ABSTRACT

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Five experiments were performed to determine the importance of chitin and cellulose in the diet of juvenile *C. sapidus*. A compartmentalized recirculating system was established to provide optimal conditions, maintaining the animals with little mortality. The appropriate ration, compartment size, and an adequate baseline diet were established. We replaced 20% of a commercial diet with varying amounts of chitin and cellulose. Crabs fed the cellulose-containing diet had higher growth rates, conversion efficiencies, molt increments and frequencies than crabs fed the chitin-containing diet, but were equal to the control diet. We then assayed for chitinase and cellulase in gut tissues. Chitinase had lower specific activity (0.072 ± 0.159 mU $mg^{-1}min^{-1}$) than cellulase (3.52 ± 0.16 mU $mg^{-1}min^{-1}$) in the foregut and hepatopancreas. There was no effect of diet on specific activity. The results show juvenile *C. sapidus* is capable of utilizing cellulose, but not chitin, when delivered as 20% of a diet.

FIBER DIGESTION IN THE JUVENILE BLUE CRAB, *CALLINECTES SAPIDUS* RATHBUN

By

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Chapter 1: Introduction

Stock abundance of the blue crab, *Callinectes sapidus* (Rathbun), in the Chesapeake Bay (USA) is declining. The baywide catch per unit effort (kg crabs caught per crab pot) declined by nearly 75% between 1945-95 (Rugolo, Knotts, Lange, 1998). From 1968-2000, the percent of legal males and the average size of male crabs have declined significantly near Calvert Cliffs, Maryland (Abbe, 2002). In the lower Chesapeake Bay, female spawning stock abundance has declined by 81% from 1988-2000 (Lipcius, Stockhausen, 2002). After the extremely low stock abundance in 2001, the crab population in Chesapeake Bay increased slightly, but remains at historically low levels and is still above the overfished threshold (Commission, 2005).

While overfishing has had a large part to play in the decline in stock abundance, it is unclear to what extent other factors are also involved. Many parameters affecting stock abundance are unknown. The effect of toxicants, such as pesticides, fertilizers, heavy metals, and countless other waste products has barely been investigated for blue crabs (Lee, Noone, 1995). To understand the influence of such toxicants, we must have a sufficient understanding of the basic biology of the blue crab. Little is known about the biochemical pathways influencing reproduction (McCarthy, Skinner, 1979), growth, and molting (Lee, Watson, 2002; Lee, Watson, Roer, 1998; Watson, Lee, Borders, Dircksen, Lilly, 2001) in *C. sapidus*, and until these pathways are elucidated there is no way to discern between specific environmental triggers and their effects on blue crabs.

In addition to the unknown effects of toxicants, there is a lack of understanding about the trophic niche of the blue crab. *C. sapidus* has been described as a detritivore, omnivore, scavenger, cannibal, and a predator (Clark, Wolcott, Wolcott, Hines, 1999;

Laughlin, 1982; Stoner, Buchanan, 1990). Although these studies describe the wide variety of items consumed in the natural habitat, to what extent different portions of the crabs' diet contribute to growth is uncertain. Variations in abundance of nutritionally valuable food items have a direct effect on the stock abundance of a population. In order to more accurately predict fluctuations in stock abundance, the animals' ecological niche must be taken into consideration. Fisheries managers are moving towards an ecosystem approach, taking trophic level, the effects of pollutants, and many other factors into account when determining stock abundances (Panel, 2004). As managers begin to take a multispecies, ecosystem approach for the Chesapeake Bay, providing more basic information about the factors affecting blue crabs would prove extremely useful.

To learn more about the blue crab's basic biology including endocrinology and digestive capabilities, it is necessary to maintain healthy animals in captivity. At the Center of Marine Biotechnology (Baltimore, MD), blue crab hatchery technology is being developed to produce thousands of juvenile crabs of approximately 25mm carapace width (Zmora, Findiesen, Stubblefield, Frenkel, Zohar, 2005). These crabs are involved in field experiments at the Smithsonian Environmental Research Center (Edgewater, MD) to assess the feasibility of stock enhancement (Davis, Young-Williams, Hines, Zohar, 2005). In addition to the hatchery, the Center of Marine Biotechnology has nursery and grow-out capabilities in which we can investigate basic biological questions for animals greater than 25 mm carapace width. The facilities available are an established compartmentalized recirculating aquaculture system, making full control of the environmental parameters involved in a captive rearing study attainable. In this way, the long-term effects of diet and environmental toxicants can be observed.

The maintenance and survival of juvenile crabs in a recirculating system for several months may be difficult, as stressed animals in captivity have a higher frequency of disease (Messick, Sindermann, 1992). However, much success has been achieved in maintaining juvenile crabs in recirculating systems, and this approach appears to be a useful avenue for future research (Cadman, Weinstein, 1988; Millikin, Biddle, Siewicki, Fortner, Fair, 1980; Winget, Maurer, Anderson, 1973; Winget, Epifanio, Runnels, Austin, 1976).

A number of parameters need to be investigated to provide optimal conditions for the health of animals in a recirculating system. Of the environmental parameters previously studied, temperature, salinity and dissolved oxygen have been main focal points. Temperature is crucial in determining growth rates of juvenile crabs. Within a 13-34^{o}C range, as temperature increases, molt frequency increases while molt increment (growth per molt) declines (Cadman, Weinstein, 1988; Leffler, 1972; Winget, Epifanio, Runnels, Austin, 1976). Since molt frequency contributes more to the overall growth rate than increment in these studies, growth increases with higher temperature. This needs to be applied with caution however, since increasing temperature also results in a higher mortality rate (Cadman, Weinstein, 1988; Leffler, 1972). Metabolic rate does not increase much from 20-27^{o}C, indicating that blue crabs have a rather broad thermoneutral range (Leffler, 1972). Salinity may also be important in determining growth of crabs, although it appears to be of less importance than temperature based on the literature. Cadman and Weinstein (1988) found higher growth at a salinity of 30 gL^{-1} than at 15 gL^{-1} in juvenile blue crabs. However, Guerin and Stickle (1997) found no differences in growth rates of juvenile crabs at 2.5, 10, or 30 gL^{-1}. Blue crabs appear to be relatively sensitive to

hypoxic conditions. When exposed to oxygen concentrations of 0.5 mgL^{-1}, blue crabs have an LT_{50} of 2.56 days, lower than the majority of epifaunal species tested from the York River, Virginia (Sagasti, Schaffner, Duffy, 2001). When offered a concentration gradient, blue crabs prefer, on average, 5.2 mgL^{-1} of oxygen saturation (Das, Stickle, 1994).

While temperature, salinity, and dissolved oxygen have been extensively studied for *C. sapidus*, appropriate dietary rations have never been determined. When initially studying nutritional requirements of a species, the tendency is to overfeed or deliver diets *ad libitum* (Biddle, Millikin, Fair, Fortner, 1978; Millikin, Biddle, Siewicki, Fortner, Fair, 1980; Winget, Epifanio, Runnels, Austin, 1976). This method of feeding provides more food than is needed for survival and maintenance, so it may confound growth data for treatments where nutrients are limiting (Tenore, Hanson, McClain, Maccubbin, Hodson, 1984). Overfeeding is also wasteful and expensive. In various fish species, as rations increase, growth rate increases rapidly and then approaches an asymptote at some maximal value (Wootton, 1998). The maximum growth rate corresponds to a maximal ration, C_{max}. Gross conversion efficiency (total weight gained (total weight fed)$^{-1}$) does not follow the same trend, but reaches a maximum value at some ration less than C_{max} and then decreases as rations approach C_{max}. The ration resulting in the highest conversion efficiency is designated C_{opt}, and provides the most growth per unit of diet delivered to the animals. This level of ration is of particular importance in an aquaculture production system, and should be determined for juvenile *C. sapidus*.

In addition to overfeeding, aquaculturists tend to provide a diet with more caloric and protein content than is needed for the species reared. This practice is not only

expensive, but it is also damaging to world fish supplies since 1/3 of the world fisheries are harvested for fishmeal for aquaculture and farm feeds, and 2-5 times the protein that comes out of intensive aquaculture goes into the feed (Naylor, Goldburg, Primavera, Kautsky, Beveridge, Clay, Folke, Lubchenco, Mooney, Troell, 2000). To determine the composition of a potential diet, basic nutritional requirements need to be determined. In general, crustacean diets need to be more water-stable than fish diets due to their slower feeding behavior. Crustaceans tend to pick apart the diet and consume it gradually over several hours. Water stability must be balanced, however, with the release of small amounts of substances attractive to the animals, so that the diet remains palatable (New, 1976). A few major aspects of diet have been investigated for blue crabs. Millikin, Biddle, Siewicki, Fortner, and Fair (1980) determined no significant difference in growth between crabs fed 37% and 49% protein, however growth was depressed when fed 23% protein diets. Conversion efficiency also tends to decrease in less nutritionally valuable diets (Wootton, 1998), which has been shown for *C. sapidus* fed 27% fiber, compared to 9% fiber (Biddle, Millikin, Fair, Fortner, 1978). Since specific nutritional requirements of juvenile crabs are still largely unknown, feeding experiments are an important area for future research. From a generalistic "control" diet, the specific requirements for *C. sapidus* can then be fine-tuned and the digestive capabilities defined.

In order to define trophic level, the relative importance of fiber in the diet of *C. sapidus* needs to be determined. Juvenile blue crabs use seagrass beds as nursery habitat in the Chesapeake Bay (Heck, Thoman, 1984). Examination of gut contents shows large portions of detrital material, including plant fragments, in young crabs. In a study in the Apalachicola Bay, Florida, detritus and plant matter composed 21.84% (by weight) of the

diets of juvenile crabs smaller than 31mm carapace width, decreasing with increasing carapace width to 4.74% in animals >60mm carapace width (Laughlin, 1982). Growth rates of juvenile crabs in field enclosures were faster in vegetated than unvegetated substrata in the lower York River, Virginia (Perkins-Visser, Wolcott, Wolcott, 1996). This may have been partly due to the ability of blue crabs to digest maltose, a component of one of the primary complex storage sugars in plant material, starch (α-1,4-glucan) (McClintock, Klinger, Marion, Hsueh, 1991). The fact that blue crabs are able to digest starch suggests that they are not strictly carnivores, but are rather omnivores with capabilities to derive nutritional value from an extensive range of food items. If crabs are able to digest starch, then they may also be able to digest more complex polysaccharides. Cellulose, followed by chitin, is the most abundant carbon-based biomass worldwide and is the primary source of fiber (McDonald, Edwards, Greenhalgh, Morgan, 2002). In a feeding experiment with varying proportions of dietary fiber (cellulose), Biddle, Millikin, Fair and Fortner (1978) found similar growth between blue crabs fed 3 and 9% cellulose, but low survival and depressed growth in crabs fed 27% cellulose. The high inclusion rate (9-27%) suggests that crabs may have some ability to derive nutritional value from cellulose.

Cellulose is a polymerized form of glucose with β-l,4-linkages with a complex structure of crystallized microfibrils among amorphous matrixes. This structure limits access to most hydrolyzing enzymes. However, some organisms have developed cellulases that actively hydrolyze cellulose fibrils (Watanabe, Tokuda, 2001). Usually three types of enzymes are needed to convert cellulose to glucose: endoglucanases that cleave internal β-1,4-glucosidic bonds, exoglucanases that cleave cellobiosyl units at the

ends of the cellulose molecules, and β-glucosidase that releases glucose from cellobiose and cellodextrins (Byrne, Lehnert, Johnson, Moore, 1999). Cellulases have been known to be produced by symbiotic protozoans living within the hindgut of termites and cattle, but it was not believed until recently that higher animals could produce cellulases endogenously (Watanabe, Tokuda, 2001). Cellulase has been discovered in a variety of crustaceans and mollusks, including the freshwater prawn *Macrobrachium rosenbergii* (Gonzalez-Pena, Anderson, Smith, Moreira, 2002), six species of Tasmanian crabs (Johnston, Freeman, 2005), and the spiny lobster *Jasus edwardsii* (Johnston, 2003). The hepatopancreas is the site of endogenous carbohydrase synthesis in crustaceans, which is then released into the foregut in the form of gastric juice (Johnston, Freeman, 2005; Johnston, Yellowlees, 1998). The cDNA encoding endogenous endoglucanases have recently been discovered and characterized in the red claw crayfish *Cherax quadricarinatus* (Byrne, Lehnert, Johnson, Moore, 1999; Xue, Anderson, Richardson, Xue, Mather, 1999), the abalone *Haliotis discus* (Suzuki, Ojima, Nishita, 2003), and in the blue mussel, *Mytilus edulis* (Xu, Janson, Sellos, 2001; Xu, Hellman, Ersson, Janson, 2000). All except the mussel endoglucanase belong to the same glycosyl hydrolase family (9) (Henrissat, 1991), and exhibit similar characteristics of being relatively small proteins (181-594 a.a. residues) lacking cellulose-binding domains (with the exception of abalone) and having high thermostability (30-50^{o}C temperature optima)(Byrne, Lehnert, Johnson, Moore, 1999; Suzuki, Ojima, Nishita, 2003; Xu, Hellman, Ersson, Janson, 2000; Xue, Anderson, Richardson, Xue, Mather, 1999).

Chitin forms the exoskeleton of arthropods in combination with proteins and salts such as calcium carbonate. It consists of monomers of N-acetyl-D-glucosamine,

connected by β(1-4) bonds. In its natural form, it is composed of crystalline fibers embedded in a matrix (much like cellulose), which reduces the accessibility of chitinolytic enzymes to the molecule (Esaiassen, Myrnes, Olsen, 1996). At 10 days postmolt, the cuticle of *C. sapidus* consists of 11.4% chitin (Vigh, Dendinger, 1982) and approximately 73% mineral salts (Cameron, 1985). During molting, chitinase is necessary in order to break down the old cuticle to allow resorption of organic content. Approximately half of the organic content of the shell is absorbed prior to molting (Cameron, Wood, 1985). Because of this, all crustaceans are able to synthesize some level of chitinase (Warner, 1977). In the shrimp *Palaemon serratus*, chitinase activity in the gut fluctuates with molt cycle (Spindler-Barth, Van Wormhoudt, Spindler, 1990). While we can assume that *C. sapidus* has some chitinase for resorption of chitin prior to molting, it remains to be determined whether blue crabs can derive nutritional value from the chitin in their diet. Blue crabs have been observed consuming their own exoskeletons after molting, from the larval stages (Sandoz, Rogers, 1944) to juveniles and adults (A. Allman, pers. obs.). Chitin is also consumed when eating other crustaceans and when cannibalizing each other (Laughlin, 1982; Stoner, Buchanan, 1990). Since salts such as calcium carbonate are absorbed directly from the surrounding water via the gills (Cameron, Wood, 1985), the potential value of consuming shed exuviae exists in the resorption of chitin and the various proteins associated with the molecule.

Generally, two types of chitinolytic enzymes are expressed in crustaceans: chitinase, which degrades chitin to soluble oligosaccharides; and β-N-acetylhexosaminidase, which hydrolyzes the oligosaccharides to N-acetylglucosamines (Watanabe, Kono, Aida, Nagasawa, 1998). However, a system of several chitinases is

often required to react with chitins of differing substrate specificities (Esaiassen, Myrnes, Olsen, 1996). Endogenous chitinases have been purified from the hepatopancreas of the prawns *Penaeus japonicus* and *Pandalus borealis* (Esaiassen, Myrnes, Olsen, 1996; Kono, Matsui, Shimizu, Koga, 1990; Watanabe, Kono, Aida, Nagasawa, 1998), and from the American lobster *Homarus americanus* (Lynn, 1990). These chitinases have molecular weights from 37000-150000, with widely varying pH and temperature optima.

Depending on the species, the addition of dietary fiber may or may not influence the production of carbohydrases. In the prawn *Macrobrachium rosenbergii*, cellulase activity positively correlated to the level of dietary cellulose (Gonzalez-Pena, Anderson, Smith, Moreira, 2002). In the mud crab, *Scylla serrata*, crabs fed 47% starch or cellulose had a fourfold higher specific activity than when fed 29% starch (Pavasovic, Richardson, Anderson, Mann, Mather, 2004). However, starvation had little effect on specific activity of proteases in *Penaeus vannamei* (Lee, Smith, Lawrence, 1984). Species capable of altering their specific activities in response to diet exhibit high phenotypic plasticity.

It is important to make the connection between growth rates and enzyme activity in order to determine at what level specific activity is no longer important to digestion. There are also many other factors involved in assimilation besides the presence of enzyme, such as food particle size, structural components of the diet inhibiting the availability of substrate to the enzyme, and potential antinutrients associated with the diet (Francis, Makkar, Becker, 2001). As a result, there is not always a direct link between enzyme activity and growth when the substrate is present in the diet. Very few studies cover both growth rates and enzymatic profiles, but linking these two subjects is the only way to determine whether an enzyme is metabolically valuable to the animal.

A series of experiments were performed in order to define the importance of chitin and cellulose in the diet of juvenile *C. sapidus*, beginning with the determination of the appropriate ration and compartment size. A compartmentalized recirculating system was established to provide optimal conditions according to previous literature values, and maintained the animals with little mortality for the time period studied. The first experiment compared three rations and two compartment size treatments. We predicted that as rations increased, growth would also increase while conversion efficiency peaked, and then dropped. We also predicted that crabs would have higher growth rates and conversion efficiencies when maintained in larger compartments. The appropriate compartment size and an adequate baseline diet were established. To better model the effect of ration on growth rates, a second experiment was performed. We delivered fractions of the lowest ration from the previous experiment to juvenile *C. sapidus*. We predicted a continuation of the trends seen in the previous experiment, with decreasing rations resulting in decreasing growth rates and a peak in conversion efficiencies at the C_{opt}. The "optimal" ration determined from this experiment was not necessarily practical, since rapid growth rates are more cost-effective in our grow-out system.

The third and fourth experiments involved formulating diets supplemented with chitin and cellulose. We set out to determine if the addition of fiber in the diet affects growth and survival of juvenile *C. sapidus*. If digestible, the addition of dietary chitin and cellulose should have no deleterious effects on growth of juvenile crabs in the laboratory. Since there is evidence that other crustaceans produce endogenous carbohydrases, we predicted no suppression of growth or decrease in conversion efficiency with the addition of dietary cellulose or chitin.

In the fifth experiment, gut and hepatopancreas samples from the fourth experiment were assayed for chitinase and cellulase activity. This was done to ascertain the presence of enzyme, their location in the gut, and whether dietary composition influenced the concentration of enzyme. We predicted that we would find both chitinase and cellulase, since all crustaceans synthesize chitinase for molting, and crabs had similar growth rates when fed cellulose-containing diets as when fed the control. We expected to find more cellulase activity than chitinase, since crabs had higher growth rates when fed 20% cellulose than when fed 20% chitin diets. Since carbohydrases are produced in the hepatopancreas and then secreted into the foregut via gastric juice (Johnston, Freeman, 2005; Johnston, Yellowlees, 1998), we expected to see the highest specific activities in the hepatopancreas and foregut, with decreased specific activity in other portions of the gut. We expected enzyme activity to vary with diet, with presence of a particular substrate in the diet resulting in higher corresponding enzyme activity.

Chapter 2: The effect of cage size and ration on growth of the juvenile blue crab, *Callinectes sapidus* (Rathbun), in a recirculating system

Summary

Blue crab (*Callinectes sapidus* (Rathbun)) hatchery technology is being developed at the Center of Marine Biotechnology (Baltimore, MD, USA). To more efficiently produce juveniles and adults for research purposes, the appropriate cage size and ration need to be determined. We performed a 21-week experiment, using a commercial pelletized shrimp diet and three rations representing a range of recommended rations for penaeid shrimp. We developed a compartmentalized recirculating system with two cage sizes (10x10x10cm and 15x15x10cm) to determine if smaller cages limit growth. Final wet weights (using initial weight as a covariate) and gross conversion efficiencies were significantly affected by cage size and ration. Percent of food consumed was significantly different among treatments, due to a difference within the small cages where the intermediate ration was significantly higher than the other two rations. From this data we concluded that small cages inhibit growth. In addition, all the rations were sufficient to maintain basic metabolic functions, but a sufficient range of rations was not tested to determine the optimum ration (C_{opt}). Genotyping of the brood revealed evidence of multiple paternity, and the food conversion efficiency was significantly different between crabs with one of two different fathers.

Since C_{opt} was not determined from the first experiment, we performed another experiment in which we fed crabs 100%, 75%, 50%, 25% and 0% of the lowest ration from the previous experiment. Crabs in the 25% and 0% ration treatments had consistently lower wet weights than the other treatments throughout the experiment. The

specific growth rate ($(\ln(ww_1) - \ln(ww_0))\ time^{-1}$) and molt increment exhibited the same pattern, both increasing as rations increased up to the 50% ration, and then leveling off. The average time required to reach the first molt decreased linearly between the 0% and 100% ration treatments. Food conversion efficiency (weight gained (weight fed)$^{-1}$) peaked at the 50% ration, and then decreased with further increase in rations. From this, we concluded the 50% ration to be the C_{opt} for juvenile *C. sapidus* in our system. Overall, growth rates were lower than in the first experiment. In our system, faster growth is more cost-effective and shows treatment effects more quickly, so a ration higher than C_{opt} is more appropriate.

Introduction

Nutrition experiments with blue crabs have involved feeding *ad libitum* only (Biddle, Millikin, Fair, Fortner, 1978; Millikin, Biddle, Siewicki, Fortner, Fair, 1980; Winget, Epifanio, Runnels, Austin, 1976). This method of feeding provides more food than is needed for survival and maintenance, so it may confound growth data for treatments where nutrients are limiting (Tenore, Hanson, McClain, Maccubbin, Hodson, 1984). Proper rations are necessary to evaluate the sufficiency of dietary components in future feeding experiments. From an energetic viewpoint, the optimal ration is the ration resulting in the highest gross conversion efficiency (C_{opt}), providing the most growth per unit of diet fed. This level of ration is normally less than the ration resulting in the highest growth rate (C_{max}). Both of these rations should be determined for farmed species, and the ideal ration chosen according to the needs and goals of the establishment.

To date, no studies have investigated the parameter of compartment size and its effects on juvenile crabs. It has been noted that crabs grown in a compartmentalized

system have lower growth rates than crabs grown in ponds (Ju, Secor, Harvey, 1999), however, this is not necessarily a result of the size of the container. Other factors such as diet and cannibalism may have a role in enhancing growth in communal ponds. Small compartment size is known to impinge upon growth in the redclaw crayfish, *Cherax quadricarinatus* (Manor, Segev, Leibovitz, Aflalo, Sagi, 2002). Putting juvenile blue crabs into separate cages is important since they are extremely cannibalistic starting from the megalopa stage (Moksnes, Lipcius, Pihl, Van Montfrans, 1997), and competition and predation make controlled feeding experiments impossible. However, if cage size is limiting in the blue crab, this may obscure treatment effects on growth.

While ration and compartment size have not been studied, environmental factors such as temperature and salinity have been extensively investigated for *C. sapidus*. Both molt frequency and mortality increase with increasing temperature between 13 and 34^{o}C, and metabolic rate changes little between 20-27^{o}C (Leffler, 1972). The highest molt increment for juvenile crabs occurs at 23^{o}C, and then decreases as temperature increases (Cadman, Weinstein, 1988). From salinity studies investigating cation concentrations in *C. sapidus* serum, the approximate isoosmotic point is 27 gL^{-1} (Colvocoresses, Lynch, Webb, 1974). These values give us a reference from which we can investigate our topic of interest.

We set out to determine adequacy of a pelletized commercial diet, as well as to determine an appropriate ration and cage size for optimal growth and survival of these animals in a recirculating system. We started with a basic commercial shrimp diet to evaluate diet adequacy. Diet adequacy was defined by high survival and the ability to maintain normal metabolic functions such as molting. We attempted to determine what

rations corresponded to the highest conversion efficiency (C_{opt}) and highest growth (C_{max}). We predicted that as rations increased, growth would also increase while conversion efficiency peaked, and then dropped. The system we established had individual compartments to avoid cannibalism during molting. Using two cage sizes, we examined whether growth was more limited in the smaller versus the larger compartments. We predicted that crabs would have higher growth rates and conversion efficiencies when maintained in larger compartments.

Since growth and conversion efficiency exhibited linear relationships with ration, we determined that the range of rations tested was not broad enough to show the overall nature of these relationships. We performed a second experiment, lasting for 6 weeks, delivering fractions of the lowest ration from the previous experiment to juvenile *C. sapidus*. We predicted decreased rations to result in decreasing growth rates and a peak in conversion efficiencies at the C_{opt}.

Materials and Methods

For both experiments, juvenile *C. sapidus* were maintained in a recirculating system of seven tanks. Each tank held a water volume of 297 L with dimensions 60x90x55 cm; one of these tanks was used as a sump containing mechanical and biological filtration. The biological filtration was KMT (Water Management Technologies, Baton Rouge, LA, USA) media filling approximately 100L of the sump with an established community of denitrifying bacteria. The flow rate from the sump to each tank was approximately 1.3 $Lmin^{-1}$, and a 20% water change was performed every three weeks. Approximately 38 tank volumes per day passed through the biological filter. Three diffusers in each tank provided aeration. Water depth in each cage was

approximately 5 cm, and the cage bottom was covered with nylon screening material (1 mm mesh) to allow flow through the tank while retaining all but particulate food in the cages.

We added brine and trace minerals to dechlorinated city water to make the saltwater. Saltwater was formulated as a compromise between mimicking natural seawater as closely as possible while still allowing for cost-efficiency (Table 2). The light source was fluorescent lights in the ceiling on a controlled photoperiod, which mirrored the daily photoperiod in Baltimore, Maryland. We considered this acceptable since no significant difference was found in growth of crabs when 8-hour versus 16-hour daylengths were tested (Winget, Maurer, Anderson, 1973). Light intensity at the water surface during the day was 300 Lux.

Table 1. Essential ion composition mgL^{-1} of saltwater from our hatchery, compared to literature values of seawater from Bowen (1979).

Element	Chemical Species	Hatchery Saltwater	Seawater (Bowen)
Cl	Cl^-	19574	19000
Na	Na^+	9560	10500
Mg	Mg^{2+}	2135	1350
S	SO_4^{2-}, $NaSO_4^-$	1066	885
Ca	Ca^{2+}	462	400
K	K^+	343	380
C	HCO_3^-, CO_3^{2-}, CO_2	39.7	28
Sr	Sr^{2+}	8.3	8.1
B	$B(OH)_3$, $B(OH)_4^-$	8.5	4.6
Li	Li^+	0.18	0.18
Mo	MoO_4^{2-}	0.006	0.01

We used a commercial pelletized 1/8 inch (3.13 mm) diameter shrimp diet (Ziegler Bros, Inc., Gardners, PA, USA). Millikin (1980) found no difference in growth rates of juvenile crabs when fed 37 or 49% protein diets but depressed growth at 23%

protein. From this study, we selected 30% protein to be an acceptable level for optimal growth and lowest protein content. The stated protein: fat: fiber ratio, measured in dry weight as fed, was 30: 7: 4. Proximate analyses were performed (New Jersey Feed Lab, Inc., Trenton, NJ, USA) to confirm the stated values, and all were within an acceptable range for our study (Table 1). We chose a shrimp diet because shrimp and blue crabs exhibit similar feeding behaviors, both feeding intermittently and leaving large portions of their food for later consumption. Therefore, water stability is an important factor in diet choice. However, more water-stable diets may not leach enough nutrients into the surrounding water to make them attractive to the animals, so a balance between water stability and palatability is required (New, 1976). The commercial shrimp diet chosen is water stable for several hours, and is a diameter that is easily consumed by the crabs. The crabs consumed it readily, so it appeared to have a good compromise between palatability and water stability to meet the needs of the animals.

