ABSTRACT

Title of Document: REPRODUCTIVE PHYSIOLOGY OF THE FEMALE BLUE CRAB, *CALLINECTES SAPIDUS*: SPAWNING INDUCTION AND VITELLOGENESIS

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In aquaculture, spawning is the baseline for production; therefore, the optimization of spawning conditions will directly increase production. The current study aims to optimize spawning conditions for *Callinectes sapidus* using environmental manipulations of photoperiod and temperature for induction while monitoring the physiological vitellogenin (VtG) levels during ovarian development and maturation. The photothermal manipulations for this study resulted in increased spawning events in 21^{o}C temperatures (compared to 11^{o}C and 15^{o}C) and complete darkness (0L:24D; compared to 8L:16D, 16L:8D, and 24L:0D) while 24L:0D and 11^{o}C suppressed spawning. When assessing the VtG levels in the hemolymph prior to, during, and after all spawning events, the VtG showed a decrease prior to spawning, and significant VtG activity was seen in 21^{o}C for all photoperiods. Overall, spawning and vitellogenesis are temperature dependent events with 67% of the females spawning in 21^{o}C. Photoperiod also has an effect on spawning, but not on vitellogenesis.

REPRODUCTIVE PHYSIOLOGY OF THE FEMALE BLUE CRAB, *CALLINECTES SAPIDUS*: SPAWNING INDUCTION AND VITELLOGENESIS

By

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ABBREVIATIONS

Hormones and Proteins

Anti-VT	Anti-Vitellin
CHH	Crustacean Hyperglycemic Hormone
GIH	Gonad-Inhibiting Hormone
GSH	Gonad-Stimulating Hormone
MIH	Molt-Inhibiting Hormone
VIH	Vitellogenesis Inhibiting Hormone
VT	Vitellin
VtG	Vitellogenin
VtGR	Vitellogenin Receptor

Experimental Methodology

ELISA	Enzyme Linked Immunosorbent Assay
RACE	Rapid Amplification of cDNA Ends

Other

ANOVA	Analysis of Variance
COMB	Center of Marine Biotechnology
BCARC	Blue Crab Advanced Research Consortium
NOAA	National Oceanic Atmospheric Administration
XO-SG	X-Organ-Sinus Gland Complex

SPECIES

	Common Name	Scientific Name
Crab	Blue Crab	*Callinectes sapidus*
	Rock Crab	*Cancer antennarius*
	Asian Shore Crab	*Hemigrapsus sanguineus*
	Golden King Crab	*Lithodes aequispinus*
	Stone Crab	*Menippe mercenaria*
		Ocypoda macrocera
		Ocypoda platytarsis
	Red King Crab	*Paralithodes camtschaticus*
	Blue King Crab	*Paralithodes platypus*
	Blue Swimmer Crab	*Portunus pelagicus*
	Japanese Blue Crab	*Portunus trituberculatus*
		Potamon potamios
	Mud Crab	*Scylla paramamosain*
	Mud Crab	*Scylla serrata*
Crayfish	Crayfish	*Cambarus virilis*
	Red Claw Crayfish	*Cherax quadricarinatus*
		Orconectes nais
	Northern Crayfish	*Orconectes virilis*
	Red Swamp Crayfish	*Procambarus clarkii*
		Procambarus simulans
Insect	Mosquito	*Aedes aegypti*
	American Dog Tick	*Dermacentor variabilis*
	Migratory Locust	*Locusta migratoria*
	American Cockroach	*Periplaneta americana*

	Common Name	Scientific Name
Lobster	American Lobster	*Homarus americanus*
	European Lobster	*Homarus gammarus*
	Spiny Rock Lobster	*Jasus edwardsii*
	Rock Lobster	*Jasus verreauxi*
	Caribbean Spiny Lobster	*Panulirus argus*
	Australian Spiny Lobster	*Panulirus cygnus*
	Spiny Lobster	*Panulirus elephas*
	Scalloped Spiny Lobster	*Panulirus homarus*
	California Spiny Lobster	*Panulirus interruptus*
	Japanese Spiny Lobster	*Panulirus japonicus*
	Ornate Rock Lobster	*Panulirus ornatus*
	Mud Spiny Lobster	*Panulirus polyphagus*
	Blue Spiny Lobster	*Panulirus versicolor*
	Mediterranean Slipper Lobster	*Scyllarides latus*
	Flathead Lobster	*Thenus orientalis*
Prawn / Shrimp	Freshwater Prawn	*Fenneropenaeus chinensis*
	Banana Shrimp	*Litopenaeus merguiensis*/ *Penaeus merguiensis*
	Giant Freshwater Prawn	*Macrobrachium rosenbergii*
	Witch Prawn	*Penaeus canaliculatus*
	Fleshy Prawn	*Penaeus chinensis*
	Brown Tiger Prawn	*Penaeus esculentus*
		Penaeus kerathurus
	Giant Tiger Prawn	*Penaeus monodon*
	Eastern King Prawn	*Penaeus plebejus*
	Green Tiger Prawn	*Penaeus semisulcatus*
	Blue Shrimp	*Penaeus stylirostris*
	Pacific White Shrimp	*Penaeus vannamei*
	Oriental River Shrimp	*Macrobrachium nipponense*
	Kuruma Shrimp	*Marsupenaeus japonicus*
	Grass Shrimp	*Palaemonetes paludosus*
	Grass Shrimp	*Palaemonetes pugio*
	Ridgeback Rock Shrimp	*Sicyonia ingentis*

CHAPTER 1: INTRODUCTION

ASPECTS OF AQUACULTURE

The diversity and quantity of the global fisheries have been declining rapidly since the 1950s (FAO, 2008). This rate of decline is due to: the increase in import/export around the world; lack of government implications to reduce the impact; and increased fishing pressure from the consumption of aquatic species (fish, crustaceans, mollusks, etc.) by underprivileged countries since these goods are labeled as inexpensive and healthy foods. Nonetheless, the global fisheries statistics being published by the FAO are often misleading due to China's overestimation of catch numbers and the connection made between declining wild populations and increasing aquaculture (Nomura, 2008; Pauly, 2008).

An increase in fishing pressure documented by the FAO since the 1950s has eliminated several species from the world's fisheries. The documented numbers for fish, crustaceans, mollusks, and other groups suggest that the aquaculture production is able to replenish the harvest. For example, when comparing the fisheries harvest of shrimp and prawns in 2006 (3,460,003 tons) with the aquaculture production of the same year (3,164,384 tons) (FAO, 2008), it appears that the input from aquaculture and harvest are almost equal; thus, there is no harm done to the wild population. However, aquaculture input for carp is about 27 times greater than the wild harvest. For tuna, the wild harvest is almost 450 times greater than the aquaculture production input. Thus, fisheries statistics starting in the 1950s show that the harvest has increased. However, for some

species, aquaculture production has allowed for a greater exploitation of reared species resulting in a reduction in the wild harvest (FAO, 2008).

CRUSTACEAN AQUACULTURE

Crustaceans (shrimp, prawns, lobsters, and crabs) are actively reared in aquaculture facilities throughout the world largely for consumption and/or research. The techniques and procedures for rearing animals have been designed and optimized through understanding the animals' behavior, physiology, and ecological requirements in order to allow for a seamless hatchery operation to occur with occasional modifications (Wickins and Lee, 2002). Gathering ecological information required for reproduction is essential and needs to be available to the hatchery prior to the rearing process to gain knowledge of the organism in the wild as a baseline for evaluating its behavior in captivity (Wickins and Lee, 2002). Currently, scientists are able to utilize environmental manipulations and molecular techniques to optimize production in the hatcheries.

Shrimp and prawn aquaculture is common worldwide. In many aquaculture facilities, the brood stocks are typically purchased from watermen with or without spawns (egg masses) present (Wickins and Lee, 2002). Females with egg masses are then acclimated in tanks for hatching, while females without egg masses are typically manipulated to shorten the ovarian maturation period by eyestalk ablation. This technique consists of removing one or both eyestalks of the animals by cauterizing, ligating, pinching, or slitting the eyestalk to remove the contents of the eye and destroy the eyestalk ganglia. The females are then acclimated and distributed to spawning tanks for copulation and spawning. Eyestalk ablation induces ovarian maturation by removing

the reproductive inhibitory hormones by the X-organ-sinus gland complex (XO-SG) located within the eyestalk (Bray and Lawrence, 1992; Kelemec and Smith, 1980; Liao and Chen, 1983; Okumura, 2004; Okumura and Aida, 2001; Okumura et al., 2007; Okumura et al., 2006; Sagi et al., 1997; Santiago, 1977; Webb, 1983; Wilder et al., 2002). Eyestalk ablation is an invasive technique which frequently compromises the quality of the eggs and larvae (Choy, 1987; Emmerson, 1980; Lawrence et al., 1980; Vaca and Alfaro, 2000) and the fitness of the female, often leading to her death (Choy, 1987; Suko, 1958). Therefore, several other forms of noninvasive manipulation have been explored.

Ovarian maturation has been indirectly and directly manipulated in crustacean aquaculture. It is thought that indirect environmental manipulations are better for inducing the females since there is less handling of the animals, thus, less stress resulting in a higher survival rate of the brood stock (Aktas et al., 2003; Crocos and Kerr, 1986; Primavera and Caballero, 1992). The indirect manipulations which have been tested include: modification of environmental factors encompassing light color (Hillier, 1984; Primavera and Caballero, 1992; Wang et al., 2003); light intensity (Chamberlain and Lawrence, 1981; Hoang et al., 2002; Nadarajalingam and Subramoniam, 1987; Pillai et al., 1988); photoperiod (Castanon-Cervantes et al., 1995; Dendy, 1978; Stevens, 1955); temperature (Hamasaki et al., 2004; Luis and Ponte, 1993; McConaugha et al., 1980; Sulkin et al., 1976); salinity (Yen and Bart, 2008); and changes in diet (Djunaidah et al., 2003; Luis and Ponte, 1993; Millamena and Quinitio, 2000). Direct forms of manipulation include: injection of serotonin (Meeratana et al., 2006; Wongprasert et al., 2006) or gonadotropin-releasing hormone (GnRH) (Ngernsoungnern et al., 2008) or the physical action of probing (the insertion of the tip of a forcep into the ovipores of a

mature female which is ovulating to release the eggs and sperm for spawning) (Pillai et al., 1988). According to these findings, it seems that the success rate of spawning largely depends on the species since all have different life cycles and stages that require different environmental changes to promote growth and reproduction. The majority of the studies focus on the manipulation of temperature and light intensity/photoperiod for shrimp (*Sicyonia ingentis*, *Penaeus merguiensis*, *Penaeus monodon*, *Fenneropenaeus chinensis*, *Penaeus stylirostris*, *Penaeus vannamei*, *Penaeus kerathurus*, *Palaemonetes pugio*, *Penaeus semisulcatus*, and *Penaeus esculentus*). It appears that these shrimp species stimulate ovarian development in conditions of low light intensities and/or warm water temperatures. Similarly, the majority of other studied crustaceans respond to warmer water temperatures for the stimulation of reproduction, especially lobsters (Aiken and Waddy, 1985; Hedgecock, 1983; Smith et al., 2003; Waddy, 1988).

Lobsters have been successfully reared in aquaculture to compensate for the declining populations, as seen in the American lobster (*Homarus americanus*) (Herrick, 1893). In these hatcheries, clawed (*H. americanus* and *Homarus gammarus*) and spiny lobsters (*Panulirus argus*, *Panulirus cygnus*, *Panulirus elephas*, *Panulirus homarus*, *Panulirus interruptus*, *Panulirus japonicus*, *Panulirus ornatus*, *Panulirus polyphagus*, *Panulirus versicolor*, *Jasus edwardsii*, *Jasus verreauxi*, *Scyllarides latus*, and *Thenus orientalis*) have been cultured; however, each species presents its own unique challenges (Wickins and Lee, 2002). For example, clawed lobsters need to be separated or restrained to reduce cannibalism and fighting during grow out, which makes the process of growing larger animals dependent on the amount of tank space available. Spiny lobsters are difficult to rear in the phyllosoma larval stages in captivity and juvenile

stages are not readily available from the wild for brood stock conditioning or grow out (Wickins and Lee, 2002). However, in facilities where spawning and grow out of both clawed and spiny lobsters occurs, the brood stock are induced to reproduce by photoperiod and/or temperature changes (MacDiarmid and Kittaka, 2000; Waddy, 1988). Once hatched, the larvae are reared in tanks until they reach juvenile stages where they remain in tanks for grow out or are released into the wild on reefs (Selgrath et al., 2007).

Several crab species are also important in aquaculture. In Asia, *Portunus trituberculatus* and *Scylla* sp. are either grown out for consumption or release into the wild to support the local fishery (Wickens and Lee, 2002). In crab aquaculture, the brood stocks are typically spawned when brought into the hatchery, and those which do not bear egg masses may undergo eyestalk ablation as described previously. This procedure is most commonly used since the spent female is normally removed from the production thus increasing genetic variation and quality in batches (Wickens and Lee, 2002). Interestingly, unlike shrimp and prawn aquaculture, there has been no mention of the quality of the eggs of these ablated crabs in the literature. Once the larvae are hatched, they are reared to juveniles for release into the wild or grown out in ponds or contained ecosystems to market size for sale. The Japanese release the juvenile crabs to help repopulate the area for commercial and recreational harvest. These animals are not tagged and thus are not monitored for their successful maturation to adulthood (Secor et al., 2002). However, the system of crab grow out in the Philippines and Japan has been very successful for several different crab species which allows the commercial watermen to rely less on the wild crab stock for harvest (Quinitio and Parado-Estepa, 2003; Secor et al., 2002).

In the United States, commercial crab species showing similar population declines include the red and blue king crabs (*Paralithodes camtschaticus* and *Paralithodes platypus*, respectfully) and the blue crab (*Callinectes sapidus*). In 1983, two years after the peak harvest season of *P. camtschaticus*, the population crashed and continues to remain low; similar historical occurrences are documented for *P. platypus* (Blau, 1997; NPFMC, 1998). These declines possibly occurred due to a period of poor recruitment caused by high fish predation and warmer climates (Blau, 1997). Since the decrease, the commercial fishing of these species has been prohibited in certain areas and limited in others; thus, watermen are forced to harvest other species, such as the golden king crab (*Lithodes aequispinus*), and/or move to different fishing grounds. Harvest limits have been set by the Alaska Department of Fish and Game for *P. camtschaticus*, *P. platypus*, and *L. aequispinus* to inhibit further or new population crashes within these harvestable species (NPFMC, 1998). The limitations and spot closures of harvesting in Alaska have allowed the populations to naturally replenish themselves permitting some of the closed fishing grounds to reopen based on yearly crab survey figures (NPFMC, 1998). However, in some locations, the population recruitment is still low; thus, the Alaskan Department of Fish and Game and NOAA began implementing king crab aquaculture for enhancement of the stock in 1998 for *P. camtschaticus* and 2004 for *P. platypus* (Persselin, 2006). The concept of stock enhancement involving these cold water species is complex since it takes about five to seven years for these species to mature. They carry their spawns for up to one year and produce hundreds of thousands of larvae which have slow development and minimal survival once hatched (Blau, 1997; NPFMC, 1998; Persselin, 2006). With a long life span of 20-30 years and a large size of up to a five foot

leg span (Blau, 1997; NPFMC, 1998), the time frame and space to make this concept feasible would be very costly (Stevens, 2006).

The hatchery production of *P. camtschaticus* and *P. platypus* is in the preliminary stages; however, a significant amount of information needs to be acquired about these species before full production of a hatchery is feasible (Stevens, 2006). To date, the hatchery has been able to successfully rear larvae to the settlement stage, but many questions have arisen beyond this point. The settled larvae engage in cannibalism, which decreases the production numbers significantly; therefore, a method to deter this activity needs to be developed. Also, once the larvae are large enough for release, a tagging system (microwire, elastomer, or genetic) and habitat release study need to be established to follow these crabs to determine the potential outcome of the project (Stevens, 2006). With a high rate of cannibalism and a very slow growth rate, a significant amount of research still needs to be conducted to increase this stock enhancement.

The Chesapeake Bay is also experiencing a decline in an important commercial species, *C. sapidus*. The crab population fluctuated in the Chesapeake Bay from 1968 to 2004 with the lowest abundance of exploitable crabs occurring in 1998 (Miller et al., 2005). With the overall population showing a decline, it can be stated that the recruitment in the Bay is also declining, which, as seen with the king crabs, can determine the fate of the species. An assessment of *C. sapidus* recruitment has been determined by surveying the mature female population in the Bay. Lipcius and Stockhausen (2002) found that between 1992 and 2000 the mature female population in the Bay decreased by 81% (Figure 1.1) which corresponds to the total population decline. After 2002, the mature female population showed an insignificant increase, but began to

decrease again in 2004 (Miller et al., 2005) with the second lowest harvest of *C. sapidus* in the Maryland region of the Chesapeake Bay occurring in 2007 (MDDNR, 2008a). This decline in mature female crabs could have a potentially harmful outcome for the production of the Chesapeake Bay if the population is not able to rebound. Hence, many conservation efforts are being studied and implicated, such as restoring crab habitats and strict fishing regulations (Lipcius et al., 2003; Secor et al., 2002). In 2008, Maryland enforced the following season and quantity regulations to aid the Chesapeake Bay crab population: mature female harvest limits were enforced from September 1st to October 22nd and the harvest of mature females was not allowed from October 23rd to December 15th. Harvest limits are based on the previous year's catch records that in some cases limit watermen to ten less bushels during the limited harvest period. Recreational female crabbing has also been prohibited (Griffin, 2008). Overall, the goal of the regulations is to reduce the harvest by 34% to allow the wild population to reestablish naturally (MDDNR, 2008c).

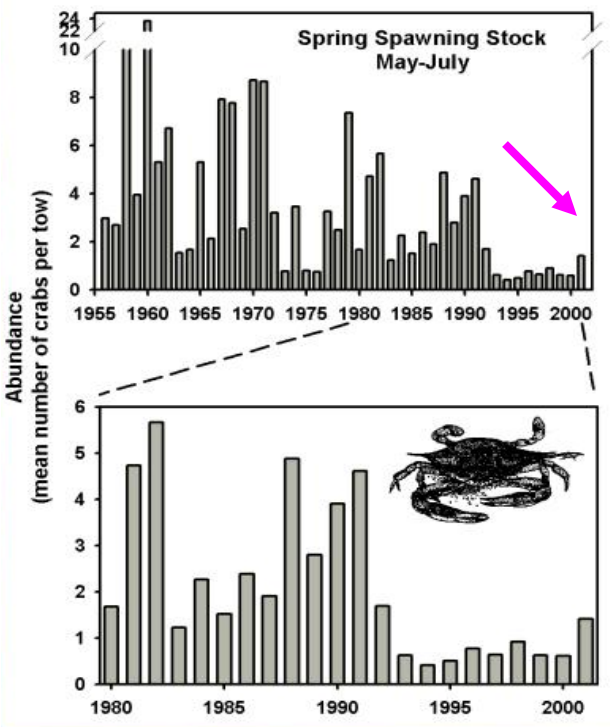


Figure 1.1. *C. sapidus* population decline in the Chesapeake Bay from 1992-2000. An 81% decrease occurred during the documented period which has not allowed population to rebound. Modified from Lipcius and Stockhausen (2002).

