. 2010; Webster et al. 2012; Katayama et al. 2013). This suggests a physiological role for CHH in the control of carbohydrates metabolism in crustaceans.

Throughout life, crustaceans may experience deficiency in nutrition for different reasons. It is presumed that a certain reserve of energy must be accumulated before molting can occur (Adelung, 1971). A reduction in growth rate is observed when nutrition is reduced below the optimal level (Hartnoll, 1982) which can cause an extended intermolt period and/or a reduced molt increment (Chittleborough, 1975; Minagawa & Murano, 1993).

In this study, we examined if chitin as a dietary supplement affected the growth of juvenile *C. sapidus*sized 37 ± 10 mm CW that were raised in the hatchery. The animals, beginning and ending in intermolt stage, underwent three molts while fed different % of shrimp chitin. At each molt, they were measured for molt interval and increment. On the 3rd day after the 3rd molt, the animals were dissected for tissue collection (eyestalk ganglia and hepatopancreas) for expression analysis. Carbohyrdate (glucose, trehalose, and glycogen) was measured in hemolymph and hepatopancreas It was hypothesized that chitin supplementation may be contributing factors to growth.

2. Materials and Methods

2.1 Experimental animals

Juveniles (carapace width 37 ± 10 mm and 4.5 ± 0.4 g wet weight) were randomly selected from the blue crab hatchery in the Aquaculture Research Center IMET, Baltimore, MD, US]. They were placed individually in experimental compartments (14 × 13 cm) in tanks holding about 200 liters of ~25 ppt artificial seawater (ASW) at 21 ºC -23ºC (14 light :10 dark photoperiod). Experiments were repeated three times using batches of animals (*N* = 13-16) produced by three different females from 2015 to 2016.

2.2 Treatments

Artifical wet diets compounded of frozen squid were prepared as reported by Quintana et al., (2008); the resultant squid paste was separated into four parts and the required amounts of chitin (0, 5, 10, and 20% of wet weight of squid paste) was blended into the paste for each diet. Practical grade chitin powder from shrimp shells was purchased (Sigma Aldrich, C7170, Lot # SLBR6796V). Then, the paste was agglutinated by hand with a gelatin from porcine skin (Sigma Aldrich) of equal weight of the paste. Afterwards, these agglutinated diets were stored in -10ºC until further use. Diet composition including moisture, ash and crude protein, fat and fiber content was analyzed by New Jersey Feed Laboratory, Inc (Table 1).

Table 1. Composition of the diet fed to *C*. *sapidus* juveniles

Treatment (% Chitin)	Moisture%	Crude Protein%	Crude Fat%	Crude Fiber%	Ash%
Squid	79.4 ± 0.1 [a]	16.6 ± 0.2 [a]	0.49 ± 0.3 [a]	1.38 ± 0.9 [a]	0.38 ± 0.4 [a]
0	83.4 ± 0.8 [bc]	13.9 ± 0.5 [b]	0.84 ± 0.1 [a]	0.5 ± 0.4 [a]	0.20 ± 0.1 [a]
5	85.0 ± 0.0 [b]	13.4 ± 0.2 [b]	0.26 ± 0.03 [a]	1.3 ± 0.1[a]	0.20 ± 0.2 [a]
10	82.5 ± 0.3 [c]	13.5 ± 0.5 [b]	0.47 ± 0.1 [a]	3.0 ± 0.4 [ab]	0.02 ± 0.02 [a]
20	79.8 ± 0.2 [a]	14.6 ± 0.02 [b]	0.22 ± 0.01 [a]	6.3 ± 0.2 [b]	0.11 ± 0.1 [a]

The data are presented as mean ± SE. letters indicate significant difference

2.3 Feeding experiment

Feeding experiment consisted of 5 different treatments: squid control and 0, 5, 10, and 20% chitin supplemented diet (as stated above). Previous research (David, 2009; Muralisankar et al., 2014) and preliminary data showed that a daily feed ~10% of wet body weight was sufficient to guarantee positive growth. Juveniles in the squid control treatment were fed daily squid ~10% of wet body weight while those animals in 0, 5, 10 and 20% chitin supplemented treatment were fed daily a piece of formulated diet ~20% of wet body weight to compensate for half of the formulated diet consisting of gelatin. The experiment concluded once juveniles completed three molts on designated diet. The experiment was repeated three times using the animals produced by three different females.

Before the juveniles began the experiment, animals molted once in tank compartments to record initial molt date and size (wet weight and carapace width). The experimental period allowed for three molt cycles. Date of each molt thereafter was recorded to calculate molt interval presented as mean ± 1SE (days). On third day after each molt, the crabs wet weight and carapace width were measured. Hemolymph (250 µL) was collected using a U-100 insulin syringe (Becton Dickinson) containing an equal volume of ice-cold modified anticoagulant without glucose (Söderhäll & Smith, 1983) and placed in a 1.5mL microcentrifuge tube and placed on dry-ice.

Three days after the third molt, the hemolymph was collected from each animal as stated above and the animals were dissected for tissue collection. The hepatopancreas, eyestalk ganglia, and sinus gland were collected in a 1.5mL tube and were immediately placed on dry ice to be stored in -80^{o}C until further use.

2.4 Neuropeptide quantification

Neuropeptides from the sinus glands were purified on a C18 Gemini column (4.6 × 150 mm, 5 µm particle size, and 110 Å pore size, Phenomenex) using RP-HPLC, as described in (Chung & Webster, 1996) and presented as mean ± 1SE (pmol/sinus gland).

2.5 Protein estimation

Protein concentration was measured in the hemolymph and hepatopancreas using a Pierce™ 660nm Protein Assay kit by following the

manufacturer's instructions. Each sample was assayed in duplicate and the plate was read at 660 nm (M5 Spectra Molecular Device). Data was presented as mean ± SE (mg/ml hemolymph and mg/ 100 mg wet weight hepatopancreas).

2.6 Carbohydrate analysis

Glucose was measured in the hemolymph and hepatopancreas using a glucose oxidase assay kit (Sigma-Aldrich) (Chung & Zmora, 2008; Shi & Chung, 2014). Data was presented as mean ± 1SE µg glucose/ 100 µl in hemolymph or µg/ 100 mg hepatopancreas wet weight. The levels of trehalose were determined using Anthrone reagent as described (Roe, 1955) in the hemolymph and hepatopancreas estimated as described (Chung, 2008; Chung 2014). The amount of trehalose in the samples was calculated by subtracting the level of glucose measured in the same samples from the total sugar that was measured using Anthrone assay (Roe, 1955). Data was presented as mean ± 1SE µg trehalose/ 100 µl in hemolymph or µg/ 100 mg hepatopancreas wet weight. The concentration of glycogen was measured in the hepatopancreas (Shi & Chung, 2014; Vinagre & Chung, 2016) and presented as mean ± 1SE µg/ 100 mg hepatopancreas wet weight.

2.7 Expression analysis using qRT-PCR assays

The total RNA of eyestalk ganglia was extracted for cDNA synthesis. The expression of CHH in the eyestalk ganglia was determined using a qRT-PCR assay with each sample cDNA containing 12.5 ng total RNA using Fast SYBR and a 7500 Fast Real Time PCR system (ABI). All samples were assayed in

duplicate. The qRT-PCR standards of CHH and AK was prepared as described (Chung et al., 2006; Chung et al., 2011; Chung et al., 2012). The levels of AK expression in the same tissue cDNAs were examined as a reference gene.

For chitinase expression, hepatopancreas samples were homogenized in 200 µl ice-cold DEPC treated PBS (pH 7.4) using Pellet Pestle® motor to be divided between carbohydrate assays and RNA extraction (Zmora & Chung, 2014; Techa & Chung; 2015). Only 50% of sample was used for RNA extraction. Total RNA of the hepatopancreas was extracted using Qiazol (Qiagen) and quantified using a NanoDrop Lite (Thermo Scientific). ~1.5 µg total RNA was subjected to cDNA synthesis using PrimeScript™ RT PCR Kit (TaKaRa).

A chitinase was identified from transcriptomic analysis of *C. sapidus* (unpublished data) and the sequence was confirmed by re-sequencing of the amplicon using ChaF5'ATGAAGTTACTCCTGCCTCTCTTGG3' and ChaR 5' CTATGGGCAGTCATTGGGGCAC3'. The chitinase sequence was analyzed using MEGA 7.0 to a create phylogenetic tree (Fig 1) showing sequences with high similarity (alignment scores >=200).