Table 2. Ingredients in Ziegler 1/8" pelletized shrimp diet, expressed as percent of the total diet, fed to juvenile blue crabs, *Callinectes sapidus* (Rathbun).

Ingredient	Guaranteed	% As Fed	% Proximate
protein	min	30.0	33.6
fat	min	7.0	4.31
fiber	max	4.0	4.74
moisture	-	-	11.71
ash	-	-	6.41

Proximate column represents results of proximate analyses performed on diet.

Experimental Design

Experiment 1: Ration / cage size

Two hundred and ten hatchery-reared crabs from a single brood were obtained from the Center of Marine Biotechnology. These crabs were on average 24 mm (18-39 mm range; standard error = 0.15 mm) carapace width and 1 gram wet weight (0.5-4.5 g range; mean = 1.24 g). Eight weeks after commencing the experiment, cages were covered with a plastic grid and attached to the sides of the tank with a crossbar to prevent crabs from escaping.

Water temperature was maintained at 21 $\pm$ 1°C. After week 15, temperature was raised to 26°C to facilitate molting. Salinity was maintained at 28 $\pm$ 2gL^{-1}. Ammonia, nitrite, and pH levels were measured daily, while nitrate was measured monthly (Table 3). Dissolved oxygen was measured in each individual cage on August 28, 2003, September 8, 2003, and January 2, 2004. Mean dissolved oxygen values were significantly different in the large versus small cages (Table 4). However, the average values were only 0.2 mgL^{-1} different from each other (6.41 versus 6.65 mgL^{-1}), so this is not likely to have any significant physiological effect on the crabs.

Table 3. Water parameters for the course of Experiment 1(21 week duration) and Experiment 2 (6 week duration).

Parameter	Experiment 1	Experiment 2
NH_3	0.285 $\pm$ 0.023	0.309 $\pm$ 0.045
NO_2	0.782 $\pm$ 0.107	0.78 $\pm$ 0.11
NO_3^-	26.7 $\pm$ 2.6	12.3
Dissolved oxygen	6.40 $\pm$ 0.03	5.9 $\pm$ 0.3
pH	7.85 $\pm$ 0.01	8.35 $\pm$ 0.01
Salinity	28 $\pm$ 0.3 gL^{-1}	29 $\pm$ 0.4 gL^{-1}
Temperature	23 $\pm$ 0.2°C	23 $\pm$ 0.2°C

Values are in mgL^{-1} unless otherwise stated; pH is unitless.
SEM = standard error of the mean.

Table 4. Mean dissolved oxygen for each cage size and ration combination.

Cage	Ration	% saturation	mgL^{-1}	Sig at p < 0.05	
small	-	88.1	6.41	a	
large	-	91.5	6.65	b	
small	low	88.4	6.43		a
small	intermediate	88.7	6.45		a
small	high	87.3	6.35		a
large	low	92.2	6.70		a
large	intermediate	90	6.55		b
large	high	92.5	6.72		a

Values with the same letter are not significantly different from each other at $p < 0.05$.

Rations were based on the recommended rations for Penaeid shrimp, expressed as a range of percent dry food per gram biomass per day (Guillaume, Kaushik, Bergot, Metailler, 1999). The minimum and maximum recommended values were designated "low" and "high" rations respectively, and the median was designated the intermediate ration (Figure 1). Crabs were fed once a day, six days a week. When excess food accumulated in the cages from previous days, it was removed.

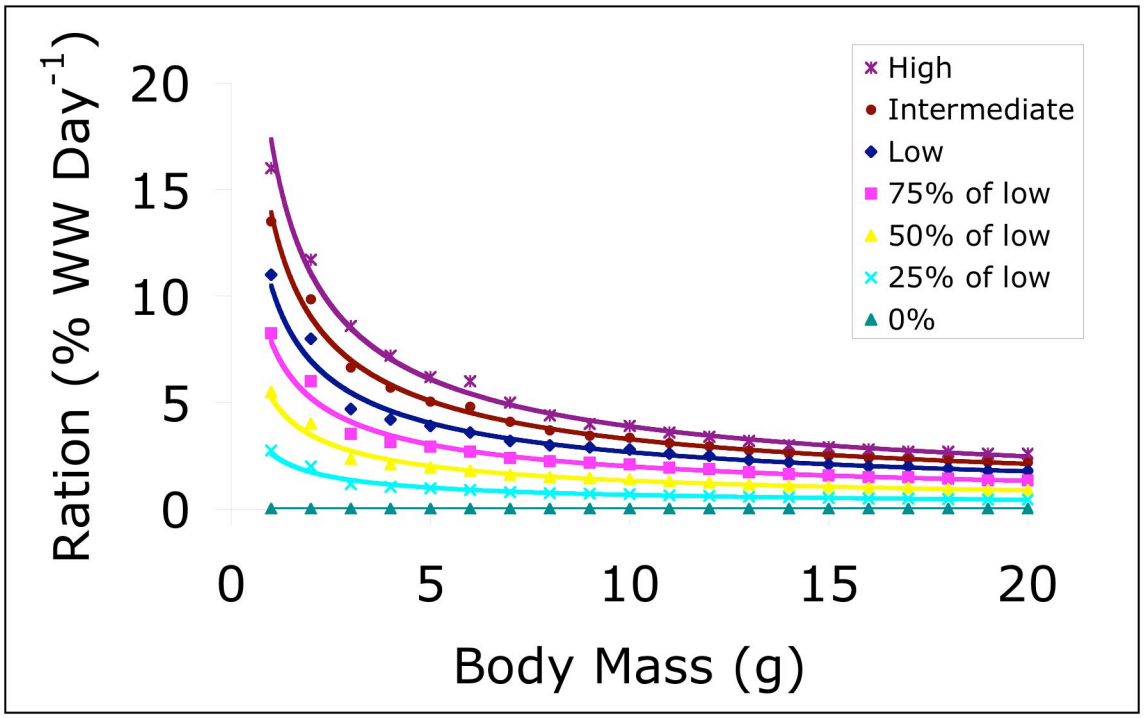

Figure 1. Rations fed to juvenile blue crabs, *Callinectes sapidus* (Rathbun), based on the recommended rations for *Penaeus* shrimp (Guillaume, Kaushik, Bergot, Metailler, 1999). DW= dry weight; WW= wet weight.

The crabs were randomly assigned to treatments of a combination of cage size and ration. Cage sizes were 10x10x10 cm, designated "small", and 15x15x10 cm, designated "large". The tank system was arranged as a nested experimental design. Six of the tanks were divided in half, with half of each tank containing large cages and the other half containing small cages. Due to size constraints, there were 24 small cages and 12 large cages per tank. Each tank half was randomly designated a ration, so that each combination of ration and cage size was assigned to two tanks (Figure 2). The experiment commenced on August 13, 2003. Wet weights (g) and carapace widths (mm) were measured for all crabs every two weeks until after week 12, when they were measured every three weeks. To measure wet weights, crabs were blotted with a paper towel and placed on a scale. Carapace widths were measured across on the dorsal side of the

carapace with a ruler, from spine to spine. When a crab molted, the date was recorded. Molts were left in the cages to be consumed by the crabs. If a crab molted on the day of measuring weights and widths, it was left to harden for 24 hours and then measured.

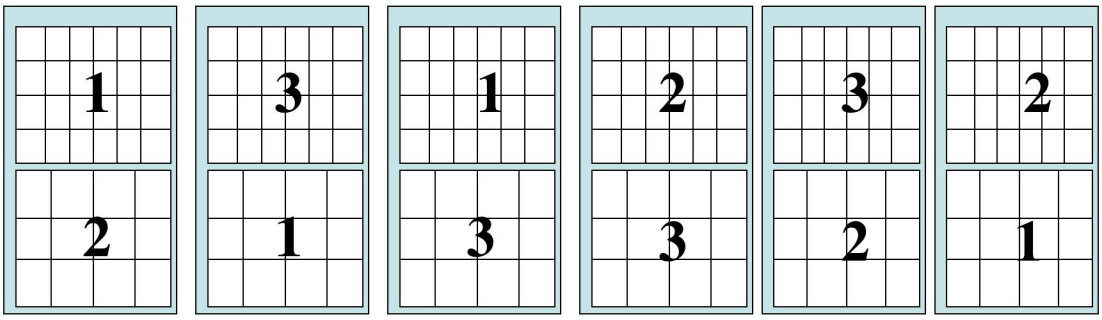

Figure 2. Layout of recirculating, compartmentalized tank system (not including sump). Numbers indicate ration delivered to each tank half.

On August 28, thirteen crabs (including ten crabs that were being held in the sump as "extras") were sacrificed and freeze-dried to get initial dry weights. The experiment terminated on week 21, January 9, 2004, and all crabs were removed, sexed, sacrificed, and freeze-dried to get dry weights (g). Crabs were sacrificed by submersion in ice water for 5 minutes. All dry weight values were pooled to make linear regressions determining the relationship between dry weight (DW) and wet weight (WW), and dry weight and carapace width (CW).

Experiment 2: Ration cutting

For the second experiment, 36 hatchery-reared crabs from a single brood were obtained from the Center of Marine Biotechnology. These crabs were on average 26 $\pm$ 1(standard error) mm carapace width (range = 19 - 34 mm) and 1.5 $\pm$ 0.11 grams wet weight (range = 0.54 - 3.13 g). Cages were covered with a plastic grid and attached to

the sides of the tank with a crossbar from the onset to prevent crabs from escaping. Prior to both experiments, crabs were acclimated to the experimental salinity and temperature for two weeks in a communal tank, and then were moved to the experimental system and allowed to acclimate to tank conditions for two days.

Water temperature was maintained at 23 $\pm$ 0.2^{o}C. Salinity was maintained at 29 $\pm$ 0.4 gL^{-1}. Ammonia, nitrite, and pH levels were as before, but dissolved oxygen was measured once, on March 11, 2004 (

Table 3). Mean dissolved oxygen was 5.9 $\pm$ 0.3 mgL^{-1}, an acceptable level according to literature values (Das, Stickle, 1994), and there was no significant difference in dissolved oxygen among rations.

The "low" ration from Experiment 1 was the highest (100%) ration delivered in Experiment 2. The other treatments delivered were fractions (75%, 50%, 25% and 0%) of this ration (Figure 1). Rations were fed to the crabs according to the crab's wet weight, in increments of 1 gram. The actual weight of the pellets fed was corrected to account for 11.71% water weight determined experimentally. Crabs were placed in 15x15x10 cm "large" cages only in two adjacent tanks, and randomly assigned one of the five ration treatments. Rations were delivered and animals measured the same way as in Experiment 1. All of the crabs were weighed and measured at 0, 2, 4, and 6 weeks. The experiment commenced on February 10, 2004. On March 24, 2004, crabs were weighed, measured, sexed, and sacrificed by submersion in icewater for 5 minutes.

Growth

All data analyses were performed using the SAS system V.8 (SAS 2000). When significant differences were found in the main effects, pairwise comparisons within cage

sizes using Tukey's HSD were performed. For both experiments, ANOVAs on initial wet weight and carapace width showed no significant difference between treatment groups (data not shown). However, the initial size of the animals contributed the majority of the variance in size. For this reason, analysis of covariance (ANCOVA) was performed for the final weights and carapace widths, using initial values as a covariate. Tests for normality and homogeneity of variance were applied to data prior to analysis, and data was transformed when appropriate. Statistical tests were performed on both wet weight and carapace width when both could be applied, but for the sake of brevity, only the methods for using wet weight will be discussed. Means are reported $\pm$ the standard error.

If significant differences were found in growth between the treatments by ANCOVA, these differences were further investigated. Differences in growth could arise as a result of differences in molt frequency or molt increment (increase in carapace width (carapace width prior to molt)$^{-1}$). Therefore, time to each molt and percent increase in carapace width (increment x 100%) for each molt was analyzed by ANCOVA. If molting is so infrequent that the total number of molts is less for a treatment, then molt frequency becomes a poor measure of treatment effects. Therefore, the total number of molts per crab was analyzed by ANOVA. Since animals were maintained in a communal tank prior to the start of the experiment, the instar molt is unknown, and may be highly variable from animal to animal. Therefore, all measures of molting are relative to the start of the experiment.

Linear measures of growth rates using the specific growth rate ((log WW_1 – log WW_0) time^{-1}) was attempted for Experiment 1. However, the frequency distribution of

data for virtually all time increments was bimodal, so the data could not be analyzed statistically.

Growth was analyzed the same way in the second experiment as in the first, with the following exceptions. Linear measures of growth rates using the specific growth rate ((log WW_1 – log WW_0) $time^{-1}$) had a normal distribution, so it was also used as a parameter for data analysis. Since specific growth rate inherently includes the initial size in the values, it was analyzed by ANOVA. The total number of molts per crab was analyzed by a Chi-squared test.

Conversion Efficiency

Gross conversion efficiencies were calculated by dividing the total change in wet weight by the total amount of food given per animal over the course of the experiment. An ANOVA was performed on the raw (wet weight) values, followed by pairwise comparisons within each cage size. The conversion efficiencies are also reported as dry weight gained and dry weight fed (DW) values (in grams) since these values are more relevant from an energetic viewpoint. Dry weight gained was calculated from the regression relationship found between wet weight and dry weight, using the following equations ($\pm$ standard error).

For Experiment 1:

$$DW = 0.3757(\pm 0.0219)(WW)^{0.9661(\pm 0.0241)}; \quad r^2 = 0.95.$$

For Experiment 2:

$$DW = 0.368\ (\pm\ 0.028)\ (WW)^{0.826\ (\pm\ 0.060)}; \quad r^2 = 0.861.$$

Final calculated dry weight was subtracted from initial calculated dry weight to estimate the dry weight gained. Dry weight fed was calculated from the 11.71% water weight value found in the proximate analysis.

Consumption

For Experiment 1 only, a 4-hour consumption experiment was performed to determine what portion of the diet the crabs were eating. Rations were weighed and delivered to the crabs in the morning, including three "control" rations delivered to empty cages. All rations were removed four hours later, rinsed with fresh water, and placed in a drying oven at 60^{o}C for three days until a constant weight was reached. Then all diets were weighed, and the control diets were subtracted from and then divided by the initial calculated dry weight values. This gave an average value of 4.75($\pm$0.57)% of the dry weight that was lost due to leaching. The proportion of dry weight consumed for the rest of the crabs was determined by the following equation:

Proportion consumed = ((0.883($\pm$0.002) x ration WW) – (DW x 0.0475($\pm$0.57)) – leftover DW) ((0.883($\pm$0.002) x ration WW) – (DW x 0.0475($\pm$0.57)))$^{-1}$

A nested ANOVA was performed on the proportion of diet consumed by the crabs. The proportion of diet consumed was not relevant in Experiment 2, since the animals appeared to consume their entire diet within 4 hours.

Genetic analysis

After terminating Experiment 1, a random sample of sixty-eight of the freeze-dried crabs were genotyped for a routine screening of hatchery-reared crabs. One claw from each crab was removed, and DNA was extracted using the FastDNA kit (QBIOgene, Carlsbad, CA, USA). To determine the genotype of a crab or ejaculate,

approximately 20 ng of total genomic DNA was used as template in a PCR with one of five microsatellite locus-specific primers (Steven, Hill, Masters, Place, 2005). PCR protocol was followed according to Hill, Masters, and Place (In Review). The resulting data was analyzed with GeneScan 3.1 software and scored with GenoTyper v2.1 software (Applied Biosystems, Foster City, CA).

Results

Survival was high for both experiments. Out of 210 crabs in Experiment 1, only 12 crabs died. Three of these were cannibalized by neighbors that escaped, two were sacrificed for initial dry weights, and three were taken for use in another experiment, leaving only four that died from unknown causes. Twenty crabs escaped, most of these prior to placing lids on the cages. Five crabs were outside an initial carapace width range of 20-30 mm, so they were not included in the data analysis. Out of 36 crabs in Experiment 2, only 4 crabs died during the experiment (1 in the 0% ration, 1 in the 50% ration, and 2 in the 75% ration). Crabs that died from unknown causes or were used in another experiment were not included in the data analysis. When their data was available, crabs that escaped, were sacrificed, or were cannibalized were included in all of the following data analyses because they were considered healthy before their date of death or disappearance.

Of the 173 crabs that were present throughout Experiment 1, all of the crabs molted at least twice, 96.5% of the crabs reached the third molt, 63.0% reached the fourth molt, and 7.5% reached the fifth molt. Of the 32 crabs that were present throughout Experiment 2, all of the crabs molted once, and 10 crabs molted twice. For both experiments, the molt frequency decreased with each successive molt (Table 5). The

percent increase in carapace width did not show an obvious trend, but was maintained around 20% for each molt (Table 5).

Table 5. Mean growth values for all juvenile *C. sapidus*, ± standard error.

Mean growth values for all juvenile *C. sapidus* , ± SEM

	Molt	% of crabs molted	Time to molt (days)	Increment
Experiment 1				
	1	100	10 ± 0.5	0.19 ± 0.01
	2	100	24 ± 1.0	0.26 ± 0.01
	3	96.5	48 ± 1.7	0.21 ± 0.01
	4	63.0	57 ± 1.3	0.18 ± 0.01
	5	7.5	45 ± 3.0	0.20 ± 0.01
Experiment 2				
	1	92	14 ± 1.8	0.19 ± 0.01
	2	28	29 ± 1.8	0.18 ± 0.03

CW = carapace width (mm).

The relationship between wet weight and carapace width was plotted for both experiments, and a regression curve was found an $r^2 = 0.99$ (Figure 3). The regression also included the initial (week 0) values for crabs that escaped, were sacrificed, or were cannibalized by escaped neighbors, and therefore did not have final values.

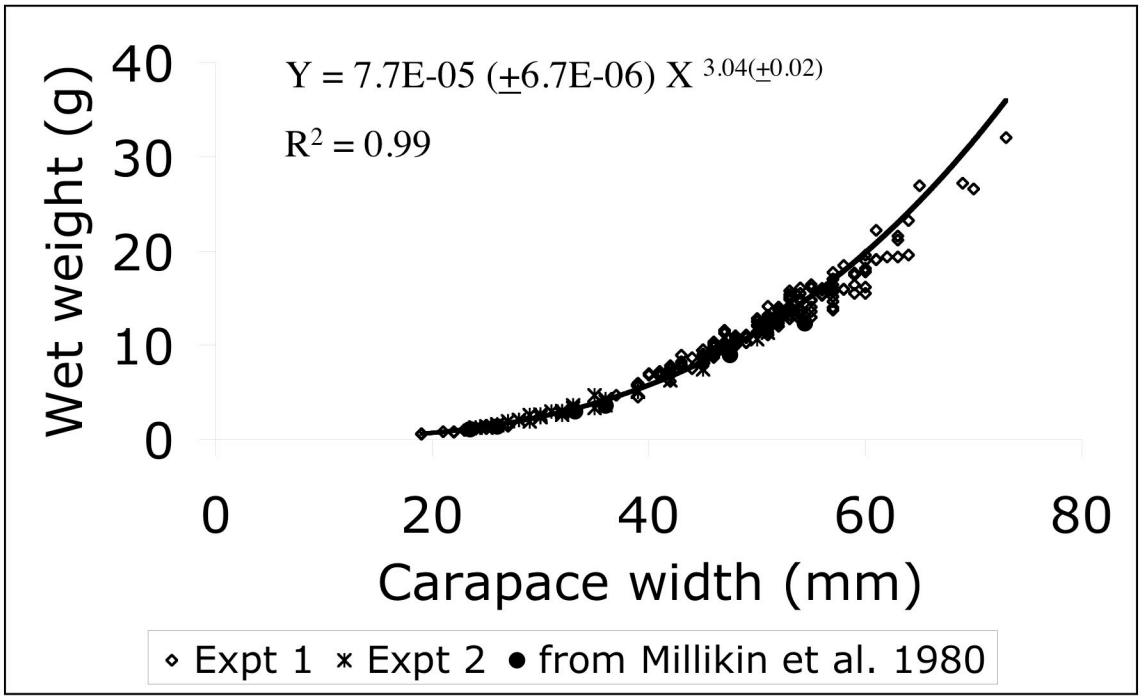

Figure 3. Wet weight versus carapace width regression for Experiment 1 and 2 values of intermolt juvenile *C. sapidus*; N = 207 and 36, respectively. No repeated measures. Standard error of the estimate = 0.094. Mean values from (Millikin et al. 1980) juxtaposed for comparison.

Although the relationship between wet weight and carapace width is the same between the first and second experiments, dry weights tend to fall slightly lower in the second experiment for the same carapace widths ($DW = 2.5 \times 10^{-4}(\pm 1.9 \times 10^{-4})(CW^{2.4(\pm 0.21)})$, $r^2 = 0.81$) than in the first experiment ($DW = 9 \times 10^{-5}(\pm 2 \times 10^{-5})(CW^{2.74(\pm 0.074)})$, $r^2 = 0.95$). There is no significant difference between the regressions when analyzed by ANCOVA, but when plotted, a difference in slopes can be visualized (Figure 4).

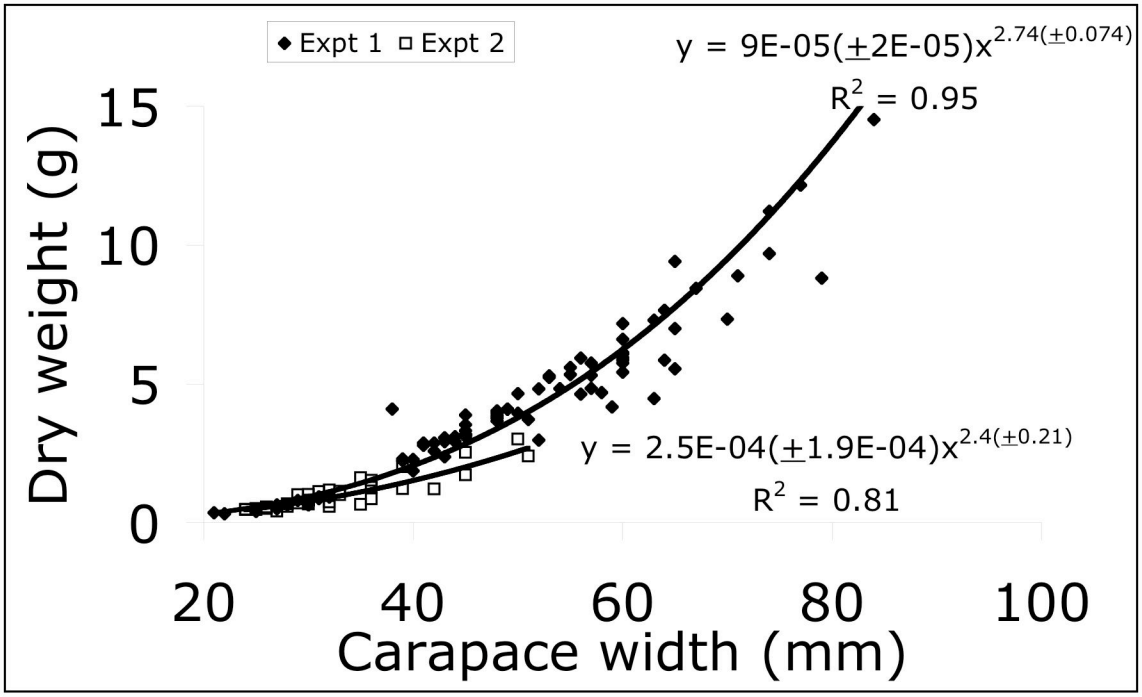

Figure 4. Dry weight versus carapace width regressions for Experiment 1 and 2 values of intermolt juvenile *C. sapidus*; N = 176 and 32, respectively.

Since the regressions had high r^2 values, wet weight, dry weight, and carapace width all appeared to be equal measures in determining statistical differences between treatment groups. Therefore, when all three could potentially be the dependent variable, only the results for wet weight values will be reported. There was no significant difference between growth of male and female crabs in any of the statistical tests performed.

Growth

Size showed increasingly large variance over time in both experiments (Figure 5, Figure 6). Within a brood, animals that started at a range of 0.72-1.52 g (Experiment 1) and 0.54-3.10 g (Experiment 2) ended the experiments between 4.68-32.07 g and 1.24-11.34 g respectively.

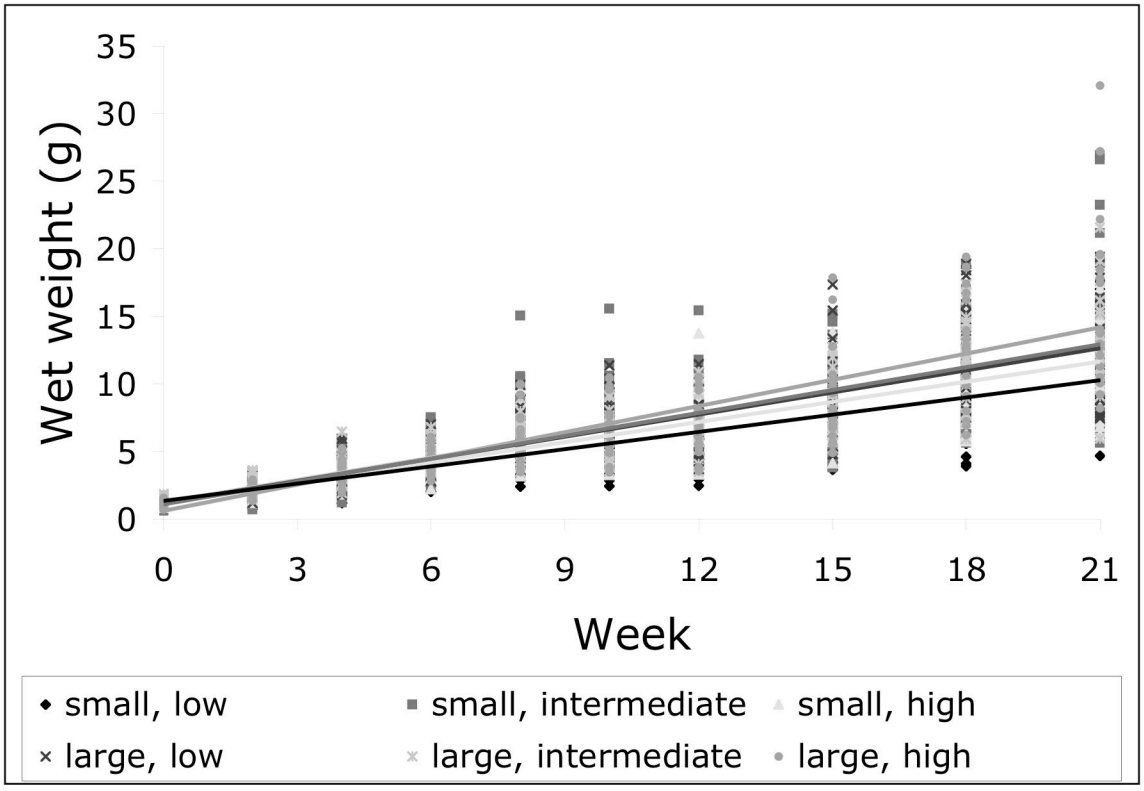

Figure 5. Wet weights for all juvenile *C. sapidus* over the course of Experiment 1. Linear regressions are plotted for each ration and cage size tested.