The limitation of harvest by the Department of Natural Resources maybe a viable option, but another approach is being evaluated by the Blue Crab Advanced Research Consortium (BCARC). This Consortium is aiming to increase *C. sapidus* breeding stock with a main focus on the Chesapeake Bay area by enhancement of the understanding of *C. sapidus* life cycle and biology, formation of a crab hatchery and nursery, closure of *C. sapidus* life cycle in captivity, and use of hatchery crabs to increase the wild population (Zohar et al., 2008). The ultimate objective for this program is to enhance the breeding stock in the Chesapeake Bay through scaling up the mass production of juveniles and allowing the watermen to manage the practice. However, the current and future spawning stocks need to be protected so the goals of BCARC can be achieved; therefore, support for the stock enhancement requires fishing regulations to be implemented.

Similar stock enhancement approaches have been applied to different shrimp, lobster, and crab species (Bell et al., 2005). The most successful accomplishments have been with the shrimp *P. japonicus* in Japan and *Penaeus chinensis* in China. In these locations, techniques have been applied to the populations since the 1960s, yet there is little information on the number of released and recovery rates until the 1990s when longer term tagging methods were available (Hamasaki and Kitada, 2006). The results from recapture studies show that with an increase in stock enhancement, there is an equal increase in harvest (Bell et al., 2005; Wang et al., 2006). However, Hamasaki et al. (2006) have seen a large die off of the released juvenile *P. japonicus* due to unsuitable and sparse shrimp nursery grounds which leads to the low numbers of recaptured and surviving animals. Neither increased harvesting nor fatality promote a positive stock enhancement environment which indicates that other regulations need to be put in place to allow for an advancement in the replenishment of the population (Bell et al., 2005; Hamasaki and Kitada, 2006).

In addition to shrimp, Bell et al. (2005) discusses stock enhancement methods used for spiny (*P. cygnus, P. ornatus,* and *J. edwardsii*) and clawed lobsters (*H. gammarus* and *H. americanus*). In these cases, it seems that the spiny lobsters are too expensive to grow and use for stock enhancement. To circumvent these issues, countries such as Australia and New Zealand are taking different approaches to increase the populations. In these countries, permits are given to farmers to obtain and grow spiny lobsters from larval stages for release, but the quota is dependant upon the country (Bell et al., 2005). These growth trials are still experimental and no significant data has been published to support the cause. The clawed lobsters, on the other hand, were first

cultivated in France in 1858 in an attempt to replenish the population and stimulate the fishery (Latrouite and Lorec, 1989). Almost 100 years after the first attempts to grow and release larvae within France, Norway, Canada and the United States, little information was available about the success of these lobsters once they were released and then the hatcheries were shut down (Nicosia and Lavalli, 1999). After several years of no hatchery production, the populations needed stimulation and the stock enhancement trials began again (Bell et al., 2005). However, these techniques became expensive and the results were not significant to allow for stock enhancement even though regulations were in place for the harvest of spawned females.

For shrimp, lobsters, and crabs, the cost and benefit analysis is important in the success of these programs. Without strict harvest regulations in place, as described above for *C. sapidus*, the aquaculture production and release of these species is negated, as seen with the *P. japonicus* populations in Japan (Bell et al., 2005).

CALLINECTES SAPIDUS AQUACULTURE

BCARC has approached the decline of *C. sapidus* breeding stock by producing a hatchery modeled after the shrimp, prawn, and crab facilities of Japan, Australia, and the Philippines (Secor et al., 2002; Zmora et al., 2005; Zohar et al., 2008). In this hatchery, female behavior and spawning activities are observed and manipulated with environmental conditions resulting in the production of spawns which, when hatched, allow for the growth and production of crabs year round. The female brood stock hatchery crabs are caught by watermen in the fall and brought into the facility. The female crabs are either kept in 16 hours of light and 8 hours of dark (16L:8D) at 21^{o}C

which simulates the summer conditions in the Chesapeake Bay (15L:9D, 27^{o}C) or 8L:16D at 15^{o}C which energetically arrests the crabs, allowing for a 'back-up' stock of females for continual production throughout the year. The summer exposure in the hatchery is limited to a maximum temperature of 21^{o}C due to an increase in the development of shell disease at higher temperatures. With these manipulated conditions, Zmora et al. (2005) are capable of producing broods year round allowing for a full year of larvae production in the hatchery (Zohar et al., 2008).

Several experiments utilizing environmental manipulations to induce spawning have been conducted in *C. sapidus* and other crustaceans as mentioned before. Of these experiments, temperature and photoperiod manipulations are most common. One such experiment was conducted by Sulkin et al. (1976) in which *C. sapidus* females were held at 15^{o}C and 19^{o}C in 10.25L:13.75D and 9.75L:14.25D over the winter period which resulted in two egg masses in the warmer condition and zero in the colder condition. The females were dissected after they died and mature ovaries were observed in the warmer crabs and undeveloped ovaries in the colder crabs. This suggests that temperature may be a key factor to initiate the development of the ovaries, while photoperiod may not be as significant (Sulkin et al., 1976). Likewise, temperature and photoperiod are important for the induction of spawning in the swimming crab, *Portunus trituberculatus,* when comparing 21^{o}C to natural temperatures from October (autumn) to May (spring) with photoperiods increasing from 12L:12D to 14L:10D. The results show that *P. trituberculatus* is stimulated to spawn when exposed to 21^{o}C and longer day lengths (Hamasaki et al., 2004). Similar findings were described in the grass shrimp, *Palaemonetes pugio* – a change in both temperature and photoperiod induced spawning

during the winter season for the shrimp (Little, 1968). However, the spawning events in all cases were not predictable.

Prawn and shrimp tend to show rapid ovarian development and spawn two to four weeks after eyestalk ablation (Choy, 1987; Kelemec and Smith, 1980; Okumura et al., 2007; Sagi et al., 1997). Conversely, in the adult female *C. sapidus*, which undergoes a terminal molt, eyestalk ablation is not effective since it induces molting and can result in mortality (Havens and McConaugha, 1990). In shrimp, crayfish, and lobsters it has been documented that successful eyestalk ablation can result in spawning; however, the larvae are often less viable than larvae from intact animals (Choy, 1987; Emmerson, 1980; Fast, 1992; Lawrence et al., 1980; Nakamura, 2000; Vaca and Alfaro, 2000). Since a variety of species, including *C. sapidus*, have been manipulated to spawn in captivity by altering the environmental conditions, the change in photoperiod and/or temperature may be used as an induction cue for ovarian maturation in aquaculture in place of eyestalk ablation.

Identifying the optimal manipulation of environmental conditions to regulate the physiology of reproduction in *C. sapidus* may allow for spawning at specific times in aquaculture. Spawning in the mouth of the Chesapeake Bay occurs in the spring (11-15^{o}C, 12L:12D), throughout the summer (27^{o}C, 15L:9D), and in the fall (17^{o}C, 12L:12D) (Hines et al., 2003). Further understanding of the reproductive biology of *C. sapidus* will allow for the control of spawning in captivity by simulating the natural environmental conditions. For the first aspect of this research, induction cues of manipulated light length and temperature regimes in a light tight, temperature controlled, closed recirculating system were assessed. Manipulation of the environmental cues of temperature to simulate the Chesapeake Bay warm months (21^{o}C) and colder months

(15°C and 11°C); and light during the longest day (16L:8D), shortest day (8L:16D), and constant darkness (0L:24D) and light (24L:0D) through a gradual acclimation period should provide a better induction indicator for spawning in *C. sapidus*.

CRUSTACEAN REPRODUCTION AND VITELLOGENESIS

The second aspect of this research will focus on the ovarian development of *C. sapidus* utilizing molecular techniques. In prawn and shrimp, the ovarian development can be visualized by shining a light through the transparent carapace of the animal; in many crab species, the carapace is opaque and this technique cannot be applied. The crab species *Ocypoda platytarsis*, *Ocypoda macrocera*, and *Scylla serrata*, which also have opaque carapaces, have been analyzed by drilling a small hole or cutting a flap in the carapace to observe the stage of ovarian development. The wound is then treated with antibiotics and closed securely with glue or wax (Nadarajalingam and Subramoniam, 1987; Nagabhushanam and Farooqui, 1982). When this technique was attempted with *C. sapidus*, it resulted in high mortality several days after the procedure was performed, possibly due to the lack of antibiotic treatment (O. Zmora, personal communication). It is necessary for this technique to be modified or a new technique to be developed to determine the stage of the ovarian development in the female crabs to predict the time of spawning in captivity.

The reproduction of *C. sapidus* begins when the immature female is preparing for her final molt to adulthood. The female and male release pheromones in their urine to form a mating pair so the male can carry the female before her terminal molt (Bushmann, 1999; Gleeson, 1991; Warner, 1977). The male senses her pheromone and performs a

copulation dance for the prepubertal female. The female then moves under the male where he will protect her during her molt; afterwards she will turn upside down under the male and mating will occur (Gleeson, 1991). During the mating, which may last 5-12 hours, the male injects his spermatophores and seminal products into the female's spermathecae. The male will then turn the female right side up and cradle her until her shell hardens (Gleeson, 1991). After the female hardens the male will release her and the female will begin developing her ovaries (Warner, 1977). During the ovarian development period in the Chesapeake Bay, the female migrates from the low salinity upper bay estuaries to the saltier mouth of the lower Bay. This migration benefits the larval development since less predation and osmotic pressure is present in the saltier waters for the larvae to develop and hatch (Carr et al., 2004; Morgan, 1990; Sandoz and Rogers, 1944). After the ovaries are developed and the female is ready to spawn, the eggs are fertilized internally before being extruded onto the sand. They are then picked up and attach to the ovigerous hairs on the pleopods (Warner, 1977). These egg masses can be composed of an average of three million eggs (Prager et al., 1990). Females are capable of producing more then one egg mass per spawning season as observed in two separate experiments where females were capable of spawning up to eight times in one season (Hines et al., 2003; Dickinson et al., 2006).

Ovarian development occurs in two phases: primary and secondary vitellogenesis (Charniaux-Cotton, 1985; Quakenbush, 1986). Primary vitellogenesis is the preparatory stage of oocyte development. During this stage, the oocytes grow slowly and start to accumulate yolk; ribosomes accrue in the cells and the rough endoplasmic reticulum prepares for the accretion of yolk by multiplying and producing glycoproteins internally

(Meusy and Charniaux-Cotton, 1984; Van Herp and Soyez, 1998). On the outer layer of the oocyte, the follicle cells tightly embrace the oocyte and will aid in the uptake of proteins for accumulation within the cell (Adiyodi and Subramoniam, 1983; Van Herp and Soyez, 1998). The oocytes then undergo a resting period before secondary vitellogenesis occurs (Meusy and Charniaux-Cotton, 1984).

In *C. sapidus* primary vitellogenesis is divided into three stages. Stage 1 begins when immature female ovaries are white (Lee et al., 1996a). Immediately after mating, the ovaries are white/pinkish and the spermathecae are swollen due to the insertion of the male spermatophores and seminal products which is termed stage 2 (Hard, 1942; Lee, 1994; Lee et al., 1996a; Lee et al., 1996b). The oocyte diameter varies from 30-60 μm (Lee et al., 1994). Stage 2 oocytes begin to absorb vitellogenin (yolk proteins; VtG) and accumulate 1.1 ± 0.5 ng of the protein per oocyte (Lee and Walker, 1995). In stage 3, the ovarian tissue develops into a yellow color and grows in size with oocytes 66-100 μm in diameter (Lee et al., 1994). The vitellin (VT) increases absorption to 3.0 ± 2.0 ng of protein per oocyte (Lee and Walker, 1995). The golden coloration of the ovaries is the primary color of advanced ovaries in many crustaceans due to the protein accumulating as a glycolipoprotein in the oocyte which is conjugated to carotenoid pigments (Adiyodi, 1969; Meusy and Charniaux-Cotton, 1984; Meusy and Payen, 1988). The carotenoid pigments, both in primary and secondary vitellogenesis, are thought to protect the ovaries and embryos from visible light and radiation and possibly serve as metabolites for embryonic development due to their antioxidant properties (Sagi et al., 1997).

During secondary vitellogenesis in crustaceans, the oocytes begin to increase in size before oviposition. Prior to vitellogenesis, the oocytes will develop microvilli to aid

in the absorption of the VtG into the cell. To increase the uptake of the VtG from extraovarian sources transported in the hemolymph, the follicle cells develop into a tubular network (Van Herp and Soyez, 1998). This network of cells remains within the ovaries and embraces a new batch of primary oocytes after each spawning event, which allow for oocyte development to occur during the maturation of the egg mass on the female (Meusy and Charniaux-Cotton, 1984). Then, under a process that is not thoroughly understood, the VtG breaks down before, during, or after absorption into the cell to form VT (Van Herp and Soyez, 1998). In some animals VT can primarily or secondarily be produced in the oocyte; thus, this breakdown does not occur (Wilder et al., 2002). With the absorption of VtG, carotenoids are brought into the growing oocyte, as mentioned above, producing the bright orange hue of the developed ovaries (Adiyodi, 1969; Meusy and Charniaux-Cotton, 1984; Meusy and Payen, 1988). Once the oocyte has acquired the yolk proteins from the VtG absorption, the microvilli disappear and the germinal vesicle breaks down. The oocyte then undergoes a brief maturation period that will result in spawning (Van Herp and Soyez, 1998).

For example, in *C. sapidus*, the mid-ovarian development stage 4 is recognized by bright orange, large ovaries that have an oocyte diameter of 103-160 μm (Lee, 1994) with the VT accumulation of 78.0 ± 15.0 ng per oocyte at this stage (Lee and Walker, 1995). The last stage (5) of development results in bright orange-red, spongy ovaries that occupy the gastric, posterior, and intestinal cavities. The oocytes are about three times their original size ranging from 168-288 μm (Lee, 1994). Once again the VT levels have significantly increased by about three fold to 250.0 ± 37.0 ng per oocyte which is about 35% of the protein in the oocyte (Lee and Walker, 1995). After the female spawns (stage

6), the ovaries stay orange but are reduced in size. The oocytes resemble stage 3 oocytes with a diameter ranging from 86-127 μm (Lee, 1994). These orange ovaries will eventually become gray and collapse over time, which is thought to indicate the time that the female has completed her lifetime of reproduction (Hard, 1942).

VtG is the primary yolk protein in crustaceans comprising 60-90% of the egg proteins (Quackenbush, 2001). It is a glycolipoprotein which can be derived in the hepatopancreas and/or ovary of crustaceans. If derived externally, it is transported in the hemolymph to the ovaries for absorption. While in the hemolymph, VtG breaks down into two subunits which are uptaken by the ovaries. While in the ovary, the protein is broken down further into three subunits known as VT. In many species such as *C. sapidus* (Lee and Walker, 1995; Zmora et al., 2007), *Potamon potamios* (Pateraki and Stratakis, 1997), and *H. americanus* (Tsukimura et al., 2002), the VtG and VT subunits have been determined. However, for some species it is not known whether this dissociation occurs in the hepatopancreas, hemolymph, or ovaries. In species where the synthesis of yolk protein is internal, there is no precursor of VtG; VT subunits are produced by the follicular cells and directly absorbed by the cell (Yano, 1987). In *C. sapidus*, immediately upon synthesis in the hepatopancreas, VtG undergoes cleavage breaking down into two subunits (200 kDa and ~79 kDa) (Zmora et al., 2007). These two subunits of VtG are transported through the hemolymph to the ovaries. The ovaries then absorb the VtG via receptor mediated endocytosis (Schneider, 1996) where the larger subunit is further broken down forming three subunits of VT (Zmora et al., 2007). The VT is accumulated in the yolk proteins for later use by the embryos as mentioned above (Lee and Walker, 1995; Tufail and Takeda, 2005).

VtG expression levels vary during synthesis with ovarian development stage. In *C. sapidus*, like many other crustaceans, VtG levels increase in the hemolymph with the delivery of the proteins from the hepatopancreas to the ovaries in the primary vitellogenesis (Zmora et al., 2007). During secondary vitellogenesis similar levels of VtG/VT are present in the hepatopancreas and ovary with VtG levels peaking in the hemolymph. Then, the hepatopancreas VtG and hemolymph VT levels decrease while ovarian concentrations remain high from the previous absorptions. According to Zmora et al. (2007), the VtG levels in the hemolymph of *C. sapidus* are relatively low for the first 2.5 weeks after their final molt; then, these levels increase over the next 2.5 weeks and decrease slightly when the female reaches stage 4 ovarian development (Figure 1.2). The location of VtG concentrations in the animal allows for an insight as to where the proteins are synthesized and how they are transported to various tissues. In the shrimp, *Litopenaeus merguiensis*, enzyme linked immunosorbent assays (ELISA) were performed on various stages of ovaries to analyze the synthesis, transport, and accumulation of VtG and VT in the body (Auttarat et al., 2006). During rapid ovarian development, VT concentrations were highest in the ovaries while VtG concentrations in the hemolymph were declining due to removal from the hemolymph by the oocytes for development. On the other hand, low VtG levels in the hepatopancreas were detected indicating that the VtG is rapidly removed from the tissue into the hemolymph directly after synthesis or that the hepatopancreas is not the site of synthesis. Another suggestion for this lack of a full length VtG in the hepatopancreas may be due to a rapid cleavage process in the tissues before being released into the hemolymph (Auttarat et al., 2006; Zmora et al., 2007).

VtG endocytosis occurs when the VtG receptor (VtGR) recognizes the modified VtG in the hemolymph and engulfs the protein into the ovary. This relationship is not clearly defined in many crustacean species, including *C. sapidus*. As a result the amplification, cloning, and sequencing of the VtGR in *C. sapidus* was attempted to gain a better understanding for the reproduction and absorption of the modified VtG into the ovaries; however, inconclusive results require further studies to be conducted (Appendix 1).