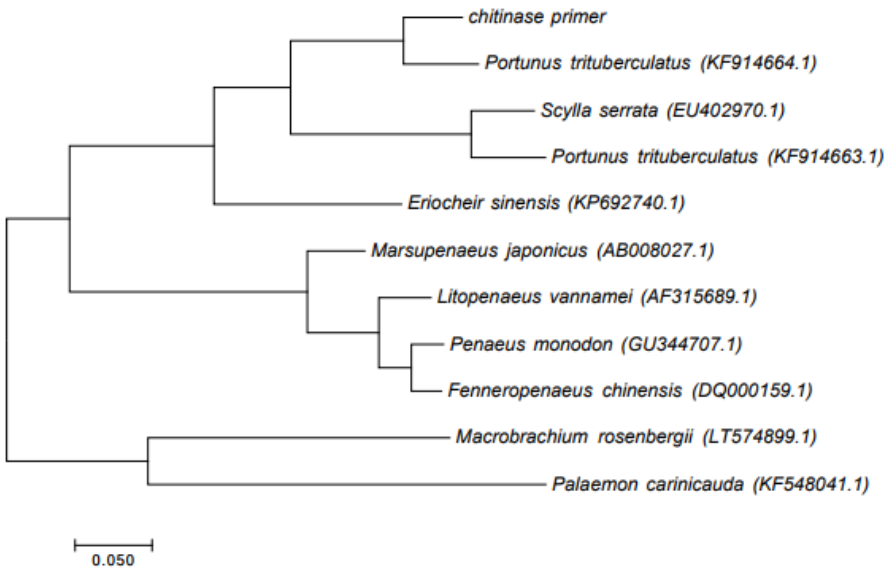


Figure 1. Phylogenetic tree comparing similarities of chitinase primer against other crustacean sequences with alignment scores >=200.

The tissue specific expression in hepatopancreas was also examined using the same primers at the following PCR conditions: 94 °C for 3 min followed by 35 cycles of 94 °C for 30 s, 60 °C for 60 s, 72 °C for 30 s and the final extension at 72 °C for 7 min. As a reference gene, AK was amplified in the same tissue cDNAs. Chitinase standards were prepared for qRT-PCR assay similarly, as described (Chung, 2008; Chung & Webster, 2003). The hepatopancreas cDNAs were assayed the same as above. The data were presented as mean ± 1SE copies/ µg total RNA.

2.8 Statistical analysis

Results were expressed as the mean ± 1SE. One-way analysis of variance (ANOVA) and post hoc Tukey and Scheffe' test (unequal sample size)

were used to determine the statistical significance ($P < 0.05$). Additionally, ANCOVA was used to determine significance of between different carbohydrates. Additionally, $\chi 2$ test was performed to determine if there was a relationship between protein concentration and the number of molts.

3. Results

3.1 Effect of chitin supplementation on CHH in the eyestalk ganglia and sinus gland

3.1.1 CHH transcript levels in the eyestalk ganglia

Chitin supplementation did not affect levels of *CHH* expression in the eyestalk (One-way ANOVA, Fig 2). The levels of *CHH* with 5, 10 and 20% chitin supplementation were 10.7 ± 5.5 ($N = 5$), 4.9 ± 2.8 ($N = 4$) and 15.0 ± 7.5 ($N = 5$) $\times 10^7$ copies/µg total RNA compared to 17.5 ± 5.2 ($N = 9$) and 10.5 ± 4.7 ($N = 6$) $\times 10^7$ copies/µg total RNA. *AK* expression in the same cDNAs showed no significant differences among treatments ranging from 11.9 ± 1.9 to 35.8 ± 7.0 $\times 10^7$ copies/µg total RNA.

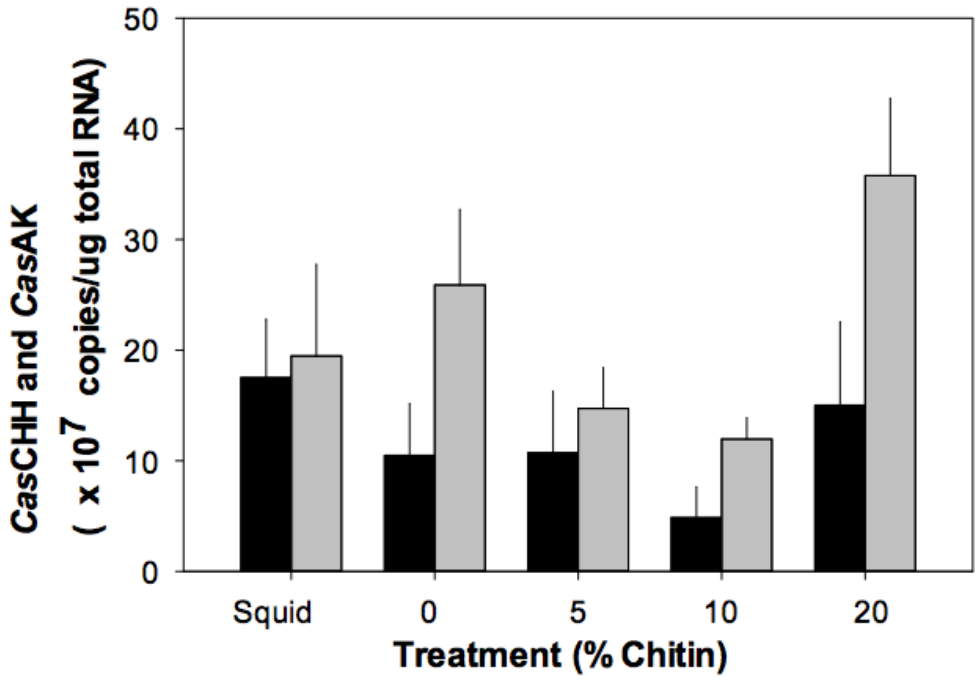


Figure 2. Expression levels (mean ± 1SE) of juvenile *C. sapidus* crustacean hyperglycemic hormone (black bars) and arginine kinase (*AK*) (gray bars). Juveniles obtained from three batches produced by different females over 2 years. The data are presented as mean ± 1SE copies/ug total RNA. No significant difference in *CHH* among treatments (One-way ANOVA followed by Scheffé post hoc test at $P < 0.05$).

3.1.2 CHH levels in the sinus gland

The levels of neuropeptides in the sinus gland were determined to see if chitin supplementation affected CHH neuropeptides. Chitin supplementation did not significantly affect CHH levels (One-way ANOVA, Table 2). The levels of CHH2, the main CHH form, with 5, 10 and 20% chitin supplementation were 83.4 ± 10.9, 105.6 ± 23.9, and 91.6 ± 10.7 pmol/sinus gland compared to 101.3 ± 8.3 and 84.2 ± 9.7 pmol/sinus gland with squid control and 0% chitin supplementation. The levels of CHH1 with 5, 10 and 20% chitin supplementation ranged from 20-30% of CHH2 and were 21.0 ± 1.1, 19.1 ± 0.5, and 23.5 ± 1.3

pmol/sinus gland compared to 26.8 ± 3.9 and 19.9 ± 0.8 pmol/sinus gland with squid control and 0% chitin supplementation.

Chitin supplementation did not significantly affect CPRP levels (One-way ANOVA, Table 2). The levels of CPRP with 5, 10 and 20% chitin supplementation were 90.8 ± 10.5, 102.3 ± 17.5 and 100.1 ± 8.7 pmol/sinus gland compared to 122.5 ± 10.5 and 85.5 ± 9.1 pmol/sinus gland with squid control and 0% chitin supplementation. There was a statistical significance among the squid control and 0% chitin supplementation ($P < 0.05$, Student's *t*-test) in the levels of CPRP. When comparing the squid control against 5, 10 and 20% chitin supplementation and the 0% chitin supplementation against 5, 10 and 20% chitin supplementation, there was no statistical difference (One-way ANOVA).

Table 2. Levels of neuropeptides in the sinus gland of juvenile *C. sapidus* with varying concentrations of chitin supplementation

Treatment (% Chitin)	Squid	0	5	10	20
CPRP (pmol/sinus gland)	122.5 ± 10.5 nd	85.5 ± 9.1 nd	90.8 ± 10.5 nd	102.3 ± 17.5 nd	100.1 ± 8.7 nd
CHH1 (pmol/sinus gland)	26.8 ± 3.9 nd	19.9 ± 0.8 nd	21.0 ± 1.1 nd	19.1 ± 0.5 nd	23.5 ± 1.3 nd
CHH2 (pmol/sinus gland)	101.3 ± 8.3 nd	84.2 ± 9.7 nd	83.4 ± 10.9 nd	106.5 ± 23.9 nd	91.6 ± 10.7 nd
***N* #**	6	5	7	4	5

The data are presented as mean± SE. nd=no difference.

3.2 Effect of chitin supplementation on protein concentration in the hemolymph and hepatopancreas

3.2.1 Hemolymph

The levels of protein in the hemolymph were analyzed after each molt. The protein concentration in the hemolymph after the first molt with 5, 10, and 20% chitin supplementation, was 1.3 ± 0.4, 1.5 ± 0.1, and 1.5 ± 0.1 mg/ml compared to 0.7 ± 0.09 and 1.0 ± 0.1 mg/ml with squid control and 0% chitin supplementation. According to One-way ANOVA, the squid control was significantly lower than the 10 and 20% chitin supplementation ($P < 0.05$) possibly due to high variation. The protein concentration in the hemolymph after the second molt with 5, 10, and 20% chitin supplementation, was 1.4 ± 0.1, 1.4 ±

0.1 and 0.8 ± 0.02 mg/ml which were similar to 1.2 ± 0.3 and 1.1 ± 0.1 mg/ml with squid control and 0% chitin supplementation. Only after three molts, protein levels were significantly lower in all treatments with chitin supplementation.