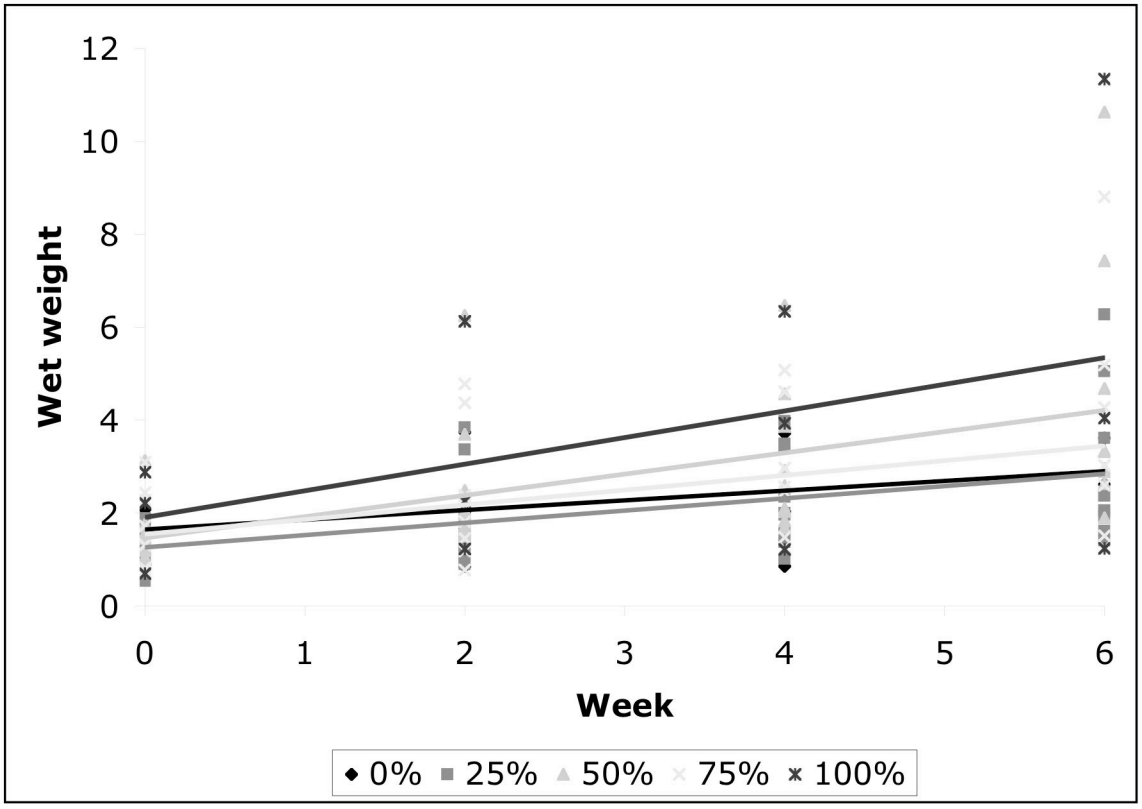

Figure 6. Wet weights for all juvenile *C. sapidus* during Experiment 2, measured every two weeks. Linear regressions are plotted for each ration treatment.

There was a significant difference between ration (Nested ANCOVA, $F_{(4,167)} = 3.44$, $p = 0.01$) and cage size (Nested ANCOVA, $F_{(1,167)} = 7.59$, $p = 0.01$) treatments at the end of Experiment 1 (Figure 7). In the small cages, crabs in the low ration were significantly smaller (10.38 $\pm$ 0.77 g) than crabs in the intermediate ration (13.12 $\pm$ 1.01 g). In the large cages, crabs in the low ration were significantly smaller (12.83 $\pm$ 1.22 g) than crabs in the high ration (15.01 $\pm$ 1.54 g).

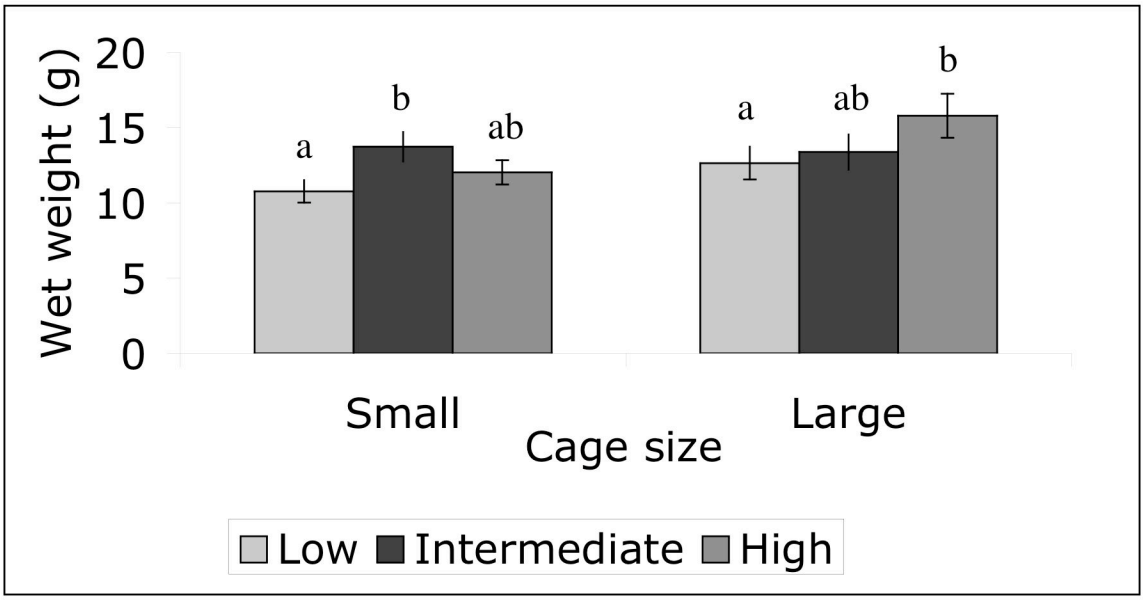

Figure 7. Weight of juvenile *C. sapidus* for each ration and cage size combination at week 21. Legend indicates ration fed. Error bars= LSD. Statistical tests were performed on LSM. Bars with different letters = sig. diff. at $p < 0.05$ (Tukey's HSD).

There was no significant ration effect on size of the animals at the end of Experiment 2 ($F_{(4,26)} = 1.67$, $p = 0.19$). But as early as week 2, crabs fed the 0% and 25% rations had reduced wet weights compared to crabs fed higher rations (Table 6).

Table 6. Least-squares mean wet weights (g) over the course of 6 weeks, ± SEM.

Ration (%)	Wet weight at week		
	2	4	6
0	1.60 ± 0.84	1.63 ± 0.83	1.91 ± 0.50
25	1.76 ± 0.32	2.34 ± 0.32	2.96 ± 0.51
50	2.06 ± 0.47	2.54 ± 0.50	3.70 ± 0.94
75	2.11 ± 0.47	2.50 ± 0.52	2.99 ± 0.91
100	2.06 ± 1.49	2.55 ± 1.48	3.04 ± 3.01

Increments and frequencies are similar between experiments for the first two molts for all treatments with the exception of the 25% and 0% rations in the second experiment (Table 7). Similar values for crabs in the same treatment (the "low" ration, "large" cages in Experiment 1 vs. the 100% ration in Experiment 2) show that growth was consistent between the two experiments.

In the second experiment, only 10 crabs molted twice (2 in the 25% ration, 5 in the 50% ration, 2 in the 75% ration, and 1 in the 100% ration) (Table 7), so statistics were only performed on data regarding molt 1. The average time required to reach the first molt decreased with higher ration, from 21 ($\pm$ 15) days at 0% ration to 7 ($\pm$ 4) days at the 100% ration. Despite the lack of significant statistical difference ($F_{(5,27)} = 1.22$, $p = 0.33$), the trend is obvious and consistent among all treatments. A trend of larger molt increment with higher ration was also apparent, although not significant ($F_{(5,26)} = 1.63$, $p = 0.19$). For the first molt, juveniles increased from 0.12 ($\pm$ 0.05) at the 0% ration to 0.21 ($\pm$ 0.05) at the 100% ration (Table 7).

Table 7. Growth parameters during molt 1 and 2 for juvenile *C. sapidus* in experimental system, ± SEM. Within each molt, top half shows Experiment 1 and bottom half shows Experiment 2 data.

	Cage	Ration	Time to molt *	Increment	N
Molt 1					
	small	high	11 ± 1.2	0.18 ± 0.01	38
	small	intermediate	10 ± 1.2	0.19 ± 0.01	34
	small	low	9 ± 0.8	0.20 ± 0.02	36
	large	high	9 ± 1.3	0.18 ± 0.01	20
	large	intermediate	9 ± 1.6	0.2 ± 0.01	23
	large	low	11 ± 1.8	0.20 ± 0.02	22
	large	100% of low	7 ± 4	0.21 ± 0.05	3
	large	75%	11 ± 2	0.20 ± 0.02	9
	large	50%	12 ± 3	0.21 ± 0.01	9
	large	25%	20 ± 3	0.17 ± 0.02	10
	large	0%	21 ± 15	0.12 ± 0.05	2
Molt 2					
	small	high	25 ± 2	0.26 ± 0.01	38
	small	intermediate	22 ± 2	0.26 ± 0.01	34
	small	low	22 ± 1	0.25 ± 0.01	36
	large	high	20 ± 1	0.27 ± 0.02	20
	large	intermediate	28 ± 4	0.25 ± 0.01	23
	large	low	24 ± 2	0.27 ± 0.02	22
	large	100% of low	37	0.24	1
	large	75%	26 ± 2	0.15	2
	large	50%	29 ± 3	0.17 ± 0.05	5
	large	25%	31 ± 5	0.17	2
	large	0%	-	-	0

*Time to molt expressed in days; Increment = (increase in carapace width / carapace width prior to molt)

Neither molt frequency nor molt increment was significantly different among treatments when looking at the overall average values or any of the individual molts except molt 3 in Experiment 1 (Table 8). There were significant ration effects in the ANCOVA model for both time to molt 3 ($F_{(6,169)} = 3.78$, $p = 0.02$), and the percent increase in carapace width for molt 3 ($F_{(6,161)} = 3.05$, $p = 0.01$). In both the frequency and increment values for molt 3, significant differences appear between the low and intermediate rations within the small cages. There was also a significant difference in the total number of molts per crab in Experiment 1 ($F_{(5,167)} = 2.30$, $p = 0.047$). Tukey's post-hoc test indicated that this difference was in the large cages, between the high ration (4.1 ± 0.2) versus the intermediate (3.7 ± 0.2) and low ration (3.6 ± 0.1).

Table 8. Growth parameters for molts 3-5 for juvenile *C. sapidus* in experimental system, ± SEM.

	Cage	Ration	Time to molt *		Increment		N
Molt 3							
	small	high	49 ± 3	ab**	0.21 ± 0.01	a	36
	small	intermediate	42 ± 4	a	0.25 ± 0.01	b	33
	small	low	57 ± 4	b	0.21 ± 0.01	a	36
	large	high	40 ± 5	A	0.21 ± 0.01	A	19
	large	intermediate	44 ± 5	A	0.20 ± 0.01	A	21
	large	low	50 ± 4	A	0.20 ± 0.01	A	22
Molt 4							
	small	high	56 ± 2		0.16 ± 0.01		23
	small	intermediate	59 ± 3		0.19 ± 0.01		24
	small	low	60 ± 3		0.19 ± 0.02		18
	large	high	51 ± 4		0.17 ± 0.02		16
	large	intermediate	58 ± 4		0.19 ± 0.01		15
	large	low	54 ± 2		0.20 ± 0.01		13
Molt 5							
	small	high	42		0.21		1
	small	intermediate	54 ± 7		0.21 ± 0.01		4
	small	low	-		-		0
	large	high	40 ± 2		0.20 ± 0.02		6
	large	intermediate	47 ± 7		0.15		2
	large	low	-		-		0

*Time to molt expressed in days; Increment = (increase in carapace width / carapace width prior to molt)

**Values with different letters denote significant difference at $p < 0.05$. Letters in different cases cannot be compared due to Nested design.

Specific growth rate ((log (final weight) – log (initial weight)) time^{-1}) was examined for Experiment 2. The specific growth rate, despite having no significant ration effect ($F_{(4,27)} = 1.76$, $p = 0.17$), had a general trend of increasing as rations increase up to the 50% ration. At the two higher rations, the growth rate appeared to level off or decrease (Figure 8). This may be attributed to the fact that more crabs reached the second molt in the 50% ration than in any other ration.

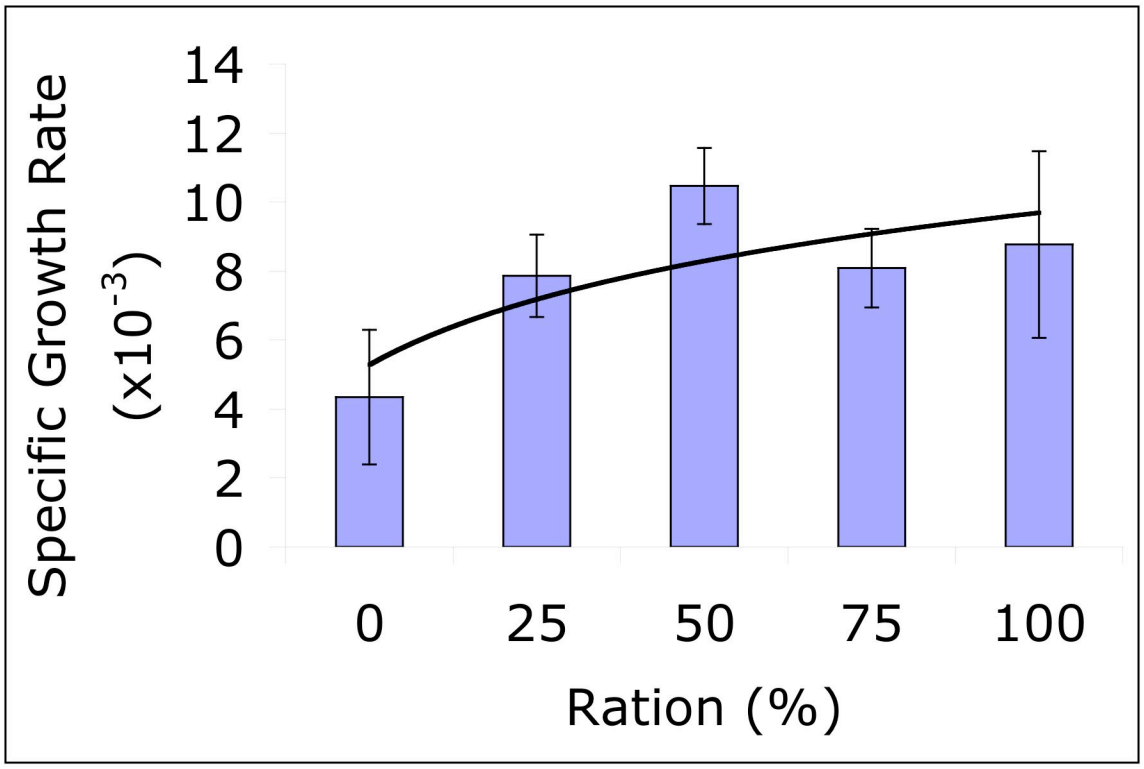

Figure 8. Mean specific growth rate (log (final weight) – log (initial weight)) time^{-1} (days) for all rations tested in Experiment 2. Error bars represent standard error.

Conversion Efficiency

In Experiment 1, there was a significant treatment effect on food conversion efficiency ($F_{(5,168)} = 7.79$, $p < 0.0001$), since efficiency tends to decrease as rations increase (Figure 9). The mean food conversion efficiency was 40% (0.40 $\pm$ 0.01; wet weight gained (wet weight fed)$^{-1}$). The conversion ratio in the large cages was significantly higher than in the small cages ($F_{(1,168)} = 5.01$, $p = 0.03$). Within the large cages, crabs fed the low ration had a significantly higher efficiency (0.48 $\pm$ 0.03) than the intermediate (0.40 $\pm$ 0.02) and high rations (0.40 $\pm$ 0.03). In the small cages, crabs fed both the low (0.44 $\pm$ 0.02) and intermediate rations (0.42 $\pm$ 0.02) have significantly higher efficiencies than the high ration-fed crabs (0.31 $\pm$ 0.01).

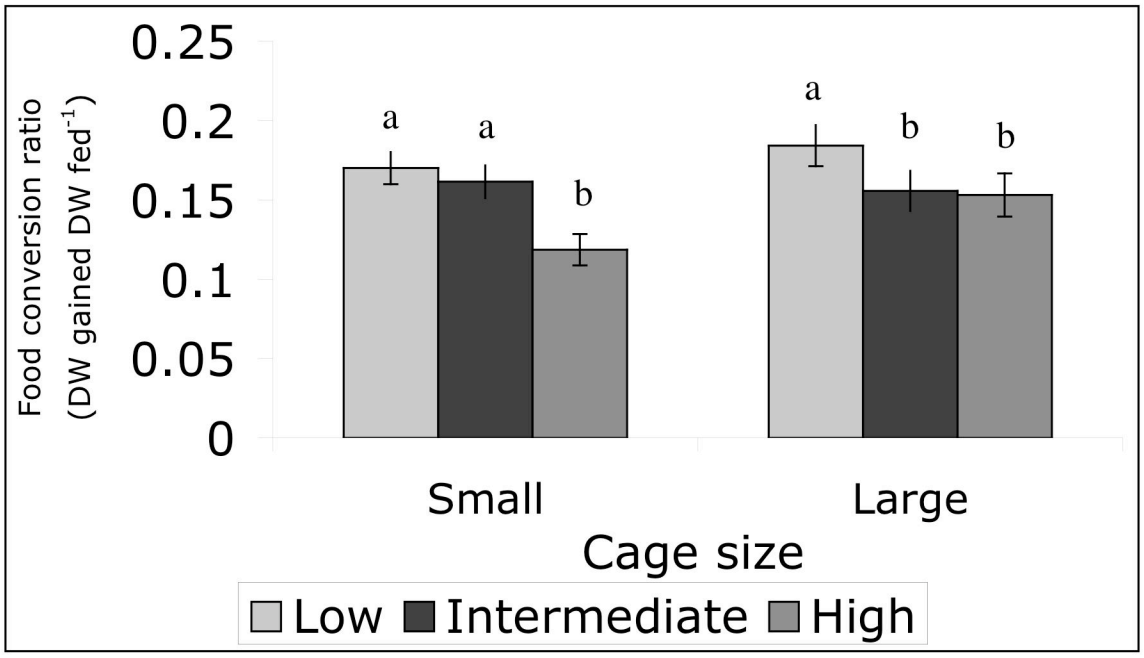

Figure 9. Mean conversion efficiency for each ration x cage size for juvenile *C. sapidus*. Test performed on LSM wet weight gained (wet weight fed)$^{-1}$; reported as dry weight (DW gained (DW fed)$^{-1}$). Error bars = LSD; letters = sig. diff. at p< 0.05 (Tukey's HSD).

Food conversion efficiency appeared to peak at the 50% ration, and then decrease with additional increase in rations. There was no significant difference in conversion efficiencies (dry weight gained (dry weight fed)$^{-1}$) when comparing all rations in Experiment 2 by ANOVA ($F_{(3,26)} = 2.75$, $p = 0.06$). But when the 0% and 100% ration treatments were eliminated (due to the low number of replicates in either treatment), a significant ration effect was found ($F_{(2,24)} = 3.64$, $p = 0.042$). The pairwise comparisons revealed this difference to exist between the 25% (0.275 ± 0.054) and 75% (0.184 ± 0.041) ration treatments (Figure 10). The overall average food conversion efficiency in wet weight: wet weight values was 0.86 ± 0.12.

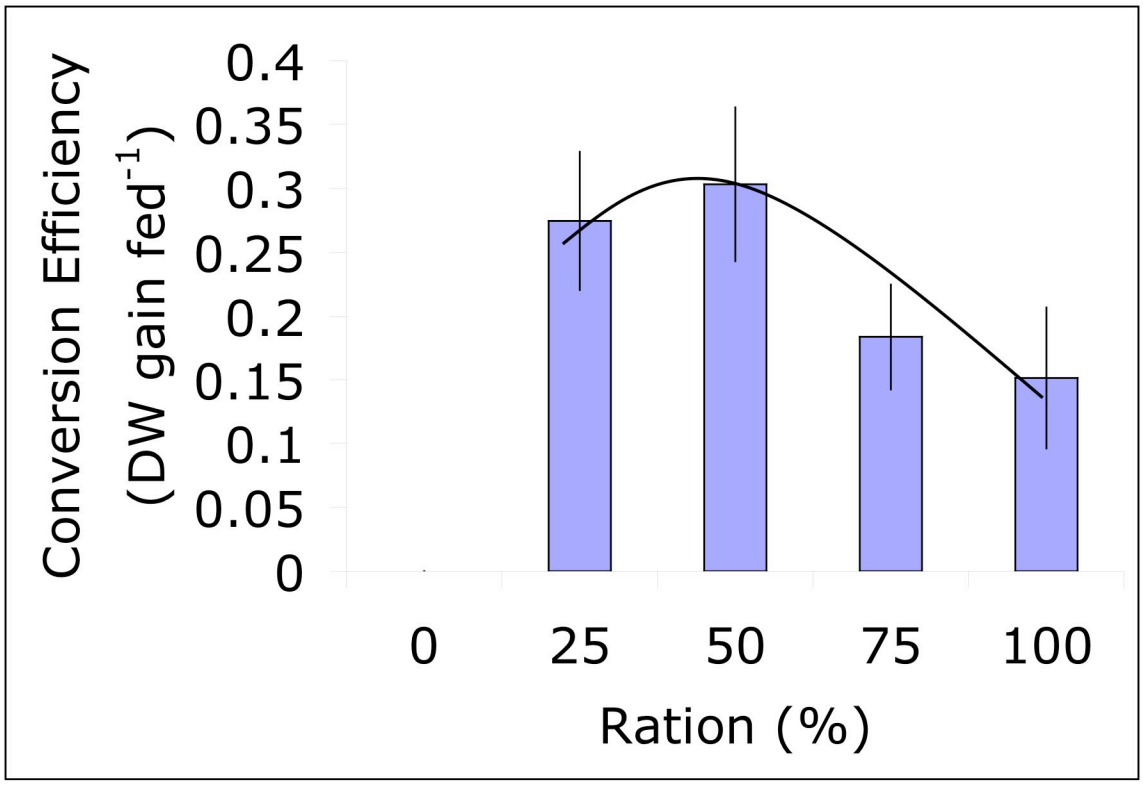

Figure 10. Mean food conversion efficiency (dry weight gained (dry weight fed)$^{-1}$) for each ration tested. Error bars represent standard error.

Consumption

There was a significant ration effect on the proportion of food consumed ($F_{(4,168)}$ = 3.36, p = 0.01) (Figure 11). This was only apparent in the post-hoc tests for the small cages, in which crabs fed the intermediate ration consumed a significantly higher proportion of food (0.61 $\pm$ 0.06) than both the low (0.38 $\pm$ 0.06) and the high (0.36 $\pm$ 0.05) rations. In fact, this treatment group had the highest proportion of food consumed than any of the other treatment combinations. Aside from this anomaly, the lack of a significant difference among the other treatments indicates that the animals were not compensating for the quantity of the food given to them by consuming more or less of it.

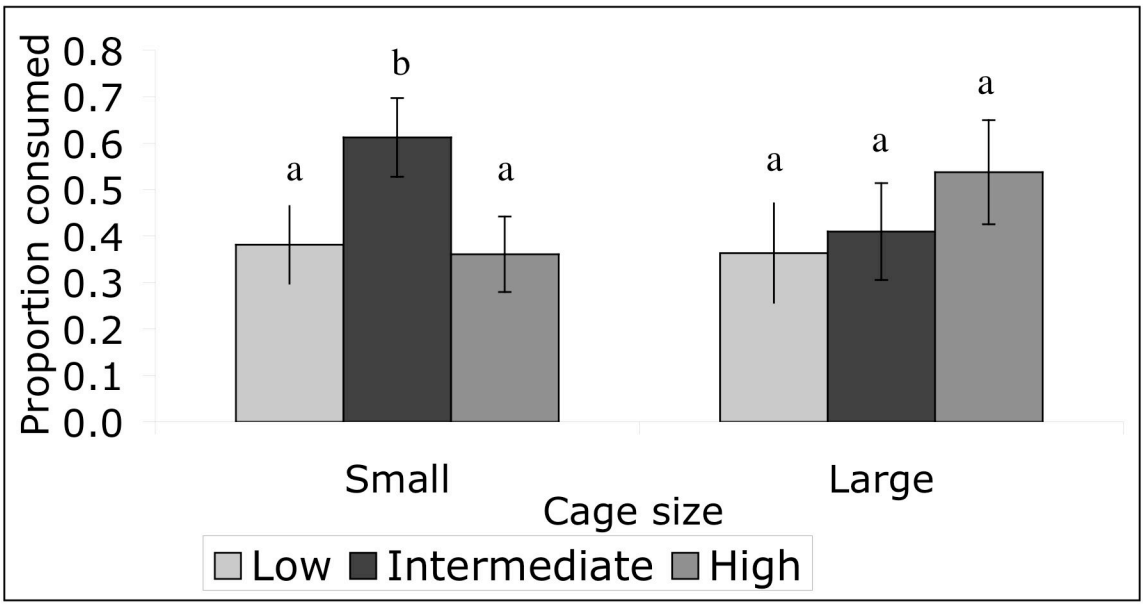

Figure 11. Mean proportion of food consumed in 4 hours for each ration x cage size for juvenile *C. sapidus*. Legend indicates ration fed. Error bars = LSD. Statistical tests were performed on LSM. Bars with different letters = sig. diff. at $p<0.05$ (Tukey's HSD).

Genetic analysis

While it is known that blue crab females can undergo multiple inseminations (Jivoff, 1997), the possibility of multiple fathers in the effective brood has not been investigated. However, a routine DNA screening of the crabs used in Experiment 1 revealed more than two parents. Of the 68 crabs that were genotyped, 53 were successfully discriminated by determining the alleles present at the MIH-SSR locus (Steven, Hill, Masters, Place, 2005). With two alleles contributed from each parent, we would expect four alleles total and four possible combinations of these alleles. However, we found five alleles total and eight possible combinations present at this locus. Analysis of the mitochondrial genome (data not shown), which is passed from mother to offspring, established that the crabs shared the same mother so the extra alleles must come from a

second father. This finding indicates that it is possible for a single brood to have multiple paternity.

When comparing growth parameters between crabs with different fathers, a significant difference was found in the food conversion efficiency (oneway ANOVA, $F_{(1,51)} = 4.59$, $p = 0.04$). The conversion ratio for 22 crabs with one father was 0.17 $\pm$ 0.01, while the 31 crabs with the other father was 0.14 $\pm$ 0.01 (Table 7).

Table 9. Mean gross conversion efficiency for juvenile *C. sapidus* according to MIH-SSR locus in crab genome.

Parent	Allele	Mean GCE $\pm$ SEM	N
Mother	146 171		
Father-1	144 146	0.14 $\pm$ 0.01	31
Father-2	160 200	0.17 $\pm$ 0.01	22

GCE = gross conversion efficiency, in (dry weight gained) (dry weight fed)$^{-1}$.
SEM = standard error of the mean.

Discussion

Rearing juvenile blue crabs in a compartmentalized recirculating system was successfully undertaken for a period of 21 weeks. This can be difficult to achieve with blue crabs, and high mortality is common. Winget et al. (1976) reported mortalities of up to 50%, and the presence of a herpes-like virus in sick crabs reared in a recirculating system. It is important, however, to maintain animals for several months in feeding experiments, since treatment effects may not manifest for months (Millikin, Biddle, Siewicki, Fortner, Fair, 1980). The extremely low mortality in our studies and absence of

any indication of disease demonstrated that this is an excellent system for future long-term feeding experiments.