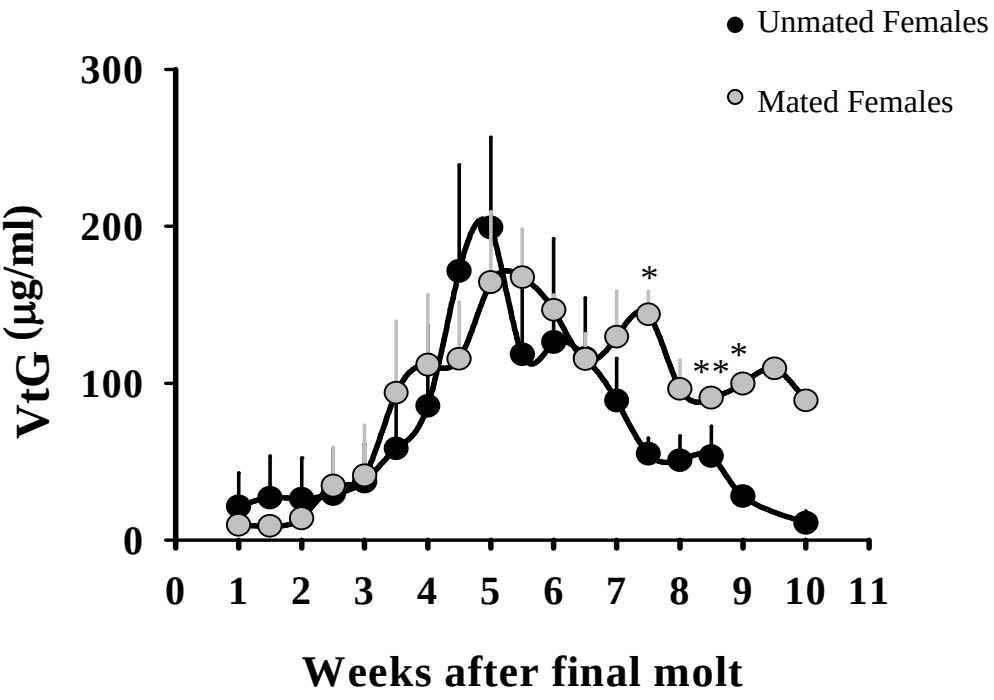


Figure 1.2. VtG levels in the hemolymph of mated and unmated female *C. sapidus.* A competitive ELISA was used to assess the VtG levels of *C. sapidus* after the terminal molt in captivity. After five weeks, the VtG levels peak and begin to drop. The VtG concentrations stabilize in mated females while unmated crabs show a continuing decrease throughout weeks 5-10 (Zmora et al., 2007).

VtG is transported from the hepatopancreas to the ovaries through the hemolymph which can allow for observation of maturation as observed by Lee and Puppione (1988),

Auttarat et al. (2006), and Zmora et al. (2007). These observations agree with the previous statements that crustacean reproduction occurs in two stages: primary and secondary vitellogenesis (Charniaux-Cotton, 1985; Quakenbush, 1986). The change in VtG levels in the hemolymph allow for the validation of the ovarian development stage without sacrificing the crab and allowing for each crab's progress to be charted. VtG concentrations determined by Lee and Puppione (1988) and Zmora et al. (2007) show an increase in VtG concentration after the terminal molt over a 10 week (70 day) period with levels reaching maximum at 5 weeks (35 days) (Zmora et al., 2007) and 8.5 weeks (60 days) (Lee and Puppione, 1988); then these levels decline. However, few studies have been conducted to assess the levels of further development of the ovaries up to and through the time of spawning (Lee et al., 1996a; Lee and Puppione, 1988). While the levels of the VtG in the hemolymph are increasing, the VtG levels in the hepatopancreas are showing a trend very similar – stage 1 development is low, stage 3 is very high, and then a slight decrease during stage 4. This decrease at stage 4 can be correlated with the increase of VT in the ovaries over the development period (Zmora et al., 2007). Thus, monitoring VtG in the hemolymph from mature females throughout the developmental period, spawning, and post-spawning may aid in the prediction of the precise timing of these events. With this knowledge a correlation may be established between maturation and spawning that further expands our understanding in the ovarian development of *C. sapidus*.

PROJECT OBJECTIVES AND ORGANIZATION OF THE STUDY

This work analyzes the reproductive physiology of *C. sapidus* by observing spawning and monitoring VtG concentrations to determine ovarian development. These experiments are documented in Chapters 2 and 3 with Chapter 4 discussing the possible design of a reproductive time frame using molecular ecology for the enhancement of production in crustacean aquaculture.

Chapter 2 will discuss the ecological aspects of spawning in *C. sapidus* exposed to different photoperiods and temperatures. Using wild caught crabs acclimated to recirculating aquaculture conditions, the crabs were monitored for 4-6 months during which the spawning activity was observed and recorded allowing for a detailed reproductive history for each crab. With the individual crab history and experimental conditions, specific environmental conditions were established for optimal spawning of first and subsequent egg masses.

Chapter 3 focuses on the physiology of ovarian development by monitoring the VtG levels of female *C. sapidus* over a 19 week period. The crabs observed in Chapter 2 were sampled twice a week by removing 100 μL of hemolymph. The hemolymph was mixed with a marine anticoagulant (1:1) and the samples were analyzed for VtG levels using a competitive ELISA. The data presents a physiological history for each female crab for the development of the ovaries during the maturation, spawning, and successive spawning stages.

Ultimately, by identifying the optimal environmental and physiological components for spawning in *C. sapidus* and their ovarian development in captivity, the generation of a specific time frame for predicting spawning was attempted. The

environmental manipulations allow for the optimization of development and spawning, while the ELISA determines the VtG synthesis by the hepatopancreas and uptake by the ovaries. With these two experiments, optimal conditions of ovarian development, including spawning, and further knowledge of *C. sapidus* reproduction will be gained.

CHAPTER 2: ENVIRONMENTAL MANIPULATIONS TO INDUCE SPAWNING

INTRODUCTION

Environmental factors, such as temperature and photoperiod, largely regulate the life cycles of many terrestrial and aquatic organisms. For example, growth of the blue crab, *Callinectes sapidus*, in the Chesapeake Bay region is largely regulated by temperature. Warm temperatures in the Bay stimulate the immature females to undergo their final molt to maturity after which they mate with mature males (Millikin and Williams, 1984). This action is then followed by a migration of the females to the spawning grounds at the mouth of the Bay which occurs twice a year, where warmer temperatures and saltier waters are present for reproduction and larval growth (Carr et al., 2004; Morgan, 1990; Sandoz and Rogers, 1944). The larvae hatch during the warmer summer months of the Bay (McConaugha et al., 1983) when rapid growth occurs, but beginning in the fall and continuing through the cooler months of the region, they slow their metabolic rate and bury in the sediment. This colder period results in no growth or reproduction until spring (Churchill, 1917; Havens and McConaugha, 1990; Van Engel, 1958). Thus it is apparent that growth and reproduction are seasonal and dependent on natural environmental conditions.

To avoid seasonally dependent reproduction in crustacean aquaculture, eyestalk ablation has been adopted for inducing ovarian development and spawning (Wickins and Lee, 2002). This traditional procedure removes the source of neuropeptides (vitellogenesis/gonad-inhibiting hormone (VIH/GIH)) that inhibit ovarian development

resulting in the induction of ovarian maturation including vitellogenesis (Bray and Lawrence, 1992; Kelemec and Smith, 1980; Liao and Chen, 1983; Okumura, 2004; Okumura and Aida, 2001; Okumura et al., 2007; Okumura et al., 2006; Sagi et al., 1997; Santiago, 1977; Webb, 1983; Wilder et al., 2002). However, the outcome of eyestalk ablation often depends upon the species. It has been noted that *Penaeus monodon* (Chen, 1979), *Penaeus canaliculatus* (Choy, 1987), *Penaeus plebejus* (Kelemec and Smith, 1980), *Marsupenaeus japonicus* (Okumura et al., 2007), and *Cherax quadricarinatus* (Sagi et al., 1997) females will begin developing their ovaries within two weeks of ablation and spawn in less than one month. Despite the rapid development of the ovaries, the eggs and larvae produced from these ablated animals are of lesser quality and have lower survival rates than larvae from intact animals (Choy, 1987; Emmerson, 1980; Lawrence et al., 1980; Vaca and Alfaro, 2000).

Alternatively, ovarian development and spawning induction in crustacean species have been achieved by modification of temperature and photoperiod. Photoperiod manipulation was first portrayed in the fresh water shrimp, *Palaemonetes paludosus* (Paris and Jenner, 1952), suggesting that the longer the exposure to light (18 hours of light with 6 hours of dark per 24 hour period; 18L:6D), the greater the ovarian development compared to 9L:15D. In addition, it was noted that the dark period is just as important for survival and growth. Few other photoperiod studies have followed Paris and Jenner (1952), yet all demonstrate the importance of the dark period during ovarian maturation or spawning. The ovarian manturation of the crayfish, *Cambarus virilis*, increased when exposed to dark conditions (Stephens, 1952). Similar results were seen in the crayfish, *Procambarus clarkii*, in which spawning increased after periods of short

light exposure (Dendy, 1978) while light interruptions during 12L:12D exposures were beneficial for ovarian maturation (Castanon-Cervantes et al., 1995). Lastly, the shrimp *Penaeus kerathurus* showed increased ovarian development under 10L:16D conditions (Luis and Ponte, 1993). In addition to photoperiod, light intensity can also affect ovarian development and spawning. The following assessments of light intensity indicate that lower light intensity results in better production: *Penaeus vannamei* 15 $\mu E/m^2$/sec (~749 lux) vs. 53 $\mu E/m^2$/sec (~2650 lux) (Wyban et al., 1987) and *P. merguiensis* 2 lux vs. 1100 lux (Hoang et al., 2002).

A few thermal manipulating experiments have also been conducted to induce spawning: *P. trituberculatus* at 21^{o}C (Hamasaki et al., 2004), *C. sapidus* at 19^{o}C (Sulkin et al., 1976), and *Menippe mercenaria* at 25^{o}C (McConaugha et al., 1980). However, several experiments combining altered temperature and photoperiod conditions have been performed: *Palaemonetes pugio* – 25^{o}C, 14.5L:9.5D (Little, 1968); *Penaeus merguiensis* – 22^{o}C and 27^{o}C, 10L:14D and 14L:10D (Hoang et al., 2002; Hoang et al., 2003); *Penaeus semisulcatus* – 20-28^{o}C, 10L:14D and 14L:10D (Aktas et al., 2003); *Penaeus esculentus* – 26^{o}C, 14L:10D (Crocos and Kerr, 1986); *Jasus edwardsii* – natural vs. compressed 9 month treatment (Smith et al., 2003); *Homarus americanus* – 9.8-15^{o}C, 8L:16D and 16L:8D (Nelson et al., 1983) and 13-14^{o}C, 8L:16D (Waddy and Aiken, 1992); *Panulirus japonicus* – 13^{o}C, 19^{o}C, and 25^{o}C, 10L:14D and 14L:10D (Matsuda et al., 2002); and *C. quadricarinatus* – 24.5-27.6^{o}C, 14L:10D (Jones, 1995). All cited manipulated environmental conditions resulted in some degree of successful ovarian maturation of the respected species; however, these experiments were short term and

cannot be used to determine the effect of year round manipulation on production as aspired for in aquaculture.

Photothermal manipulation of *C. sapidus* females can accelerate reproduction (vitellogenesis and spawning) in closed recirculating aquaculture (Zmora et al., 2005; Zohar et al., 2008). Mature females collected from the wild were exposed to conditions mimicking summer lower Bay conditions for the induction of spawning (16L:8D, 21^{o}C, 30 ppt) or a colder, energetically arresting condition (8L:16D, 15^{o}C, 30 ppt) to allow for year round production (Zmora et al., 2005; Zohar et al., 2008). Summer temperatures in the lower Bay can increase to 28^{o}C (Sandoz and Rogers, 1944), yet in captivity temperatures above 21^{o}C increase the outbreak of shell disease on the crabs and leads to mortality (personal observation). The exposure to the colder conditions allows the crab's metabolism to slow, which allocates a supply of crabs for the summer induction during the natural winter season. This technique has been successfully inducing female *C. sapidus* reproductively in closed, recirculating aquaculture for six years (Zohar et al., 2008).

This study was designed to define the optimal environmental conditions, photoperiod and temperature, for ovarian development and spawning. By introducing female *C. sapidus* into the following controlled conditions: photoperiods of 24L:0D, 16L:8D, 8L:16D, and 0L:24D and temperatures of 21^{o}C, 15^{o}C, or 11^{o}C, ovarian development, spawning, and spawning interval experiments were conducted over a two year period for 4-6 month intervals using three batches of wild caught female crabs. By identifying the optimal environmental cues for *C. sapidus'* reproductive cycle, crustacean

aquaculture will be able to incorporate photothermal manipulations to increase productivity.

MATERIALS AND METHODS

Animal Collection

Mature *C. sapidus* females were obtained with a local waterman using crabpots on three separate dates: (1) November 17, 2006, (2) April 27, 2007, and (3) November 24, 2007. Batches 1 and 3 were caught south of the Chesapeake Bay Bridge, MD near Sharps Island lighthouse (76^{o}20'60" W, 38^{o}35'15" N). Batch 2 was obtained due to low survival of Batch 1. The harvest location was farther south in the Chesapeake Bay between Cedar Point and Point No Point Lighthouse (76^{o}30'60" W, 38^{o}15'22" N) due to low harvest numbers in the aforementioned location (Figure 2.1). The requirements for crab selection were: no more than two legs were completely missing, both chelae were present, no wounds were visible on the carapace and limbs, and all present limbs were fully intact. The crabs were placed in wet burlap in coolers aboard the vessel and the salinity and temperature of the harvesting location were documented using a refractometer (VitalSine, model SR-6) and thermometer. Upon return to the dock (3-5 hours after the start of collecting), the crabs were transferred to coolers (10-15 crabs per cooler) which contained 15-20 L of aerated water from the Bay and Nitex netting for shelter. The crabs were transported 1-2 hours by van to the Center of Marine Biotechnology (COMB, Baltimore, MD), where they were immediately placed into a 3800 L closed, recirculating tank at similar salinity and temperature conditions as the harvesting areas (Batch 1: 30 ppt, 16^{o}C after 3 days of a 5 ppt/day salinity increase; Batch

2: 11 ppt, 13°C; and Batch 3: 18 ppt, 13°C) with Nitex for shelter. The photoperiod in the holding tanks reflected the natural conditions of the wild.

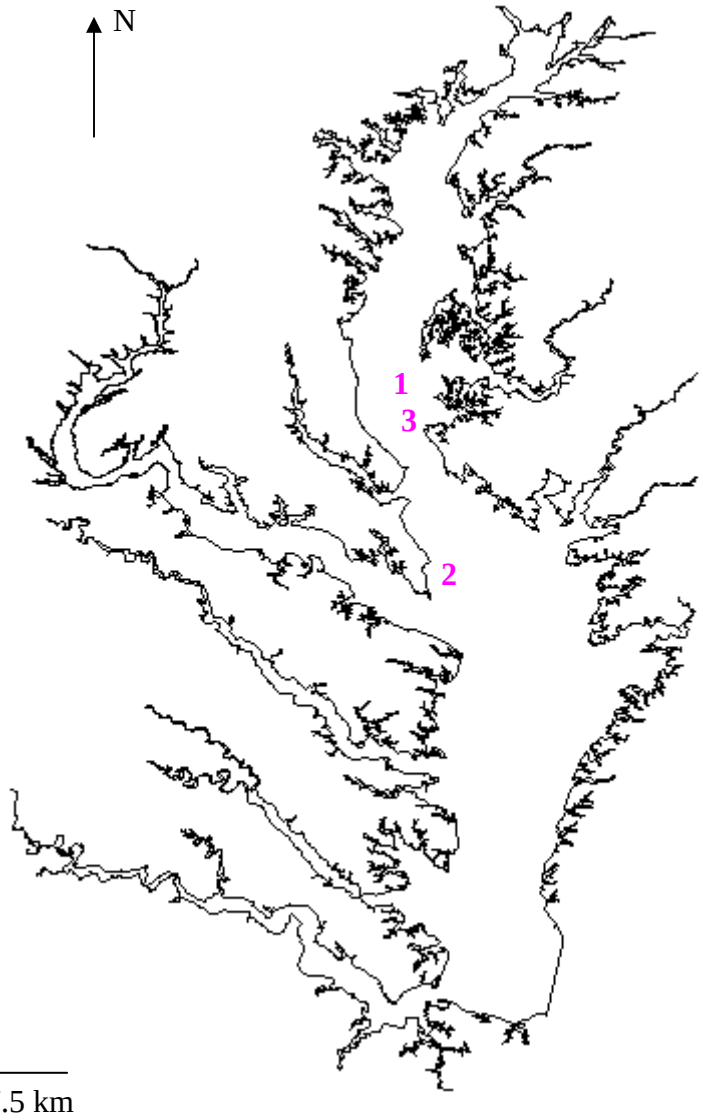


Figure 2.1. Location of harvesting sites. The three harvesting sites located within the Chesapeake Bay varied. The first Batch (1) location was harvested on November 17, 2006, Batch 2 on April 27, 2007, and Batch 3 on November 24, 2007. (www.mgs.md.gov)

Acclimation of Wild Crabs

The crabs were maintained for 2-5 months by feeding cut up frozen squid and pelleted food (EWOS Brood, 9 mm). The crabs were held for these lengths of time due to preparation of the tank system and photoperiod acclimation after the shortest day of the year (see below). Water temperature and salinity were monitored on a daily basis using a hand held refractometer and thermometer, while nitrite and ammonia were monitored several times a week and pH was documented once a week. The pH was measured using an Orion expandable ionAnalyzer (EA 940), while ammonia and nitrite were analyzed by flow injection. After two weeks, the water salinity was increased gradually (2 ppt/day) up to 30 ppt. Day length during the acclimation period was regulated using a computer regulated daylight cycler (Energy Control Systems) to obtain a natural photoperiod until December 21, 2007 and 2008 when the conditions were kept at 8L:16D to reflect the shortest day of the year. However, since Batch 2 crabs were brought in after the shortest day of the year, the day length was decreased 30 min/day for 2.5 weeks to reach 8L:16D. Once the established conditions were met, the crabs were kept for 2-5 months before being moved into the experimental tank systems.