The levels of protein were statistically different with 5, 10 and 20% chitin supplementation compared to squid and 0% chitin supplementation (One-way ANOVA, $P < 0.01$) (Fig 3) after three molting events. Levels of protein in the hemolymph with 5, 10, and 20% chitin supplementation were 0.53 ± 0.07 (N = 10), 0.62 ± 0.06 (N = 8), and 0.56 ± 0.05 (N = 12) mg/ml compared to 1.1 ± 0.11 (N = 8) and 1.3 ± 0.11 (N = 9) mg/ml with the squid control and 0% chitin supplementation. According to χ2 test, treatment significantly influenced the protein concentration as the number of molts increased ($P < 0.01$).

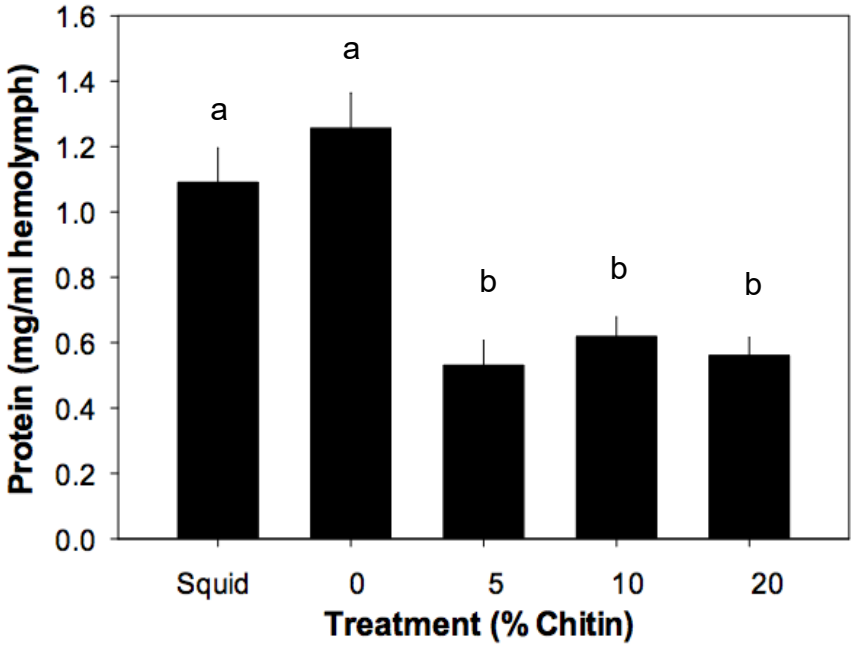


Figure 3. Protein levels (mean ± 1SE) in the hemolymph of juvenile *C. sapdius* fed squid (*N* = 8), and 0 (*N* = 9), 5 (*N* = 10), 10 (*N* = 8), and 20% (*N* = 12) chitin supplementation. Juveniles obtained from three batches produced by different females over 2 years. Letters indicate significant difference according to One-way ANOVA followed by Scheffé post hoc test ($P < 0.05$).

3.2.2 Hepatopancreas

Chitin supplementation did not affect protein concentration in the hepatopancreas (One-way ANOVA, Table 3). Protein concentration in the hepatopancreas with 5, 10, and 20% chitin supplementation, was 9.6 ± 0.8, 12.2 ± 4.2, and 9.8 ± 1.3 mg/100 mg wet weight hepatopancreas compared to 9.6 ± 1.1 and 8.2 ± 1.5 mg/100 mg wet weight hepatopancreas with squid control and 0% chitin supplementation after three molts.

Table 3. Protein levels in the hepatopancreas of juvenile *C. sapidus* with varying concentrations of chitin supplementation

Treatment (% Chitin)	**Squid**	**0**	**5**	**10**	**20**
Protein mg/100 mg hep w.w.	9.6 ± 1.1 nd	8.2 ± 1.5 nd	9.6 ± 0.8 nd	12.2 ± 4.2 nd	9.8 ± 1.3 nd
***N* #**	8	8	10	8	12

The data are presented as mean ± SE. w.w.= wet weight. nd=no difference.

3.3 Effect of chitin supplementation on carbohydrate in the hemolymph and hepatopancreas

3.3.1 Hemolymph

The levels of trehalose and glucose in the hemolymph were not significantly different among treatments (One-way ANOVA, Table 3). Trehalose was the major sugar in the hemolymph. The levels of trehalose in the hemolymph with 5, 10, and 20% chitin supplementation were 532.4 ± 166.4, 783.7 ± 229.2, and 585.4 ± 73.2 µg/ 100µl hemolymph compared to 350.8 ± 55.8 and 872.8 ± 92.6 µg/ 100µl hemolymph with squid control and 0% chitin supplementation. There was a statistical significance among the squid control and 0% chitin supplementation ($P < 0.05$, Student's *t*-test) in the levels of trehalose. When comparing the squid control against 5, 10 and 20% chitin supplementation and the 0% chitin supplementation against 5, 10 and 20% chitin supplementation, there was no statistical difference (One-way ANOVA).

Glucose levels in the hemolymph with 5, 10, and 20% chitin supplementation were 30.0 ± 5.0, 31.9 ± 5.7 and 40.5 ± 10.7 µg/ 100µl hemolymph compared to 22.0 ± 7.2 and 39.1 ± 10.3 µg/ 100µl hemolymph with squid control and 0% chitin supplementation after three molts. According to ANCOVA, there was no statistically significant difference in trehalose levels among the different diets once adjusted for glucose levels.

Table 4. Glucose concentration in the hemolymph of juvenile *C. sapidus* with varying concentrations of chitin supplementation

Treatment (% Chitin)	**Squid**	**0**	**5**	**10**	**20**
Glucose (µg/ 100µL)	21.9 ± 7.2 nd	39.1 ± 10.3 nd	30.0 ± 5.0 nd	31.9 ± 5.7 nd	40.5 ± 10.7 nd
Trehalose (µg/ 100µL)	350.8 ± 55.6 nd	872.8 ± 92.6 nd	532.4 ± 166.4 nd	783.7 ± 229.2 nd	585.4 ± 73.2 nd
N #	9	10	11	9	11

The data are presented as mean ± SE. nd=no difference.

3.3.2 Hepatopancreas

Glycogen was the major sugar in the hepatopancreas. According to One-way ANOVA and post hoc Scheffé test, the level of glycogen with a 5% chitin concentration was significantly higher than the squid control (Table 4). The levels of glycogen in the hepatopancreas with 5, 10, and 20% chitin supplementation were 11044.5 ± 1106.7, 10784.9 ± 3459.0, and 7415.4 ± 735.5 µg/ 100 mg wet weight hepatopancreas compared to 5473.9 ± 651.5 and 7415.4 ± 735.5 µg/ 100 mg wet weight hepatopancres with squid control and 0% chitin supplementation. According to ANCOVA, there was no statistically significant difference in glycogen levels among the different diets once adjusted for glucose and trehalose levels.

The levels of glucose and trehalose in the hepatopancreas were not significantly different among treatments (One-way ANOVA, Table 4). The levels of glucose in the hepatopancreas with 5, 10, and 20% chitin supplementation

were 60.0 ± 12.9, 70.8 ± 15.4, and 63.7 ± 18.5 µg/ 100 mg wet weight hepatopancreas compared to 29.3 ± 4.1 and 83.0 ± 22.3 µg/ 100 mg wet weight hepatopancreas with squid control and 0% chitin supplementation. There was a statistical significance among the squid control and 0% chitin supplementation ($P < 0.05$, Student's *t*-test) in the levels of glucose. When comparing the squid control against 5, 10 and 20% chitin supplementation and the 0% chitin supplementation against 5, 10 and 20% chitin supplementation, there was no statistical difference (One-way ANOVA).

The levels of trehalose in the hepatopancreas with 5, 10, and 20% chitin supplementation were 652.8 ± 280.5, 573.7 ± 126.2, and 655.8 ± 49.9 µg/ 100 mg wet weight hepatopancreas compared to 349.9 ± 90.7 and 454.0 ± 102.0 µg/ 100 mg wet weight hepatopancreas with squid control and 0% chitin supplementation. There was a statistical significance among the squid control and 20% chitin supplementation ($P < 0.05$, Student's *t*-test) in the levels of trehalose. There was no statistically significant difference in trehalose levels among the different diets once adjusted for glucose levels (ANCOVA).