Growth parameters were similar to literature values for blue crabs reared in captivity. Carapace width and wet weight values were similar to Millikin et al. (1980), with mean values falling roughly along the same regression curve (Figure 3). The relationship between dry weight and carapace width for intermolt crabs had a high r^2 value, which was seen as unlikely by Cadman and Weinstein (1985) due to the large proportion of weight that is carapace. But since our weight to carapace width correlation was so high, we saw no problem in measuring the crabs during intermolt. Like Cadman and Weinstein (1988), we saw no effect of sex on growth of juveniles. Molt frequency was comparable to other studies with similar-sized crabs, both in actual values (about once a month) and in the trend of frequency decreasing with each successive molt (Cadman, Weinstein, 1988; Guerin, Stickle, 1997; Leffler, 1972; Millikin, Biddle, Siewicki, Fortner, Fair, 1980). Percent increase in carapace width is comparable to published values at 20-27^{o}C, which fall between 12-20% (Guerin, Stickle, 1997; Leffler, 1972; Winget, Epifanio, Runnels, Austin, 1976). The comparison to other captive studies shows that our crabs exhibited normal growth and molting patterns.

Growth rates are substantially lower in laboratory experiments than they are in the field. Crabs in field enclosures at Point Lookout, Maryland starting at approximately 4.0 g wet weight and 35 mm carapace width (determined using our allometric relationship between wet weight and carapace width) molted twice in 35 days (Brylawski, Miller, 2003). This is a substantially higher molt frequency than we found for animals of the same size in our study (once every 20-37 days). There are various reasons why animals

grow faster in the field. Among them is temperature, which reaches several degrees (celcius) higher in the Chesapeake Bay in summer than in our laboratory experiments (Cowan, Boynton, 1996). Preventing activity in the compartmentalized system also decreases metabolic rates, which may depress growth rates in the laboratory compared to the field (Brylawski, Miller, 2003). A more widely varied diet in the field may supply additional essential nutrients to *C. sapidus* that are largely unknown, which limits our ability to properly formulate diets for laboratory experiments. These various obstacles may be difficult to overcome in rearing studies, and should be further investigated to improve growth in the laboratory.

Feeding a commercial pelletized shrimp diet proved a practical option for rearing *C. sapidus* juveniles. High survival, combined with growth and molting comparable to those found in other laboratory experiments showed the diet to be adequate for the animals' needs. It is easier to achieve a balanced diet including all essential amino acids, lipids, sterols, vitamins and minerals in a multi-ingredient diet containing different protein sources, such as the commercial diet we used (New, 1976). The combination of water stability and palatability of the diet proved effective in providing for the animals' needs. Water stability proved desirable not only because of the crabs' feeding behavior of gradually consuming it over several hours, but it also helped to preserve water quality in the recirculating system (New, 1976). Since the diet was a dry pellet, it may have been less efficient than a moist diet, however, the practicality of having an easily produced and storable diet proved important for a long-term feeding experiment. The production cost of the diet was not high, because the company formulated it in bulk. If the intermediate ration was fed to crabs growing at our rates, we could supply food for juvenile crabs

starting at 25mm carapace width for five months at the cost of $0.04 per crab. In the future, various aspects of the diet composition should be investigated so that the diet is not providing more energetic and protein content than is needed, as this is wasteful and fish meal is expensive (Naylor, Goldburg, Primavera, Kautsky, Beveridge, Clay, Folke, Lubchenco, Mooney, Troell, 2000). However, using a commercial shrimp diet from the beginning gives us a jump-start for achieving the goal of economic feasibility.

All parameters of growth measured were significantly influenced by ration. This was not a residual effect of different percentages of the diet being consumed, most likely because we used low enough rations that the animals were food-limited for all of them. There was a trend towards less and less growth with lower ration, with the 25% and 0% rations showing the most dramatic decreases in wet weight, specific growth rate, increment, and molt frequency when compared to the other rations (Table 6, Table 7, Figure 7, Figure 8). Molt frequency and increment are both influenced by ration when juvenile crabs are seriously deprived of food. Some studies have determined only frequency and not increment to vary in response to different levels of dietary protein (Millikin, Biddle, Siewicki, Fortner, Fair, 1980), dietary fiber (Biddle, Millikin, Fair, Fortner, 1978), and salinity (Guerin, Stickle, 1997). Although differences in increment were apparent in our experiments, they were still subtle (range: 12-29%) and may be difficult to detect in a lower-resolution study.

Depending on the priorities of the grow-out facility, optimal rations can be determined in various ways. From an energetic viewpoint, the ration resulting in the highest conversion efficiency is the best ration to deliver. The food conversion efficiency (dry weight gained (dry weight fed)$^{-1}$) increased rapidly as ration increased up to the 50%

ration, and then declined. This conversion efficiency continued to decline from the low to the high rations in Experiment 1, showing a continuum of conversion efficiency between the two experiments. We can therefore conclude that the optimal ration to use (C_{opt}) is the 50% ration in Experiment 2. This results in the most economical use of diets, when diet is the highest cost involved in grow-out. However, we have determined diet to be a fairly inexpensive commodity, while electrical costs including water heating, aeration and water pumps for running the recirculating systems are relatively high. To reduce these costs, the priority is to grow animals as quickly as possible to maturity, and to perform experiments with equal speed. Therefore, the 50% ration is not the ideal ration for our purposes. Instead, we should use the intermediate or high ration from Experiment 1 for future diet studies, as they resulted in the highest growth rates. Even though the differences in growth were not as obvious for the rations delivered in Experiment 1, they could still be statistically distinguished from one another, so any treatment effects should be apparent in future dietary studies.

Juvenile crabs were able to survive and molt, even in starvation conditions. Although the relationship between wet weight and carapace width are virtually the same between Experiments 1 and 2, dry weights in Experiment 2 are lower for the same carapace widths. This is a result of less body tissue in animals when fed lower rations, but the water weight in the body cavities masks the loss of tissue when considering wet weight. Therefore, even when animals were surviving and molting, their live tissues may have been degenerating. In *C. sapidus*, the RNA: DNA ratio (an indicator of protein production and therefore growth) drops by 31% within 3 days of starvation, and by 61% after 30 days of starvation (Wang, Stickle, 1986). In our starvation treatment, one crab

molted at the beginning of the experiment, on day 6, while the other did not molt until day 36. This suggests that it is possible that cues for molting proceed regardless of nutritional condition.

Cage size had a significant effect on growth (Figure 7, Figure 9) and produced apparent differences in molt frequency. The total number of molts in the small cages was 3.62, versus 3.75 molts in the large cages. The mean time to molt 3 in the small cages was 48 days, versus 45 days in the large cages. Within the small cages, the intermediate ration had both a higher frequency and increment than the low ration. The intermediate ration also had a higher increment than the high ration, which is difficult to explain. Water quality did not seem to be the issue, since dissolved oxygen was not significantly different between any of the rations in the small cages. It may be that the crabs are inhibited in some way by the combination of plentiful food and limited space. This is very important in future experiments, and needs to be further investigated to determine at what point cage size is no longer a limiting factor in the growth of juvenile crabs. The variable *k*, representing the ratio of compartment area to carapace width squared (cm^2cm^{-2}), is a common factor used to indicate when cell size inhibits growth. For the lobster *Homarus americanus*, growth was inhibited at $k \leq 33$ (Van Olst, Carlberg, 1978), while for the crayfish *Cherax quadricarinatus*, growth was inhibited at $k \leq 50$ (Manor, Segev, Leibovitz, Aflalo, Sagi, 2002). In this study, the initial *k* values for the small and large cages were 17 and 38, respectively, while the final *k* values for the small and large cages were 4 and 8, respectively. If the *k* that is limiting to growth in blue crabs is similar to that of other crustaceans, this may help explain why a cage effect was significant

throughout the experiment. In the future, a larger range of cage sizes should be studied to determine the true limiting *k*.

Other factors besides the ratio of cage size to animal size also come into play when determining what compartment size to ultimately use. Water depth may significantly affect growth in *C. quadricarinatus* (Manor, Segev, Leibovitz, Aflalo, Sagi, 2002). If this is true for blue crabs, then it may be possible to use a smaller size cage in combination with deeper water to achieve the same growth. We plan to investigate the effect of water depth on the growth of blue crabs in future studies. Practicality may also come into play when determining what cage size to use. When analyzing the overall yield in *C. quadricarinatus*, smaller compartments produced more mass per unit area than larger compartments even though the individual animals were smaller (Manor, Segev, Leibovitz, Aflalo, Sagi, 2002). Depending on the goals of grow-out production, an aquaculturist may prefer to rear a larger biomass per unit area rather than larger individuals.

Evidence of multiple mating was discovered by the presence of two ejaculates in 12.4% of wild females (Jivoff, 1997). Physiologically, both ejaculates have access to the eggs, since the seminal fluid disappears over time, allowing spermatophores to mix in the spermathecae (Jivoff, 1997). However, subsequent fertilization of the eggs, sponge production, hatching, and survival of offspring have never been monitored to determine if the two fathers both played a role in brood production. This study proves that two males can both have offspring from a dual-inseminated female, and in a single brood.

Chapter 3. The effect of dietary fiber on growth of the juvenile blue crab, *Callinectes sapidus* (Rathbun), in a recirculating system: comparing four combinations of chitin and cellulose

Summary

Recent work indicates that many species of crustaceans have the capability to digest various forms of fiber. Cellulose, followed by chitin, composes the largest biomass worldwide, posing an energetic advantage for animals that can digest them. To more efficiently produce juveniles and adults for research purposes, the appropriate diet needs to be formulated. A six-month experiment was performed where we replaced 20% of a commercial diet with varying amounts of chitin and cellulose. The diet was coated with nutritional oil to improve water stability. During the first three months of the experiment, crabs fed 20% chitin had lower wet weights and specific growth rates ($(\ln(ww_1) - \ln(ww_0))\ time^{-1}$) than crabs fed 20% cellulose after the second and third molts, while the 14% cellulose / 6% chitin and 14% chitin / 6% cellulose diets had intermediate growth rates. Time to molt was longer and molt increment was lower for the 20% chitin diet than for the other diets for three successive molts. No crabs fed 20% chitin molted a fourth time. After three months, we switched the 20% chitin and 20% cellulose dietary treatments. During the second half of the experiment, crabs fed the 20% cellulose diet had significantly higher specific growth rates (0.00668 ± 0.00054) than either crabs fed 20% chitin (0.00273 ± 0.00065) or the control diet (0.00318 ± 0.00120), indicative of compensatory growth. Only the crabs fed 20% cellulose molted a second time. These findings suggest that cellulose is digestible in blue crabs, while chitin is either indigestible or is inhibitory if digested.

Introduction

Biddle, Millikin, Fair and Fortner ((1978) performed an *ad libitum* feeding experiment with varying proportions of cellulose in the diet of juvenile *C. sapidus*. Similar growth was found between crabs fed 3 and 9% cellulose, but there was low survival and depressed growth in crabs fed 27% cellulose. In the wild, approximately 20% of the diet of juvenile crabs (< 80 mm carapace width) consists of detritus and plant material (Laughlin, 1982; Stoner, Buchanan, 1990). We performed a feeding experiment where we replaced 20% of a commercial shrimp diet with chitin and cellulose, and delivered the diet according to appropriate rations (see Chapter 2). We set out to determine if the addition of fiber in the diet affects growth and survival of juvenile *C. sapidus*. If digestible, the addition of dietary chitin and cellulose should have no deleterious effects on growth of juvenile crabs in the laboratory.

Methods

One-hundred and forty-four juvenile crabs from a single hatchery-reared brood were obtained from the Center of Marine Biotechnology. The crabs averaged 1.04 ($\pm$ 0.02) grams wet weight and 23 ($\pm$ 0.2) mm carapace width. We used a compartmentalized recirculating system consisting of seven tanks (described in Chapter 2).

The crabs were placed in the experimental system in 15x15x10 cm "large" compartments with walls constructed of PVC board. Treatments were assigned randomly to the compartments within each tank, according to the random complete block design (with the tanks as blocks). Wet weights and carapace widths were measured 24 hours after each molt, and the date of each molt was recorded.

Water temperature was maintained at 25 $\pm$ 0.4 ^{o}C as a compromise between rapid growth and high survival (Cadman, Weinstein, 1988; Leffler, 1972). Saltwater was formulated as in the ration and cage size experiment (Chapter 2). Salinity was maintained at 28 $\pm$ 1 gL^{-1} according to the approximate isoosmotic point (Colvocoresses, Lynch, Webb, 1974). Fluorescent lights in the ceiling were on a controlled photoperiod equivalent to the photoperiod in Baltimore, Maryland from July 8, 2004 to December 5, 2004. Ammonia (0.250 $\pm$ 0.029 mgL^{-1}), nitrite (0.524 $\pm$ 0.060 mgL^{-1}), and pH (8.08 $\pm$ 0.02) levels were measured daily, while salinity and temperature were measured weekly. Dissolved oxygen for this system has been previously measured as 5.9 $\pm$ 0.3 mgL^{-1} at approximately the same temperature and salinity.

The experiment was started on July 8, 2004. The crabs were acclimated to the same temperature and salinity (25^{o}C and 28 gL^{-1}) in a communal tank for 1 week before they were acclimated to the system for 2 days prior to the start date. Five experimental diets were delivered to the crabs, containing the following variations: 20% cellulose (by wet weight), 20% chitin, 14% cellulose / 6% chitin, 14% chitin / 6% cellulose, and a control diet (no chitin or cellulose). On September 29 and 30, all crabs in the two diet treatments that contained both chitin and cellulose were weighed, measured, and sacrificed. Five crabs randomly sampled from each of the other three treatments were also measured and sacrificed. Crabs were sacrificed by submersion in ice water for 5 minutes. Half of the sacrificed crabs were freeze-dried to obtain dry weights. After removing the two treatments containing a combination of chitin and cellulose, the 20% cellulose and 20% chitin treatments were switched to determine the effect of changing diets on the growth of the crabs. The experiment was terminated on December 5, 2004,

and remaining crabs were weighed, sexed, measured and sacrificed. The animals were then freeze-dried for obtaining dry weights. All dry weight values were pooled to make linear regressions determining the relationship between dry weight (DW) and wet weight (WW), and dry weight and carapace width (CW). A regression relationship was also found between wet weight and carapace width. Values for allometric relationships were collected at the time of sacrifice, therefore no repeated measures were used.

Diet Formulations

Experimental diets were formulated by replacing 20% by weight of the ground wheat and fiber portions of a commercial shrimp diet with varying amounts of chitin and cellulose (Table 10). The wheat portion of the diet is considered "filler", but blue crabs have the ability to digest maltose, a component of starch (α-1,4-glucan) (McClintock, Klinger, Marion, Hsueh, 1991). Therefore, we are replacing a portion of the diet that supplies some nutritional value to the animals, without removing crucial components such as protein. The diets were based on Melick Aquafeed's (Catawissa, PA) 1/8" shrimp pellets, which have a similar formulation to the shrimp diet that was proven successful in maintaining juvenile blue crabs (Chapter 2). Juvenile *C. sapidus* readily consumed the Melick shrimp diet. The cellulose used was Solka-floc cellulose (International Fiber Corporation, North Tonawanda, NY), standard in many dietary formulations. The chitin was made from deproteinated, demineralized blue crab shells (ChitinWorks, Cambridge, MD). Fiber sources were sent to the Melick Aquafeed facilities, and the company produced the diets in bulk according to the formulations. Proximate analyses were performed on the diets by New Jersey Feed Lab, Inc. (Trenton, NJ) (Table 11a), and show the fiber content to be a lot less than was expected, so ADF

(acid-detergent fiber) analysis was then performed, resulting in more accurate numbers. This is due to the small cellulose particles (35 *u*m diameter) passing through the filters used when performing proximate analyses, but they were retained in ADF analysis.

Table 10. Formulations for juvenile *C. sapidus* diets, produced by Melick Aquafeed (Catawissa, PA). Ingredients expressed in percent by weight. Highlighted portions were varied between diets.

Ingredient	Diet 1 20% cellulose	Diet 2 14% cell/6% chi	Diet 3 6% cell/14% chi	Diet 4 20% chitin	Diet 5 Control
Fish oil, unspec.	2.425	2.425	2.425	2.425	2.425
Corn grain	25	25	25	25	25
Fish menhaden	10	10	10	10	10
Poultry feathers	5	5	5	5	5
Soybean meal	30	30	30	30	30
Pegabind	1.25	1.25	1.25	1.25	1.25
Wheat Ground	2.5	2.5	2.5	2.5	20
Mineral Mix 3	0.1	0.1	0.1	0.1	0.1
Dical	1.25	1.25	1.25	1.25	1.25
DL Meth	0.75	0.75	0.75	0.75	0.75
Vit Pak 1	0.3	0.3	0.3	0.3	0.3
Choline CL 70%	0.15	0.15	0.15	0.15	0.15
Ascorbic acid	0.025	0.025	0.025	0.025	0.025
Limestone	1.25	1.25	1.25	1.25	1.25
Red Dog (fiber)	0	0	0	0	2.5
Crab Chitin	0	6	14	20	0
Solka-Floc Cellulose	20	14	6	0	0

The experimental diet, however, was not water-stable and fell apart within 5 minutes of contact with tank water. After testing different concentrations, volumes, and proportions of potential stabilizing additives, we developed a protocol for coating diets with nutritional oil to maintain their water stability for a minimum of 2 hours. Pellets were coated with a 1:2 (by volume) combination of pure lecithin "Yelkinol TS" (Archer Daniels Midland Corp., Decatur, IL) and menhaden oil (Omega Protein, Reedville, VA). Pellets were weighed, arranged in a single layer on a flat baking tray, sprayed using compressed nitrogen with a paint sprayer containing the oil mixture on both sides for a specific amount of time and reweighed, and then placed in a drying oven at 50^{o}C for 8 hours. The lecithin and menhaden oil coating comprised 13.15% of the wet weight of the initial coated diet, and then was decreased to 7.38% after 6 weeks (on August 25, 2004)

by cutting the spraying time in half. The final proximate analyses and ADF values after coating with the latter amount of oil are shown in Table 11b. When the oil coating was decreased, pellets were water-stable for approximately 30 minutes, after which they visibly fell apart. Crabs readily consumed the oil-coated diet. The majority of the animals, however, left large portions of their diet to be consumed over a period longer than the water-stability of the diet, indicating that the pellets should be made more water-stable in the future.

Table 11 a, b. Proximate analyses of diets with and without a nutritional oil coating, expressed in mean percentages ± SEM. N=3 for all except control, where N=1. ADF performed at a later date than proximates.

a. *Uncoated*

Diet	Moisture	Protein	Fat	Fiber	Ash	ADF
20% cellulose	10.32±0.21	30.73±0.35	4.43±0.12	7.96±0.77	5.86±0.10	16.12±1.11
14% cell/ 6% chi	10.43±0.15	35.67±0.15	5.60±0.08	8.64±0.22	6.37±0.06	13.54±0.56
6% cell/ 14% chi	9.49±0.31	40.93±0.20	5.39±0.02	8.61±0.21	6.40±0.04	12.73±0.55
20% chitin	9.29±0.02	35.63±0.52	4.24±0.02	15.62±0.04	6.42±0.08	17.97±0.15
control	10.79	33.60	4.31	4.74	6.41	7.05

b. *Coated*

Diet	Moisture	Protein	Fat	Fiber	Ash	ADF
20% cellulose	3.27±0.07	32.30±0.12	6.59±0.05	13.68±0.23	6.49±0.01	18.21±0.23
14% cell/ 6% chi	3.22±0.16	37.40±0.15	8.22±0.06	10.29±0.35	6.72±0.05	14.18±0.07
14% cell/ 6% chi	2.81±0.04	42.30±0.10	8.88±0.03	10.37±0.23	6.81±0.03	13.39±0.65
20% chitin	3.15±0.35	36.70±0.06	7.18±0.07	17.87±0.12	6.55±0.03	19.90±0.33
control	3.60±0.15	34.43±0.09	7.99±0.05	5.35±0.04	6.77±0.05	7.72±0.42

The animals were fed the median ration from a range of recommended rations for shrimp of the genus *Penaeus* (See Chapter 2) during the week, but were fed *ad libitum* on the weekend. Rations are expressed in grams dry weight per 100 grams body weight per day, and vary according to the crabs' wet (body) weight. The actual weight of the pellets was corrected to account for moisture content.

Growth

The SAS system software V.8 was used for data analyses (SAS 2000). When assumptions of normality and homogeneity of variance were not met, data was transformed as appropriate. Data was divided into two time periods and those periods

analyzed separately: prior to switching diets (from the start to September 30), and after switching diets (from September 30 to December 5). The size of the animals at September 30 was the "initial" size for the second half of the experiment, and the number of molts started over at 1 for the second half. This is to prevent confounding of the data due to switching treatments. When a significant diet effect was found, pairwise comparisons were performed between diets using Tukey's HSD at an experimentwise error rate of $p < 0.05$.

There was no significant difference between diets for wet weight ($F_{(4,139)} = 0.26$, $p = 0.91$) or carapace width ($F_{(4,139)} = 0.22$, $p = 0.93$)at the beginning of the experiment when analyzed by ANOVA. There was a significant difference between diets at the beginning of the second half of the experiment, however, for both wet weight ($F_{(2,40)} = 17.88$, $p < 0.0001$) and carapace width ($F_{(2,40)} = 17.24$, $p < 0.0001$). The majority of the variance in wet weight and carapace width at the end of each time period was related to the size at the beginning of the period. Because of this, analysis of covariance was performed using the size at the beginning of the period as a covariate for the size at the end of the period. Statistical results for wet weight and carapace width were redundant, so only the results for wet weight will be reported.

A linear measure of growth rate, specific growth rate ($(\log WW_1 - \log WW_0)$ $time^{-1}$) was also used to discern differences between treatments. Since specific growth rate inherently includes the initial size in the values, it was analyzed by ANOVA.

If significant differences in growth between diets were found, these differences were further investigated. Growth can differ as a result of molt increment ((increase in carapace width) previous carapace $width^{-1}$)) or molt frequency (time to each molt in

days). Both of these parameters were found to covary with size prior to molt, so they were analyzed by ANCOVA. The total number of molts per crab was analyzed by ANOVA. Molt numbers are relative to the start of the experiment.

Consumption

An experiment was conducted to determine if all of the crabs were consuming the same proportion of their diet. On August 31, rations were delivered as normal in the morning, and then removed four hours later with a turkey baster. The food was placed in aluminum cups, rinsed with fresh water, and placed in a 50^{o}C drying oven until they reached a constant weight (72 hours). The moisture of the initial diets (as measured by the drying oven) was calculated by placing preweighed pellets directly into the drying oven and removing them 72 hours later. Control rations were placed in empty cages to account for leaching and other loss of the diet during immersion in the tanks. The percent of diet consumed could then be calculated by subtracting the final oven-dried diet from the calculated dry weight of the initial diet (subtracting 1.93 ($\pm$ 0.26)% moisture), accounting for the average percent of food leached (38 ($\pm$ 9)% of the dry weight). The effect of diet on the percentage of food consumed was examined by ANOVA.

Results

Out of 144 crabs, 25 crabs died during the experiment. Fourteen of these died during a molt, and 11 died for unknown reasons. Five crabs escaped, and one crab was taken for use in another experiment. When their data was available, crabs that died or escaped were included in all of the following data analyses because they were considered healthy before their date of death or disappearance. The molt frequency and percent

increase in carapace width decreased with each successive molt (Table 12). Variability in animal size was high throughout the experiment (Figure 12).

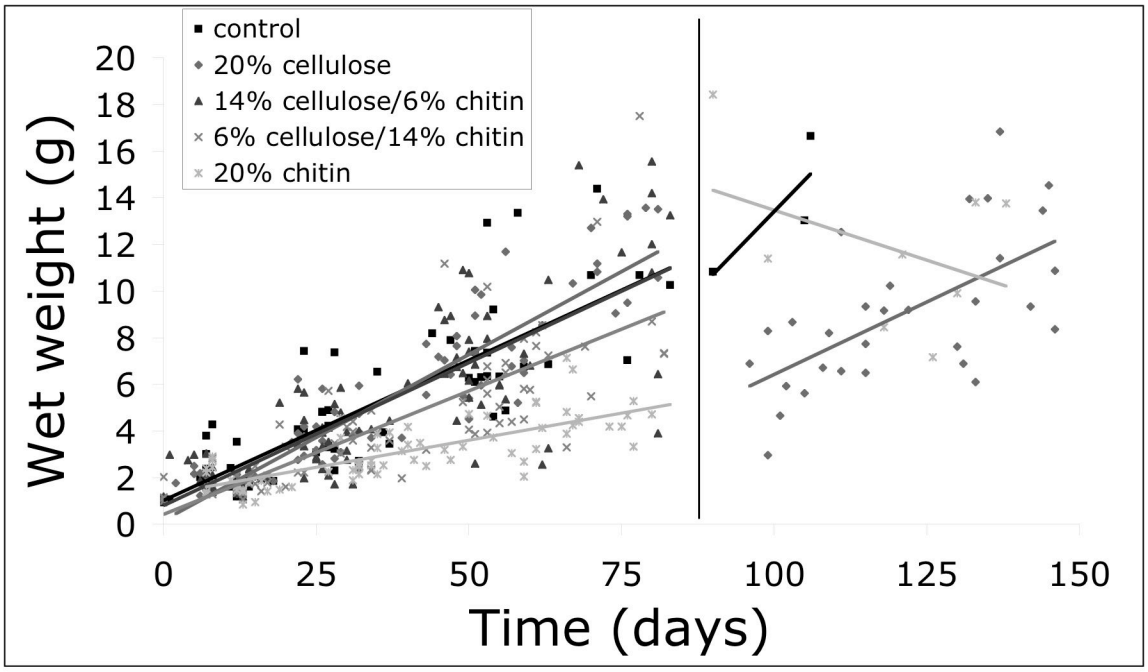

Figure 12. Wet weight of all juvenile *C. sapidus* during the experiment, measured 24 hours post-molt. Vertical division in the plot represents switching of diets at day 88. Linear regressions are plotted for each dietary treatment.

Table 12. Mean growth values for all juvenile *C. sapidus*, ± SEM.

Molt	N of crabs molted	% increase CW	mean time to molt (days)
1	139	27 ± 1	16 ± 1
2	131	27 ± 1	26 ± 1
3	100	25 ± 1	31 ± 1
4	40	25 ± 2	38 ± 2
5	5	19 ± 4	35 ± 8

Power regressions provided the best fit for the allometric data when plotted. Wet weight appeared to be a more accurate predictor of dry weight than carapace width, with an r^2 value of 0.82, compared to 0.75. Regression relationships for calculating dry weight were as follows:

$$DW = 0.402(\pm 0.044)WW^{0.863(\pm 0.051)}$$

$$DW = 0.00055(\pm 0.00035)CW^{2.2(\pm 0.17)}$$

Wet weight and carapace width, however, had a very close relationship, with an r^2 of 0.94 and the following regression equation:

$$WW = 0.00038\ (\pm 0.00009)\ CW^{2.6(\pm 0.1)}$$

Growth: prior to switching diets

Significant diet effects were found ($F_{(4,109)} = 18.05$, $p < 0.0001$) using ANCOVA. Crabs in the 20% chitin diet (4.14 ± 0.25 g) were significantly smaller than crabs in the 20% cellulose diet (9.34 ± 0.73 g), the 14% cellulose / 6% chitin diet (9.00 ± 0.72 g), the 14% chitin / 6% cellulose diet (7.47 ± 0.55 g), and the control diet (8.78 ± 0.87 g) (Table 13).