Photoperiod and Temperature Manipulations

The experimental tank systems comprised of ten enclosed tanks: DB – five tanks of 91 x 112 cm with a depth of 58 cm and DT – five tanks of 91 x 112 cm with a depth of 41 cm which were individually enclosed using black PVC sheeting (US Plastics, 3.2 mm) (Figure 2.2). Each tank contained 15 cm of washed play sand with PVC piping with holes underneath to allow for water to flow up from the bottom of the tank through the

sand (Figure 2.3). The photoperiod was regulated in each enclosed tank by a light fixture with a timer (Intermatic Heavy Duty Digital Timer, Model DT27C). Each system possessed a bubble bead filter (Aquatic System Technology), protein skimmer (RK2 Systems, Model RK25PE), temperature controller (Process Technology, Model DQ15D) with heat exchanger, and fluidized sand biofilter (QuikSand Filters). The tank systems were manually backwashed and refilled every day with a daily water change of 3.4% in the DB tank system and 5.5% in the DT tank system. Also, temperature and salinity were recorded every day, ammonia and nitrite were measured every other day, and pH was checked weekly.

A.

B.

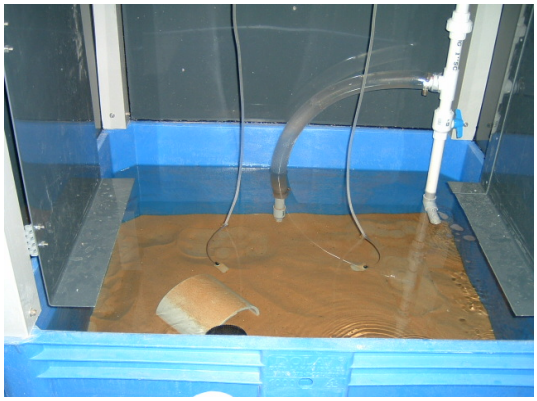


Figure 2.2. Experimental tank system with individually enclosed tanks. (A) The blue tanks on the bottom are the DB system while white tanks on the top are the DT system. (B) Each tank was enclosed with black plastic PVC sheeting (0.32 cm thick) to create a light tight environment.

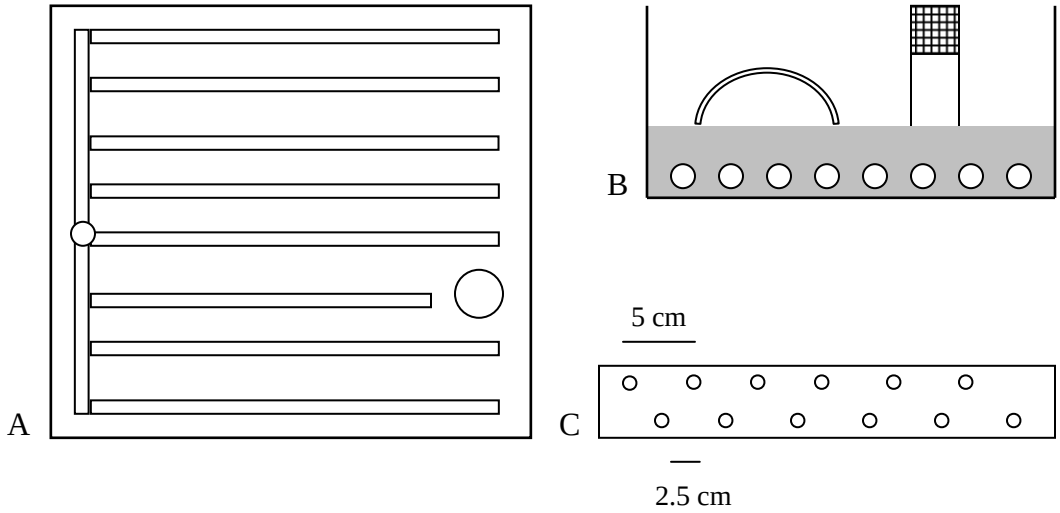


Figure 2.3. Internal filtration for the experimental tanks. PVC pipe was assembled to create an upwelling of water underneath the sand layer to allow for filtration of the sand. A. Top view of tank with filtration pipes. Pipes were spread to create a uniform disturbance in the sediment. B. Side view of the tank to illustrate the complete setup of the tank system. The pipes were laid underneath the 15 cm of sand. Netting was placed on the standpipe to increase the water height in the tank while half of a PVC pipe was provided for shelter. C. Schematic of the filtration pipe placed under the sand. Two rows of staggered holes were drilled so each hole was 5 cm apart in the row on one side of the pipe. The rows were 90^{o} apart to provide an outward and upward flow of water from the pipe.

The crabs were weighed and labeled using wire tags wrapped around their carapace spines to monitor each female's activity (Figure 2.4). They were then placed into the experimental tanks at a density of 4-5 crabs per tank. The crabs were moved into the experimental tanks at the same conditions as they were experiencing in the holding tanks: 30 ppt, 15^{o}C, and 8L:16D for all batches.

Figure 2.4. Wire label attached to crab for identification. A 30 cm piece of plastic coated copper wire (14 gauge) was cut, the ends of the wire were hot glued so the copper was not exposed, and loops were created on each end of the wire to slide over the carapace tips. A labeled tag (Brother P-touch labeler) was attached to the wire for identification.

After transfer to the experimental tanks, the crabs were exposed to light and temperature changes to obtain the predetermined experimental conditions as follows: Batch 1 were exposed to 21^{o}C with lighting of 24L:0D, 16L:8D, and 8L:16D; half of Batch 3 was exposed to 21^{o}C, 24L:0D, 16L:8D, 8L:16D, and 0L:24D while the other half of Batch 3 experienced decreased temperature of 11^{o}C and photoperiods of 24L:0D, 16L:8D, 8L:16D, and 0L:24D; and Batch 2 experienced no temperature increase (remained at 15^{o}C) and light changes resulting in 16L:8D and 8L:16D (Table 2.1). The exterior of the 0L:24D tanks were covered in black plastic sheeting (FilmGard, 6 mm) to prevent any light from entering the enclosed tanks during feeding and observation during which a red light (General Electric, 25W) was used. All lights were positioned 61 or 76 cm above the surface of the water. The light was increased or decreased gradually in each tank by 30 min/day until the desired photoperiod was obtained. The temperature

increase for the warmer water systems occurred at 1°C/day until 21°C was reached. Lighting intensities were measured in each tank using a Milwaukee lux meter (SM700) (Table 2.2).

Table 2.1. Harvest information for the three sampling dates. Experimental setup for each collection date including batch number, total number of crabs, temperature, and photoperiod manipulations.

Batch	Catch Date	Location	Date of Transfer to Experimental System	Number of Crabs	Temperature (°C)	Light Treatments
1	Nov. 17, 2007	76°20'60" W 38°35'15" N	April 17, 2007	21	21	8L:16D 16L:8D 24L:0D
2	Apr. 27, 2007	76°30'60" W 38°15'22" N	June 26, 2007	20	15	8L:16D 16L:8D
3	Nov. 24, 2007	76°20'60" W 38°35'15" N	January 10, 2008	25	21	0L:24D 8L:16D 16L:8D 24L:0D
				25	11	0L:24D 8L:16D 16L:8D 24L:0D

Table 2.2. Light intensities for the experimental tanks during each trial run. Light intensities were measured with a lux meter (Milwaukee, SM700) placed on top of the sand surface underwater for each experimental tank.

	2007		2008	
Tank	Lux	Treatment	Lux	Treatment
DT1	9-11	16L:8D	9-11	24L:0D
DT2	10-12	16L:8D	10-12	16L:8D
DT3	16-18	16L:8D	16-18	8L:16D
DT4	10-12	8L:16D	0	0L:24D
DT5	15-17	8L:16D	0	0L:24D
DB1	6-7	16L:8D	6-7	24L:0D
DB2	5-8	16L:8D	5-8	16L:8D
DB3	6-8	8L:16D	6-8	8L:16D
DB4	8-9	8L:16D	0	0L:24D
DB5	0	0L:24D	0	0L:24D

Spawning Observations

Observations during the experimental period occurred for six months for Batches 1 and 3 while Batch 2, which was introduced into the system two months later then Batch 1, was monitored for only four months. During the experimental period, crabs were observed to determine if an egg mass had been produced. If a female was thought to be carrying an egg mass and could not be confirmed by visual observation of the crab in the tank, the crab was gently lifted into the water column by its wire tag using a hook to observe the abdomen of the crab. The crab was then released so as not to cause a lot of stress. If an egg mass was present, the date of spawning and female label was recorded. The female was monitored for the next 2-3 weeks to watch the development of the embryos in the experimental tank. Approximately 12-14 days when the spawn became dark brown, a piece of the egg mass was taken to assess the development of the embryos in more detail. If heartbeats were observed in the embryos under a dissecting microscope, the female was transferred to a container with 15 L or 30 L of tank water and sufficient aeration. The female was not fed while in the hatching container. If the female was from the 0L:24D tank, the container was covered with a sheet of black plastic under the plastic sheeting that shaded the tanks to maintain complete darkness and monitored with a red light for hatching progress. After 2-4 days, the egg mass would hatch and the female was placed back into her original tank. The hatched larvae were then counted using a 1 mL pipet (Pyrex, 13-666-7 Series). The hatching container was subjected to strong aeration so there were no areas where the larvae could congregate. The pipet was immersed into the container of water and stopped; when removed the larvae were counted in 1 mL. The sample was then placed back into the container, let mix, and

another 1 mL sample was taken and counted. Overall, ten samples were taken, averaged, and the total number of larvae was calculated within 15 L or 30 L of water depending upon the size of the hatching container.

Biopsy of Ovarian Tissue

Several females that died during the experimental period were dissected to determine the ovarian development using the guidelines of Lee and Puppione (1988). A fraction of their ovaries were removed for oocyte diameter measurement to determine the development stage. Female oocyte stages were designated as described by Lee and Puppione (1988). The stages are as follows: Stage 1 oocytes 50-60 μm in diameter; Stage 2 – 140-180 μm in diameter; Stage 3 – 200-250 μm in diameter; and Stage 4 – 330-350 μm in diameter. As the oocytes develop they grow larger; thus, Stage 1 is a premature stage while Stage 2 and 3 are maturing and Stage 4 is mature (Lee and Puppione, 1988; Zmora et al., 2007).

Long-term Hatchery Data

Brood stock data was obtained from *C. sapidus* hatchery at COMB for the time frame of 2001-2006. The data was tabulated and compared to the results of the current experiment. All data documented female, date of spawning, spawn number, hatch date, number of zoea released, and date of placement into experimental treatment (Appendix 2). This allowed for the calculation of the embryo development period, fecundity, ovary development period from move into the experimental conditions to spawn and between spawns, and the occurrence of multiple spawns by a single female.

Statistical Analysis

Data was analyzed using SAS (ANOVA and ANCOVA) and GraphPad (linear regression) statistical programs. The spawned females analyzed from the hatchery (Appendix 2) were animals which spawned in captivity.

RESULTS

Temperature vs. Photoperiod

The number of spawned females varied significantly between the three temperature treatments ($p = 0.02$, two-way ANOVA) (Figure 2.5); the greatest number of spawned females occurred in 21^{o}C, while no females spawned in 11^{o}C ($p < 0.05$, Tukey Studentized Range Test). No significant variation was present between photoperiod treatments ($p = 0.21$, two-way ANOVA) and no interaction occurred between temperature and photoperiod ($p = 0.30$).

Survival showed significant variation between temperature regimes ($p = 0.04$, ANOVA). The greatest survival occurred at 15^{o}C which was only observed for four months compared to six months for 21^{o}C and 11^{o}C. A significant difference in survival was present between 15^{o}C and 11^{o}C ($p < 0.05$, Tukey Studentized Range Test). There was no interaction present between temperature and photoperiod ($p = 0.98$, two-way ANOVA) and no significant difference between photoperiods ($p = 0.62$ ANOVA) (Figure 2.6).

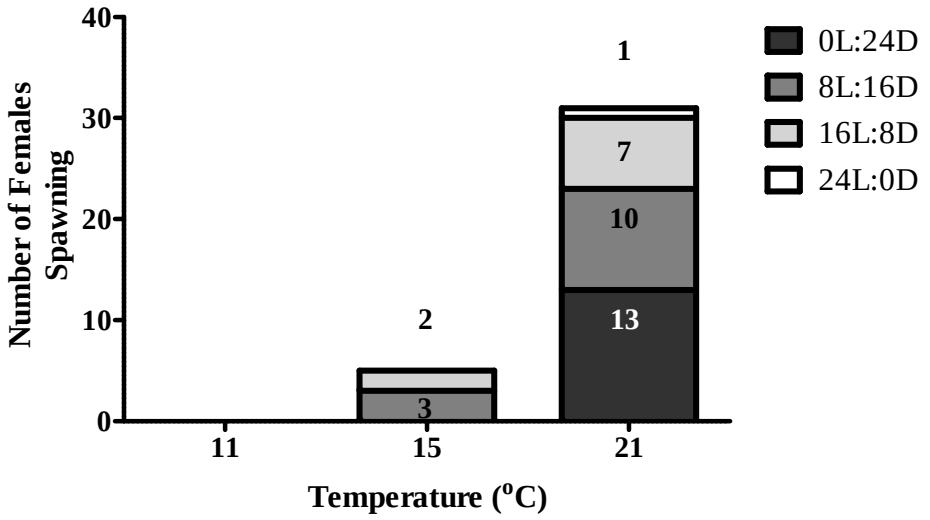


Figure 2.5. Total spawns produced for each temperature treatment. The greatest spawning occurred in 21°C which varied significantly ($p = 0.02$). The 15°C treatment was also capable of producing spawns while the 11°C reproductively arrested the crabs. The number of females per treatment are as follows: 21°C: 0L:24D = 15, 8L:16D = 13, 16L:8D = 13, 24L:0D = 5; 15°C: 8L:16D = 8, 16L:8D = 12; and 11°C: 0L:24D = 10, 8L:16D = 5, 16L:8D = 5, 24L:0D = 5.

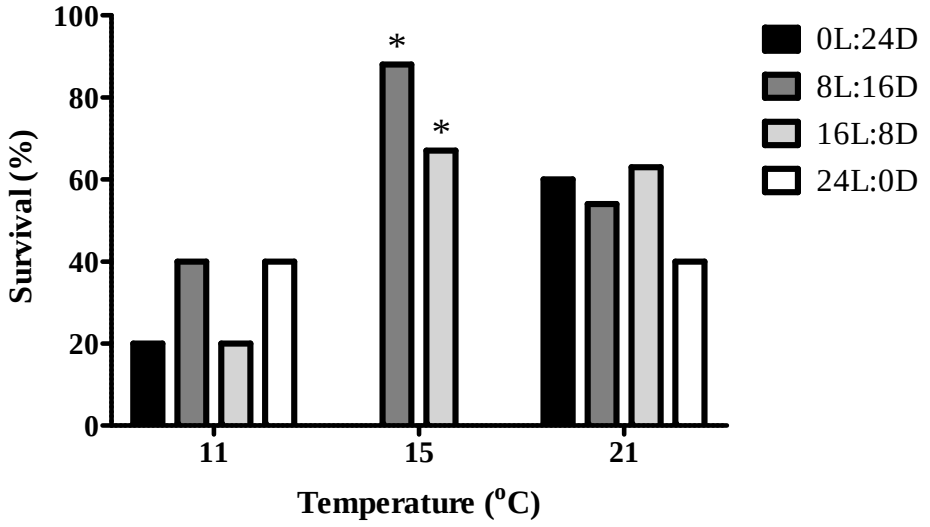


Figure 2.6. Survival during the experimental periods for each temperature and photoperiod treatment. Temperature treatments varied statistically ($p = 0.04$) with 15°C and 11°C varying significantly ($p < 0.05$). Photoperiod did not vary ($p = 0.62$) and no interaction was present between the photoperiod and temperature ($p = 0.98$). *The 15°C experimental period was observed for four months while 11°C and 21°C were observed for six months. The total number of females per treatment are: 21°C: 0L:24D = 15, 8L:16D = 13, 16L:8D = 13, 24L:0D = 5; 15°C: 8L:16D = 8, 16L:8D = 12; and 11°C: 0L:24D = 10, 8L:16D = 5, 16L:8D = 5, 24L:0D = 5.

Ovarian and Embryo Development

For each female, the embryonic developmental period varied with maturation of the ovaries; however, there was no interaction between spawning intervals at 21°C and the photoperiod treatments ($p = 0.95$, two-way ANOVA, Figure 2.7) or between photoperiods ($p = 0.49$, two-way ANOVA). The spawning intervals between the spawning events varied significantly ($p < 0.0001$, ANOVA) with the greatest variation occurring between the first-second spawning event and no variation between the second-third spawning event ($p > 0.05$, Tukey's Studentized Range Test).

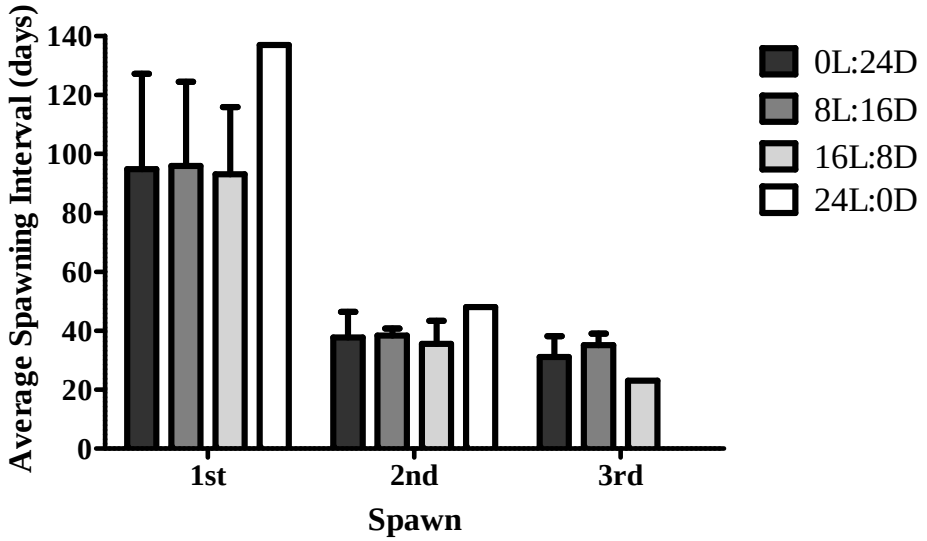


Figure 2.7. Spawning intervals between 1st, 2nd, and 3rd spawns for 21°C crabs. Spawning intervals varied significantly between the first spawn and the second ($p < 0.05$) while the second and third were similar ($p > 0.05$). The longest developmental period occurred before the first spawning event (~100 days). No deviation was present for 24L:0D since only one female spawned in this treatment. Total females in each photoperiod: 1st: 0L:24D = 13, 8L:16D = 10, 16L:8D = 7, 24L:0D = 1; 2nd: 0L:24D = 6, 8L:16D = 4, 16L:8D = 2, 24L:0D = 1; and 3rd: 0L:24D = 2, 8L:16D = 3, 16L:8D = 2, 24L:0D = 0.