Table 5. The amount of carbohydrates in the hepatopancreas of juvenile *C. sapidus* with varying concentrations of chitin supplementation

Treatment (% Chitin)	Squid	0	5	10	20
Glucose (µg/100 mg hep w.w.)	29.3 ± 4.1 nd	83.0 ± 22.3 nd	60.0 ± 12.9 nd	70.8 ± 15.4 nd	63.7 ± 18.5 nd
Trehalose (µg/100 mg hep w.w.)	349.9 ± 90.7 nd	454.0 ± 102.0 nd	652.8 ± 280.5 nd	573.7 ± 126.2 nd	655.8 ± 49.9 nd
Glycogen (µg/100 mg hep w.w.)	5473.9 ± 651.5 ^{a}	7415.4 ± 735.5 ^{ab}	11044.5 ± 1106.7 ^{b}	10784.9 ± 3459.0 ^{ab}	7415.4 ± 735.5 ^{ab}
***N* #**	11	6	5	4	6

The data are presented as mean ± SE. nd=no difference. a, b indicates significant difference

3.4 Effect of chitin supplementation on chitinase expression

Tissue distribution was performed to examine expression of chitinase in 17 different tissues from an adult female *C. sapidus:* eyestalk ganglia, thoracic ganglia complex, brain, pericardial organ, Y-organ, mandibular organ, hepatopancreas, foregut, hindgut, antenna gland, gill, abdominal brown muscle, hypodermis, ovary, spermatheca, heart, hemocytes (Fig. 4). Chitinase expression was found in two tissues, showing greatest expression in the hepatopancreas followed by the mandibular organ.

Chitin supplementation did not affect expression analysis of chitinase in the hepatopancreas (One-way ANOVA, Fig. 5). The levels of chitinase with 5, 10 and 20% chitin supplementation was 8.3 ± 4.3, 17.0 ± 12.5 and 23.2 ± 8.7 $\times 10^6$ copies/µg total RNA compared to 22.8 ± 7.8 and 16.6 ± 4.3 $\times 10^6$ copies/µg total

RNA. The arginine kinase showed no significant differences among treatments ranging from 1.2 ± 0.5 to 23.1 ± 15.2 ×10^6 copies/µg total RNA.

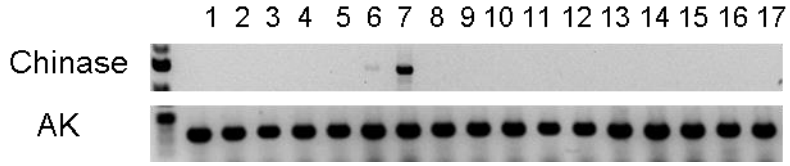


Figure 4. Spatial expression pattern of chitinase in various tissues of an adult female *Callinectes sapidus*: 1. eyestalk ganglia, 2. thoracic ganglia complex, 3. brain, 4. pericardial organ, 5. Y-organ, 6. mandibular organ, 7. hepatopancreas, 8. foregut, 9. hindgut, 10. antenna gland, 11. gill, 12. abdominal brown muscle, 13. hypodermis, 14. ovary, 15. spermatheca, 16. heart, 17. hemocytes. Each tissue cDNA sample containing 12.5 ng of total RNA equivalent was amplified using an end-point PCR assay. The expression of arginine kinase (AK) in the same sample cDNAs was examined as a reference gene.

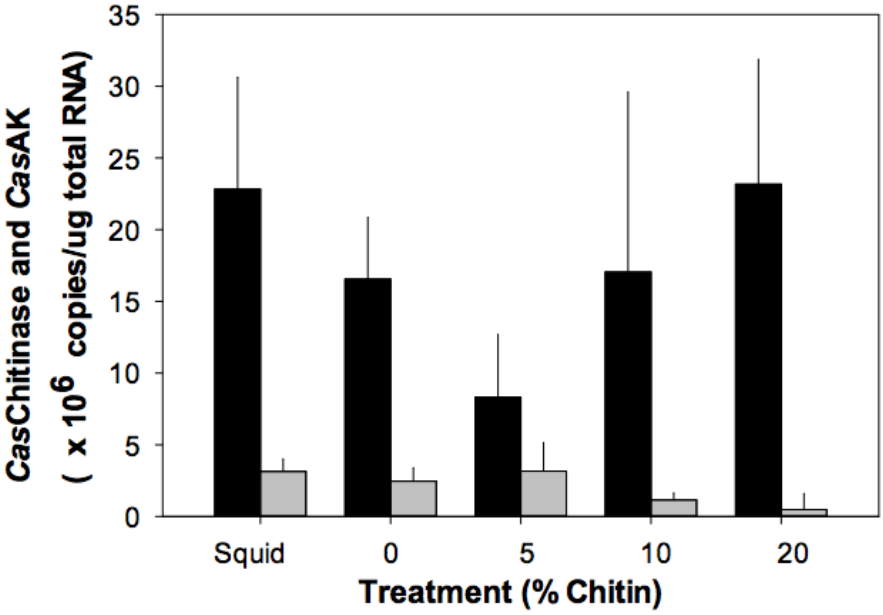


Figure 5. Expression levels (mean ± 1SE) of juvenile *C. sapidus* chitinase (black bars) and arginine kinase (*AK*) (gray bars). Juveniles obtained from three batches produced by different females over 2 years. The data are presented as mean ± 1SE copies/ug total RNA. No significant difference in chitinase among treatments (One-way ANOVA followed by Scheffé post hoc test at $P < 0.05$).

3.5 Effect of chitin supplementation on survival, molt increment and interval

3.5.1 Survival

The survival with 5, 10 and 20% chitin supplementation was 100 ± 0.0% (*N* = 13 out of 13), 86.7 ± 13.3% (*N* = 11 out of 13), and 94.4 ± 5.6% (*N* = 14 out of 15) which were similar to 95.8 ± 4.2% (*N* = 15 out of 16) and 94.4 ± 5.6% (*N* = 13 out of 14) with squid control and 0% chitin supplementation.

3.5.2 Molt increment

Chitin supplementation did not significantly affect molt increment over three molting events (One-way ANOVA, Table 6). For the first molt, molt increments for 5, 10 and 20% chitin supplementation were 17.4 ± 1.2, 20.8 ± 1.9, 20.7 ± 1.2% compared to 22.2 ± 1.0 and 20.6 ± 1.3% with squid control and 0% chitin supplementation. For the second molt, molt increments for 5, 10 and 20% chitin supplementation were 22.2 ± 1.0, 22.2 ± 2.1, 19.0 ± 1.5% compared to 23.6 ± 1.0 and 23.8 ± 1.1% with squid control and 0% chitin supplementation. For the third molt, molt increments for 5, 10 and 20% chitin supplementation were 21.0 ± 1.3, 20.3 ± 1.7, 25.5 ± 1.4% compared to 20.0 ± 2.5 and 23.2 ± 3.4% with squid control and 0% chitin supplementation

3.5.3 Molt interval

Overall, chitin supplementation did not shorten molt interval across three molting events (One-way ANOVA, Fig 6) due to high variation but molt interval was statistically different ($P < 0.05$) as the number of molts increased. For the

first molt of the experiment, the molt intervals with 5, 10, and 20% chitin supplementation were 23.9 ± 1.8, 30.2 ± 4.2, and 26.7 ± 2.3 days compared to 18.2 ± 0.7 and 24.7 ± 2.2 days with squid control and 0% chitin supplementation. Only diet supplemented with 10% chitin showed slightly longer molt interval ($P < 0.05$) compared to squid control during first molt (One-way ANOVA, Fig. 6A) according to One-way ANOVA and post hoc analysis. However, there is a statistical difference between the squid and 0% chitin supplemented treatment ($P < 0.05$, Student's *t*-test). When comparing 5, 10 and 20% chitin supplementation against squid control only, there is a statistically difference among treatments in molt interval ($P < 0.01$). But when comparing 5, 10 and 20% chitin supplementation against 0% chitin supplementation only, there is no statistically difference among treatments.

Table 6. Molt increment (%) for juvenile *C. sapidus* fed squid control and varying levels of chitin supplementation

Molt	Treatment	Molt increment (%)	*N* #
1	Squid	22.2 ± 1.0 [nd]	16
	0	20.6 ± 1.3 [nd]	14
	5	17.4 ± 1.2 [nd]	13
	10	20.8 ± 1.9 [nd]	12
	20	20.7 ± 1.2 [nd]	15

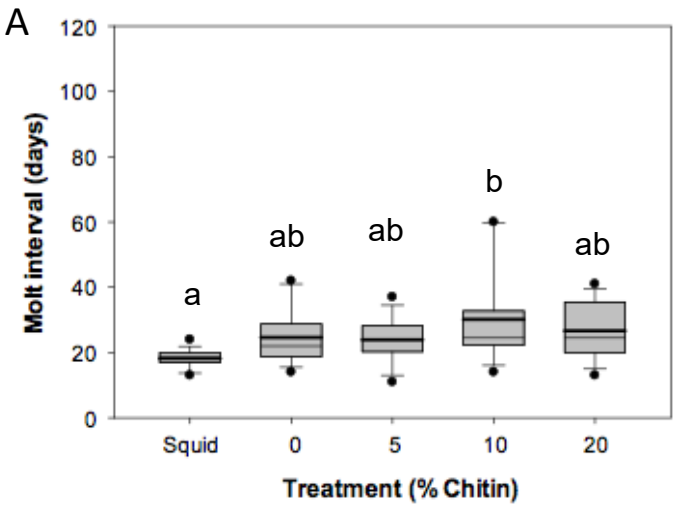
	Squid	23.6 ± 1.0 nd	16
	0	23.8 ± 1.1 nd	14
2	5	22.2 ± 1.0 nd	13
	10	22.2 ± 2.1 nd	12
	20	19.0 ± 1.5 nd	14
	Squid	20.0 ± 2.5 nd	15
	0	23.2 ± 3.4 nd	13
3	5	21.0 ± 1.3 nd	13
	10	20.3 ± 1.7 nd	11
	20	25.5 ± 1.4 nd	14