Table 13. ANCOVA performed on final ln(wet weight) using initial ln(wet weight) as a covariate for the first time period (prior to switching diets).

Effect	Num DF	Den DF	F	Pr > F
diet	4	109	18.05	< 0.0001
initial ln wet wt	1	111	16.44	< 0.0001

Significant Pairwise Comparisons (Tukey's HSD)

Diet	N	Mean ± SEM*	Sig at p < 0.05**
20% cellulose	22	9.34 ± 0.73	a
14% cell / 6% chi	26	9.00 ± 0.72	a
6% cell / 14% chi	28	7.47 ± 0.55	a
20% chitin	28	4.14 ± 0.25	b
control	15	8.78 ± 0.87	a

* Means reported are mean wet weight (g), SEM = standard error of the mean
**Treatments with different letters denote significant difference at p < 0.05

Specific growth rate was significantly different among diets ($F_{(4,64)} = 4.22$, $p = 0.004$), and the difference existed between the 20% cellulose (0.0127 ± 0.00091) and the 20% chitin diets (0.00794 ± 0.00046). There was also a significant difference between the 14% cellulose/ 6% chitin diet (0.0110 ± 0.00037) and the 20% chitin diet (Figure 13).

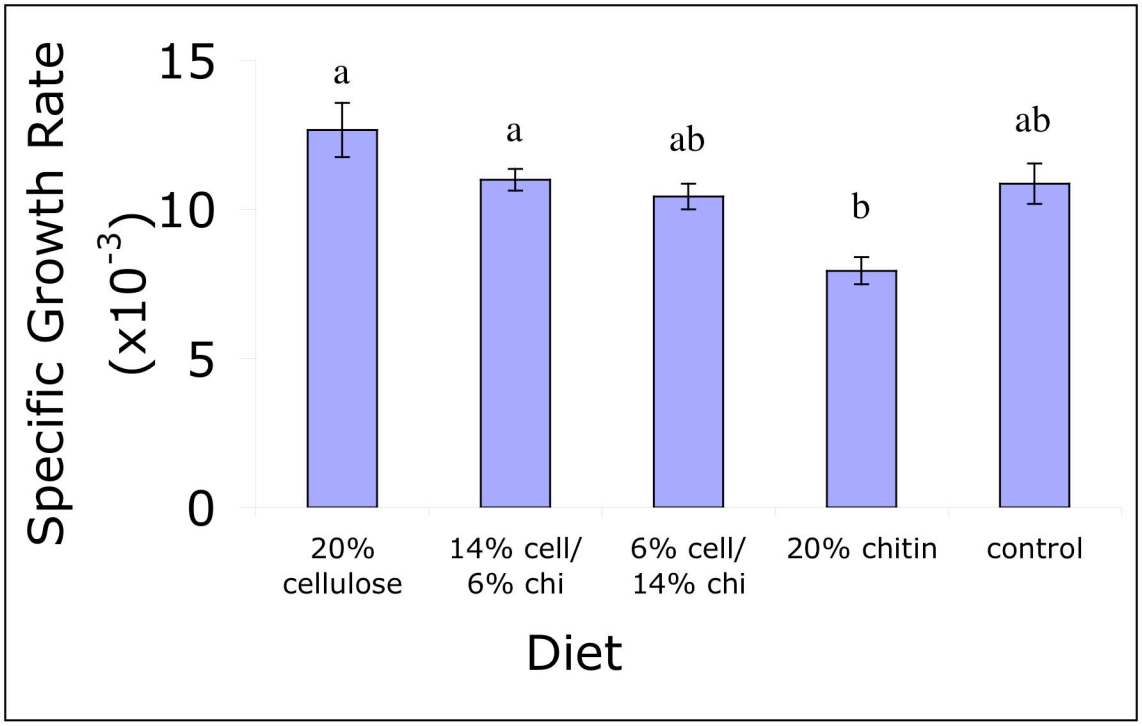

Figure 13. Specific growth rate ((log(final wt.) – log (initial wt.)) days^{-1}) of juvenile *C. sapidus* in fed different diets for the first half of the experiment. Error bars = SEM. Bars with different letters = sig. diff. at $p < 0.05$ (Tukey's HSD).

When comparing diet effects on molt frequency, a significant diet effect on the time to molt 2 was found ($F_{(4,116)} = 4.64$, $p = 0.002$). During the second molt, crabs in the 20% chitin treatment (31 $\pm$ 2 days) molted less frequently than crabs in the 20% cellulose treatment (21 $\pm$ 1 days), the 14% cellulose / 6% chitin treatment (23 $\pm$ 2 days), and the control (19 $\pm$ 2 days). In addition, the 14% chitin / 6% cellulose treatment (26 $\pm$ 2 days) was significantly different than the control. Although there were no significant differences for the other molts, a trend was present for molts 1 and 3, in which the time to molt was longer for the 20% chitin diet than for the other diets (Figure 14). In addition, no crabs reached molt 4 for the 20% chitin diet. Only 2 crabs in the control group reached molt 5.

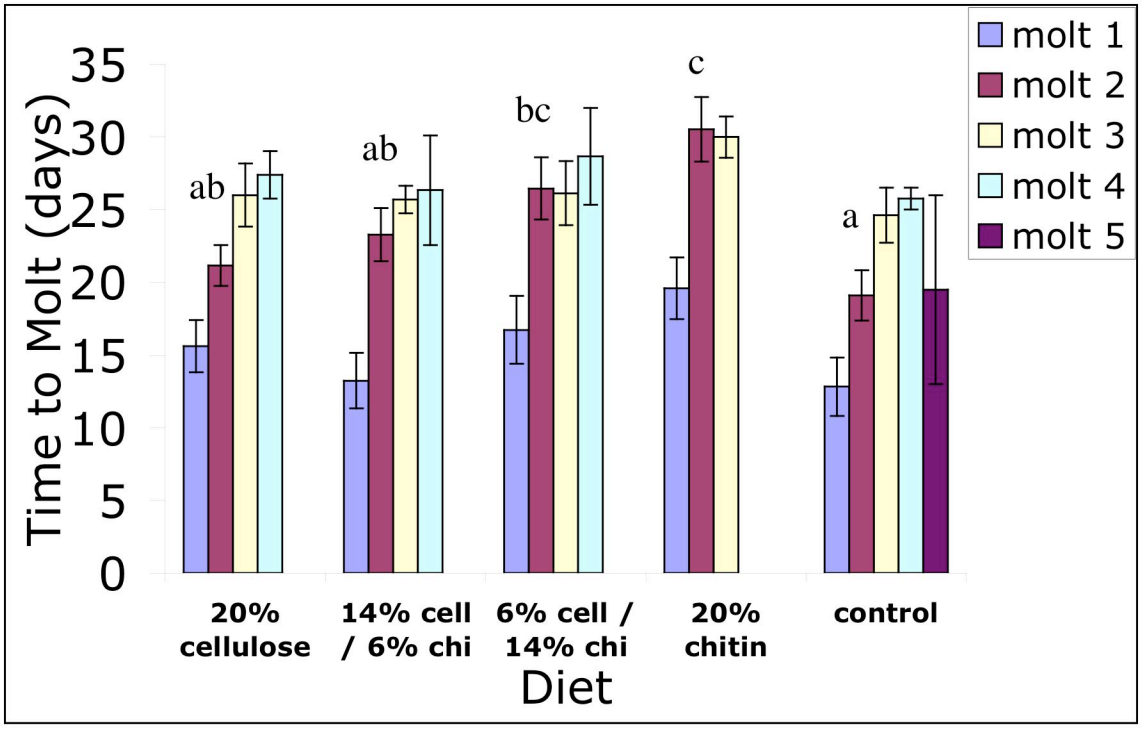

Figure 14. Mean time to molt for juvenile *C. sapidus* fed different diets for the first half of the experiment. Error bars represent standard error of the mean. For molt 2, bars with different letters = sig. diff. at $p < 0.05$ (Tukey's HSD).

There were significant differences between the molt increments of crabs fed different diets for molt 2 ($F_{(4,120)} = 5.15$, $p = 0.0007$) (Figure 15). The 20% chitin-fed crabs had a significantly smaller molt increment than any of the other treatments (0.19 ± 0.02). Although there were no significant differences for the other molts, the values of the molt increments during molts 1 (0.23 ± 0.02) and 3 (0.20 ± 0.02) for the 20% chitin diet were less than for the other diets. There was no significant diet effect on the total number of molts per crab ($F_{(4,115)} = 2.25$, $p = 0.07$).

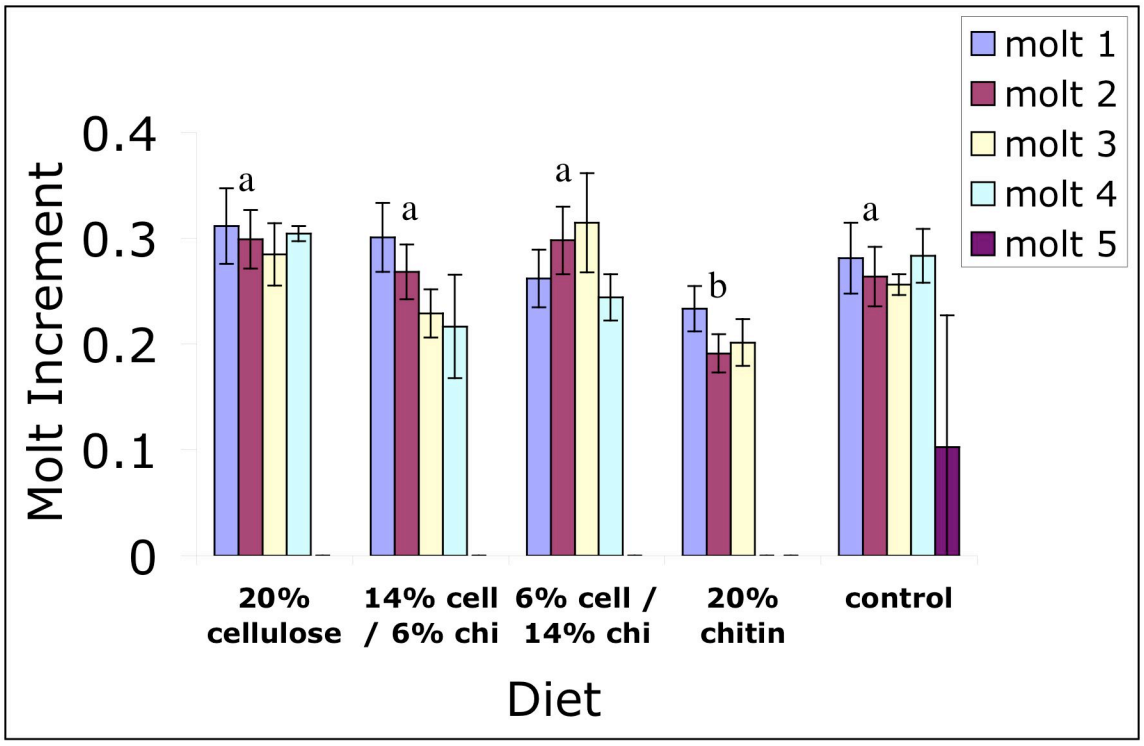

Figure 15. Mean increment ((increase in carapace width)(carapace width prior to molt)$^{-1}$) for juvenile *C. sapidus* fed different diets for the first half of the experiment. Error bars = SEM. For molt 2, bars with different letters = sig. diff. at $p < 0.05$.

Growth: after switching diets

There was no significant difference in final wet weights ($F_{(2,38)} = 1.20$, $p = 0.31$) between treatments using ANCOVA. The size of the animals at the end of the first half of the experiment (range 25-59 mm carapace width) accounted for the majority of the variance in the second half of the experiment.

There was a significant diet effect on specific growth rate (ANOVA, $F_{(2,37)} = 15.04$, $p < 0.0001$). Crabs fed the 20% cellulose diet had significantly higher specific growth (0.00668 ± 0.00054) than either crabs fed 20% chitin (0.00273 ± 0.00065) or the control diet (0.00318 ± 0.00120) (Figure 16).

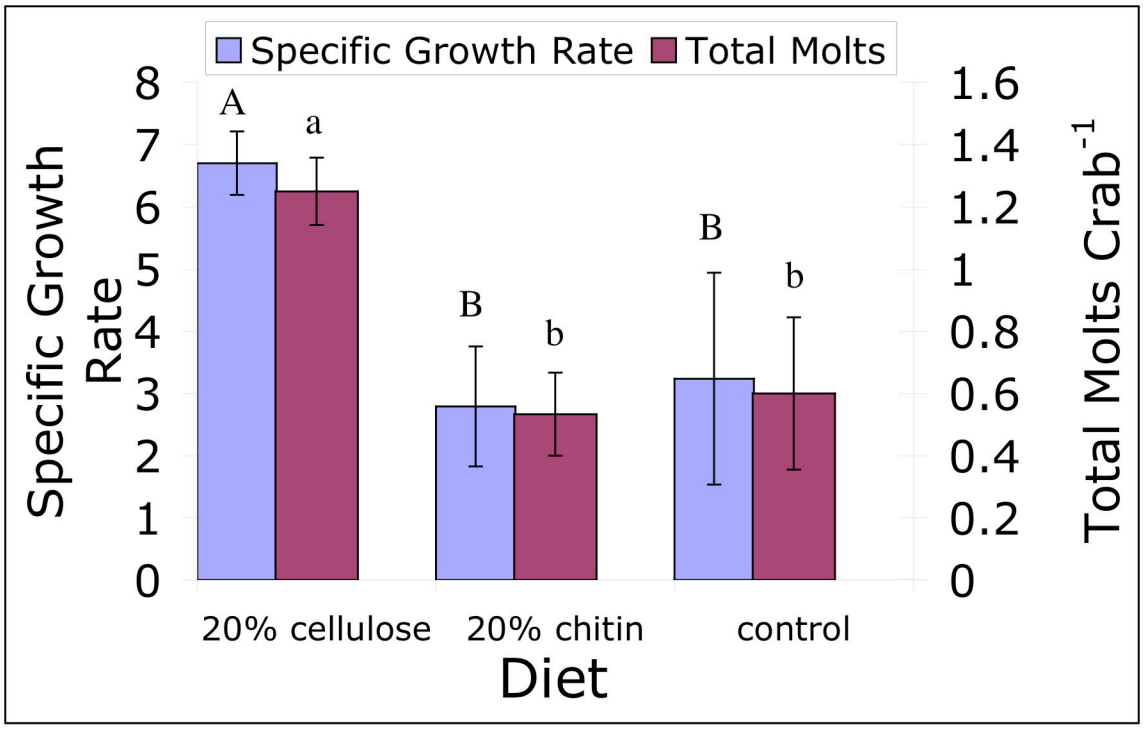

Figure 16. Specific growth rate ((log(final wt.) – log (initial wt.)) time^{-1} (days)) and total molts crab^{-1} of juvenile *C. sapidus* in different diet treatments for the second half of the experiment. Error bars = SEM. Bars with different letters = sig. diff. at $p < 0.05$ (Tukey's HSD).

There was no significant difference between diets for frequency (ANCOVA, $F_{(2,26)} = 2.56$, $p = 0.10$) or increment (ANCOVA, $F_{(2,29)} = 0.05$, $p = 0.95$) during molt 1. There was insufficient data to compare means for molt 2, since only the crabs fed 20% cellulose molted a second time. But because of this, the total number of molts per crab was significantly different between diets (ANOVA, $F_{(2,37)} = 11.52$, $p = 0.0001$). Crabs fed the 20% cellulose diet had a significantly higher number of molts (1.2 ± 0.1) than crabs fed the 20% chitin (0.5 ± 0.2) or control diets (0.6 ± 0.2) (Figure 16).

Consumption

There were no significant diet effects on the proportion of food consumed ($F_{(4,125)} = 1.71$, $p = 0.15$). The average proportion of food consumed (taking leaching into account) was 0.73 ± 0.03.

Discussion

Altering the components of a commercially available feed was a feasible method for diet studies of juvenile blue crabs. The combination of high survival and good growth and molting of the animals showed the diet to be adequate for the animals' needs. By combining water stability and palatability of the diet, we effectively provided for the animals' metabolic requirements. Although the diet was not initially water-stable, providing a nutritional oil coating improved the stability to a certain extent. Normally, higher growth rates are correlated to firmer diets (New, 1976), but we actually observed higher growth rates in this experiment with less stable diets (stability lasting approx. 30 minutes) than in the initial experiment (stability > 4 hours) (Chapter 2). For crabs with the same approximate starting size and the same total number of molts, the average increment was 0.26 ± 0.01 for this experiment versus 0.22 ± 0.01 for the initial experiment, and the average time to molt was 25 ± 1 days for this experiment versus 31 ± 1 days for the initial experiment. Since the proximate analyses of the Melick control diet and the Ziegler 1/8" diet (Chapter 2) were virtually the same aside from fat content, this provides evidence that the nutritional content of the oil coating over-compensated for lack of water-stability. The oil coating improved palatability so that the animals consumed the diet more quickly. It also provided an extra energy source to counteract

the loss of nutrients due to leaching, and in fact increased the overall available energy to enhance growth.

The two types of nutritional oils were chosen in combination because the menhaden oil has a high omega-3: omega-6 fatty acid ratio (approximately 4:1 by weight) while the lecithin has a low ratio (approximately 1:4 by weight). Combining the two ensures that the animals receive a proper balance of omega-3: omega-6 highly unsaturated fatty acids, containing slightly more omega-3 than omega-6 fatty acids, which is beneficial for growth of shrimp (Guillaume, Kaushik, Bergot, Metailler, 1999; New, 1976). For our study, the ratio of omega-3: omega-6 fatty acid was approximately 1.4: 1.

The rations fed were sufficient to maintain normal metabolic functions such as growth and molting, but were minimal enough to show diet effects. This confirms that the intermediate ration tested in the initial experiment (Chapter 2) is acceptable for diet experiments.

The proportion of the diet consumed was the same among treatments, showing that the growth differences between diets are a result of diet composition, not palatability. In general, crabs in this experiment consumed a higher proportion of their diet (0.73 ± 0.03) than in the initial experiment (0.44 ± 0.03) (Chapter 2). This may be a result of improved palatability as a result of the addition of nutritional oil to the diet, which may also have resulted in the higher growth rates observed.

Diet affected molt increment, frequency, and the total number of molts (Figure 14, Figure 15, Figure 16). For blue crabs, previous studies have determined only frequency and not increment to vary in response to diet (Millikin, Biddle, Siewicki, Fortner, Fair, 1980). In temperature and salinity studies that determined both frequency and increment

to vary, molt frequency was more important in determining the overall growth rates of blue crabs than increment (Cadman, Weinstein, 1988; Leffler, 1972). It is possible that the previous diet studies were unable to detect the more subtle differences in molt increment because the animals were being fed *ad libitum* instead of specific rations, making the studies less sensitive to treatment effects.

The inclusion of 20% and 14% cellulose had no effect on growth of juvenile *C. sapidus* when compared to the control diet (Figure 13, Table 13). The acid-detergent fiber content in the oil-coated control diet is 7.72%, compared to 13.39-19.90% in the experimental diets (Table 11). If the cellulose were indigestible, the inclusion of twice as much fiber in the experimental diets would depress growth. In addition, the portion of the diet that we replaced had a high starch component, which blue crabs have the capacity to digest (McClintock, Klinger, Marion, Hsueh, 1991), so we replaced a portion of the diet containing energetic value with a potentially indigestible substance. This indicates that blue crabs can, in fact, digest cellulose. In shrimp, starch and carbohydrates are hydrolyzed to form glucose for use in the Krebs cycle, glycogen storage, chitin synthesis, and the formation of sterols and fatty acids (New, 1976). It is possible that juvenile blue crabs utilize complex carbohydrates for these purposes as well, and is a potential subject for further study.

In the wild, juvenile crabs less than 80 mm carapace width consume detritus and plant material that constitutes approximately 20% of their diet (Laughlin, 1982; Stoner, Buchanan, 1990). In light of this feeding experiment, the consumption of plant material containing starch and cellulose may be an energetically important part of a juvenile blue crab's diet in the wild. In a study involving juvenile *C. sapidus* fed 100% detrital

material, the animals grew, however slowly, and had 90% survival in the 3 week time-frame of the experiment (Dittel, Epifanio, Schwalm, Fantle, Fogel, 2000). Even though these crabs did not grow as quickly as those fed diets containing protein, the study suggests that detritus may provide a secondary source of nutrition when food is scarce. From the wide range of food items consumed by juvenile crabs and the broad descriptions of their dietary niche (Laughlin, 1982; Stoner, Buchanan, 1990), we can safely describe them as omnivorous. Therefore it is not surprising that the animals would grow slowly on 100% detritus. Although they may derive energetic value from cellulose and starch in detrital material, detritus does not provide enough of the necessary proteins for growth and building of body mass needed by an omnivorous animal.

In Biddle et al. (1978), growth was not depressed at 3% or 9% inclusion of cellulose, but was inhibited at 27% inclusion. It is possible that the reduction in growth with a 27% cellulose diet was a result of problems with the water-stability of the diet. When the diet contained 27% cellulose it fell apart rapidly in water, which may have introduced some bias into the study. This was a problem with the extrusion-based production of the diet, and it may be possible to include more cellulose if the processing is improved.

Crabs fed 20% chitin had lower mean wet weights (Table 13), specific growth rates (Figure 13), increments (Figure 15), and frequencies (Figure 14) than crabs fed 20% cellulose, while the crabs fed a combination of chitin and cellulose had growth parameters in between the 20% chitin and 20% cellulose treatments. The addition of chitin in the diet has been found to depress growth in tilapia (Shiau, Yu, 1999). A possible reason for this is that chitin is known to interfere with fat digestion and absorption (Deuchi, Kanauchi, Imasato, Kobayashi, 1994). In the current study, that is of

particular importance since chitin may have behaved differently towards the oil coating than cellulose. As a result, some bias may have been introduced into the experiment, and the oil coating may have been differentially absorbed between treatments. Although the nutritional oil had the advantage of compensating for the rapid leaching and instability of the diet, a neutral stabilizer that truly provides water stability (on the order of hours) should be used in the future.

If chitin is indigestible, this provides little explanation for why crabs consume their shed exuviae. Blue crabs have been observed consuming their own exoskeletons after molting, from the larval stages (Sandoz, Rogers, 1944) to juveniles and adults (A. Allman, pers. obs.). Chitin is also consumed when eating other crustaceans and when cannibalizing each other (Laughlin, 1982; Stoner, Buchanan, 1990). Since salts such as calcium carbonate are absorbed directly from the surrounding water via the gills (Cameron, Wood, 1985), the potential value of consuming shed exuviae exists in the resorption of chitin and the various proteins associated with the molecule. It is possible that *C. sapidus* consumes their exoskeleton for the nutritional value of the protein portion only, which is less than 20% of the dry weight (Vigh, Dendinger, 1982).

Upon switching the diets, crabs in the 20% cellulose treatment molted more and had a higher specific growth rate (Figure 16) than either the 20% chitin or control group. Since they were growing poorly on 20% chitin diet prior to the switch, the accelerated growth beyond that of the control treatment is indicative of compensatory growth. This has been indicated for *C. sapidus* by Wang and Stickle (1986), who found that the RNA: DNA ratio (starting at 2.92 ± 0.09) decreased to 1.15 ± 0.09 after 30 days of starvation and then increased to 1.74 ± 0.13 within 5 days of feeding. The rapid increase in the RNA: DNA

ratio after 5 days of feeding indicates that the animals have a high capacity for recovery, and can recover at a more rapid rate than the decline in protein production that they experienced upon starvation. Compensatory growth is common in fish (Ali, Nicieza, Wootton, 2003) and has also been identified in the Chinese shrimp *Fenneropenaeus chinensis* (Wu, Dong, 2002; Wu, Dong, Wang, Tian, Ma, 2001). That juvenile *C. sapidus* appears to "make up" for loss of growth from previous nutrient limitation is an interesting avenue for future research, and may have management implications.

Chapter 4. The effect of dietary fiber on growth of the blue crab, *Callinectes sapidus* (Rathbun), in a recirculating system: using alginate to improve water stability of a pelletized diet

Summary

The addition of a coating of nutritional oil may have introduced bias into the previous experiment, so we replicated the experiment using a neutral stabilizer instead of oil. We performed a 12-week experiment, using a commercial pelletized shrimp diet for which we replaced 20% of the wet weight (comprised of ground wheat derivatives) with either chitin or cellulose, and coated with sodium alginate to provide water stability. We used a recirculating, compartmentalized system that we developed to maintain near optimal water quality values while preventing cannibalism. Specific growth rate ((log (final weight) – log (initial weight)) time^{-1}) was significantly lower in the chitin-fed crabs ($3.77 \times 10^{-3} \pm 0.18 \times 10^{-3}$) than in either the cellulose-fed crabs ($5.81 \times 10^{-3} \pm 0.22 \times 10^{-3}$) or the control group ($6.20 \times 10^{-3} \pm 0.26 \times 10^{-3}$). There was a substantial difference in food conversion efficiency (dry weight gained (dry weight fed)$^{-1}$) between crabs fed chitin (0.0750 ± 0.0056) and either cellulose (0.144 ± 0.009) or the control diet (0.161 ± 0.017). All of the growth parameters investigated (molt increment, molt frequency, and total number of molts per crab) were lower in the chitin-fed treatment than the other two treatments. These findings are similar to those of the previous experiment, suggesting that cellulose is digestible in blue crabs, while chitin is not. There was no bias introduced in the previous experiment by the usage of nutritional oil, however alginate is the preferred stabilizing additive due to its higher water-stability and energetically neutral character.

Introduction

Chitin is known to interfere with fat digestion and absorption (Deuchi, Kanauchi, Imasato, Kobayashi, 1994), and may have behaved differently towards the nutritional oil coating in the previous experiment (Chapter 3) than cellulose, introducing bias into the experiment. In addition, the oil-coated pellets were only water stable for approximately 30 minutes. Therefore, we set out to replicate the experiment with a coating of some non-nutritive, water-stable substance instead of oil. We performed a 12-week experiment, using the same commercial pelletized shrimp diet, which we coated with sodium alginate and calcium chloride instead of oil. Divalent calcium ions form ionic bonds between adjacent alginate molecules, forming a water-stable complex (Meyers, Butler, Hastings, 1972). After eliminating the potential bias from the oil coating, we set out to determine if the addition of fiber in the diet affects growth and survival of juvenile *C. sapidus*. If digestible, the addition of dietary chitin and cellulose should have no deleterious effects on growth of juvenile crabs in the laboratory.

Methods

Ninety juvenile crabs from a single hatchery-reared brood (167 days old) were obtained from the Center of Marine Biotechnology. The crabs averaged 3.99 ($\pm$ 0.11) grams wet weight and 34 ($\pm$ 0.3) mm carapace width. We used a compartmentalized recirculating system consisting of seven tanks (described in Chapter 2).

The crabs were placed in 15x15x10 cm "large" compartments with walls constructed of PVC board in 5 of the 6 available tanks. Treatments were randomly assigned to the compartments within each tank, according to the random complete block

design (with the tanks as blocks). Wet weights and carapace widths were measured 24 hours after each molt, and the date of each molt was recorded.