The ovaries of the dissected animals showed development throughout the first 100 days of the experimental period with a positive correlation of oocytes size with time (r = 0.70, Figure 2.8). The first 100 days were chosen since the first spawn was produced about 50 days after exposure to the experimental conditions and the spawning interval to the first spawn was ~100 days. From the first spawning events at 21°C, the number of larvae hatched increased positively with the weight of the female (r = 0.43) (Figure 2.9).

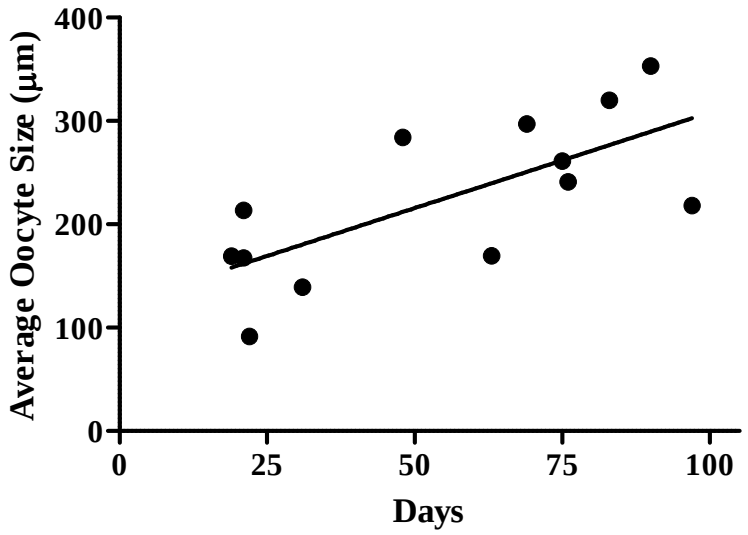


Figure 2.8. Oocyte development during the first 100 days of the experiment. Dead females were biopsied to determine the rate of ovarian development. The females represented were removed from the 21°C experimental treatment. Oocyte diameter positively correlates with time (r = 0.70, n = 13).

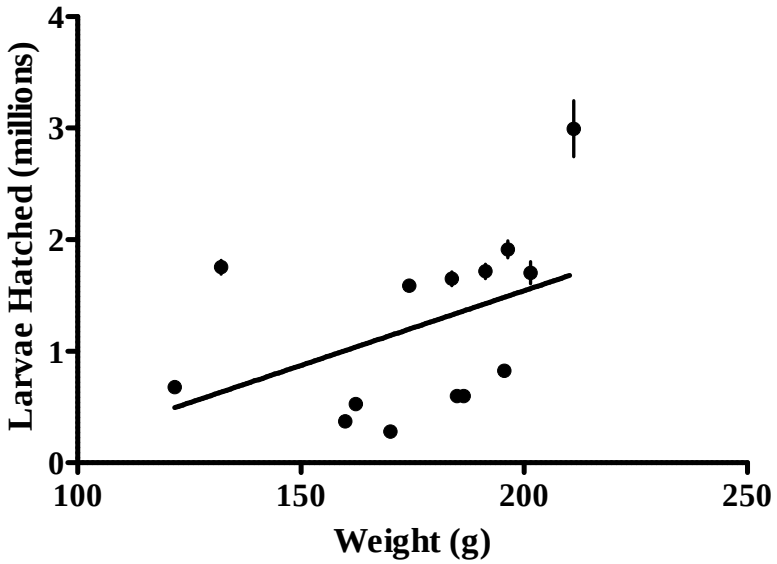


Figure 2.9. Number of larvae hatched correlated with body weight. The larvae hatched from the first spawning event shows a positive correlation with the weight of the female (r = 0.43, n =14).

Multiple spawns produced in 21^{o}C crabs did not vary significantly between spawn number and production (p = 0.06, ANCOVA) (Figure 2.10). However, when adjusting for weight of the female, the spawning events varied significantly (p = 0.03, ANCOVA). The analysis of the least squares means for the dataset indicate a significant different between the second spawn and the third with respect to the amount of larvae hatched (p = 0.01, ANCOVA). On average, the heavier females produced more larvae with all females bearing smaller third spawns and larger second spawns (Figure 2.10).

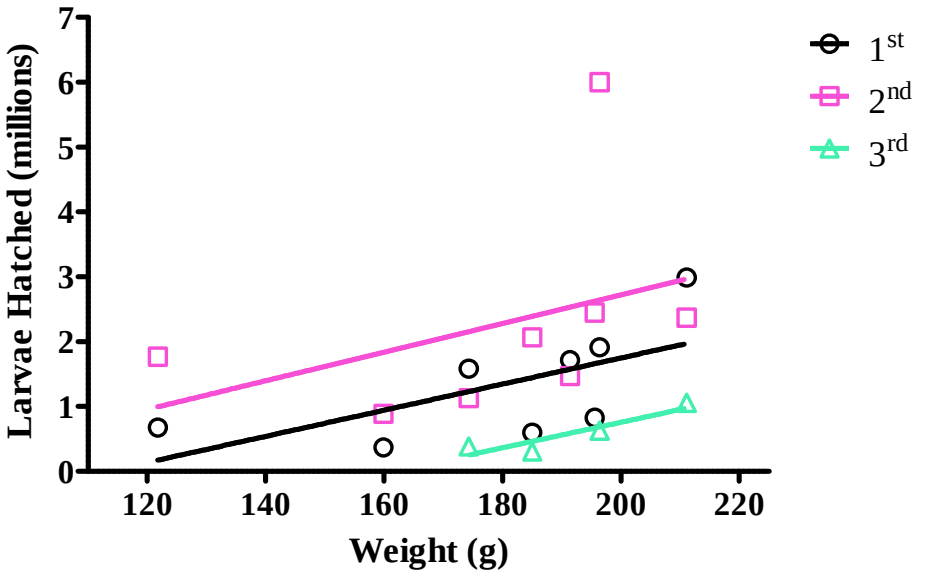


Figure 2.10. Total larval release numbers for eight crabs producing 2+ spawns. Multiple spawns were analyzed by spawning event and hatch number with an adjustment for weight of the female ($p = 0.03$). Results showed increased hatch numbers for the second spawn which different significantly from the third spawn ($p = 0.01$) and increased hatch numbers for heavier crabs.

Spawning Data Comparison

Spawning tables were obtained from the blue crab hatchery at COMB for 2001-2006 (Appendix 2). From this large dataset, correlations were made between the current data and the hatchery data which resulted in a varied developmental period between transfer into the system and the first spawning event ($r = 0.78$). In this regression, year 2003 was removed as an outlier (42 days). Similar developmental periods occurred between multiple spawning events ($r = 0.29$) with the shortest move to spawn time of 1 day and 14 days between multiple spawns (Figure 2.11A). The production by the females between the different years was also similar for the total number of spawns produced ($r = 0.19$) and the total number of spawning females ($r = 0.12$) (Figure 2.11B). The data for female spawns shows that the hatchery and experimental crabs are capable

of spawning an average of 110 days (~15.7 weeks) after exposure to 'summer' conditions and embryo development periods of 47 days (6.7 weeks) were obtained. Multiple spawns can be produced by females held in captivity resulting in the production of up to 5 spawns from one female with an average of 1.5 spawns per female (Figure 2.12).

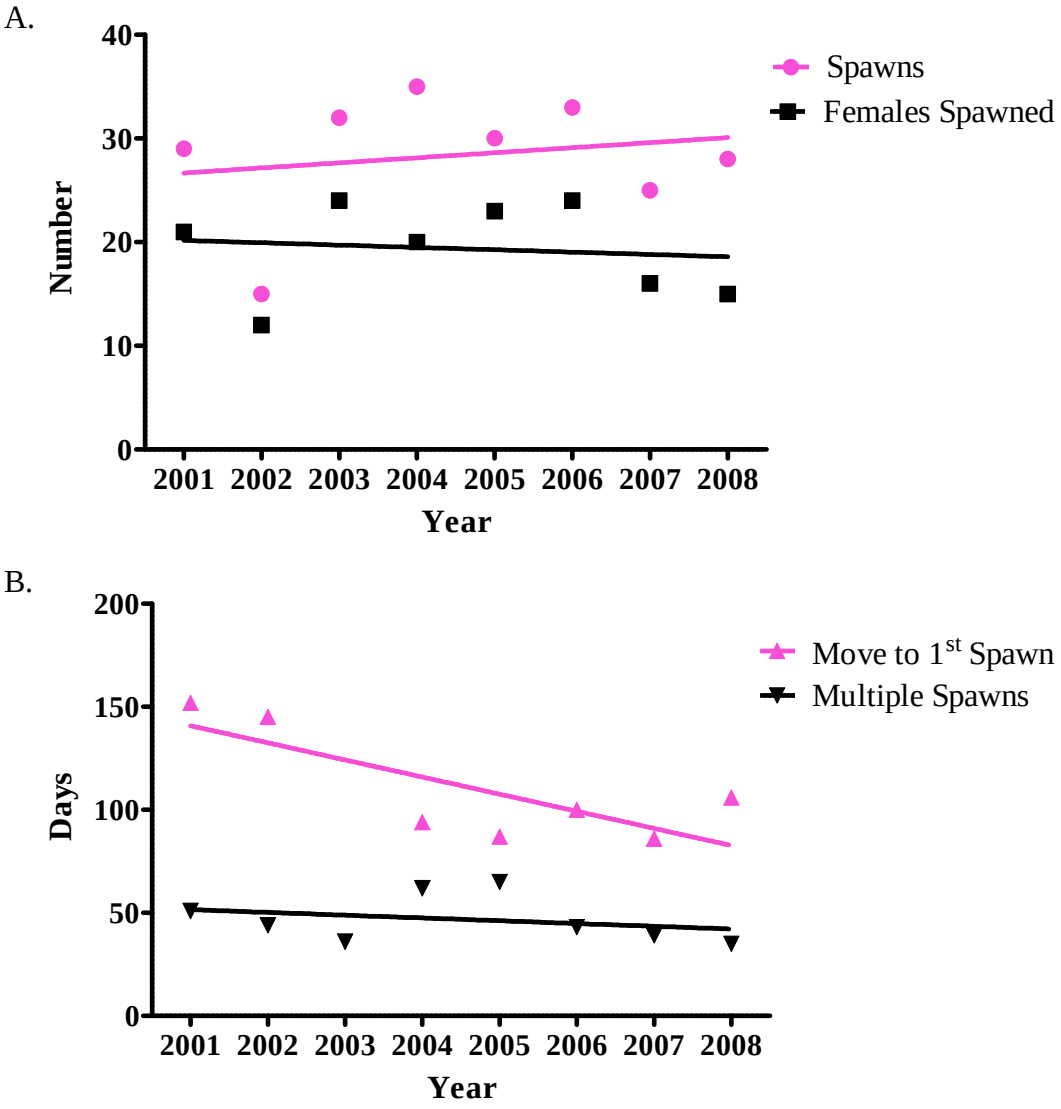


Figure 2.11. Correlation of *C. sapidus* hatchery data to experimental results. Comparisons were assessed for 21°C conditions for 2001, 2003, 2005, 2006, 2007, and 2008 while 2002 was 23°C and 2004 was 22°C. Years 2001-2006 are compiled from the hatchery while 2007 and 2008 are from the current experiment. (A) Developmental periods between the datasets show variation for the number of days to the first spawning event (r = 0.78) while similar periods of development occurred between multiple spawns (r = 0.29). Year 2003 was removed as an outlier for the development of the first spawn. (B) No significant variation occurred between years for the number of spawns produced (r = 0.19) nor the number of spawned females (r = 0.12).

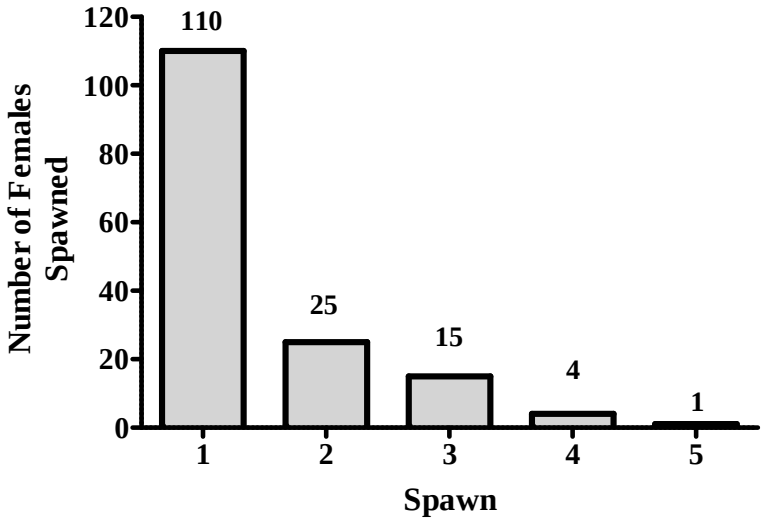


Figure 2.12. Multiple spawns produced by hatchery and experimental animals from 2001-2008. Females were capable of producing up to five spawns in captivity with an average of 1.5 spawns per female (n = 155).

DISCUSSION

Photothermal Manipulations

Photoperiod and temperature conditions were manipulated for ovarian development and spawning in *C. sapidus* in captivity. The experimental temperature conditions of 11^{o}C, 15^{o}C, and 21^{o}C allow for a range of temperatures for observation which are both achievable in captivity and experienced in the wild by *C. sapidus*. Since wild *C. sapidus* larvae hatch in 21-28^{o}C (Sandoz and Rogers, 1944) and prolonged exposure to temperatures above 21^{o}C has previously led to shell disease in *C. sapidus* (personal observation), the highest temperature exposure for this experiment was 21^{o}C.

In *C. sapidus* ovarian development and spawning were temperature dependent (Figure 2.5). As expected, the 11^{o}C treatment arrested the metabolism of the crabs which

resulted in no spawns over the experimental period, while 15^{o}C was able to halt spawning, but not completely arrest the activity. Finally, 21^{o}C was the best temperature treatment in which the highest production occurred with 86% of the crabs spawning. *C. sapidus* exposed to 19^{o}C increased ovarian development during the experimental period (76 days) leading to spawning in captivity (Sulkin et al., 1976). Of the spawns obtained in this study at 21^{o}C, the majority were produced in the constant dark condition (0L:24D), which is supported by the practice of the Chinese in *P. trituberculatus* (O. Zmora, personal communication). This is reasonable since mud crabs, crayfish, spiny lobster, and shrimp in captivity spawn primarily during dark periods with only a few documentations of spawning during daylight exposure for each species (Andrews, 1906; Bray and Lawrence, 1992; Dall et al., 1990; Nakamura, 2000; Zeng, 2007; personal observation).

Based on these results, temperature, photoperiod, and/or a combination of both, affect the reproduction in *C. sapidus*. Sastry (1983) noted that temperature and photoperiod could be manipulated amongst species to influence reproduction in crustaceans. However, in other species, temperature has a greater influence on spawning than photoperiod as seen in the northern crayfish, *Orconectes virilis* (Aiken, 1969); mud crabs, *Scylla serrata* (Hamasaki, 2003) and *Scylla paramamosain* (Zeng, 2007); and blue shrimp, *Penaeus stylirostris* (Robertson et al., 1991). In these experiments, the animals were exposed to constant light conditions with different temperatures which all resulted in higher production at higher temperature. The importance of photoperiod for reproduction was noted by Dendy (1978) in the red swamp crayfish (*P. clarkii*) which produced more spawns during shorter day length treatments than ambient light under

constant temperature exposure. Similar findings have also been described for ovarian maturation with the influence of photoperiod (Perryman, 1969; Rice and Armitage, 1974; Stephens, 1952; Suko, 1958). In these experiments, prawn and crayfish (*Procambarus simulans*, *Orconectes nais*, and *Cambarus* sp.), which do not experience a terminal molt, exhibited greater reproductive output with less or halted molting during shorter day length treatments. In constant darkness or shorter day length, the ovaries mature and are not reabsorbed (Perryman, 1969; Stephens, 1952; Suko, 1958) as seen during longer photoperiod treatments (Perryman, 1969). However, this contradicts the previous findings of Paris and Jenner (1952) in the shrimp *P. paludosus*, whereas longer day length has a higher induction rate of spawning.

The light intensity used for the current study (0-18 lux) was low compared to previous experiments. With this low light intensity, a greater number of spawning events occurred in the lowest lux exposure which has also been noted for other species. The light illuminance documented for the current experiment ranged from 0-12 lux (Table 2.2). In *P. vannamei*, dim light induced spawning as seen by Wyban et al. (1987) with increased reproduction in an aquarium with significant algal mat growth causing a decrease in the light transmission through the water column. The light intensity was measured at 15 uE/m^2/sec (~749 lux) at the water surface, but as mentioned in the reference, it was less at the bottom of the algal mat tank (Wyban et al., 1987). Hoang et al. (2003) also saw increased ovarian development and spawning in *P. merguiensis* at lower lux exposure. By decreasing the light intensity from 360 to 2 lux and keeping a constant 2 lux, reproductive performance significantly increased with increased temperature and photoperiod (Hoang et al., 2003). *P. merguiensis* migrates to deep

waters to spawn; thus, dimmer light conditions simulate their reproductive grounds (Dall et al., 1990). The same migration is seen by *C. sapidus,* whereas once they mate in shallower estuarine waters, they migrate to the lower Bay to spawn and hatch their larvae (Churchill, 1917; Van Engel, 1958). According to a mark and recapture study by Turner et al. (2003), the majority of the recaptured females were located close to the shoreline in 5-10 m of water in the middle Bay. Similar results were obtained by Aguilar et al. (2005) and Lipcius et al. (2003) in the lower Bay. As for the spawning areas of the Chesapeake Bay, about half of the mature females are located in 10-26 m (Lipcius et al., 2003). According to the Chesapeake Bay Program, the sunlight penetration in this area from 2003-2008 was very low with Secchi disk readings averaging 1.4 m implying that the light is diffused before reaching the bottom of the Bay (10-23 m) (CBP, 2009).