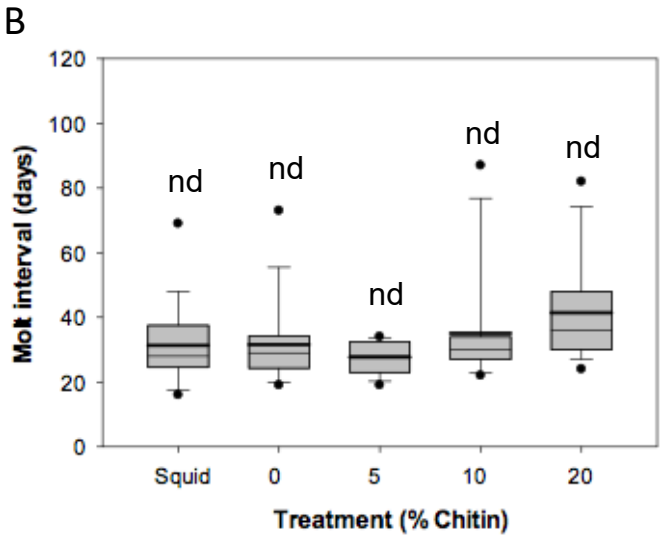
The data are presented as mean ± SE. nd=no difference.

There was no statistical difference in the molt interval among the diets for the second and third molt. For the second molt, the molt interval with 5, 10, and 20% chitin supplementation was 27.7 ± 1.3, 35.1 ± 4.9, and 41.4 ± 4.5 days compared to 31.3 ± 3.4 and 31.6 ± 3.6 days with squid control and 0% chitin supplementation (One-way ANOVA, Fig 6B). For the third molt, the molt interval with 5, 10, and 20% chitin supplementation, was 37.6 ± 3.8, 55.1 ± 8.1, and 59.4 ± 6.7 days compared to 60.2 ± 6.4 and 49.4 ± 6.8 days with squid control and 0% chitin supplementation for third molting event (One-way ANOVA, Fig 6C).

As expected, the molt interval increased as the animals grew. Overall, three molts occur in 108.5 ± 3.5 days in all experimental and control groups. For 5, 10, and 20% chitin supplementation, three molts occur in 88.9 ± 4.8, 107.7 ± 9.3 and 127.8 ± 7.0 days compared to 109.1 ± 7.1 and 107.2 ± 7.8 days with squid control and 0% chitin supplementation. One-way ANOVA followed by Scheffé post hoc test showed that a 5% chitin supplement is significantly lower than a 20% chitin supplement suggesting that a lower chitin supplement may positively affect growth.

The molt interval with a squid control increased from 18.2 ± 0.7 to 31.3 ± 3.3 to 60.2 ± 9.5 days to the last molt. The molt interval with 0% chitin supplementation increased from 24.7 ± 2.2 to 31.6 ± 3.7 to 49.4 ± 9.9 days to the last molt. The molt interval with 5% chitin supplementation increased from 23.9 ± 1.8 days to 27.7 ± 1.4 days to 37.6 ± 4.5 days to the last molt.


A
Molt interval (days)
120
100
80
60
40
20
0
a
ab
ab
b
ab
Squid
0
5
10
20
Treatment (% Chitin)


B
Molt interval (days)
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Squid
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Treatment (% Chitin)

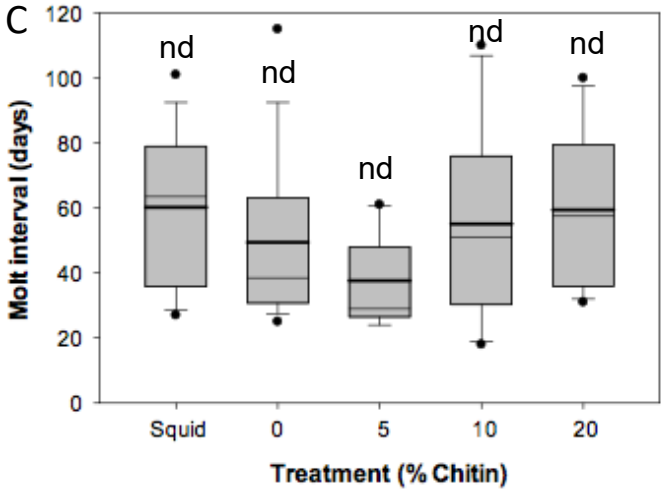

C
Molt interval (days)
120
100
80
60
40
20
0
nd
nd
nd
nd
nd
Squid
0
5
10
20
Treatment (% Chitin)

Figure 6. Molt interval in days of juvenile *C. sapdius* following [A] first [B] second and [C] third molt fed squid and 0, 5, 10, and 20% chitin supplementation. Juveniles obtained from three batches produced by different females over 2 years. Letters indicate significant difference according to One-way ANOVA followed by Scheffé post hoc test ($P < 0.05$).

The molt interval with 10% chitin supplementation increased from 30.2 ± 4.0 days to 35.1 ± 6.3 days to 55.1 ± 10.0 days to the last molt. The molt interval with 20% chitin supplementation increased from 26.7 ± 2.3 days to 41.4 ± 4.7 days to 59.4 ± 9.8 days to the last molt. There was no statistically difference in molt interval between molting events for each treatment except in the case of 10% chitin supplementation; molt 1 and 2 were statistically different from molt 3 but not each other.

The change in molt interval from the first to second molt and second to third molt was also not significantly different among treatments. The change in molt interval from the first to second molt for 5, 10 and 20% chitin supplement was 9.1 ± 1.3, 8.8 ± 2.1 and 17.3 ± 4.6 which were similar to 13.1 ± 2.7 and 11.8 ± 1.6 for squid control and 0% chitin supplementation. The change in molt interval from the second to third molt was for 5, 10 and 20% chitin supplement was 11.8 ± 2.9, 29.6 ± 8.9 and 30.2 ± 6.3 which were similar to 32.9 ± 5.6 and 22.4 ± 6.5 for squid control and 0% chitin supplementation.

4. Discussion

In this study, we examined the effects of chitin supplementation on juvenile *C. sapidus*. In general, chitin supplementation does not affect

development but the levels of protein in the hemolymph were decreased with chitin supplementation over three molting events.

4.1 Dietary components on neuropeptide levels

CHH release caused by hemolymph glucose via a feedback mechanism indicates that a nutritional status may affect the CHH expression and translation of CHH (Glowik et al., 1997; Chung & Webster, 2005;) In this study, CHH expression and neuropeptide levels, ratios of CHH1/2 and CPRP were estimated in the animals fed with a 5, 10, or 20% chitin supplement. Chitin supplementation had no effect on the levels of CHH1, CHH2 and CPRP in the sinus gland or CHH expression in eyestalk ganglia.

Gelatin from porcine skin (Sigma Aldrich) was used to formulate the 0, 5, 10 and 20% chitin supplemented diet. Addition of gelatin itself does not affect the growth of *C. sapidus* in general, similar to previous findings in *Octopus vulgaris* and *Carcinus maenas* (Powell & Rowley, 2007; Quintana et al., 2008). Interestingly, there was a statistical significance between the squid control and 0% chitin supplementation ($P < 0.05$, Student's *t*-test) in the levels of CPRP in the sinus gland, trehalose in the hemolymph, and glucose in the hepatopancreas in the hepatopancreas. CPRP is known as a circulating hormone, while the exact function of CPRP is clearly not defined to date (Wilcockson et al., 2017). The difference among the two control groups: with and without gelatin used here implies that CPRP may have a modulatory role in the digestion of gelatin like materials.

4.2 Chitin supplementation on development

We found that a 5, 10, or 20% chitin supplement to a squid-based diet did not significantly affect development (molt interval and increment) unlike previous literature which saw deleterious effects on *C. sapidus* growth with increasing amounts of chitin form 6-20% (Allman et al., 2017) and significantly higher body weight gains of *P. monodon* fed a 5% chitin supplement. (Shiau & Yu, 1998). The method of chitin delivery could be an ineffective ration delivery. However, our molt intervals were comparable to previous studies conducted on *C. sapidus* of similar size (Leffler, 1972; Allman et al., 2017) implying our animals grew normally even with chitin supplementation and artificial we formulated diets.

In this study molt increment ranged from 17.4 ± 1.2 -25.5 ± 1.4% across all experimental and control treatments (Table 2). Our molt increments were comparable to previous studies conducted in the laboratory at 20°C-24°C who saw a molt increment ranging from 18-27% (Millikin et al., 1980; Guerin & Stickle, 1997; Allman et al., 2017) implying our animals grew normally even with chitin supplementation.

Unlike the previous finding by (Powell & Rowley, 2007) that had increased survival with 5% chitin supplement, chitin supplementation did not affect survival in *C. sapidus*. Additionally, all mortality was observed during ecdysis with majority occurring during in the third molt (Table 2).