Water temperature was maintained at 23 $\pm$ 0.2 ^{o}C as a compromise between rapid growth and high survival (Cadman, Weinstein, 1988; Leffler, 1972). Saltwater was formulated the same way as in the previous experiments (see Chapter 2). Salinity was maintained at 29 $\pm$ 1 gL^{-1}, near the approximate isoosmotic point for *C. sapidus*, 27 gL^{-1} (Colvocoresses, Lynch, Webb, 1974). Fluorescent lights in the ceiling were on a controlled photoperiod equivalent to the photoperiod in Baltimore, Maryland from March 14, 2005 to June 28, 2005. Ammonia and nitrite were measured daily, while pH, salinity and temperature were measured weekly from the sump. Dissolved oxygen was measured on June 8, 2005 in one compartment, chosen randomly, within each tank (N=5) (Table 14).

Table 14. Water quality parameters during the course of the experiment.

Parameter*	Mean $\pm$ SEM
NH_3	0.122 $\pm$ 0.008
NO_2	0.175 $\pm$ 0.026
Dissolved Oxygen	5.6 $\pm$ 0.2
Salinity (gL^{-1})	29 $\pm$ 1
pH	8.04 $\pm$ 0.04
Temperature (^{o}C)	23 $\pm$ 0.2

*Values in mgL^{-1} unless otherwise stated; pH is unitless.

The experiment was started on March 14, 2005. The crabs had been in the experimental system since December 18, 2004, at the same salinity and temperature. Prior to the experiment, they were fed a commercial shrimp diet (Ziegler 3/32" pellets) *ad libitum* daily.

Diet Formulations

Formulated diets fell apart within 5 minutes of contact with the tank water. I set out to find a coating for the pellets that provided water stability but was metabolically neutral. After testing different concentrations of sodium alginate, gum Arabic, gum Ghatti, and gelatin, I developed a protocol for spraying diets with sodium alginate and calcium chloride based on the methods of Meyers (1972) to visibly maintain their water stability for a minimum of 8 hours, providing enough time for the animals to consume the entire diet that they were given without losing the soluble nutrients in the diet to leaching. The crabs consumed it readily, showing it to be a palatable diet.

Sodium alginate was dissolved in deionized water overnight to make a 2% solution (by weight). Calcium chloride was also dissolved in deionized water to make a 0.5% solution. Pellets were weighed, arranged in a single layer on a flat baking tray, sprayed on both sides using compressed nitrogen with a paint sprayer for a specific amount of time with sodium alginate solution followed by calcium chloride solution, weighing after each addition. The sodium alginate solution was highly viscous and had to be warmed to 50^{o}C in order for it to spray evenly on the diets. The pellets were then placed in a drying oven at 55^{o}C for 8 hours. The alginate and calcium chloride coating comprised 27 ($\pm$ 1)% of the wet weight of the coated diets, prior to placing in the drying oven. There were 3.67×10^{-3} ($\pm$ 2.8×10^{-4}) g sodium alginate added to every gram of pelletized diet, and 9.88×10^{-4} ($\pm$ 9.1×10^{-5}) g calcium chloride added to every gram of pelletized diet. The diet was sprayed in batches, approximately once a month, and the coated diet was kept refrigerated at 4^{0}C to prevent mold spoilage. Triplicate samples of each of the experimental diets were sent to New Jersey Feed Lab, Inc. (Trenton, NJ) for performing

proximates and acid-detergent fiber (ADF) analyses (Table 15). Crabs readily consumed the alginate-coated diet. To increase the amount of replicate samples per treatment, I reduced the number of treatments to 3 (compared to Chapter 3), using only the 20% cellulose, 20% chitin, and control diets.

Table 15. Proximate analyses of alginate-coated diets, expressed in percent composition ± standard error. N = 3 for each diet.

Diet	Moisture	Protein	Fat	Ash	ADF*
20% cellulose	8.02 ± 0.74	31.9 ± 0.5	4.46 ± 0.09	6.22 ± 0.02	14.07 ± 0.79
20% chitin	8.21 ± 1.88	36.6 ± 0.6	4.20 ± 0.06	6.70 ± 0.13	18.31 ± 0.48
control	7.64 ± 1.45	34.8 ± 0.5	4.32 ± 0.08	6.83 ± 0.09	8.20 ± 0.15

*ADF = acid-detergent fiber analysis

The animals were fed the median ration from a range of recommended rations for shrimp of the genus *Penaeus* (Guillaume, Kaushik, Bergot, Metailler, 1999) (see Chapter 2) 6 days a week. Crabs were fed double rations the following day when a day was missed. Rations are expressed in grams dry weight per 100 grams body weight per day, and vary according to the crabs' wet (body) weight. The weight of the pellets fed was corrected to account for an estimated 10% water weight, which was later found to be 7.96% (determined from the proximates). This was considered unimportant since the rations were fed consistently according to the estimated water weight, and the difference in rations according to the estimated versus actual wet weights was never more than 0.01 g day^{-1}.

On June 8, all crabs were weighed and measured to determine the relationship between wet weight and carapace width. On June 28, thirty-eight crabs (randomly chosen from each treatment) were weighed, measured, and sacrificed and the experiment terminated. The remaining crabs were used in another experiment. Crabs were sacrificed by submersion in ice water for 5 minutes. Seventeen of the sacrificed crabs were freeze-

dried to obtain dry weights. Twenty-one of the sacrificed crabs (7 from each treatment) were used for gut and hepatopancreas sample collection for future enzyme assays. Dry weight values were used to make linear regressions determining the relationship between dry weight (DW) and wet weight (WW), and dry weight and carapace width (CW). Values for allometric relationships were collected at the time of sacrifice, therefore no repeated measures were used.

Growth

All measures of growth were performed on the data collected from March 14 to June 8, when all of the animals were measured for final values. The SAS system V.8 software was used for data analyses (SAS 2001). When assumptions of normality and homogeneity of variance were not met, data was transformed as appropriate. Tukey's HSD was performed post-hoc to examine specific differences between treatments, with an experimentwise error rate of $p < 0.05$.

There was no significant difference in wet weight and carapace width between diets at the beginning of the experiment (ANOVA, $F_{(2,86)} = 0.49$, $p = 0.61$ and $F_{(2,86)} = 0.55$, $p = 0.58$, respectively). Throughout the experiment, the majority of the variance in wet weight and carapace width after each molt was related to the size prior to molting. Because of this, analysis of covariance was performed using the previous size as a covariate for the size at each successive molt. Statistical results for wet weight and carapace width were redundant, so only the results for wet weight will be reported.

A linear measure of growth rate, specific growth rate ($(\log WW_1 - \log WW_0)$ $time^{-1}$) was also used to discern differences between treatments. Wet weights measured

on June 8 were used as the final wet weights for this parameter. Since specific growth rate inherently includes the initial size in the values, it was analyzed by ANOVA.

When dietary treatments significantly affected growth, the differences in growth were further investigated. Growth can differ as a result of molt increment ((increase in carapace width) (previous carapace width^{-1}) or molt frequency (time to each molt in days). Both of these parameters were also found to covary with size prior to molt, so they were also analyzed by ANCOVA. The total number of molts per crab was analyzed by ANOVA. Since the instar is unknown, molts are counted relative to the start of the experiment.

Conversion Efficiency

We calculated food conversion efficiencies by subtracting the initial wet weights from the final wet weights (measured on June 8), and then dividing that by the total amount of food given per animal over the course of the experiment. An ANOVA was performed on the raw (wet weight) values, followed by pairwise comparisons between diets (Tukey's HSD). The conversion efficiencies are also reported as dry weight gained and dry weight fed (DW) values since these values are more relevant from an energetic viewpoint. Dry weight gained was calculated from the regression relationship found between wet weight and dry weight, using the equation below:

$$DW = 0.0922(\pm 0.0314)(WW)^{1.43(\pm 0.133)}$$

This regression had an r^2 of 0.89. Final calculated dry weight was subtracted from initial calculated dry weight to estimate the dry weight gained. Dry weight fed was calculated from the 7.96% water weight value found in the proximate analysis.

Consumption

On May 23, an experiment was conducted to determine if all of the crabs were consuming the same proportion of their diet (see Chapter 2 for methods). The percent of diet consumed was calculated by subtracting the final oven-dried diet from the calculated dry weight of the initial diet (subtracting 4.62 ($\pm$ 0.81)% moisture), accounting for the average percent of food leached (25 ($\pm$ 3)% of the dry weight). The effect of diet on the percentage of food consumed was examined by ANOVA. Tukey's HSD was performed post-hoc to examine specific differences between treatments, with an experimentwise error rate of $p < 0.05$.

Results

No crabs died during the course of the experiment. One crab escaped on June 8. Data prior to June 8 for this crab was used in the data analysis because the crab was considered healthy before it's date of disappearance. Variability in size of the individuals was high throughout the experiment (Figure 17). When comparing overall average values, the molt frequency decreased between the first (20 $\pm$ 2 days) and second molts (40 $\pm$ 1 days) and then leveled out at 38 $\pm$ 1 days during molt 3, while the percent increase in carapace width increased from molt to molt (Table 16).

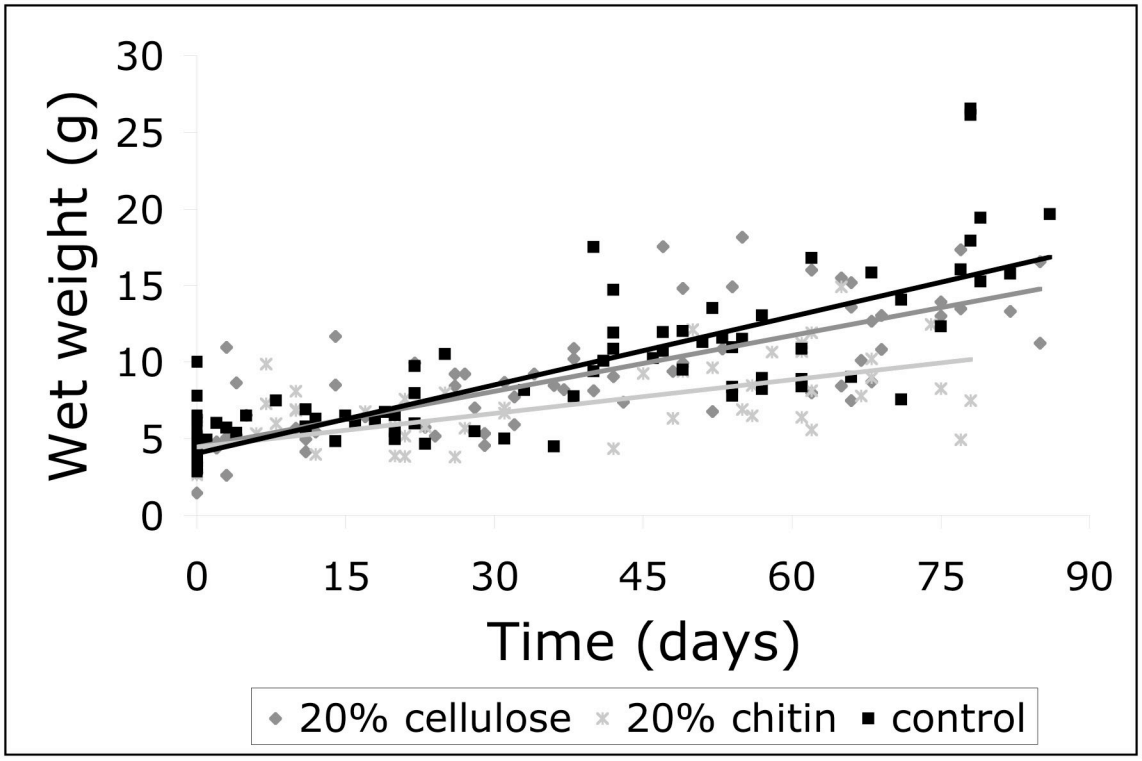

Figure 17. Wet weight of all juvenile *C. sapidus* throughout the experiment, measured 24 hours post-molt. Linear regressions are plotted for each dietary treatment.

Table 16. Mean growth values for juvenile *C. sapidus*, ± standard error.

molt	N of crabs molted	% increase in CW	time to molt (days)
1	90	17 ± 1	20 ± 2
2	78	18 ± 1	40 ± 1
3	15	19 ± 1	38 ± 1

Based on r^2 values, power regressions provided the best fit for the data. Wet weight appeared to be a more accurate predictor of dry weight than carapace width, with an r^2 value of 0.89, compared to 0.72. Regression relationships for calculating dry weight were as follows:

$$DW = 0.0922(\pm 0.0314)WW^{1.43(\pm 0.133)}$$

$$DW = 4.8x10^{-6}(\pm 1.0x10^{-5})CW^{3.4(\pm 0.55)}$$

Wet weight and carapace width, however, had a very close relationship, with an r^2 of 0.97 and the following regression equation:

$$WW = 0.0003 (\pm 6.4x10^{-5}) CW^{2.7(\pm 0.1)}$$

Growth

Significant diet effects were found using ANCOVA ($F_{(2,85)} = 26.78$, $p < 0.0001$). Crabs in the 20% chitin diet (8.73 $\pm$ 0.45 g) were significantly smaller than crabs in the 20% cellulose diet (12.64 $\pm$ 0.61 g) and the control diet (13.63 $\pm$ 0.91 g) (Table 17).

Table 17. ANCOVA for final wet weight, using initial wet weight as a covariate.

Effect	Num DF	Den DF	F	Pr > F
Diet	2	85	26.78	< 0.0001
Initial wet weight	1	85	50.99	< 0.0001

Significant Pairwise Comparisons (Tukey's HSD)

Diet	N	Mean $\pm$ SEM*	Sig at p < 0.05**
20% cellulose	30	12.64 $\pm$ 0.61	a
20% chitin	30	8.73 $\pm$ 0.45	b
Control	29	13.63 $\pm$ 0.91	a

* Means reported are mean wet weight (g), SEM = standard error of the mean
**Treatments with different letters denote significant difference at p < 0.05

There was a significant diet effect on specific growth rate ((log(final weight) – log(initial weight)) $time^{-1}$) (ANOVA, $F_{(2,85)} = 34.5$, $p < 0.0001$). Specific growth rate was significantly lower in the chitin-fed crabs ($3.77x10^{-3} \pm 0.18x10^{-3}$) than in either the cellulose-fed crabs ($5.81x10^{-3} \pm 0.22x10^{-3}$) or the control group ($6.20x10^{-3} \pm 0.26x10^{-3}$) (Figure 18).

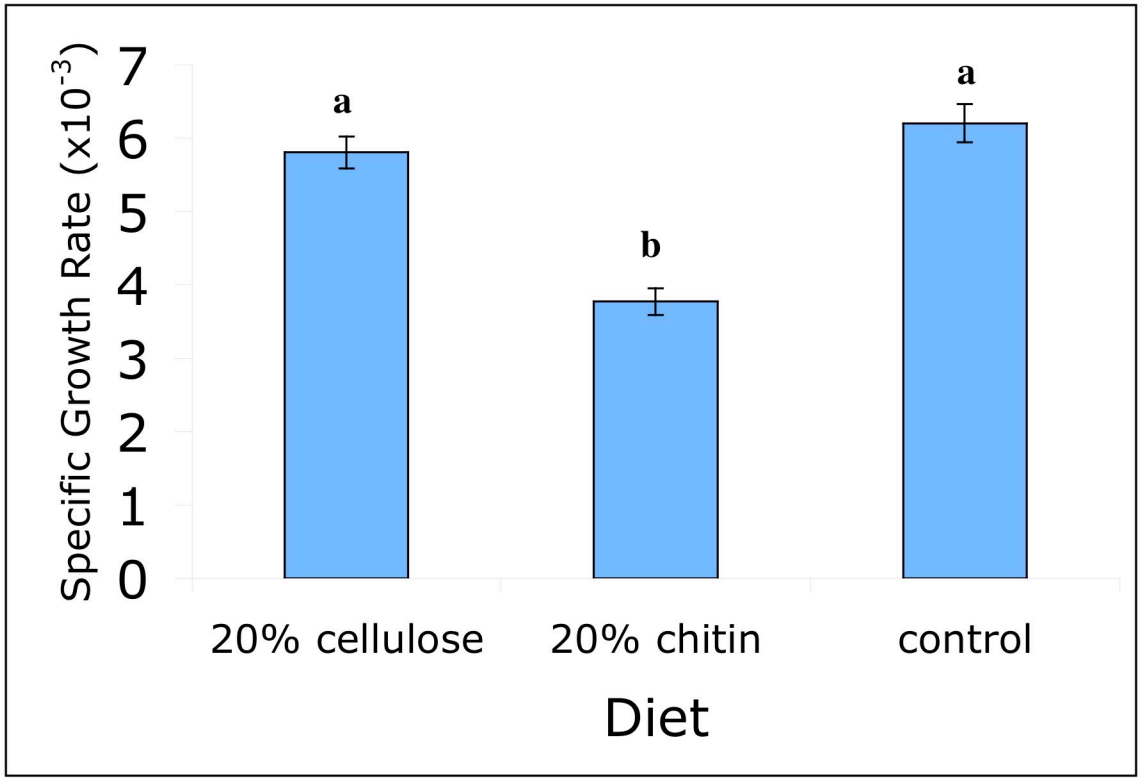

Figure 18. Mean specific growth rate ((log (final wt.) – log (initial wt.)) time^{-1} (days)) for experimental diets. Error bars = SEM. Bars with different letters denote sig. diff. between treatments at $p < 0.05$ (Tukey's HSD).

Molt increment ((increase in CW)(CW prior to molt^{-1})) was significantly affected by diet during molt 2 (ANCOVA, $F_{(2,73)} = 13.5$, $p < 0.0001$). Crabs in the 20% chitin diet had a significantly lower increment (0.14 $\pm$ 0.01) than crabs in either the 20% cellulose (0.19 $\pm$ 0.01) or the control treatment (0.20 $\pm$ 0.01). There was no significant difference in increment between treatments during molt 1 (ANCOVA, $F_{(2,84)} = 1.18$, $p = 0.31$), but a trend was present where the 20% chitin diet had a smaller increment (0.16 $\pm$ 0.01) than either the 20% cellulose (0.18 $\pm$ 0.01) or the control (0.17 $\pm$ 0.01) (Figure 19).

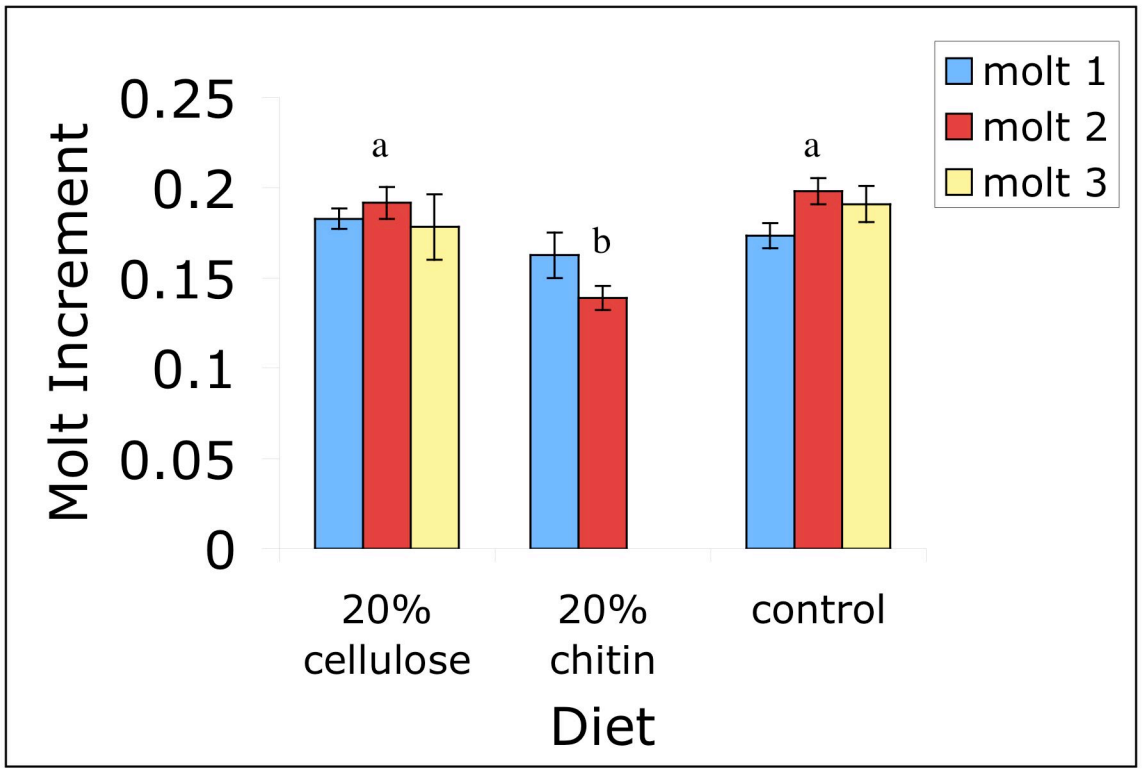

Figure 19. Mean molt increment ((increase in carapace width)(carapace width prior to molt)$^{-1}$) for experimental diets, measured molt-by-molt. Error bars = SEM. Within each molt: bars with different letters denote sig. diff. between treatments at $p < 0.05$ (Tukey's HSD).

Time to molt (days) was significantly different among diets during molts 1 and 2 (ANCOVA, $F_{(2.85)} = 6.43$, $p = 0.003$ and $F_{(2,69)} = 8.16$, $p = 0.0007$, respectively). The post-hoc test indicated that crabs in the 20% chitin diet took significantly longer to reach molt 1 (29 $\pm$ 4 days) than in either the 20% cellulose (18 $\pm$ 2 days) or the control diets (15 $\pm$ 2 days). For molt 2, the same pattern appears: the 20% chitin-fed crabs took significantly longer to molt (46 $\pm$ 2 days) than either the 20% cellulose-fed (39 $\pm$ 1 days) or the control-fed crabs (38 $\pm$ 1 days) (Figure 20).

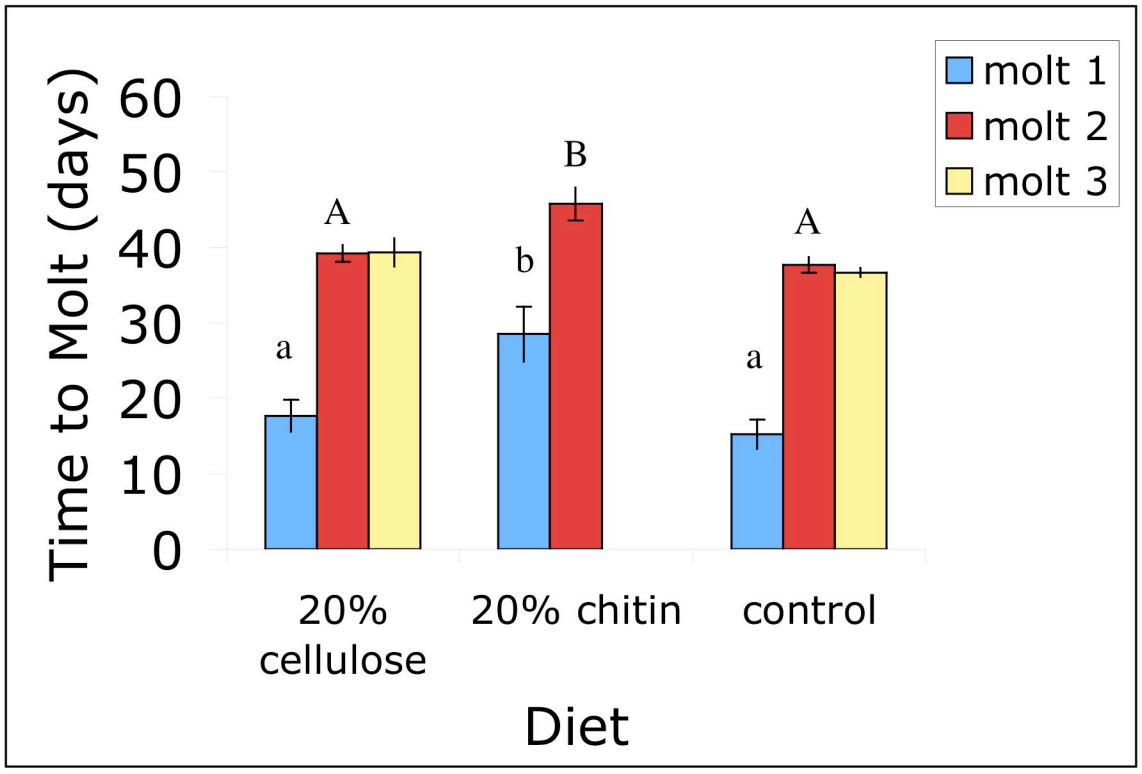

Figure 20. Mean time to molt for experimental diets, measured molt-by-molt. Error bars = SEM. Within each molt: bars with different letters denote sig. diff. between treatments at $p < 0.05$ (Tukey's HSD).

No crabs in the 20% chitin group molted a third time. This resulted in a significant difference between diets in the total number of molts per animal (ANOVA, $F_{(2,87)} = 20.4$, $p < 0.0001$). The average number of molts in the 20% chitin group (1.6 ± 0.1) was significantly lower than in the 20% cellulose (2.2 ± 0.1) or the control groups (2.3 ± 0.1) (Figure 21).

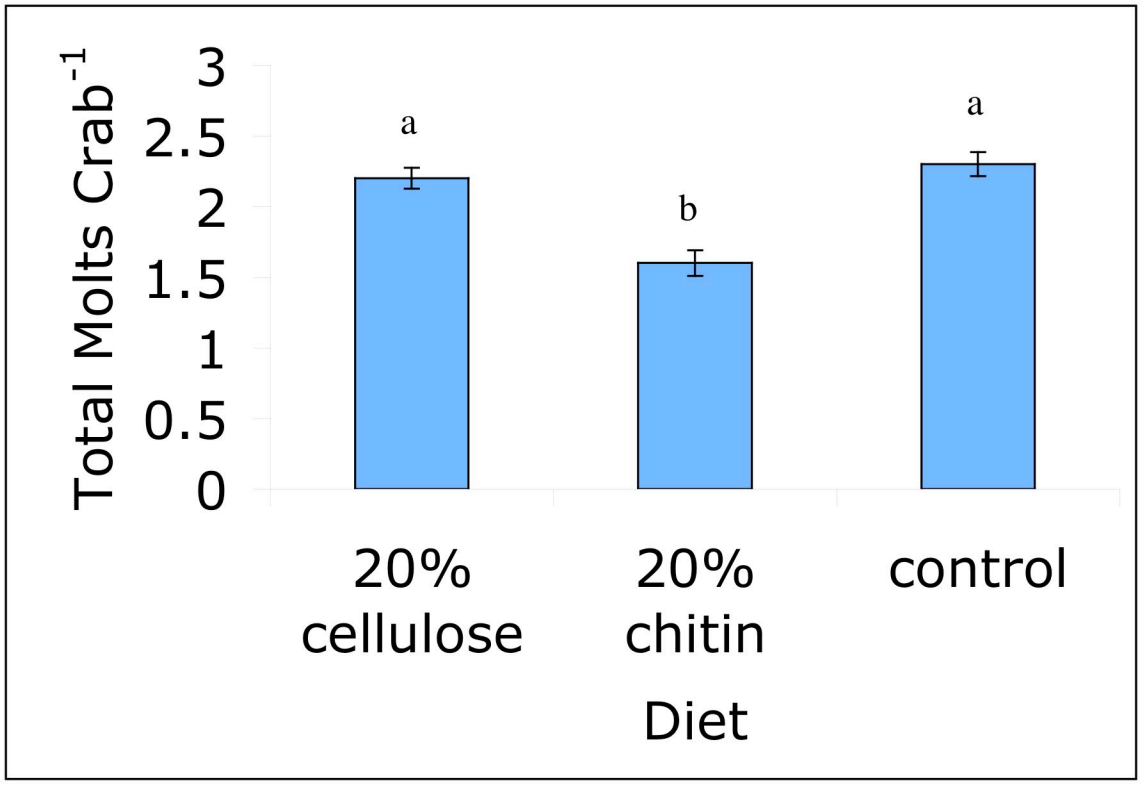

Figure 21. Mean total molts per crab for experimental diets. Error bars = SEM. Bars with different letters denote significant differences between treatments at $p < 0.05$ (Tukey's HSD).