With the combination of light intensity and photoperiod, many of the aforementioned species respond similarly. From the current study, *C. sapidus* seems to be influenced by both factors but mainly by temperature, which is comparable to previous results (Sulkin et al., 1976). At higher temperatures (21^{o}C) and constant dark (0L:24D), the reproductive output of *C. sapidus* was significantly higher in this experiment than at other photoperiods. In many other species, higher temperatures are important, but longer day length also benefits spawning. In the spiny lobster, *Panulirus argus*, long day length and warm water increases ovarian development (Lipcius and Herrnkind, 1987). For other decapods, higher temperature is the predominant cue while photoperiod supports the induction: *C. quadricarinatus* (Jones, 1995), *H. americanus* (Hedgecock, 1983), *P. japonicus* (Matsuda et al., 2002), *P. merguiensis* (Hoang et al., 2003), *P. pugio* (Little, 1968), *P. esculentus* (Crocos and Kerr, 1986), and *P. semisulcatus* (Aktas et al., 2003).

The increase in water temperature simulates the onset of spring/summer conditions when reproduction in the wild typically occurs. However, Waddy and Aiken (1992) found that *H. americanus* needs to experience the colder winter temperatures to be capable of spawning in the spring/summer. Similar results were found by Aktas et al. (2003) in the shrimp, *P. semisulcatus*, when cycling temperatures between 20-28^{o}C stimulated spawning. In both experiments photoperiod exposure was also pertinent to the reproductive output of the decapods as similarly observed in the current experiment (Aktas et al., 2003; Waddy and Aiken, 1992).

Each species requires different environmental signals for ovarian development and reproduction, thus the results of the current study using *C. sapidus* will have to be optimized for other species and experimental conditions. The king crabs living near the Arctic will not have the same favorable requirements as the mud crab living near the equator (Sastry, 1983).

Ovarian and Embryo Development

Embryonic development after spawning was also examined in this study. By recording the date of spawning and hatching, a comparison could be made for the period of ovarian development during the different photoperiods at 21^{o}C. As stated earlier, a significant difference was found between the first and second spawning events and with no variation occurring between the different light treatments. The initial development of the ovaries for the production of the first spawn takes ~4 months (Dickinson et al., 2006). During the development of the first batch of oocytes for spawning, subsequent batches of oocytes are being prepared thus shortening the intervals between spawning events

(Charniaux-Cotton, 1985). The ovarian development was examined (Lee and Puppione, 1988) during the interval of 110 days to the first spawn by dissecting dead crabs removed from the tank system. The crabs which had recently died were used for determination of ovarian development since biopsies can not be performed on *C. sapidus* without sacrificing the animal. The oocytes increased in size over the 100 day growth period (Figure 2.8). Maturation of oocytes has previously been observed in *C. sapidus* for a 30 day period after molting in which animals were sacrificed to measure the oocyte diameter which increased from 40 ± 11 μm to 201 ± 45 μm (Lee and Walker, 1995). This similar increase in oocyte size implies that the current experimental crab's ovarian development was at an early stage when obtained from the wild.

By counting the larvae after hatching, it was evident that warmer temperatures produced more zoea of better quality with shorter developmental periods. Similar results were obtained by Zeng (2007) and Hamasaki (2003) in two *Scylla* sp. In the current experiment, *C. sapidus* showed a three-fold increase in their developmental period during 15^{o}C as compared to 21^{o}C (an average of 57 days compared to an average of 19 days, respectfully; data not shown). The larvae at 21^{o}C were healthier with few to no prezoea present when compared to the 15^{o}C larvae. However, in the 0L:24D condition, there was a higher abundance of prezoea. Prezoea are thought to occur due to abnormal hatching (Costlow, 1965), thus having a lower survival rate (personal observation). With increased production in 21^{o}C, 0L:24D and a higher abundance of prezoea, the conditions need to be further optimized to allow for the best development of embryos. In lobster aquaculture, temperature is able to be regulated to determine the development rate and

hatching date of the egg masses which could possibly be applied to crab aquaculture after further experimentation (Aiken and Waddy, 1985).

In this study, the number of larvae produced during the spawning events increased with the weight of the female. Larger crabs are known to produce more offspring (Prager et al., 1990); however, the use of weight in the current analysis indicates heavier crabs are also capable of producing larger spawns. The amount of larvae produced in these spawns varied when analyzed for different spawning events. The second spawning event produced more larvae then the first and third events (Figure 2.10). This pattern does not support data obtained in field experiments in which hatch numbers decreased with subsequent spawns (Dickinson et al., 2006). However, with little documented information available on multiple spawning events in the wild or captivity for *C. sapidus*, it is unknown which pattern is typical for this species.

Spawning Data Comparison

The data collected during this study was compared with six years of data from the COMB blue crab hatchery to determine the behavior of crabs in captivity. By having a large dataset to compare the current results to, the spawning behavior of *C. sapidus* show no significant difference (Figure 2.11A). Spawning intervals varied between the first and second spawning event (Figure 2.11B). The period of development to the first spawn was about 110 days. For this analysis, the interval for 2003 was removed since it was an outlier. The 2003 females were obtained from Virginia in February implying migration occurred prior to the overwintering period. The short interval to the first spawn also indicates that ovarian development had begun in the wild before being captured and when

exposed to temperatures in the hatchery, the rate of the development increased. In the 2001 and 2002 females a longer developmental period was observed. These crabs were obtained from the Rhode River in Maryland, in October, where crabs are residing to molt to maturity and mate. These crabs are assumed to have mated just prior to capture without migration occurring; thus, very little ovarian development has occurred. The crabs in 2004-2008 were all obtained in the late fall (October-November) in the middle region of the Chesapeake Bay (similar locations as illustrated in Figure 2.1). It is assumed that these females have recently molted, mated, and are beginning their migration to the lower Bay for overwintering and spawning in the spring. The ovarian development at this stage is minimal; therefore, when brought into captivity manipulations can occur to increase or decrease the maturation interval. For all years, the interval between multiple spawns was similar due to the acclimation of the females to the hatchery conditions. The estimation of spawning intervals for wild crabs was calculated to be about 22 days between multiple spawns using information from Dickinson et al. (2006) in which 8 spawns were produced in 25 weeks. The shortest interval seen in the hatchery and this experiment was 20 days, while the longest was 69 days. Since these animals were in captivity, they were not exposed to the same natural conditions or temperatures as the Dickinson et al. (2006) study which could explain the difference in average interval periods between multiple spawns. This confirms that the timing of capture for the hatchery is pertinent to be able to collect animals of early ovarian development to allow for year round production.

CONCLUSION

The results of this study show the importance of temperature and photoperiod in the ovarian development and spawning of *C. sapidus*. Since environmental conditions regulate the reproductive physiology of crustaceans (Sastry, 1983), these environmental signals should be evaluated for each species to provide optimal conditions for crustaceans held in aquaculture.

CHAPTER 3: HEMOLYMPH VITELLOGENIN LEVELS DURING OVARIAN DEVELOPMENT

INTRODUCTION

Vitellogenesis in most crustacean species is known to be negatively and positively regulated by the eyestalk neuropeptides, vitellogenesis/gonad-inhibiting hormone (VIH/GIH) and the gonad-stimulating hormone (GSH), which are structurally related and belong to the crustacean hyperglycemic hormone (CHH) family. This interplay of endocrine hormones in crustaceans has been vastly studied with several detailed reviews published (Beltz, 1988; Carlisle and Knowles, 1959; Fingerman, 1987; Huberman, 2000; Quackenbush, 1986; Van Herp and Soyez, 1998).

Crustaceans undergo the onset of vitellogenesis that is usually divided into two phases: primary and secondary vitellogenesis (Charniaux-Cotton, 1985; Lee and Walker, 1995; Quackenbush, 1986). In *C. sapidus*, primary vitellogenesis involves the accumulation of glycoproteins in the oocytes which causes the oocytes to grow (Charniaux-Cotton, 1985). Once the oocytes have accumulated enough protein, the second phase of vitellogenesis begins. During this phase, vitellogenin (VtG), which is produced by the hepatopancreas, is transported to the ovaries via the hemolymph (Lee and Puppione, 1988; Zmora et al., 2007). Then, the VtG is uptaken by the ovaries and accumulated as yolk (vitellin, VT) in the oocytes which increases the size of the ovaries (Charniaux-Cotton, 1985; Lee and Walker, 1995). Similar activities also occur in the mud crab, *Scylla* sp. (Quinitio and Parado-Estepa, 2003).

Previous results obtained from *C. sapidus* reveal that VtG (~282 kDa), produced as a preproVtG in the hepatopancreas is immediately cleaved into two subunits (~78.5

kDa and 207.3 kDa) which is then secreted into the hemolymph (Zmora et al., 2007). VtG and VT subunits have also been determined in a variety of crustaceans including *Cancer antennarius* (Puppione et al., 1986), *Potamon potamios* (Pateraki and Stratakis, 1997), and *Homarus americanus* (Tsukimura et al., 2002) which show similar patterns of cleavage as *C. sapidus*.

Female *C. sapidus* can produce more than one egg mass per spawning season (Dickinson et al., 2006; Hines et al., 2003) with vitellogenesis occurring over a 30-40 day period; therefore, it is expected that VtG levels will remain high in animals producing multiple spawns to support the development of the next batch of oocytes. One way to monitor this development is to measure the VtG levels in the hemolymph as shown by Zmora et al. (2007). The hemolymph VtG levels documented by Zmora et al. (2007) steadily increased during ovarian development up to day 35 after pubertal molt and mating after which the VtG level remained stable until the end of the experiment (70 day experiment). Similar increased VtG trends were also found in *C. sapidus* by Lee and Puppione (1988) during a 70 day experiment and in *Macrobrachium rosenbergii* during a 30 day observation period (Okumura and Aida, 2001).

The monitoring of VtG levels in *C. sapidus* hemolymph have allowed for an assessment of the ovarian development up to 70 days after terminal molt (Lee and Puppione, 1988; Zmora et al., 2007). In Chapter 2, it was stated that mature female *C. sapidus* spawn after 42-152 days in captivity and subsequent spawning events can transpire an average of 47 days later. These two experiments on *C. sapidus* ovarian development were short-term observations of VtG concentrations before, during, and after molting (Lee and Puppione, 1988; Zmora et al., 2007) and through spawning (Lee

and Puppione, 1988). To better understand spawning and multiple spawning, the current experiment utilized four experimental systems during a two year period. The experimental systems allowed for the monitoring of hemolymph VtG levels in three temperature regimes (11^{o}C, 15^{o}C, and 21^{o}C) and four photoperiods (0L:14D, 8L:16D, 16L:8D, and 24L:0D) over 133 days each to determine whether, as in Chapter 2, temperature and/or photoperiod influence ovarian development.

MATERIALS AND METHODS

Animal Collection and Care

Adult female crabs were caught in crab pots with a local waterman in the Chesapeake Bay, MD. Harvest dates were as follows: (1) November 17, 2006, (2) April 27, 2007, and (3) November 24, 2007. Batches 1 and 3 were harvested near Sharps Island lighthouse (76^{o}20'60" W, 38^{o}35'15" N) while Batch 2 was obtained between Cedar Point and Point No Point Lighthouse (76^{o}30'60" W, 38^{o}15'22" N). Crabs were placed in coolers with wet burlap while aboard the vessel and transferred to coolers with Bay water, aeration, and Nitex netting upon arrival at the dock. Crabs were chosen by appearance: no less then eight legs present, both chelae intact, no scars/wounds, and no limbs were broken. Harvesting locations temperature and salinity were measured and recorded using a thermometer and refractometer (VitalSine, model SR-6). After transferring the crabs to the coolers with water from the harvesting locations, they were taken 1-2 hours by car to the Center of Marine Biotechnology (COMB) in Baltimore, MD. Upon arrival the crabs were transferred to holding tanks of 3800 L at similar conditions as their harvest locations with a natural photoperiod regime (Batch 1: 30 ppt,

16°C after 3 days of a 5 ppt/day salinity increase; Batch 2: 11 ppt, 13°C; and Batch 3: 18 ppt, 13°C). Salinity in the holding tanks was increased to 30 ppt (2 ppt/day over a two week period) for Batches 2 and 3, photoperiod decreased to 8 hours light with 16 hours dark (8L:16D) by December 21st except for Batch 2 which was reduced to 8L:16D over a 2.5 week period after arrival at the facility in April. Crabs were acclimated in the holding tank for 2-5 months while the experimental systems were prepared. Afterwards, they were transferred to the experimental systems. Throughout the period in captivity, the crabs were fed cut up squid and a pelleted diet (EWOS Brood, 9 mm). Crabs were weighed then labeled with a plastic coated copper wire bearing a tag for identification during the period of transfer to the experimental systems. While the crabs were in the experimental systems, salinity and temperature were recorded daily, while ammonia and nitrate were measured twice a week (flow injection) and pH once a week (Orion expandable ionAnalyzer, EA 940).

The experimental tank systems were designed to assess the effects of temperature and photoperiod on ovarian development; therefore, the crabs were exposed to 21°C (Batch 1 and half of Batch 3), 15°C (Batch 2), or 11°C (half of Batch 3) with a salinity of 30 ppt and photoperiods of 0L:14D, 8L:16D, 16L:8D, and 24L:0D. In the case of the 15°C condition, the crabs experienced the set temperature for 13.5 weeks before increasing the temperature to 21°C (1°C per day). The tank systems were equipped with temperature controllers (Process Technology, Model DQ15D) with heat exchangers, bubble bead filters (Aquatic System Technology), fluidized sand biofilters (QuikSand Filters), and protein skimmers (RK2 Systems, Model RK25PE). One system was 530 L

and the other was 333 L with a daily backwash exchanged 3.4% and 5.5% of the water, respectfully.

Hemolymph Sampling

Hemolymph samples were drawn twice a week for 19 weeks (38 samples) for all Batches using a 1 mL syringe (BD). Hemolymph was mixed at a 1:1 ratio with a marine anticoagulant (pH 4.0) (Söderhäll and Smith, 1983) and then stored in -20^{o}C until assayed.

Vitellin Purification

C. sapidus ovaries were dissected and stored in -80^{o}C until purified. Ovaries of stage 2 (2 grams) and stage 3 (3 grams) were placed in 15 mL Tris (20 mM, pH 7.5) with 1.5 mL protease inhibitor cocktail (Sigma, P1860). The ovaries were then homogenized using a tissue homogenizer (Ultra-Turrax T25, Janke and Kunkel IKA Labortechnik) and centrifuged for 10 minutes at 8000 g at 4^{o}C. After centrifugation, the supernatant solution was kept, while the solids and lipids were discarded. The proteins were precipitated by adding ammonium sulfate (final concentration 60%) to the supernatant solution and kept on ice for 1 hour. The sample was then centrifuged at 16,000 g at 4^{o}C for 20 minutes. The pellet was resuspended in 10 mL Tris (pH 7.5) with protease inhibitor by vortexing and protein precipitation and centrifugation was repeated as before. The pellet was resuspended in running buffer (0.01% EDTA in PBS) containing protease inhibitor.

VT was purified by size exclusion chromatography using Bio-Gel P-300 beads (size exclusion of 300,000 MW). Dry beads were hydrated in PBS for 3 hours at room temperature. A column (2.8 cm x 17 cm) was first filled with 10 mL PBS, then hydrated beads, and then washed with PBS then running buffer. The cap was applied to the bottom of the column while loading the sample. Approximately 2-4 mL of the resuspended crude protein was slowly loaded onto the column. Running buffer (~10 mL) was gently added on top of the sample as not to disturb the sample. The cap was removed and the bed was gently washed with running buffer by gravity. The first third of the column flow through (10 mL) was collected and discarded as void volume after which 14 fractions (3 mL each) were collected and stored in -20^{o}C overnight.

On the following day, a 4-15% SDS denaturing gel was run with 2 μL of each fraction. The gel was stained with Coomassie blue (BioSafe Coomassie, BioRad G-250 Stain) and analyzed for two bands: ~75 and 100 kDa (Figure 3.1) due to the denaturation of the gel. The two fractions with the most distinct ~75 and 100 kDa bands were analyzed using a BioRad D_C protein assay kit to estimate the protein quantifications. The selected fractions were pooled and divided into 1 mg aliquots. The aliquots were lyophilized (Freezone 12, Labconco) and the samples were stored in -80^{o}C until further use.

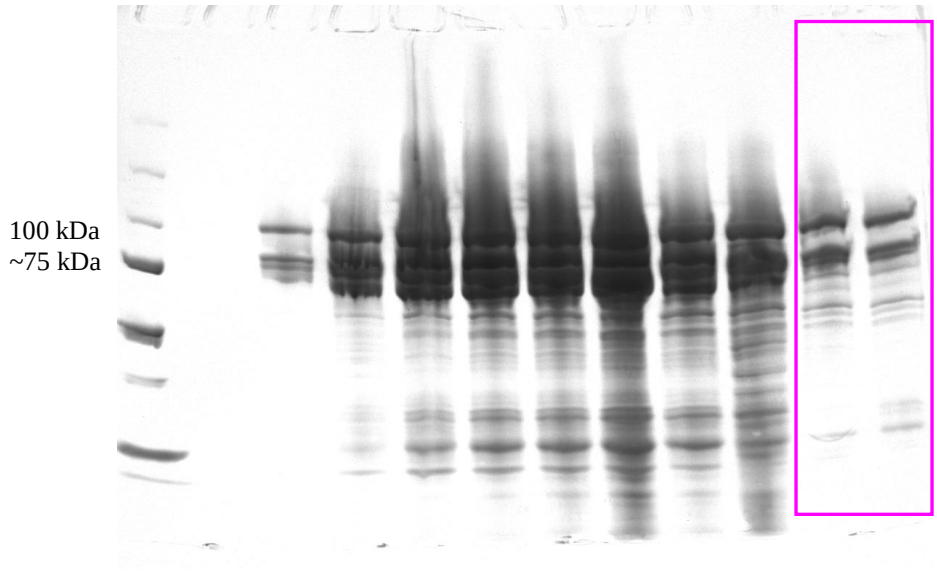


Figure 3.1. Vitellin purification gel to determine subunits for extraction. VT was separated on a 4-15% SDS denaturing gel with 2 μL of each fraction to determine the collected fractions containing the VT subunits.

Affinity Purification of Anti-VT

Rabbit Anti-VT Serum

The anti-VT was previously produced in rabbits (Sigma-Genosys Laboratories) (Zmora et al., 2007).