4.3 Chitin supplementation on protein levels

Only after three molts, protein levels in the hemolymph were significantly lower in all treatments with chitin supplementation. The crude protein levels were

also significantly lower in the formulated diets compared to squid diet (Table 1) which may have led to the reduction in protein levels in the hemolymph. This reduced protein levels may affect subsequent 4th molting event including the growth and physiological performance of the animals. It remains to be further studied on what kind of specific proteins are reduced with chitin fed *C. sapidus*.

4.4 Chitin supplementation on carbohydrates

In the current study, the addition of chitin to a squid-based diet had no effect on carbohydrate levels in the hemolymph and hepatopancreas except in the glycogen levels between the squid control and 5% chitin supplementation in the hepatopancreas which may have resulted from variation between individuals. Similarly, the addition of chitin had no significant effect on the levels of glucose in the hemolymph and glycogen in the hepatopancreas of *C. maenas* (Powell & Rowley, 2007). The rate of nutrient absorption depends on the rate of interaction of nutrients with the absorptive epithelium. Accordingly, the influence of dietary fiber such as chitin on the movement of nutrients along the gut will likely affect nutrient absorption. Diet analysis determined that fiber was only significantly different with 20% chitin supplementation implying nutrient absorption. Fecal material should be collected to determine specifically how much of diet and chitin was digested by the juvenile *C. sapidu*s which may enlighten carbohydrate results. A possible reason for lack of significance in carbohydrate levels is that the rate of production of endogenous chitinases is too slow to allow digestion of a continual supply of dietary chitin (Fox, 1993).

4.5 Chitin supplementation on chitinase expression

In addition to this study, chitinase has been found in the hepatopancreas of several species of crustaceans including *Nectocarcinus tuberculosus, Leptograpsus variegatus,* and *C. maenas* (Johnston & Freeman, 2005), *Paralithodes camtschaticus* (Novikov & Mukhin, 2003)*, Penaeus japonicas* (Koga et al., 1990) and *P. monodon* (Tan et al., 2000). In crustaceans, chitinase is also found in the hypodermis (Tom et al., 2014). Hypodermis chitinase is largely involved in the breakdown of chitin during their molt process on the other hand the chitinase in the hepatopancreas digests dietary chitin (Spindler-Barth et al., 1990; Kono et al., 1995; Peters et al., 1999).

In this study, chitinase transcript expression was not significantly different between treatments suggesting chitinase expression is not inducible via chitin supplementation. However, the chitinase enzyme activity in the hepatopancreas still needs to be determined. Previous literature found that chitinase activity was low and not significantly different between treatments containing 6-20% chitin (Allman et al., 2017). It was observed that chitinase expression peaked before ecdysis in the hepatopancreas of *P. monodon* (Tan et al., 2000). CHH is also known to mediate other metabolic functions of the hepatopancreas, the site of synthesis and secretion of digestive enzymes such as chitinase and amylase (Watanabe et al., 1998; Pan et al., 2005). Since CHH was similar across all treatments, chitinase expression was expected to be similar across all treatments.

4.6 Percent of chitin in a dietary supplement

The percent of chitin supplemented in a diet may be an important factor. In this study, although it was not significantly different to the controls, crabs fed 5% chitin supplementation completed three molts faster, 88.8 ± 4.8 days compared to 109.1 ± 7.1 and 107.2 ± 7.8 days with squid and 0% chitin supplementation, indicating that a lower concentration may have beneficial effects for *C. sapidus.* At 2 and 5% addition of chitin, *P. monodon* showed an enhanced feed efficiency, protein efficiency ratio and higher body weight gains; however, increasing chitin to 10% severely reduced growth and significantly depressed protein and lipid digestibility of the diet (Shiau & Yu, 1998).

Additionally, *C. sapidus* juveniles fed 20% chitin replacement also showed a significantly longer molt interval and lower final wet weight (Allman et al., 2017). Future studies must be conducted with 1.25 and 2.5% chitin supplements to determine if lower concentrations of chitin positively effects growth in *C. sapidus*. Furthermore, the processed shrimp form of chitin used in this study may be inhibiting the resorption of chitin or preventing nutrient uptake. It was suggested that native chitin may be of greater nutritional value to a species (Fox 1993).

4.7 Genetic diversity

Genetic diversity is notably high in *C. sapidus* populations and within the same batch of animals (Feng et al., 2017; Plough, 2017). This implicates that there may be a great variation in the growth rates of *C. sapidus*. In this study, the animals with similar sizes were used to reduce variation between the batches. This may explain that overall, the growth rates (molt interval or increment) did not

significantly differ among the batches. The animals of one batch had slightly lower survival rate than the other two; however, no statistical difference was found.

5. Conclusion

In summary, chitin supplementation does not influence development in juvenile *C. sapidus.* However, a general trend shows that a concentration of less than 5% chitin supplementation to a squid-based diet may decrease molt interval. Neither CHH levels nor carbohydrate levels were significantly different between treatments with and without chitin supplement. Our data does show that chitin supplementation decreases protein levels in the hemolymph after three molts. It remains to be further studied on what specific proteins are reduced with chitin fed *C. sapidus* but it may be related to innate immune response due to the immunomodulatory impacts of chitin on different species.

CHAPTER 4 General discussion and future direction

1. Batch variation

Individual genetic differences inherited from parents lead to batch to batch variation in development specifically maternal factors. Variability in survival and duration of development among zoeae from different broods was related to initial biomass at egg laying (Gimenez & Anger, 2001). Variation in the number of larval stages also results between batches when females collected from different sources most likely due to local condition adaptations (Criales & Anger, 1986). The high developmental variation between batches is observed in size of individuals, molting frequency, stage morphology, and survival in *Crangon sp.* (Criales & Anger, 1986). Genetic diversity is notably high in *C. sapidus* populations (Feng et al., 2017; Plough, 2017) which may account for variation in batches of *C. sapidus* when individuals are reared in the same conditions. A power analysis was performed to determine the minimal number of batches to decrease batch to batch variation for zoeal development. Power analysis showed that at least six different batches were needed.

Differences in zoeal duration can be observed in the six batches reared without PHB supplementation used in this study under high and low density (Figure 1). Mortality was also different from batch to batch without PHB supplementation (Table 1). Variation in development also occurs within the same batch, possibly resulting from differences in conditions in the egg mass; zoeae from the same batch can differ in size, larval duration, and mortality (Kunisch & Anger, 1984). Observations from this experiment as well as other studies show

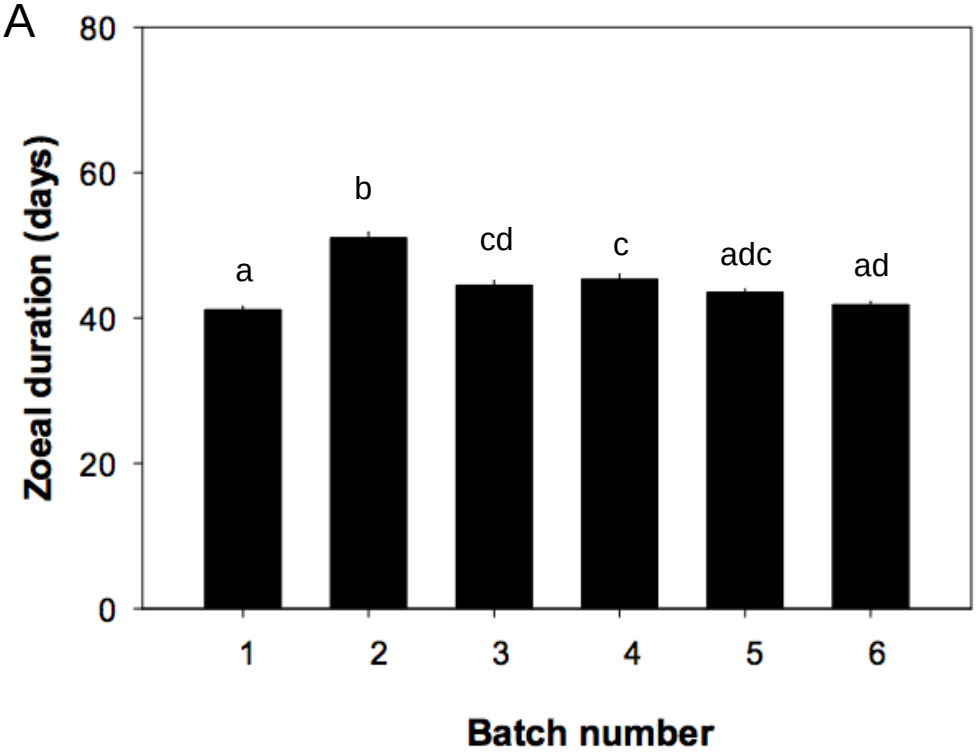
that individuals produced from a batch can vary developmentally from one another (Boyd & Johnson, 1964; Pandian, 1970; Pandian & Katre, 1972).