Conversion Efficiency

A significant difference between diets was found for conversion efficiency ($F_{(2,86)} = 33.9$, $p < 0.0001$). There was a substantial difference in food conversion efficiency (dry weight gained (dry weight fed)$^{-1}$) between crabs fed chitin (0.0750 $\pm$ 0.0056) and either cellulose (0.144 $\pm$ 0.009) or the control diet (0.161 $\pm$ 0.017) (Figure 22). The average food conversion efficiency in wet weight gained (wet weight fed)$^{-1}$ values was 0.231 ($\pm$ 0.014) and 0.403 ($\pm$ 0.019) for the chitin-fed and cellulose-fed animals, respectively (Figure 22).

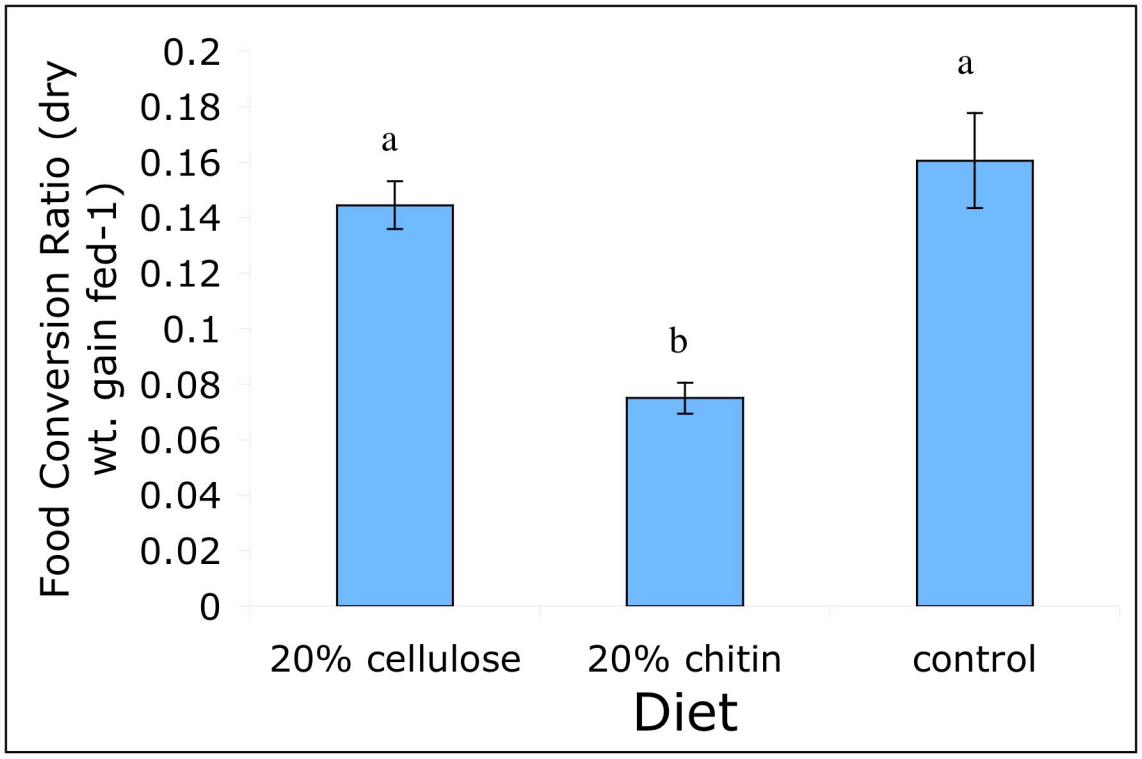

Figure 22. Mean food conversion efficiency (dry weight gained (dry weight fed)$^{-1}$) for experimental diets. Error bars represent standard error of the mean. Bars with different letters denote sig. diff. between treatments at $p < 0.05$ (Tukey's HSD).

Consumption

Diet had a significant influence on the proportion of food consumed ($F_{(2,85)}$ = 5.51, p = 0.006). Tukey's HSD indicated that the difference existed between the 20% cellulose (98.7 $\pm$ 0.7 %) and 20% chitin (91.1 $\pm$ 2.2 %) diets (Figure 23).

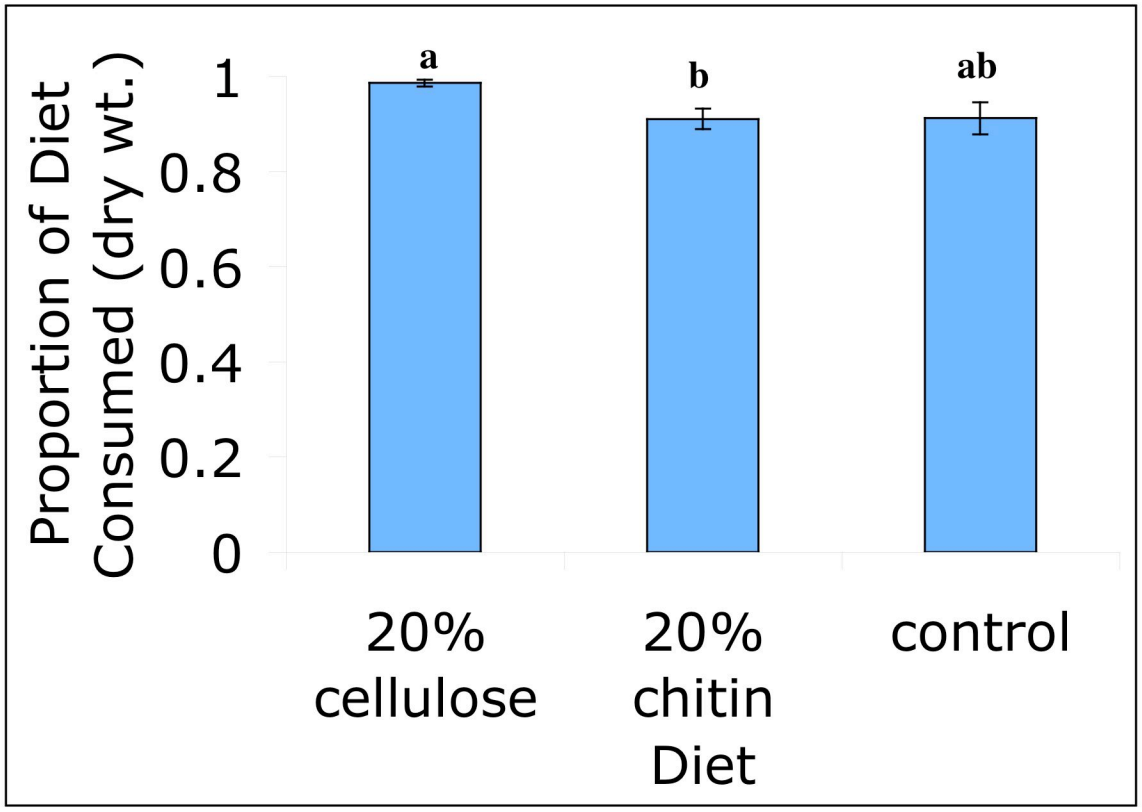

Figure 23. Mean proportion of diet consumed in 4 hours for different diets, taking leaching into account. Error bars = SEM. Bars with different letters denote sig. diff. between treatments at $p < 0.05$ (Tukey's HSD).

Discussion

Spraying diets with a combination of alginate and calcium chloride was an effective method of improving the water stability of a pelletized diet for juvenile *C. sapidus*. The high survival and growth rates showed the diet to be adequate for the animals' needs. In the literature protocol, the dry diet mixture was submerged in an alginate solution before pelletizing (Meyers, Butler, Hastings, 1972), but for our purposes we needed to develop a protocol for coating the diets after they had already been pelletized. Using our protocol, the diet retained water stability for at least 8 hours, while remaining palatable to the animals. In this way, we achieved the balance between retaining the physical structure of

the diet and releasing small amounts of substances attractive to the crabs, allowing for the crabs' gradual feeding behavior with minimal loss of nutrients due to leaching. The average proportion of the diet consumed was the highest for this experiment (0.94 ± 0.01) than any of the previous experiments, showing the highest palatability within four hours of feeding. By improving the stability of the diet, water quality in the recirculating system could also be retained. While the water quality was acceptable in the previous experiment (Chapter 3), there is a relatively low biomass for the volume of water in the system, and use of oil-coated pellets in a more intensive system could potentially cause problems with water fouling. The use of a dry, commercially-formulated diet was convenient and practical, and coating the diets with alginate could be performed on large quantities as infrequently as once a month, provided the coated diet was refrigerated.

Juvenile *C. sapidus* growth was suppressed by the addition of chitin, but there were no negative effects upon inclusion of 20% cellulose to the diet. All measures of growth (wet weights, specific growth rate, molt increment, molt frequency, and the total number of molts per crab) were significantly decreased by a 20% inclusion of dietary chitin, but not cellulose, in the diet. Since the animals had a larger starting size in this experiment (3.99 ± 0.11 g wet weight and 34 ± 0.3 mm carapace width) and the experiment lasted for 86 instead of 149 days, we could not compare specific values for growth parameters between this experiment and the previous experiment. However, when comparing treatment effects, there was not much of a difference in results when using either the oil-coated diet in the previous experiment (Chapter 3) or the alginate-coated diet. The results of the ANCOVA showed the weight of crabs fed 20% chitin to be significantly lower than for 20% cellulose or the control in both this experiment and the one previous (Table

13, Table 17). In both experiments, the 20% cellulose-fed crabs had higher specific growth rates than the 20% chitin-fed crabs, but the same as the control (Figure 13, Figure 16, Figure 18). The time to molt was significantly lower for crabs fed the 20% cellulose and control diets than the 20% chitin diet during at least one molt (Figure 14, Figure 20), and the increment was significantly higher for crabs fed the 20% cellulose and control diets than the 20% chitin diet during the second molt (Figure 15, Figure 19). This indicates that the oil coating did not obscure or bias the results of the previous feeding experiment.

Conversion efficiency was significantly decreased in crabs fed the chitin-containing diet (Figure 22). The average food conversion efficiency in wet weight gained (wet weight fed)$^{-1}$ values was 0.231 ($\pm$ 0.014) and 0.403 ($\pm$ 0.019) for the chitin-fed and cellulose-fed animals, respectively. These values are similar to the conversion efficiency values in a feeding experiment with varying proportions of cellulose (range 0.23 – 0.77 wet weight gained (wet weight fed)$^{-1}$) (Biddle, Millikin, Fair, Fortner, 1978). Conversion efficiency appears to be an effective tool in determining differences between dietary treatments, which is another reason to deliver and record specific rations when performing a feeding experiment. Much data was lost in the previous experiment as a result of feeding the animals *ad libitum* on the weekend.

Unlike in the previous experiment, the proportion of diet consumed was significantly lower for the 20% chitin diet (91.1 $\pm$ 2.2 %) than the 20% cellulose diet (98.7 $\pm$ 0.7 %) (Figure 23). While this may have accounted for some of the difference between dietary treatments, neither treatment had a significantly different proportion of the diet consumed than the control. Therefore, this difference in consumption could not account for the

difference between the 20% chitin-fed and control-fed crabs regarding wet weight, specific growth rate, molt increment, molt frequency, conversion efficiency, or the total number of molts per crab. The decline in growth and conversion efficiency in the chitin-fed crabs must therefore be attributed to diet composition rather than palatability.

The results indicate that cellulose is digestible in blue crabs, while chitin is not. While there is evidence that utilization of cellulose provides an ecological advantage for juvenile *C. sapidus* (see discussion Chapter 3), it may not be necessary for them to utilize dietary chitin, if chitin can be synthesized from glucose monomers like it is in shrimp (New, 1976). However, all crustaceans produce some chitinase for reabsorption of chitin from the exoskeleton prior to molting (Warner, 1977). In the shrimp *Palaemon serratus*, chitinase activity in the integument of the gut is very low except immediately prior to a molt (Spindler-Barth, Van Wormhoudt, Spindler, 1990). However, the chitinase activity remains at a constant, higher level in the shrimp's hepatopancreas regardless of molt. In order to further investigate this, enzyme assays will be performed on gut and hepatopancreas samples taken from animals sacrificed at the termination of this experiment.

Chapter 5. Activity of chitinase and cellulase in the gut and hepatopancreas of the juvenile blue crab, *Callinectes sapidus* (Rathbun), fed different diets

Summary

To confirm that the differences in growth rate were a result of differences in enzyme activity, chitinase and cellulase assays were performed on gut tissue samples from the previous experiment. Specific activity was highest in the foregut and hepatopancreas, with an average of 3.52 $\pm$ 0.16 (standard error) mU $mg^{-1}min^{-1}$ for cellulase, and 0.072 $\pm$ 0.159 mU $mg^{-1}min^{-1}$ for chitinase. The midgut, hindgut and gastric ceca had substantially lower specific activities overall, and were variable from tissue to tissue. Cellulase was higher in the foregut (4.54 $\pm$ 0.31 mU mg^{-1} min^{-1}) than the hepatopancreas (2.50 $\pm$ 0.30 mU $mg^{-1}min^{-1}$), whereas chitinase was higher in the hepatopancreas (0.117 $\pm$ 0.018 mU $mg^{-1}min^{-1}$) than in the foregut (0.0270 $\pm$ 0.0074 mU $mg^{-1}min^{-1}$). There was no effect of diet on specific activity of the enzymes. The levels of cellulase are similar to those of other primarily carnivorous crustaceans. Gastric juice from wild-caught adult *C. sapidus* had specific activities (cellulase = 1.92 mU mg^{-1} min^{-1}; chitinase = 0.104 mU mg^{-1} min^{-1}) similar to those of the juvenile hepatopancreas samples. The significantly lower chitinase activity suggests that chitin is not fully utilized in the diet. Both cellulase and chitinase specific activities were substantially lower in pooled samples from adult *C. sapidus*, indicating the possibility of ontogenetic shifts in enzyme activity.

Introduction

Endogenous cellulases and chitinases have been discovered in a variety of crustaceans and mollusks, including the prawns *Macrobrachium rosenbergii*, *Penaeus*

japonicus and *Pandalus borealis* (Esaiassen, Myrnes, Olsen, 1996; Gonzalez-Pena, Anderson, Smith, Moreira, 2002; Kono, Matsui, Shimizu, Koga, 1990; Watanabe, Kono, Aida, Nagasawa, 1998), six species of Tasmanian crabs (Johnston, Freeman, 2005), the lobsters *Jasus edwardsii* and *Homarus americanus* (Johnston, 2003; Lynn, 1990), the red claw crayfish *Cherax quadricarinatus* (Byrne, Lehnert, Johnson, Moore, 1999; Xue, Anderson, Richardson, Xue, Mather, 1999), the abalone *Haliotis discus* (Suzuki, Ojima, Nishita, 2003), and the blue mussel, *Mytilus edulis* (Xu, Janson, Sellos, 2001; Xu, Hellman, Ersson, Janson, 2000). While these enzymes have been positively identified in many species, very few studies examine the effects that the presence of these enzymes have on growth rates when the substrate is present in the diet. In a study involving the mud crab *Scylla serrata*, an endogenous cellulase was identified that, when fed a diet containing 47% cellulose, allowed for the same growth rates as when fed a diet high in protein and lacking cellulose (Pavasovic, Richardson, Anderson, Mann, Mather, 2004). To further complicate the issue, there is often an interaction between the diet offered and the specific activity of the enzyme. *Scylla serrata* had a fourfold higher specific activity for cellulase when fed 47% starch or cellulose than when fed 29% starch (Pavasovic, Richardson, Anderson, Mann, Mather, 2004). In the prawn *Macrobrachium rosenbergii*, cellulase activity positively correlated to the level of dietary cellulose (Gonzalez-Pena, Anderson, Smith, Moreira, 2002). The hepatopancreas is the site of carbohydrase synthesis in other crustaceans, which is then released into the foregut in the form of gastric juice (Johnston, Freeman, 2005; Johnston, Yellowlees, 1998).

We assayed for chitinase and cellulase in the hepatopancreas and different portions of the gut of intermolt crabs fed 20% chitin and 20% cellulose diets (described

in the previous chapter). We then determined the effect of different diets on specific activity, the localization of the enzymes in the gut and hepatopancreas, and compared specific activities of chitinase and cellulase. We believe we will find activity for both enzymes, since all crustaceans synthesize chitinase for molting, and crabs had similar growth rates when fed cellulose-containing diets as when fed the control. We expect these enzymes to be localized in the hepatopancreas and foregut, with higher activity in the hepatopancreas. We predict higher specific activity of enzymes in those diets containing the substrata that the enzyme degrades. We expect to find more cellulase activity than chitinase, since crabs had higher growth rates when fed 20% cellulose than when fed 20% chitin diets (Chapters 3 and 4).

Methods

Ninety juvenile crabs from a single hatchery-reared brood (167 days old) were obtained from the Center of Marine Biotechnology. We performed a 12-week feeding experiment (starting March 14, 2005: see Chapter 4) by using a commercial pelletized shrimp diet for which we replaced 20% of the wet weight (comprised of ground wheat derivatives) with either chitin or cellulose (see Chapter 4). On June 28, twenty-one crabs (7 intermolt crabs randomly chosen from each treatment) were weighed, measured, and sacrificed for tissue sample collection. Crabs were sacrificed by submersion in ice water for 5 minutes. The following tissue samples were collected from each animal under a dissecting microscope: hepatopancreas, foregut, midgut, hindgut, anterior gastric ceca, and posterior gastric cecum. Gut tissues were carefully removed of all contents before collection. Tissues were placed in 1.5 mL Eppendorf tubes and stored at -80^{o}C.

Frozen tissues were weighed and combined with extraction buffer (80 mM sodium acetate buffer, pH 4.5). The solutions were homogenized with Eppendorf micropestles for 1.5-2 mL tubes for 5 minutes and then centrifuged at 16.1K g for 10 minutes. The supernatant was placed in a clean tube and served as the extract for both the chitinase and cellulase assays. For the hepatopancreas samples, a thin layer of lipid on top of the sample required pipetting out the middle layer as the extract. Extracts were used for both enzyme assays the same day that they were made. For the midgut, hindgut, anterior and posterior gastric ceca, the samples were too small (< 10 mg) to detect enzyme activity and protein for the quantity of extract required in the following assays, so tissues were combined to make one extract per treatment. For the hepatopancreas and foregut, only 5 of the 7 animals dissected in the 20% cellulose and 20% chitin-fed treatments were used for the following enzyme assays.

Cellulase activity was quantified using a dye-linked substrate, Azo-CM-Cellulose (Megazyme, Ireland). Triplicate samples of enzyme extract were added to extraction buffer, making a total of 100 uL of enzyme solution. Substrate solution (100 uL) was then added to the extract, and the mixture was shaken at 25^{o}C for 2 hours on a thermomixer at 1400 cycles per minute. The reaction was terminated by addition of 500 uL of precipitant solution (prepared according to the assay protocol), stirred on the thermomixer at 1400 cycles per minute for 10 seconds, and allowed to equilibrate at room temperature for 10 minutes. The tubes were then centrifuged at 16.1K g for 10 minutes and 200 uL of supernatant from each tube was placed in a clear 96-well flat-bottom microplate. The blank was prepared the same as for the above samples, with the exception that the precipitant solution was added immediately after addition of the

substrate. The plate was then read on a Vmax Kinetic Microplate Reader (Molecular Devices Corp., Sunnyvale CA) at 595 nm. Optical density (O.D.) values were corrected by subtracting the mean value for the blank from the sample O.D values. Corrected O.D. values were converted to activity units in reference to a standard curve for purified *T. reesei* cellulase. One unit of activity (U) is equal to 1 uM of glucose liberated from cellulose under the conditions described above. MilliUnits of activity for the 100uL assay volume were equivalent to 148.64(O.D.). Preliminary studies were performed to ensure that the rate of reaction was proportional to time and enzyme concentration at the temperature tested.

Chitinase activity was quantified using tritium-labeled chitin as substrate, based on the methods of Molano (1977). The 100ul assay solution consisted of 5 ul of 1M ammonium acetate pH 4.5, 15 ul chitin suspension, extract and extraction buffer. Samples were run in triplicate, and the blank excluded extract. The samples were incubated on a thermomixer at 1400 cycles per minute and 25^{o}C for 2 hours. The constant action of the thermomixer kept the chitin constantly suspended and therefore available for interaction with the assay sample. The enzymatic reaction was stopped and insoluble chitin is precipitated with 300 ul of 10% TCA. The mixture was centrifuged for 5 minutes at 20.8K g and 200 ul of the supernatant was carefully removed so as not to disturb the chitin pellet. The supernatant was combined with 400 uL of BioSafe II cocktail (RPI, IL) in a liquid scintillation tube. Disintegrations per minute (DPM) for each sample was read on the Liquid Scintillation Counter 1801 (Beckman Coulter Inc., CA). DPM was then converted to ug N-acetyl-D-glucosamine produced by measuring the DPM for a known amount of tritium-labeled chitin incubated with enzyme overnight, so that all of the

substrate was digested. One unit of N-acetyl-D-glucosamine was equivalent to 1036 DPM. One unit of activity (U) is equal to 1 uM of chitobiose liberated from chitin under the conditions described above.

Protein was quantified using the Micro BCA Protein Assay Kit (#23235; Pierce, IL). The 200ul assay solution consisted of 100 ul working reagent, extract, and extraction buffer. Samples were run in triplicate, and the blank excluded extract. A set of BSA standards was run on each plate, ranging from 4-20 ug protein. After addition of the working reagent, the plate was mixed on a plate shaker for 30 seconds, covered and incubated at 37^{o}C for 2 hours. It was then cooled to room temperature and measured on the Vmax Kinetic Microplate Reader (Molecular Devices Corp., Sunnyvale CA) at 540 nm. Raw O.D. values were corrected by subtracting the average value for the blank from the sample O.D value.

Specific activity is expressed in mU mg protein^{-1} min^{-1}. The means of the triplicate samples were used for the following data analyses. A factorial ANOVA was performed on specific activity of hepatopancreas and foregut samples, with tissue type (foregut versus hepatopancreas), diet (20% cellulose versus 20% chitin), and enzyme (cellulase and chitinase) as factors. A factorial ANOVA was performed on the pooled samples for the other four tissue types, comparing the effects of diet and enzyme on specific activity. For both factorial ANOVAs, specific activity was log-transformed to meet homogeneity of variances and normality assumptions. Due to log-transformations, one sample from the foregut and hepatopancreas samples and two samples from the midgut, hindgut, and gastric ceca samples obtained values that were negative, and so were omitted from the data analysis.

Results

Specific activity was highest in the foregut and hepatopancreas, with an average of 3.52 ± 0.16 (standard error) mU $mg^{-1}min^{-1}$ for cellulase, and 0.072 ± 0.159 mU $mg^{-1}min^{-1}$ for chitinase. The midgut, hindgut and gastric ceca had substantially lower specific activities overall, and were variable from tissue to tissue (Table 18). This may have occurred because the total amount of material was so small (< 2 mg after combining samples) that the activities and protein levels were about equal to the blank, and were therefore difficult to measure accurately.

Table 18. Specific activity (mU mg^{-1} min^{-1}) of cellulase and chitinase in the gut of *C. sapidus* fed 20% cellulose vs. chitin diets, ± SEM. N=5 for hepato., foregut samples; N=1 for other tissues.

Enzyme	Tissue	20% cellulose diet	20% chitin diet
cellulase	hepato.	2.71 ± 0.53	2.29 ± 0.033
	foregut	4.29 ± 0.40	4.79 ± 0.050
	midgut	1.72	0.0856
	hindgut	0.144	0.150
	anterior g.c.*	0.764	1.86
	posterior g.c.	0	0.468
chitinase	hepato.	0.128 ± 0.026	0.106 ± 0.027
	foregut	0.0186 ± 0.0110	0.0353 ± 0.0096
	midgut	0.0407	0.0142
	hindgut	0.00317	0.0824
	anterior g.c.	0.0239	0
	posterior g.c.	0.00445	0.0511

* g.c. = gastric ceca

There was a significant model effect in the factorial ANOVA run for hepatopancreas and foregut samples ($F_{(7,31)} = 100$, $p < 0.0005$; Table 19). A significant difference existed between tissue types ($F_{(1,31)} = 6.94$, $p = 0.013$), but this was confounded by a significant interaction between tissue type and enzyme type ($F_{(1,31)} = 43.4$, $p < 0.0005$). The interaction effect is due to converse activities of the enzymes in the different tissues. Cellulase was higher in the foregut (4.54 ± 0.31 mU mg^{-1} min^{-1})

than the hepatopancreas (2.50 ± 0.30 mU $mg^{-1}min^{-1}$), whereas chitinase was lower in the foregut (0.0270 ± 0.0074 mU $mg^{-1}min^{-1}$) than the hepatopancreas (0.117 ± 0.018 mU $mg^{-1}min^{-1}$).

Table 19. Statistical results from factorial ANOVA performed on log-transformed specific activity (mU mg^{-1} min^{-1}) of foregut and hepatopancreas samples.

Source	Type III SS	df	Mean Square	F	Significance
Model	33.7	7	4.81	100	< 0.0005
Enzyme*	31.9	1	31.9	664	< 0.0005
Tissue*	0.333	1	0.333	6.94	0.013
Diet*	6.70E-05	1	6.70E-05	0.140	0.711
Enzyme x Tissue	2.08	1	2.08	43.4	< 0.0005
Enzyme x Diet	1.02E-02	1	1.02E-02	0.212	0.648
Tissue x Diet	0.101	1	0.101	2.10	0.157
Enzyme x Tissue x Diet	2.43E-02	1	2.43E-02	0.507	0.482
Error	1.49	31	4.80E-02		
Corrected Total	35.2	38			

* Enzyme = chitinase versus cellulase; Tissue = hepatopancreas vs. foregut; Diet = 20% cellulose vs. chitin

There was a significant model effect in the factorial ANOVA for midgut, hindgut, and gastric ceca pooled samples ($F_{(3,10)}$ = 8.15, p = 0.005; Table 20). The model effect was attributed to a significant main effect of enzyme type ($F_{(1,10)}$ = 21.0, p = 0.001). Cellulase had a mean specific activity of 0.636 ± 0.205 mU mg^{-1} min^{-1}, while chitin had a mean specific activity of 0.0206 ± 0.205 mU mg^{-1} min^{-1}.

Table 20. Statistical results from factorial ANOVA performed on log-transformed specific activity (mU mg^{-1} min^{-1}) of pooled midgut, hindgut, and gastric ceca samples.

Source	Type III SS	df	Mean Square	F	Significance
Model	6.96	3	2.32	8.15	0.005
Enzyme*	5.99	1	5.99	21.0	0.001
Diet*	8.28E-02	1	8.28E-02	0.291	0.601
Enzyme x Diet	0.554	1	0.554	1.95	0.193
Error	2.85	10	0.285		
Corrected Total	9.81	13			

* Enzyme = chitinase versus cellulase; Diet = 20% cellulose vs. chitin

We were also able to isolate gastric juice from the foreguts of 5 crabs (purchased at a local farmers market), which we pooled and analyzed for specific activities of chitinase and cellulase. Cellulase activity (1.92 mU mg^{-1} min^{-1}) was approximately

twenty-fold the level of chitinase activity (0.104 mU mg^{-1} min^{-1}) in the gastric juice. Specific activity was 1.47 mU mg^{-1} min^{-1} for cellulase and 0.0638 mU mg^{-1} min^{-1} for chitinase in the hepatopancreas of the same animals.