Affinity Purification Column Preparation

The anti-VT was purified to increase the specificity of the assay. First, CNBr activated Sepharose beads (0.24 g, Sigma) were placed into 10.5 mL of cold 1M HCl (pH 3.0) and mixed gently to suspend the beads for hydration and kept on ice for 30 minutes. After hydrating, the solution was centrifuged at 500 g, 4^{o}C for 5 minutes. A majority of the supernatant was removed leaving 1 mL of bed volume. The beads were once again suspended in ice cold 1M HCl, inverted for mixing, and centrifuged for another 5 minutes

as before. These steps were repeated four more times after which the fifth time only 0.5 mL of HCl was left above the bead bed. The beads were then resuspended in coupling buffer (0.1 M $NaHCO_3$, 0.5 M NaCl, pH 8.0) and centrifuged at 500 g, 4^oC for 5 minutes. The buffer was removed leaving only 0.5 mL of buffer above bed. The bed was again resuspended with coupling buffer and centrifuged another three times leaving only 0.5 mL of buffer above bed (1.5 mL total volume).

VT Purification

The freeze dried VT fraction was further purified to obtain the VT subunits. Freeze dried VT fraction (2 mg) was separated on a 5% native gel with SDS to allow for the retention of the tertiary structure, then one lane was stained with Coomassie blue (BioSafe Coomassie, BioRad G-250 Stain) to determine the location of the two subunit bands. The two distinct bands of ~75 and 100 kDa were cut from the gel and electroeluted (Centrielutor- Micro-Eluter, Millipore and Centricon 30) according to the manufacturer's instructions in non-denaturing running buffer (Tris-Glycine, pH 8.3) and quantified (BioRad D_C protein assay kit). VT was concentrated by centrifugation on a Centriplus filter (3000 Da exclusion) at 3000 g, 4^oC. The collected proteins were later released from the filter into a coupling buffer (0.1 M $NaHCO_3$, 0.5 M NaCl, pH 8.0).

Affinity Purification of Anti-VT

The purified VT in the coupling buffer was then added to the bead bed and placed on a shaker (Roto-Shake Genie, Scientific Industries, Inc.) for 4 hours at room temperature. After the allocated mixing time, 1 mL of 1M Tris (pH 8.0) was added to the

solution and mixed once again at room temperature for 2 hours on the shaker. The solution was then centrifuged at 500 g, 4°C for 5 minutes. The beads were washed three times in PBS by repeating the centrifugation step. After the final washing step, all PBS was removed except 0.5 mL above the bed which prepared the beads for the addition of the antibody.

Previously freeze dried antiserum (2 mL) was resuspended in 2 mL sterilized water and added to the beads (3.5 mL total volume). The mixture was incubated at 4°C overnight or for several days for affinity binding of VT to the antisera. After incubation, the mixture was gently mixed to resuspend the beads for transfer to a column (1 cm x 5 cm) in 1 mL increments. The flow through was collected for future use. The beads were transferred to the column and were washed with cold 1 mL PBS three times. Next, the antibody was eluted from the beads using cold 1M HCl (pH 2.5). The eluted antibody (500 μL) was collected in ten collection tubes containing 100 μL 1M Tris (pH 8.0) to buffer the 500 μL HCl solution upon elution (total volume of 600 μL) and the tubes were placed on ice for protein estimation (BioRad D_C protein assay kit). The column was then washed with PBS three times. The sample tubes with the antibody were combined and glycerol was added at a 2:1 ratio. The purified antibody was stored in 4°C until further use.

The beads were then removed from the column using PBS and placed into the flow through antibody solution collected above for further incubation. The VT coupled beads were used several times by renewing the antibody until enough purified antibody was obtained. The yield of the affinity purified anti-VT from 1 mL of antiserum was ~250 μg/mL.

Competitive ELISA

Coating Plate

The purified VT and affinity purified antibody were used to measure the VtG levels of the hemolymph samples. The competitive enzyme linked immunosorbent assay (ELISA) was performed over a two day period. The first day, a 96 well plate (Corning, Costar 3590) was coated with 100 ng of purified VT in freshly made 50 mM sodium bicarbonate buffer (pH 9.6) except for two wells which were coated with only sodium bicarbonate buffer (100 μL/well) to serve as blanks for the experiment. The plate was covered with adhesive film (Kendall Elkay Seal Plate, Tyco) and placed in 4^{o}C overnight for 18 hours.

Standard and Sample Preparation

A standard curve using purified VT in 0.01% BSA in PBS was previously determined for use (0-3200 ng/mL). The majority of the hemolymph samples were initially diluted at 1 μL/250 μL 0.01% BSA in PBS; however, samples which were out of the range of the standards were diluted accordingly and reassayed. A mixture of two randomly selected hemolymph samples from each female (n = 91) was used as a reference for all assays (197.11 ± 8.11 μg/mL) and was diluted at 0.25 μL/250 μL 0.01% BSA in PBS. The affinity purified antibody (1:250) was then added to the hemolymph samples, reference, and standards at a 1:1 ratio in 0.01% BSA in PBS. The samples, reference, and standards were vortexed and incubated at 4^{o}C overnight (18 hours).

Assay

The following day, the plate was washed three times with PBST (0.05% Tween 20 in PBS) and then blocked with 5% nonfat skimmed milk (Carnation) in PBST for 1 hour at 37^{o}C. After discarding the blocking solution, the wells were loaded with the standards, reference, and samples – 100 μL/well in duplicate. The plate was then incubated for 1 hour at 37^{o}C. After incubation, the plate was washed three times with PBST and incubated with 100 μL/well of secondary antibody (Peroxidase-conjugated AffiniPure Goat Anti-Rabbit IgG (H+L), Jackson ImmunoResearch Laboratories, Inc.) at 1:15,000 for 1 hour. The plate was washed three times with PBST after incubation. The color developing solution (TMB, KPL) was mixed (1:1) and left at room temperature for 1 hour. The color solution was loaded onto the plate at 100 μL/well and the reaction was stopped after 5 minutes with 100 μL/well of 1M H_3PO_4. The absorbance was immediately read at 450 nm using a SPECTRAmax M5 plate reader (Molecular Devices). Samples which were out of the accepted standard range (< 100 ng/mL or > 2000 ng/mL) were reassayed after further dilution. The value of EC_{50} was 397.7 ± 14.7 ng/mL (n = 93) (Figure 3.2).

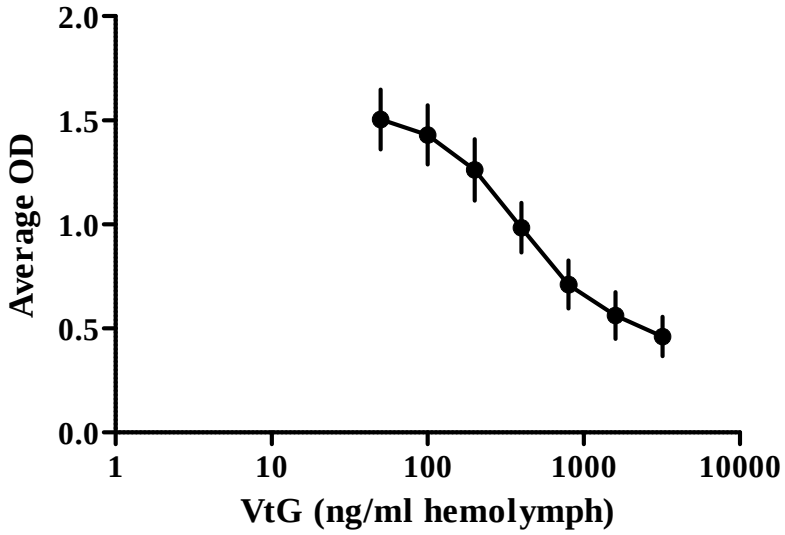


Figure 3.2. A typical standard curve for vitellogenin competitive ELISA. Competitive ELISA was used to determine the amount of VtG in the hemolymph samples using a predetermined standard range from 0-3200 ng VtG/mL hemolymph (EC_{50} = 397.7 ± 14.7 ng/mL, n = 93).

Protein Quantification

Total protein quantification was performed on selected hemolymph samples to evaluate the differences in VtG concentrations seen between the three batches of animals. Using the highest and lowest VtG concentrations per female (two samples per female) at 50x dilution, protein concentrations were determined using a BioRad D_C protein assay kit and the absorbance was read at 650 nm using a SPECTRAmax M5 plate reader (Molecular Devices).

Statistical Analysis

Hemolymph VtG concentrations were categorized by Batch into spawning and non-spawning females. The spawned females were aligned by spawning date (0) while the non-spawned females were assessed using sampling dates. The 21^{o}C Batches 1 and 3 were combined for several analyses in which the two Batches were aligned by spawning

date (0) or duration of the experiment (19 weeks). The VtG levels were averaged (mean ± SEM) and graphed (GraphPad Prism) while the EC_{50} was calculated with SigmaPlot. The spawned females were analyzed using two-way ANOVA. Female weight, number of hatched larvae, and VtG levels were correlated using Pearson correlation (GraphPad Prism). Total hemolymph VtG was calculated using the total protein quantification and then averaged for spawned and non-spawned females. Statistical analyses were accepted at $p < 0.05$.

RESULTS

Competitive ELISA

Non-spawned Females

Non-spawning animals were present in all temperature conditions (21°C, 15°C, and 11°C). In Batches 1 and 3 (21°C), the VtG concentrations fluctuated throughout the first 9 weeks of the duration of sampling after which Batch 1 levels off and Batch 3 continues to fluctuate (Figure 3.3A). When Batches 1 and 3 VtG levels were compared, there was a significant variation between the two experiments ($p = 0.03$) with Batch 3 (1340.9 ± 262.9 μg/mL) having a significantly higher VtG level than Batch 1 (309.3 ± 147.0 μg/mL) at 18.5 weeks ($p < 0.01$). Batch 2 (15°C) VtG levels increased in the beginning of the sampling period to ~850 μg/mL; then they decreased and leveled off to ~250 μg/mL with little fluctuation (Figure 3.3B). After the temperature was increased to 21°C the levels remained low, at ~250 μg/mL. Lastly, the 11°C Batch 3 females had very

low levels of VtG (maximum mean level 266.0 ± 154.2 μg/mL) throughout the 133 day sampling period (Figure 3.3C).

Spawned Females

Temperature

VtG levels in the spawned females varied by Batch, but all Batches showed a decrease in VtG levels prior to or during the time of spawning (Figure 3.4). Females producing spawns were exposed to 21°C and 15°C conditions. Batches 1 and 3 (21°C) and Batch 2 (15°C) showed similar VtG levels ($p = 0.83$) with mean VtG concentrations of 678.2 ± 140.3 μg/mL ($n = 19$) and 653.6 ± 210.3 μg/mL ($n = 5$), respectively, at the time of spawning (Figure 3.5). When analyzing Batches 1 and 3 (21°C) separately (Figure 3.6), Batch 3 females had higher levels of VtG concentrations than those of Batch 1 ($p < 0.0001$) which occurred over ten consecutive sampling points, 2.5 weeks, before and after spawning (point 0).

Photoperiod

The analysis of photoperiod in both 21°C and 15°C showed no significant interaction between photoperiods ($p = 0.99$ and $p = 0.96$, respectively) (Figure 3.7). The VtG levels for the 15°C females initially range from ~500-1000 μg/mL for 5 weeks after which an increase to ~1250 μg/mL occurs before spawning when the levels decrease to ~400 μg/mL where they stay for the remainder of the sampling points (Figure 3.7A). The

21°C exposed females show very similar VtG levels throughout the experimental period with the majority of the points between ~200-1000 µg/mL (Figure 3.7B).

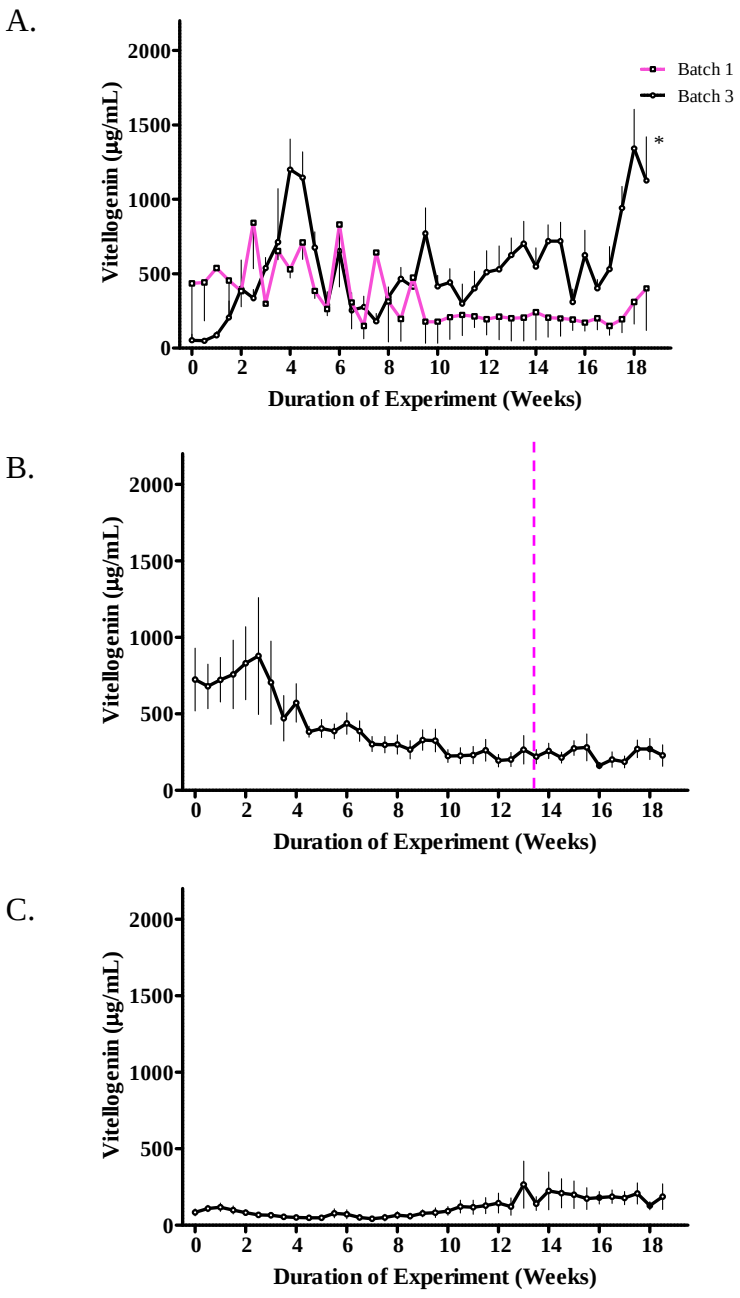


Figure 3.3. Vitellogenin levels of non-spawning females. Females in (A) 21°C, (B) 15°C, and (C) 11°C during the sampling dates show contrasting patterns. (A) Batch 1 (pink) varies significantly from Batch 3 (black) at 18.5 weeks ($p < 0.01$). (B) The 15°C females experienced 13.5 weeks of 15°C after which the temperature was increased to 21°C (indicated by the dotted line). All graphed results are represented as mean ± SEM. (A) Batch 1: n = 2 and Batch 3: n = 6, (B) n = 5 and (C) n = 10.

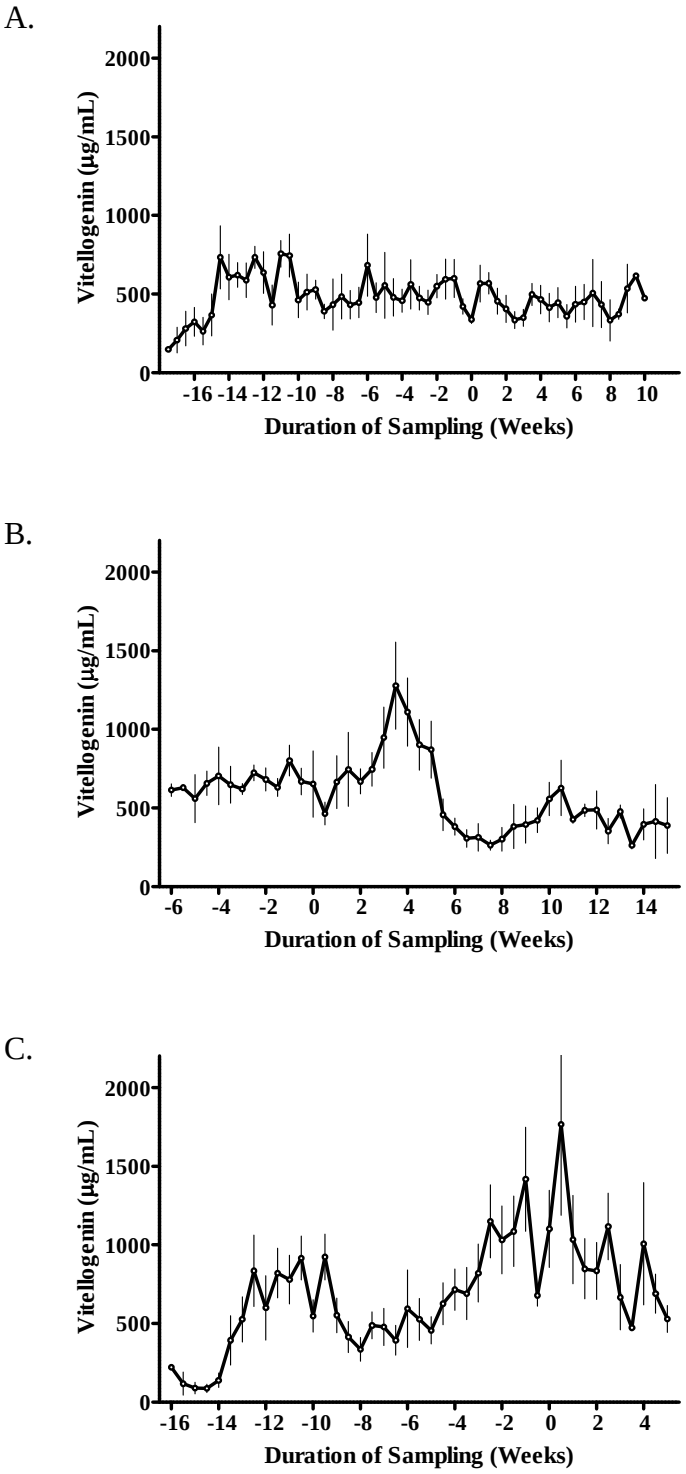


Figure 3.4. Vitellogenin concentrations determined by ELISA for all spawning animals by Batch. Concentrations for (A) Batch 1, (B) Batch 2, and (C) Batch 3 are plotted with sampling point 0 as the spawning date. Concentrations are presented as mean ± SEM. (A) n = 10, (B) n = 9, and (C) n = 5.