Variation was also observed in the three juvenile batches although only in the survival; animals of similar sizes were used to reduce their variations in development. Two of the three batches had 100% survival while one batch had 84.8% survival (*N* = 28 out of 33). There was no significant difference in days to complete three molts between the batches according to One-way ANOVA. Overall, three molts occur in 98.7 ± 6.1, 114.5 ± 5.3 and 108.8 ± 6.7 days in all experimental and control groups. There was also no significant difference in molt increment for each molting event between the batches. For each batch, molt increment was 19.3 ± 1.2, 20.9 ± 1.1 and 20.6 ± 0.8% for the first molt. For each batch, molt increment was 21.5 ± 1.6, 22.9 ± 0.9 and 21.8 ± 1.0% for the second molt. For each batch, molt increment was 22.6 ± 1.5, 23.8 ± 1.4 and 19.4 ± 1.8% for the third molt.

Table 1. Percent of survival of *Callinectes sapidus* zoea for each batch under high and low prey density

Prey Density	**1**	**2**	**3**	**4**	**5**	**6**
High	80.6	55.0	71.9	63.0	67.8	64.3
Low	59.7	38.0	25.0	44.0	28.1	34.1

A


80
60
40
20
0
Zoeal duration (days)
a
b
cd
c
adc
ad
1
2
3
4
5
6
Batch number

B

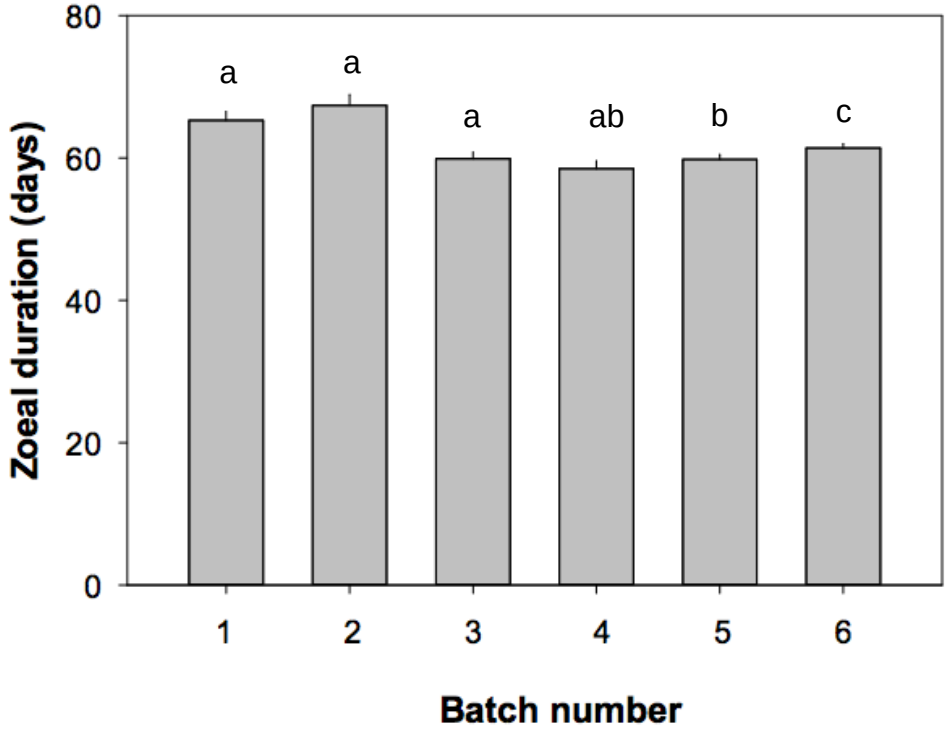

80
60
40
20
0
Zoeal duration (days)
a
a
a
ab
b
c
1
2
3
4
5
6
Batch number

Figure 1. Average zoeal duration in days for each batch of *Callinectes sapidus* (average ± SE) required to reach the megalopal stage fed a [A] high prey density (*N* = 391 zoeae survived) and [B] low prey density (*N* = 205 zoeae survived). Zoeae obtained from six batches produced by six different females over 3 years. Significant differences ($P < 0.05$).

2. Plasticity in development

Larval plasticity specifically in the number of zoeal stages required to reach the megalopal stage, not only occurs in the laboratory (Anger, 1991; Boyd & Johnson, 1963; Broad, 1957a; Costlow, 1965; Gore, 1979; Oliphant et al., 2013; Porter, 1960; Sandoz & Rogers, 1944; Templeman, 1936a) but also in the wild (Boyd & Johnson, 1963; Goy & Provenzano, 1978; Greenwood, 1965; Lebour, 1943; Pike & Williamson, 1961; Webb, 1919). Plasticity in larval development may be very important for larval success given the highly variable habitat conditions. Skipping stages in crustacean zoeal development may be driven mostly by the nutritional status of an individual as seen by more frequent stage skipping with a high prey density and dietary supplementation.

Variability in larval development could be an adaptive advantage in the wild. Variability in larval development may allow for a flexible response to suboptimal conditions, hence increasing a species ability to colonize new and better suited habitats (Sandifer & Smith, 1979). *C. sapidus* zoeae consume various zooplankton such as rotifers and nauplii and phytoplankton (Millikin & Williams, 1984) which in the wild varies seasonally (Roman et al., 2005). Seasonal abundance for rotifers is highest in summer followed by a smaller bloom in the spring in the lower Chesapeake Bay (Park & Marshall, 1993).

Blue crab zoeae may optimize development during periods of high density by skipping stages to reach the megalopal stage sooner. A large rotifer bloom in the summer would also allow zoeae to shorten development ensuring successful development to later larval or megalopae stage to take advantage of nauplii bloom in late summer/early fall (Park & Marshall, 1993). Additionally, optimal foraging theory states that a species must spend energy foraging to get energy via prey. When prey density is low, prolonging zoeal development with more stages may give zoeae time to acquire nutrients required to complete molt (Anger, 2001).

Faster metamorphosis to megalopa does offer some advantage in the wild in terms of fitness. As megalopae, *C. sapidus* acquire claws which aids in self-defense. Additionally, as megalopae, *C. sapidus* settle in the Chesapeake Bay which provides protection via bay grasses increasing local recruitment. Reaching the post larval stage sooner may ensure successful development to adulthood. Longer larval development can be disadvantageous because it increases the time zoeae are vulnerable to pelagic predators (Vaughn & Allen, 2010).

Altering the length of larval development may have an impact on post-larval survival and recruitment (Strathmann, 1985). Moreover, there can be carry-over effects from one life stage to the next due to environmental conditions experienced in the previous stage (Anger, 2006; Giménez & Anger, 2003). Whether skipping stages offers an advantage or disadvantage to *C. sapidus* megalopae/juvenile development was not monitored because the experiment concluded once zoeae reached the megalopal stage. However, larger juveniles

were reported for *Chasmagnathus granulata* zoeae that previously passed through five zoeal instars instead of four (Giménez & Anger, 2003).

3. Animal handling

The handling of animals especially during the zoeal stage can cause stress that can alter an individual's performance. Initially to complete water exchange, 50% of water was going to be removed from well with zoeae remaining; however, gently removing the zoea and placing the animal in a new plate was far less stressful and ensured 100% water exchange. A water exchange of every two days did not appear to affect development because zoeae fed a high density of prey still reached the megalopal stage in 30-50 days (Costlow & Bookhout, 1959).

As for the juvenile experiment, the crabs were handled at least once a month for hemolymph sampling and carapace width and wet weight measurements; during this time, an animal was out of water for ~3-4 minutes. During hemolymph extraction and measuring of carapace width, a wet paper towel was placed over the crab to control the chelae and cover the eyes to keep the crab calm. To measure the crabs wet weight, the crab was dried before being placed on scale. Even with the occasional handling, the zoeae and juveniles consumed diet regularly and molted in intervals similar to other studies (Tagatz, 1968; Leffler, 1972); therefore, any stress form handling and sampling did not impact the results.

4. Dietary supplementation on growth

Feeding a combination of rotifers and *Artemia*, even reared in a 24-well plate, produces the best results for rearing *C. sapidus* zoeae (Sulkin, 1978; Zmora et al., 2005). Only two densities of prey were used in this experiment: a high density (similar to that of the hatchery) and a low density (~40% of high density). Preliminary data showed that the low density was sufficient to promote growth; however, survival was not ideal, 38% compared to 64% with a high density of prey. Further research is needed to determine the minimal density of prey that promotes survival similar to that of high prey density, around 50-60%. This will result in the most economical use of the prey since rearing live feed can be costly and labor intensive.

Rotifers and *Artemia* are non-selective filter feeders, meaning they can successfully deliver dietary supplements to zoeae including PHB as shown by (Sui et al., 2014). Using PHB as a dietary supplement resulted in a significantly enhanced survival, development rate and osmotic tolerance of *Eriocheir sinensis* (Sui et al., 2014). However, results showed that PHB should only be applied as a supplement and that the availability of nutrients appears critical to induce the beneficial effects of PHB (Sui et al., 2014) which may have resulted in lack of PHB effect on *C. sapidus* zoeae when fed low density of prey.