Discussion

We found both chitinase and cellulase activity in all of the tissues examined, as well as in the hepatopancreas and gastric juice of adult wild-caught crabs (Table 18). Previously, McClintock et al. (1991) found low but detectable amounts of *B*-glucosidase activity in *C. sapidus* hepatopancreas (0.0007 ± 0.0001 mU mg^{-1} min^{-1}). While cellobiose was used as a substrate in the study, larger oligomers and cellulose chains were not tested. Our study shows a much higher specific activity for cellulase (Table 19, Table 20), indicating that the cellulase(s) in *C. sapidus* cleave glucose monomers from a mixture of complex substrates. To our knowledge, no previous studies have assayed for chitinase activity in blue crabs.

Cellulase activity was comparable to levels for other crustaceans. In hepatopancreas samples from six species of Tasmanian crabs ranging from carnivorous to herbivorous, cellulase activity ranged from 1.4 – 19 mU mg^{-1} min^{-1} respectively (Johnston, Freeman, 2005). Cellulase activity in juvenile *C. sapidus* hepatopancreas (2.50 ± 0.30 mU $mg^{-1}min^{-1}$) falls within this range, slightly higher than the cellulase activity of primarily carnivorous crabs. However, *C. sapidus* cellulase activity is approximately equal to cellulase activity in the hepatopancreas of the juvenile spiny lobster *Jasus edwarsii* (2.1 ± 0.6 mU mg^{-1} min^{-1}), also a consumer of primarily animal material (Johnston, 2003). In the spiny lobster, protease activities were 3-4 orders of magnitude higher than carbohydrases. Conversely, cellulase activity in the crayfish

Cherax quadricarinatus had a specific activity of 70 mU mg^{-1} min^{-1} in the digestive gland (homologous to the hepatopancreas), indicating that crayfish are more capable of utilizing lower trophic levels as a food source (Xue, Anderson, Richardson, Xue, Mather, 1999).

What may be regarded as surprising is that primarily carnivorous species (determined by gut contents) have any cellulase activity at all. The conclusion that the cellulase activity is simply a byproduct of the organisms consumed may be incorrect. It was previously assumed that higher animals did not produce endogenous cellulases, and that animals with this activity must acquire it from symbiotic microorganisms (Watanabe, Tokuda, 2001). However, this has been proven false for many species of crustaceans and mollusks (Byrne, Lehnert, Johnson, Moore, 1999; Suzuki, Ojima, Nishita, 2003; Xu, Janson, Sellos, 2001; Xu, Hellman, Ersson, Janson, 2000; Xue, Anderson, Richardson, Xue, Mather, 1999). Molecular characteristics of the majority of these cellulases are very similar, suggesting that they evolved from a common ancestor (Watanabe, Tokuda, 2001). Therefore, functionally carnivorous species may possess cellulase as a periphery enzyme. This can prove to be an advantage when resources are scarce (Dittel, Epifanio, Schwalm, Fantle, Fogel, 2000), so perhaps defining *C. sapidus* as carnivorous may be too limited a characterization of its trophic level. This is particularly true for juvenile *C. sapidus*, for which approximately 20% of the diet (< 80 mm carapace width) consists of detritus and plant material (Laughlin, 1982; Stoner, Buchanan, 1990). The diet experiments (Chapters 4 and 5) showed that the inclusion of 20% cellulose produced the same growth rates as the control diet. Therefore, even the low specific activity that we found in this study is enough to hydrolyze that percentage of cellulose in the diet.

Cellulase specific activity was higher in the foregut (4.54 $\pm$ 0.31 mU mg^{-1} min^{-1}) than in the hepatopancreas (2.50 $\pm$ 0.30 mU $mg^{-1}min^{-1}$). These findings are contradictory to those of McClintock et al. (1991) for *C. sapidus* carbohydrases, but consistent with cellulase in the prawn *Macrobrachium rosenbergii* (Gonzalez-Pena, Anderson, Smith, Moreira, 2002). The specific activities of *C. sapidus* carbohydrases did not include general cellulases, investigated in *M. rosenbergii*, which may account for this inconsistency. Carbohydrases originate in the hepatopancreas of crustaceans, shown by mRNA *in situ* hybridization in *Penaeus monodon* (Lehnert, Johnson, 2002). In addition, the hepatopancreas has the highest specific activity of all gut tissues in six species of crabs (Johnston, Freeman, 2005). Cellulase is then secreted into the foregut in the form of gastric juice, which has a higher specific activity than the hepatopancreas in *Cherax quadricarinatus* (Xue, Anderson, Richardson, Xue, Mather, 1999). We also found higher cellulase activity in the gastric juice than in the hepatopancreas of pooled samples for adult *C. sapidus*. The elevated specific activity in the foregut and gastric juice suggests that cellulase is concentrated in the foregut to aid in digestion of raw materials.

Cellulase activity was significantly higher than chitinase for all tissue types investigated (Table 19, Table 20). Chitinase activities in other crustaceans are variable, and less associated with trophic level than cellulase. In the same six species of Tasmanian crabs, chitinase activity ranged from 23 – 41 mU mg^{-1} min^{-1} in the hepatopancreas (Johnston, Freeman, 2005). We did not find such high specific activity in *C. sapidus* hepatopancreas (0.117 $\pm$ 0.018 mU $mg^{-1}min^{-1}$). Considering the inhibitory affect that dietary chitin had on growth in the previous experiments (Chapters 3 and 4), it appears to have no functional importance to digestion. In the shrimp *Palaemon serratus*,

chitinase activity is very low in the gut except immediately prior to a molt (Spindler-Barth, Van Wormhoudt, Spindler, 1990). Since we ensured that the animals sacrificed for tissue samples were all intermolt, this pattern seems to apply to *C. sapidus*. From this perspective, we can conclude that the primary purpose of chitinase in the blue crab is for molting.

Chitinase specific activity was higher in the hepatopancreas (0.117 ± 0.018 mU $mg^{-1}min^{-1}$) than in the foregut (0.0270 ± 0.0074 mU $mg^{-1}min^{-1}$) at intermolt. In *Palaemon serratus*, chitinase activity remains constant in the midgut gland, and high relative to the integument, except during a molt (Spindler-Barth, Van Wormhoudt, Spindler, 1990). For both of these species, it appears that chitinase is more important for molting than for daily digestion. The only inconsistency is for the gastric juice of adult *C. sapidus*, which had a higher chitinase activity (0.104 mU mg^{-1} min^{-1}) than the hepatopancreas (0.0638 mU mg^{-1} min^{-1}). The adult crabs were not molt-staged, however, and it is possible that a couple of the adult males sampled were premolt. We suggest that chitinase is stored in the hepatopancreas until molting is imminent, at which point it is released into the foregut in concentrated amounts as gastric juice to aid in the degradation of the old cuticle at the anterior and posterior ends of the gut.

We chose to focus on the chitinase enzyme that uses an endo-mechanism to hydrolyze polysaccharides because many of the mollusks and crustaceans previously investigated had incomplete enzyme systems consisting solely of endoglucanases and endochitinases (Byrne, Lehnert, Johnson, Moore, 1999; Kono, Matsui, Shimizu, Koga, 1990; Suzuki, Ojima, Nishita, 2003; Xu, Hellman, Ersson, Janson, 2000). The limited hydrolytic capacity of these invertebrates may have occurred because they do not need

the complete system, since symbiotic microflora in the gut synthesize the missing enzymes, cleaving small oligomers into individual sugar units (Tomme, Warren, Gilkes, 1995). Future work should be performed to determine whether blue crabs possess the enzymes to cleave N-acetylchitobiose.

There was no difference in specific activities of either enzyme in relation to diet (Table 19, Table 20). In the prawn *Macrobrachium rosenbergii* and the mud crab *Scylla serrata*, cellulase activity was higher with increased proportions of dietary cellulose (Gonzalez-Pena, Anderson, Smith, Moreira, 2002; Pavasovic, Richardson, Anderson, Mann, Mather, 2004). That there was no up- or down-regulation of enzymes suggests a lack of phenotypic plasticity in blue crabs. However, the rapid increase and decrease in digestive enzymes seen in these two species could reflect fluctuations in populations of endosymbionts in the gut flora. At least part of the cellulase activity in *M. rosenbergii* was attributed to microorganisms in the gut, proven when the activity dropped upon treatment with antibiotics (Gonzalez-Pena, Anderson, Smith, Moreira, 2002). Whether cellulase was endogenous or exogenous was not investigated for *S. serrata* (Pavasovic, Richardson, Anderson, Mann, Mather, 2004). That neither cellulase nor chitinase activity changed in relation to diet provides evidence that these enzymes are of endogenous origin. However, since we performed our assays on whole gut and tissue extracts of animals that were not treated with antibiotics, we cannot conclusively determine whether the cellulase and chitinase activity is endogenous or exogenous. This issue is not of primary concern to our study, since we were more interested in the functional outcome of the enzymes. The determination of origin of the enzymes can be performed in future

studies through purification, cloning and sequencing procedures (Watanabe, Kono, Aida, Nagasawa, 1998; Xue, Anderson, Richardson, Xue, Mather, 1999)

Compared to juveniles, both cellulase and chitinase specific activities are lower in adult *C. sapidus*. Since the adult samples were pooled and there are no replicate samples, this data is tentative and any conclusions should be approached with caution. It is possible, however, that there are ontogenetic changes in enzyme activity, which should be further investigated. Since the percentage of detrital and plant material declines with increasing carapace width in wild blue crabs (Laughlin, 1982; Stoner, Buchanan, 1990), it is possible that the suite of carbohydrase activities may also decline.

Chapter 6: Discussion

The experimental system

Rearing juvenile blue crabs in a recirculating system was successfully undertaken for multiple broods over the course of several months. This can be difficult to achieve with blue crabs, and high mortality and disease is common (Biddle, Millikin, Fair, Fortner, 1978; Guerin, Stickle, 1997; Winget, Epifanio, Runnels, Austin, 1976). We were able to maintain good water quality in our system throughout the feeding experiments by using of mechanical and biofiltration, in addition to partial water changes. The 15 x 15 x 10 cm compartment size and mesh compartment floors allowed for enough water exchange with the tank that the dissolved oxygen in each compartment remained high, while the mesh was small enough that food particles were retained in the cages. There was some interaction effect that restricted growth and reduced conversion efficiency in the small (10 cm^3) compartments when fed higher rations, so these compartments were not used after the initial experiment. The extremely low mortality in our studies and absence of any indication of disease demonstrated that this is an excellent system for future long-term feeding experiments. In the future, a larger range of cage sizes should be studied to determine the point where compartment size no longer limits growth. Other parameters of the system, such as water depth, production costs, and optimal yield should be investigated in the future to optimize the system.

Experimental diets

Feeding commercial pelletized shrimp diets to juvenile *C. sapidus* proved a realistic option for the rearing of crabs. The combination of high survival and good growth and molting in the feeding experiments showed the Ziegler (Ziegler Bros, Inc., Gardners, PA,

USA) formulated diet to be adequate for the animals' needs. The Melick (Melick Aquafeed, Catawissa, PA) formulated diets, once coated with water-stabilizing additives, were also adequate for maintaining juvenile crabs. It is easier to achieve a balanced diet including all essential amino acids, lipids, sterols, vitamins and minerals in a multi-ingredient diet containing different protein sources, such as the commercial diets we used (New, 1976). The practicality of having an easily produced, inexpensive, and storable diet proved prevailingly important for a long-term feeding experiment.

Water stability is desirable not only because of the crabs' feeding behavior of gradually consuming it over several hours, but it also helped to preserve water quality in the recirculating system (New, 1976). Although the Melick diets were not initially water-stable, providing an oil coating improved the stability to a certain extent, and compensated for its lack of water-stability by the added nutritional content. The oil coating improved palatability so that the animals consumed the diet more quickly, and it provided an extra energy source to compensate for nutrients lost due to leaching. Spraying the Melick diets with a combination of alginate and calcium chloride was a more effective method of improving the water stability. The combination of water stability and palatability of the diets proved effective in providing for the animals' needs, and resulted in high survival and growth rates. The average proportion of the diet consumed was the highest for the alginate-coated diets (0.94 ± 0.01), showing the highest palatability within four hours of feeding.

In the future, various aspects of the diet composition should be investigated so that the diet is not providing more protein content than is needed, as this is wasteful and fish meal is expensive (Naylor, Goldburg, Primavera, Kautsky, Beveridge, Clay, Folke,

Lubchenco, Mooney, Troell, 2000). In shrimp, it is possible to decrease the protein content if the caloric content is increased (New, 1976). This should be investigated for blue crabs in the future. However, using a commercial shrimp diet from the beginning gives us a jump-start for achieving the goal of economic feasibility.

Rations

Depending on the priorities of the grow-out facility, optimal rations can be determined in various ways. From an energetic viewpoint, the ration resulting in the highest conversion efficiency is the best ration to deliver. In Chapter 2, the food conversion efficiency (dry weight gained (dry weight fed)$^{-1}$) increased rapidly as ration increased up to the 50% ration, and then declined (Table 21). This conversion efficiency continued to decline from the low to the high rations in Experiment 1, showing a continuum of conversion efficiency between the two experiments. We can therefore conclude that the optimal ration to use (C_{opt}) is the 50% ration in Experiment 2. This results in the most economical use of diets, when diet is the highest cost involved in grow-out. However, we have determined diet to be a fairly inexpensive commodity, while electrical costs including water heating, aeration and water pumps for running the recirculating systems are relatively high. To reduce these costs, the priority is to grow animals as quickly as possible to maturity, and to perform experiments with equal speed. Therefore, the 50% ration is not the ideal ration for our purposes. Instead, we should use the intermediate or high ration from Experiment 1 for future diet studies, as they resulted in the highest growth rates. Even though the differences in growth were not as obvious for the rations delivered in Experiment 1, they could still be statistically distinguished from one another, so any treatment effects should be apparent in future dietary studies.

The intermediate ration we used for the diet experiments, based on recommended rations for penaeid shrimp, was sufficient for the crabs' metabolic needs. Rations for the crayfish *Cherax quadricarinatus* are around 4% of the animals' body weight day^{-1}, starting at 10g body weight (Manor, Segev, Leibovitz, Aflalo, Sagi, 2002). The shrimp rations we chose for our diet studies had a range of 2.8-3.9% body weight day^{-1} at 10g body weight, and then reached an asymptote at about 2% body weight day^{-1} (Guillaume, Kaushik, Bergot, Metailler, 1999), which is approximately the rate of ingestion for the juvenile shrimp *Penaeus setiferus* (Sick, White, Baptist, 1973). This appears to be a more fine-tuned method of ration calculation, since the rations continuously change as the animal increases in size. It allows the aquaculturist to be more efficient, reducing costly and wasteful overfeeding. We did not find significant differences in ingestion rates when rations increased because the rations we delivered were low enough that the animals were food-limited for all of them. Feeding specific rations helps prevent confounding of treatment effects in nutritional experiments (Tenore, Hanson, McClain, Maccubbin, Hodson, 1984).

Table 21. Growth parameters for juvenile *C. sapidus* in large cages of experimental system, ± standard error. From Chapter 2.

Ration	Time to molt 1 (days)	% Increase in CW (molt 1)	Conversion Efficiency
Experiment 1			
high	9 ± 1.3	18 ± 1	0.15 ± 0.01
intermediate	9 ± 1.6	20 ± 1	0.16 ± 0.01
low	11 ± 1.9	21 ± 2	0.18 ± 0.01
Experiment 2			
100% of 'low' (Chapter 2)	7 ± 4	21 ± 5	0.15 ± 0.06
75%	11 ± 2	20 ± 2	0.18 ± 0.04
50%	12 ± 3	21 ± 1	0.30 ± 0.06
25%	20 ± 3	17 ± 2	0.27 ± 0.05
0%	21 ± 15	12 ± 5	N/A

Conversion Efficiency = dry weight gained (dry weight fed)$^{-1}$

Measures of Growth

Growth parameters were analogous to literature values for blue crabs, showing the experimental system, diets, and rations to be acceptable for juvenile *C. sapidus*. Molt frequency was comparable to other studies with similar-sized crabs, both in actual values (range: 12.4 - 50 days; Table 22) and in the trend of frequency decreasing with each successive molt (Cadman, Weinstein, 1988; Guerin, Stickle, 1997; Leffler, 1972; Millikin, Biddle, Siewicki, Fortner, Fair, 1980) (Table 7, Table 8). Percent increase in carapace width is comparable to published values at 20-26^{o}C, which fall between 11.8-26% (Table 22). Values were also comparable to published data for similar-sized juvenile *C. similis*, a close relative to *C. sapidus* (Table 22). The comparison to previous laboratory studies shows that our crabs exhibited normal growth and molting patterns. Among the feeding trials we performed, the average intermolt period ranging from 18-32 days was related to the length of the study, due to shorter intermolt periods in smaller crabs. But when comparing our experiments to other experiments of the same duration, the intermolt periods were similar. The molt increments (x 100%) for our feeding trials, ranging from 18-26%, were higher than most of the other studies performed (Table 22).

Table 22. Intermolt periods and percent increase in carapace width (CW) for juvenile *C. sapidus* and *C. similis*: Comparative data from previous and current studies.

Study	Salinity (gL^{-1})	Temperature (^oC)	CW (mm)	Intermolt Period (days)	% Increase in CW	Experiment Duration (days)
C. sapidus						
Millikin et al. (1980)	20-25	23	24*	33.6	13.2	126
Cadman and Weinstein (1988)	3-30	19	20-30*	30.5	-	1 ecdysial cycle**
	3-30	23	20-30*	18.0	-	1 ecdysial cycle
	3-30	26	20-30*	21.0	-	1 ecdysial cycle
Das and Stickle (1993)	30	24	20*	12.4	21.7	28
Guerin and Stickle (1997)	2.5-30.0	21	25-35	33.6	11.8	67
Leffler (1972)	15-27	20	20-69	32.61	24.41	180
Winget et al. (1976)	20	20	59	50	15	60
Our animals - Chapter 2 Expt.1	28	23-26	20-30	32	22	148
Our animals - Chapter 2 Expt.2	29	23	19-34	18	19	42
Our animals - Chapter 3	28	25	18-28	25	26	149
Our animals - Chapter 4	29	23	24-40	31	18	86
C. similis						
Das and Stickle (1993)	30	24	20*	13.6	10.8	28
Guerin and Stickle (1997)	5.0-30.0	21	25-35	53.6	18.02	67

Since there was no significant salinity effect in the above studies or salinity effects were not measured, intermolt periods and increments shown are averaged across all salinities tested.

* = initial sizes were estimated from average wet weight of 1.25 g (Millikin et al. 1980), widthxweightxdepth volume of 2000-4000 mm (Cadman and Weinstein, 1988), and 686-735 mg wet weight (for *C. sapidus* and *C. similis* , respectively; Das and Stickle 1993).
** = Cadman and Weinstein (1988) study was performed for a period in which one ecdysial cycle could be completed, therefore results are not averaged across molts.

Growth rates are substantially lower in laboratory experiments than they are in the field. Crabs in field enclosures at Point Lookout, Maryland starting at approximately 4.0 g and 35 mm carapace width (determined using our allometric relationship between wet weight and carapace width) molted twice in 35 days (Brylawski, Miller, 2003). This is almost double the molt frequency than we found for animals of the same size in our experiments. There are various reasons why animals grow faster in the field. Among them is temperature, which reaches several degrees (celcius) higher in the Chesapeake Bay in summer than in our laboratory experiments (Cowan, Boynton, 1996). Preventing activity in the compartmentalized system also decreases metabolic rates, which may depress growth rates in the laboratory compared to the field (Brylawski, Miller, 2003). A more widely varied diet in the field may supply additional essential nutrients to *C. sapidus* that are largely unknown, which limits our ability to properly formulate diets for

laboratory experiments. These various obstacles may be difficult to overcome in rearing studies, and should be further investigated to improve growth in the laboratory.

Dietary Cellulose

In both dietary fiber experiments, there was no difference in growth between juvenile *C. sapidus* fed the control diet and the diets containing 14% or 20% cellulose. The acid-detergent fiber content in the oil- and alginate-coated control diet is 7.72% and 8.20% respectively, compared to a range of 13.39-19.90% in the experimental diets (Table 11, Table 15). If the cellulose were indigestible, the inclusion of twice as much fiber in the experimental diets would depress growth. In addition, the portion of the diet that we replaced had a high starch component, which blue crabs have the capacity to digest (McClintock, Klinger, Marion, Hsueh, 1991), so we replaced a portion of the diet containing energetic value with a potentially indigestible substance. This, combined with the presence of cellulase in the hepatopancreas samples, shows that blue crabs can digest cellulose. In shrimp, starch and carbohydrates are hydrolyzed to form glucose for use in the Krebs cycle, glycogen storage, chitin synthesis, and the formation of sterols and fatty acids (New, 1976). It is possible that juvenile blue crabs utilize complex carbohydrates for these purposes as well, and is a potential subject for further study.

In the wild, juvenile crabs less than 80 mm carapace width consume detritus and plant material that constitutes approximately 20% of their diet (Laughlin, 1982; Stoner, Buchanan, 1990). In light of these experiments, the consumption of plant material containing cellulose is an energetically important part of a juvenile blue crab's diet in the wild. In hepatopancreas samples from six species of Tasmanian crabs ranging from carnivorous to herbivorous, cellulase activity ranged from 1.4 – 19 mU mg^{-1} min^{-1}

respectively (Johnston, Freeman, 2005). Cellulase activity in juvenile *C. sapidus* hepatopancreas (2.50 $\pm$ 0.30 mU $mg^{-1}min^{-1}$) falls within this range (Table 18), slightly higher than the cellulase activity of the most carnivorous crab, *Carcinus maenas*, for which algae comprises approximately 10% of its diet. *C. sapidus* cellulase activity is approximately equal to cellulase activity in the hepatopancreas of the juvenile spiny lobster *Jasus edwarsii* (2.1 $\pm$ 0.6 mU mg^{-1} min^{-1}), which also has a minor algae constituent in its diet (Johnston, 2003). From the wide range of food items consumed by juvenile crabs and the broad descriptions of their dietary niche (Laughlin, 1982; Stoner, Buchanan, 1990), we can safely describe them as omnivorous. Although the majority of their diet consists of animal material, they are also able to derive energetic value from cellulose and starch in plant and detrital material, similar to other omnivorous crustaceans. Although the specific activity that we found in this study is low compared to that of the omnivorous scavenger *Cherax quadricarinatus* (70 mU mg^{-1} min^{-1}) (Xue, Anderson, Richardson, Xue, Mather, 1999), it is at least enough to provide the same level of growth in a diet containing 20% cellulose as a control diet.

Dietary Chitin

Suppression of growth by inclusion of dietary chitin and low chitinase activity suggest that chitin has little dietary contribution in *C. sapidus*. In both dietary fiber experiments, crabs fed 20% chitin had lower mean wet weights, specific growth rates, increments, and frequencies than crabs fed 20% cellulose or a combination of chitin and cellulose (Chapter 3, 4). While there is evidence that utilization of cellulose provides an ecological advantage for juvenile *C. sapidus*, it may not be necessary for them to utilize dietary chitin, if chitin can be synthesized from glucose monomers like it is in shrimp

(New, 1976). In six species of Tasmanian crabs, chitinase activity ranged from 23 – 41 mU mg^{-1} min^{-1} in the hepatopancreas (Johnston, Freeman, 2005). We did not find such high specific activity in *C. sapidus* hepatopancreas (0.117 $\pm$ 0.018 mU $mg^{-1}min^{-1}$) (Table 18). Considering the inhibitory affect that dietary chitin had on growth in the previous experiments (Chapter 3, 4) and the extremely low chitinase activity, it appears to have no functional importance to digestion. However, all crustaceans produce some chitinase for reabsorption of chitin from the exoskeleton prior to molting (Warner, 1977). In the shrimp *Palaemon serratus*, chitinase activity in the integument of the gut is very low except immediately prior to a molt (Spindler-Barth, Van Wormhoudt, Spindler, 1990). However, the chitinase activity remains at a constant, higher level in the shrimp's hepatopancreas regardless of molt. In our samples, chitinase specific activity was higher in the hepatopancreas (0.117 $\pm$ 0.018 mU $mg^{-1}min^{-1}$) than in the foregut (0.0270 $\pm$ 0.0074 mU $mg^{-1}min^{-1}$) at intermolt (Table 18). For both of these species, it appears that chitinase is more important for molting than for daily digestion.

Endogenous vs. Exogenous Origins

There was no difference in specific activities of either enzyme in relation to diet (Table 19, 20). In the prawn *Macrobrachium rosenbergii* and the mud crab *Scylla serrata*, cellulase activity was higher with increased proportions of dietary cellulose (Gonzalez-Pena, Anderson, Smith, Moreira, 2002; Pavasovic, Richardson, Anderson, Mann, Mather, 2004). That there was no up- or down-regulation of enzymes suggests a lack of phenotypic plasticity in blue crabs. However, the rapid increase and decrease in digestive enzymes seen in these two species could reflect fluctuations in populations of endosymbionts in the gut flora. At least part of the cellulase activity in *M. rosenbergii*

was attributed to microorganisms in the gut, proven when the activity dropped upon treatment with antibiotics (Gonzalez-Pena, Anderson, Smith, Moreira, 2002). Whether cellulase was endogenous or exogenous was not investigated for *S. serrata* (Pavasovic, Richardson, Anderson, Mann, Mather, 2004). That neither cellulase nor chitinase activity changed in relation to diet provides evidence that these enzymes are of endogenous origin. However, since we performed our assays on whole gut and tissue extracts of animals that were not treated with antibiotics, we cannot conclusively determine whether the cellulase and chitinase activity is endogenous or exogenous. This issue is not of primary concern to our study, since we were more interested in the functional outcome of the enzymes. The determination of origin of the enzymes can be performed in future studies through purification, cloning and sequencing procedures (Watanabe, Kono, Aida, Nagasawa, 1998; Xue, Anderson, Richardson, Xue, Mather, 1999)

Other Discoveries

In searching for the presence of chitinase and cellulase in the diet and whether the presence of these enzymes affects growth, we had some additional unlooked-for discoveries that could become topics of further research. Previously, evidence of multiple matings was discovered by the presence of two ejaculates in 12.4% of wild females. Physiologically, both ejaculates have access to the eggs, since the seminal fluid disappears over time, allowing spermatophores to mix in the spermathecae (Jivoff, 1997). However, the following fertilization of the eggs, sponge production, hatching, and survival of offspring have never been monitored to determine if the two fathers both played a role in brood production. The initial experiment proved that two males could both have offspring from a dual-inseminated female, and in a single brood. The half-

siblings had significantly different conversion efficiencies, indicating that genetics plays a role in growth (Table 9).

In addition to this finding, we provide the first evidence of compensatory growth in *C. sapidus*. In the first dietary fiber experiment, after switching diets, crabs in the 20% cellulose treatment molted more and had a higher specific growth rate than either the 20% chitin or control group (Figure 16). Compensatory growth is common in fish (Ali, Nicieza, Wootton, 2003) and has also been identified in the Chinese shrimp *Fenneropenaeus chinensis* (Wu, Dong, 2002; Wu, Dong, Wang, Tian, Ma, 2001). That juvenile *C. sapidus* appears to "make up" for loss of growth from previous nutrient limitation is an interesting avenue for future research, and may have management implications.

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