In this study, PHB supplementation increased the frequency of stage skipping may be driven by the nutritional status of the zoeae. The degradation of PHB polymers in the zoeal gut affects the gut pH (Defoirdt et al., 2007a). The reduction in the gut pH may improve the activity of digestive enzymes resulting in better digestibility and absorption of nutrients from the diet (Baruah et al., 2007)

allowing zoeae to reach nutrient threshold required to molt sooner (McConaugha, 1985). Future studies may include analyzing the levels of digestive enzymes in zoea fed PHB.

Furthermore, PHB acts as a microbial control agent. PHB supplementation has shown to affect the microbial community richness and diversity found in a species intestine (De Schryver et al., 2010; Najdegerami et al., 2012). The microbial community influenced by the PHB may also be contributing to the incidence of stage skipping observed in the zoeae.

In this experiment, PHB was not added directly to the *C. sapidus* water due to the observation that the presence of insoluble PHB particles in the water induced a stress for *E. sinensis* and PHB added directly into the zoeal rearing media did not positively affect development (Sui et al., 2014). Other studies have concluded that PHB supplemented in a bacterial carrier can increase larviculture efficiency similar to supplementation of PHB in powdered form (Thai et al., 2014; Van Hung et al., 2015). Since both modes of delivery (carrier vs powder) proved beneficial, a cost analysis would be determining factor when choosing delivery method if PHB used in large aquaculture setting. PHB can effectively be applied in *C. sapidus* larviculture as a dietary supplement however a high density of prey is required to observe enhanced zoeal growth. Further research is needed to explain the mechanism of the growth enhancing effect of PHB. A possible explanation is the microbiota associated with the zoeae and the biochemistry of PHB since PHB is a quorum sensing compound.

In the juvenile study, similar growth rates (Table 1) were obtained for juveniles fed with the formulated wet artificial diet prepared with porcine gelatin without chitin supplementation compared to juveniles fed solely squid. Similar artificial diets have been supplied to *Octopus vulgaris* and *Carcinus maenas* without depressing growth (Powell & Rowley, 2007; Quintana et al., 2008). Our molt intervals were comparable to previous studies conducted on *C. sapidus* of similar size (Leffler, 1972). Additionally, our molt increments were comparable to previous studies conducted in the laboratory at 20-24*C who saw a molt increment ranging from 18-27% (Millikin et al., 1980; Guerin & Stickle, 1997; Allman et al., 2017). It can be concluded that gelatin, used as agglutinant, did not have any negative effect on the quality of the diet.

Additionally, after a couple days on the formulated wet artificial diet, animals eagerly accepted and consumed food similarly to squid diet. The formulated wet artificial diet also held its form long enough for animals to consume food. Stability is favorable because of the crabs' feeding behavior of gradually consuming food over several hours. The cohesiveness of the formulated diet could be associated with a high percentage of collagen in squid (Ando et al., 2001) as well as the gelatin.

The influence of dietary fiber such as chitin on the movement of nutrients along the gut will likely affect nutrient absorption. Diet analysis of each formulated wet artificial diet must be conducted to determine nutritional components of each treatment. Additionally, fecal material should be collected to

determine specifically how much of diet and chitin was digested by the juvenile *C. sapidus*.

5. Growth

The crabs used in this experiment showed normal growth compared to previous literature. *C. sapidus* zoeal duration was as expected, 30-50 days (Costlow & Bookhout, 1959) which varied depending on individuals for an optimal prey density under laboratory conditions most likely due to genetic diversity. In laboratory conditions under a low prey density, zoeae experienced longer larval duration similar to other studies (Broad, 1957a; Costlow & Bookhout, 1959; Porter, 1960; Sandoz & Rogers, 1944; Templeman, 1936a; Zeng et al., 2004).

Table 2. Average molt increment in mm and molt interval in days for juvenile *C. sapidus* fed squid control and varying levels of chitin supplementation

Molt	Treatment	Molt increment (%)	Molt interval (days)	*N* #	*P* < 0.05
	Squid	22.2 ± 1.0	18.2 ± 0.7	16	nd
	0	20.6 ± 1.3	24.7 ± 2.2	14	nd
1	5	17.4 ± 1.2	23.9 ± 1.8	13	nd
	10	20.8 ± 1.9	30.2 ± 4.0	12	nd
	20	20.7 ± 1.2	26.7 ± 2.3	15	nd

	Squid	23.6 ± 1.0	31.3 ± 3.3	16	nd
	0	23.8 ± 1.1	31.6 ± 3.7	14	nd
2	5	22.2 ± 1.0	27.7 ± 1.4	13	nd
	10	22.2 ± 2.1	35.1 ± 6.3	12	nd
	20	19.0 ± 1.5	41.4 ± 4.7	14	nd
	Squid	20.0 ± 2.5	60.2 ± 9.5	15	nd
	0	23.2 ± 3.4	49.4 ± 9.9	13	nd
3	5	21.0 ± 1.3	37.6 ± 4.5	13	nd
	10	20.3 ± 1.7	55.1 ± 10.0	11	nd
	20	25.5 ± 1.4	59.4 ± 9.8	14	nd

The data are presented as mean ± SE. nd=no difference.

As expected, juvenile molt interval increased with each molt but was not statistically different between diets (Chapter 3 Figure 1, Table 2). Molt increment in carapace width for juvenile *C. sapidus* was also similar across all treatments for each molt (Table 2). Molt interval and molt increment were comparable to previous studies conducted in the laboratory at 20-24*C (Leffler, 1972; Millikin et al., 1980; Guerin & Stickle, 1997) implying our animals grew normally even with chitin supplementation. However, animals reared in the wild tend to have higher growth rates than laboratory experiments (Brylawski & Miller, 2006). This may be

due to the fact temperature remained constant in the experimental system (20-21*C) while in the wild the water temperature can reach as high as ~30*C (Cronin et al., 2003). Another possibility is the increased mobility of those animals reared in the wild. Using compartments to rear the juveniles restricted movement which may have led to decreased metabolic rates thus decreased growth (Brylawski & Miller, 2003).

5. Future Work

It was observed that a high prey density with PHB supplementation effects zoeal development in *C. sapidus* by increasing the frequency of stage skipping. However, stage skipping may generate smaller individuals at end of larval development. *Neohelice granulata* that required more zoeal stages to reach megalopa were significantly larger as juveniles (carapace width, dry biomass, carbon and nitrogen contents) than individuals who required fewer zoeal stages (Giménez et al., 2004). More research needs to be done to determine if stage skipping effects the size of the megalopa or juvenile stage in high and low prey density with and without PHB supplementation.

It was also observed that a chitin supplementation of 5, 10 or 20% to a squid based diet does not significantly affect molt interval (Table 2). At 2 and 5% addition of chitin, *P. monodon* showed an enhanced feed efficiency, protein efficiency ratio and higher body weight gains; however, increasing chitin to 10% severely reduced growth and significantly depressed protein and lipid digestibility of the diet (Shiau & Yu, 1998). Additionally, *C. sapidus* juveniles fed 20% chitin replacement also showed a significantly longer molt interval and lower final wet

weight (Allman et al., 2017). Future studies must be conducted with 1.25 and 2.5% chitin supplements to determine if lower concentrations of chitin positively effects growth in *C. sapidus.*

Additionally, only the hitinase present in the hepatopancreas was measured in the hepatopancreas of *C. sapidus*. Chitin deacetylase, which catalyzes the conversion of chitin to chitosan by the deacetylation of N-acetyl-D-glucosamine, should be measured in *C. sapidus.* Chitonsan has also shown to have many beneficial effects on growth (Cha et al., 2008; Lin et al., 2012).

The main goal in an aquaculture setting is to get the animals to maturity as quickly as possible. Even though the treatments were statistically similar in terms of molt interval, there was a general trend that a lower chitin supplementation can decrease molt interval possibly decreasing time to maturity. In the future, juveniles must be grown to maturity to determine if a chitin supplement can enhance growth.

7. General Conclusion

Crustacean development has been studied for many years especially on species of ecological or economic significance. With the variability in the *C. sapidus* population over the last twenty years, the species fate has raised concerns. A deeper understanding of blue crab development will improve management practices in the wild and future culture technologies within hatcheries to increase production for commercial endeavors. The current study obtained dietary conditions that cause variation in early development in hatchery raised *C. sapidus*.

C. sapidus zoeae exposed to a high prey density had shorter larval duration by having fewer zoeal stages and greater survival rate than those with a low prey density. Additionally, zoeae exposed to PHB supplementation with a combination of a high prey density increased the instances of stage skipping, without changing larval duration. Prey density may affect nutritional status of an individual thus increasing the influence that prey density has on the development of *C. sapidus* zoeae.

Juvenile *C. sapidus* supplemented with chitin showed no effect on development (molt increment or interval) or carbohydrate and CHH levels but decreased the levels of protein in the hemolymph after three molts. It remains to be further studied on what specific proteins are reduced with chitin fed *C. sapidus* but it may be related to innate immune response due to the immunomodulatory impacts of chitin on different species. Overall, the understanding of *C. sapidus* growth and physiology has been expanded and the dietary conditions that increases developmental plasticity has been determined.

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