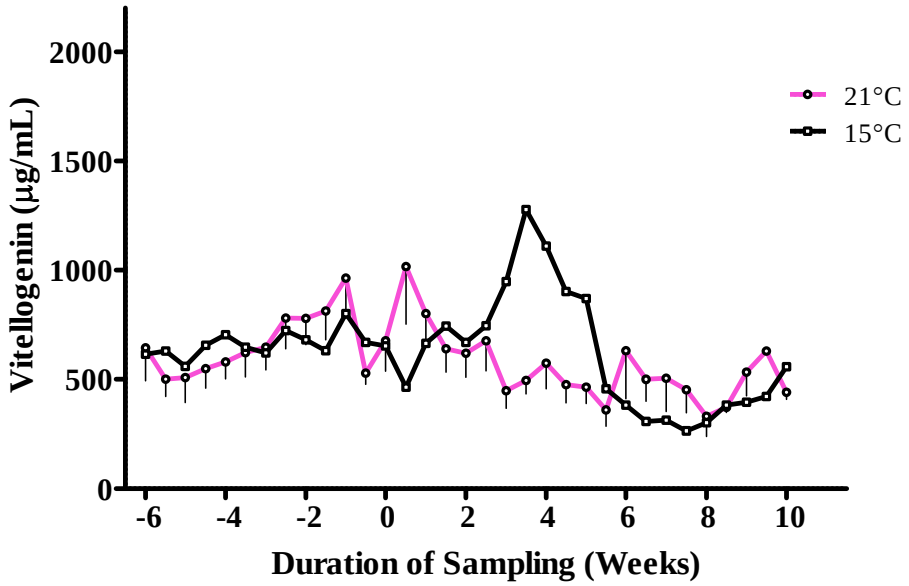


Figure 3.5. The effect of different temperatures on vitellogenin concentrations. Temperature regimes were compared for spawning animals (21°C, pink and 15°C, black) using two-way ANOVA and presented as mean ± SEM with 21°C: n = 19 and 15°C: n = 5. The time of spawning is indicated by point 0.

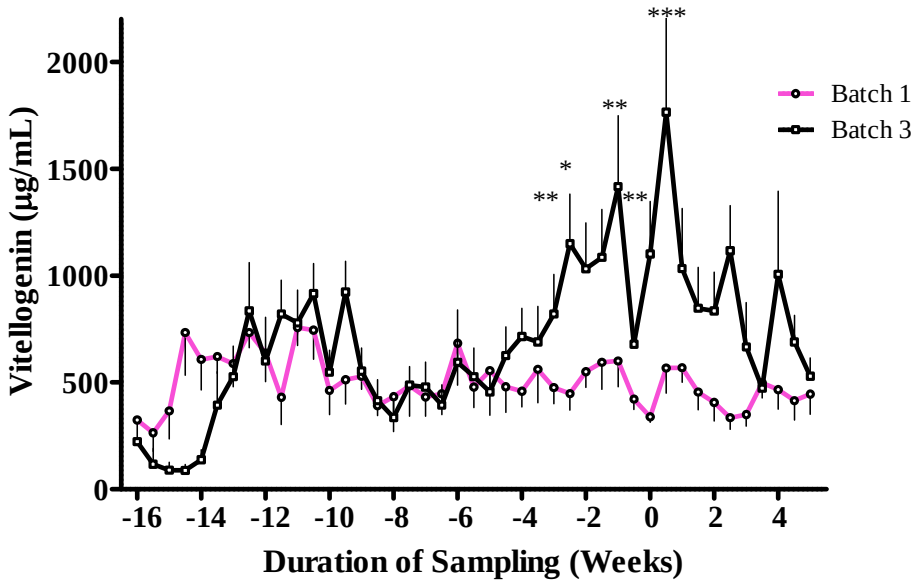
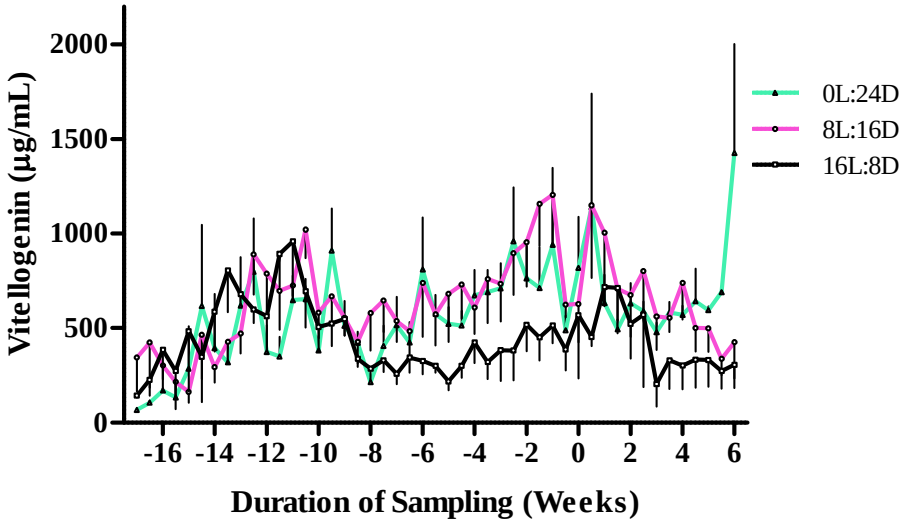


Figure 3.6. Vitellogenin concentrations between 21°C Batches 1 and 3. Females of Batch 3 (black) showed significantly higher VtG concentrations than Batch 1 (pink) ($p = 0.0017$). Results are presented as mean ± SEM with Batch 1: n = 10 and Batch 3: n = 9 with spawning occurring at point 0.

A.



B.

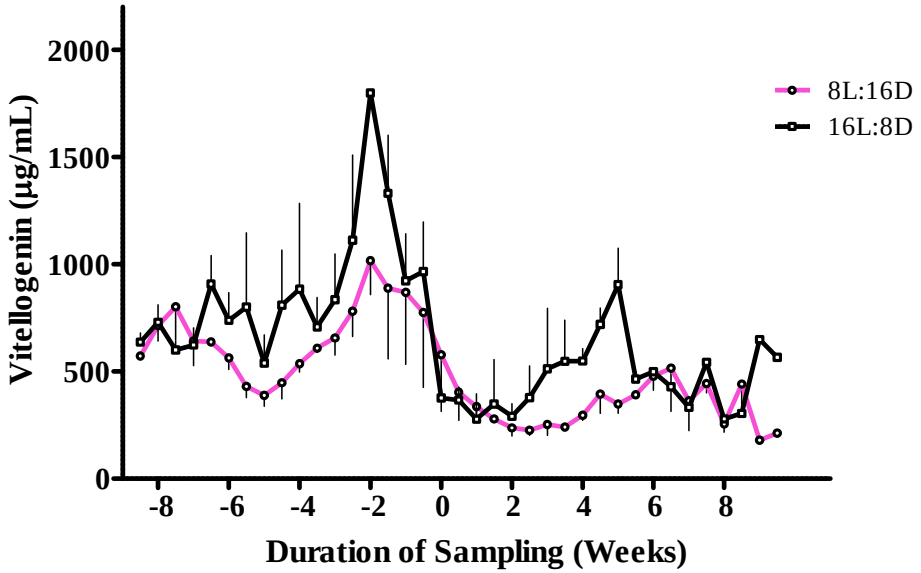


Figure 3.7. The effect of photoperiod on vitellogenin concentrations. VtG levels were compared for photoperiods in (A) 21°C and (B) 15°C. In both temperatures, VtG levels varied significantly between the photoperiods varied (A: $p < 0.0001$; B: $p < 0.0001$). VtG levels are in as mean ± SEM with (A) 0L:24D n = 6, 8L:16D n = 8, and 16L:8D n = 5 and (B) 8L:16D n = 2 and 16L:8D n = 3.

Protein Quantification

Hemolymph total protein levels in the spawned and non-spawned females were similar($p = 0.38$ and $p = 0.20$, respectively; f-test), as were protein levels between spawned Batches ($p = 0.35$, f-test) and non-spawned Batches ($p = 0.46$, f-test). The hemolymph VtG levels for spawned females were on average 1534.1 ± 157.5 μg/mL (high VtG samples) and 134.3 ± 17.2 μg/mL (low VtG samples) while non-spawned females averaged 992.5 ± 152.1 μg/mL (high VtG samples) and 82.7 ± 22.8 μg/mL (low VtG samples). The total protein levels for spawned females consist of 3.2 ± 0.5% VtG (high VtG samples) and 0.3 ± 0.0 % VtG (low VtG samples) while the percentages of non-spawned females were lower (high VtG samples: 1.8 ± 0.3% VtG and low VtG samples: 0.2 ± 0.0 % VtG).

DISCUSSION

Temperature Dependent VtG Levels

There are two major findings for this experiment: (1) hemolymph VtG levels in *C. sapidus* decrease before or during first and multiple spawning events; and (2) temperature affects the hemolymph concentration of VtG in *C. sapidus*. The VtG levels prior to and after spawning in *C. sapidus* were first documented by Lee and Puppione (1988), where VtG was termed lipoprotein II. Female *C. sapidus* hemolymph was sampled at ovarian stages 1-8 (recently molting to carrying a very well developed egg mass) with the highest level of VtG occurring at stage 6 (4.1 ± 2.1 mg/mL) when an orange spawn was present on the abdomen of the crab. The levels of VtG prior to stage 6 increased and afterwards decreased rapidly to 0 ± 0.00 mg/mL at stage 8 when the spawn was gray before hatching

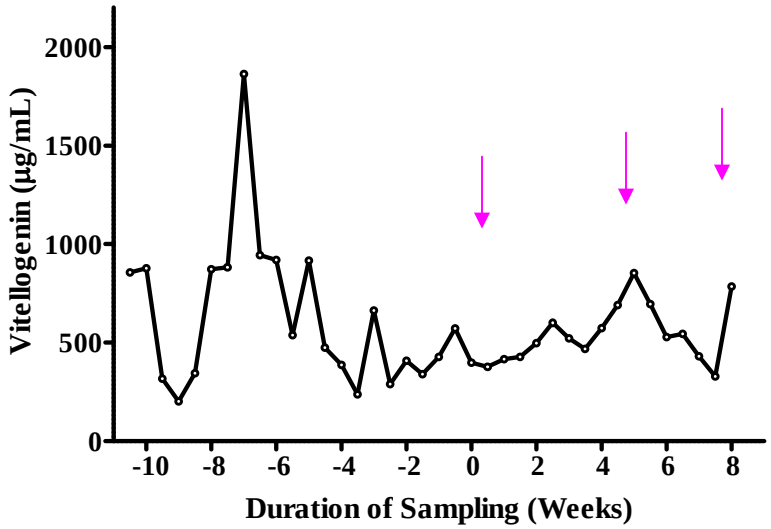
(Lee and Puppione, 1988). A second study monitoring the VtG levels of *C. sapidus* illustrate a slight increase in VtG levels from stage 1-4 (0 ± 0.00 mg/mL-0.02 ± 0.04 mg/mL) with molting occurring prior to stage 1 and spawning occurring at stage 4 followed by an increase after spawning to 0.14 ± 0.04 mg/mL (Lee et al., 1996a). In the current experiment, the VtG levels show a slight decrease prior to or during the first and subsequent spawning events (Figure 3.4) opposing the previously described results. However, decreases in VtG prior to spawning have been documented for other species: *H. americanus* (Byard and Aiken, 1984; Nelson, 1986), *Macrobrachium nipponense* (Okumura et al., 1992), and *Macrobrachium rosenbergii* (Derelle et al., 1986; Okumura and Aida, 2001).

H. americanus (Byard and Aiken, 1984; Nelson, 1986), *M. nipponense* (Okumura et al., 1992), and *M. rosenbergii* (Derelle et al., 1986; Okumura and Aida, 2001) differ from *C. sapidus* since they continue to molt while *C. sapidus* experiences anecdysis. The three species listed above illustrate VtG levels increasing after molting and decreasing prior to ecdysis and spawning during the reproductive molt cycles. In *C. sapidus*, the VtG levels increase after the terminal molt and then decrease 35 days later with mated females continuing ovarian development (~100 μg VtG/mL hemolymph) (Zmora et al., 2007). In the current experiment, the reproductive female VtG levels fluctuate, but consistently remain above ~200 μg VtG/mL hemolymph (Figure 3.4). The consistent decrease in VtG prior to or during spawning could be due to the preparation of a subsequent batch of oocytes uptaking VtG during the current spawning event (Lee et al., 1996a). However, in species which undergo continuous molting during adulthood, VtG is produced and absorbed into the oocytes to prepare for the upcoming spawning event.

When the oocytes are matured and ready for spawning the VtG production is halted and VtG is no longer absorbed by the oocytes and spawning occurs resulting in low levels of VtG in *M. nipponense* (Okumura et al., 1992). It is unknown as to whether *C. sapidus* halts vitellogenesis to prepare for spawning; however, this pattern seems to be a common feature as it was repeatedly seen in females producing multiple spawns in this study (Figure 3.8).

The levels of VtG measured in this experiment in spawned females correlates to the number of larvae hatched in Chapter 2. Females with higher averaged VtG concentrations produced more larvae (Figure 3.9A). VtG concentrations were averaged up to the point of spawning and correlated with the total larvae produced during that spawning event. Overall, there was a significant correlation between hatched number of larvae and averaged VtG level ($r = 0.67$). When comparing body weight and accumulated and averaged VtG levels, no correlation is present ($r = 0.05$) (Figure 3.9B). In Chapter 2, heavier females produced more larvae; however, by looking at a fraction of the total hemolymph different results are obtained. Assuming the total hemolymph volume is 10% of the crab's body weight, the total VtG in the body increases with weight ($r = 0.45$, Figure 3.9C). Heavier females have a larger hepatopancreas and ovary and a greater hemolymph volume therefore they are able to produce, transport, and uptake more VtG to produce larger spawns.

A.



B.

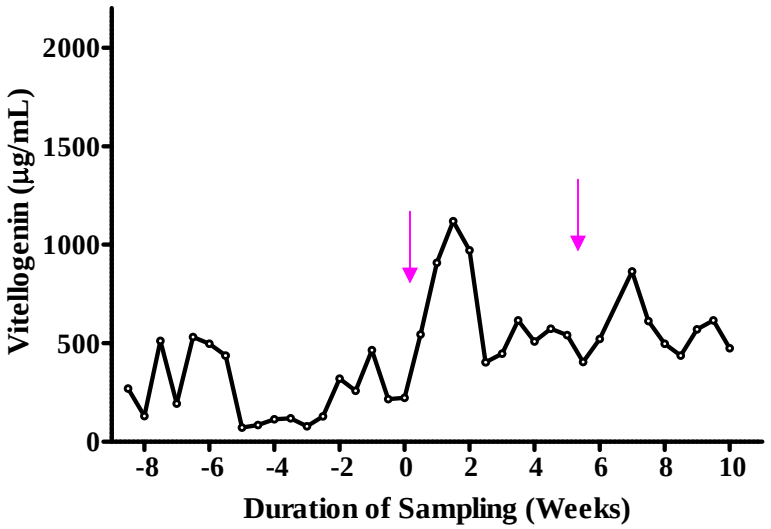


Figure 3.8. Spawning females during multiple spawning events. VtG levels are documented for multiple spawning events in two representative females: (A) B19 and (B) B20 during the sampling period. In each case, the 0 point indicates the first spawning event indicated by the first arrow while subsequent spawns are also denoted with arrows.

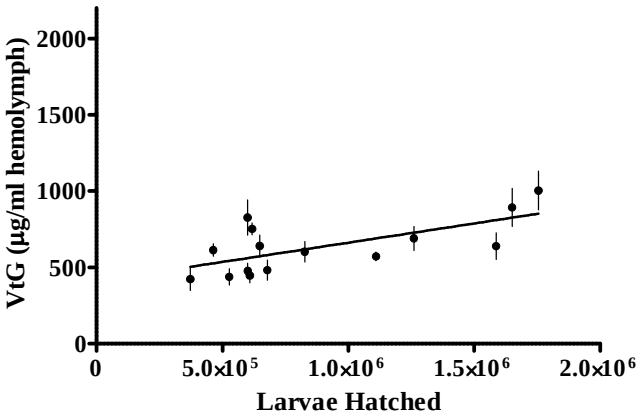
The spawning of *C. sapidus* is influenced by temperature as observed in this study which correlates with the natural population. In the Chesapeake Bay *C. sapidus* spawns during warmer temperatures (21-27^{o}C) from July to September (Sandoz and Rogers, 1944) while areas such as Florida experience similar temperatures (21-27^{o}C) and spawning occurs from April to November (Hines et al., 2003). In the Chesapeake Bay, a three month season is long enough for the crabs to produce 1-3 spawns, whereas in Florida the longer season allows for up to 7 spawns (Hines et al., 2003). During the current experiment of 19 weeks (4.75 months) at 21^{o}C, the number of spawns produced was 1-3; therefore, the crabs regulate ovarian development by continuously producing and uptaking VtG to allow for spawning intervals of 47 days as determined in Chapter 2.

In non-spawning females at 21^{o}C and 15^{o}C, VtG levels were also influenced by temperature and vary throughout the sampling period. In Batch 1 female's (21^{o}C) VtG levels remain constant after 10 weeks; then, they slightly increased at the end of the sampling period. In Batch 3 (21^{o}C) the VtG concentrations fluctuate throughout the entire sampling period with maximum levels being reached 4 weeks (1199.3 ± 205.0 μg/mL) after the sampling began and 1 week (1340.9 ± 262.9 μg/mL) before the sampling ended. Finally, in Batch 2 (15^{o}C), the VtG concentrations were similar to Batch 1 with levels fluctuating initially and then leveling off. These results are similar to the findings in non-reproductive *M. nipponense* (Okumura et al., 1992) and unmated *C. sapidus* (Zmora et al., 2007). The results of Batch 3 (21^{o}C) seem to be consistent with the results of mated *C. sapidus* (Zmora et al., 2007) since the surviving animals spawned after the experimental period (data not shown).

Non-spawning 11^{o}C females of Batch 3 showed low levels of hemolymph VtG throughout the entire sampling period suggesting little vitellogenesis and resulting in no spawns as stated in Chapter 2. In observations on *H. americanus* (Byard and Aiken, 1984) during the winter season (~0^{o}C), VtG levels range from zero to very low levels. The low levels observed in *C. sapidus* correspond with the metabolic activity decrease during low temperature overwintering periods seen in the wild (Churchill, 1917; Havens and McConaugha, 1990; Van Engel, 1958). During this overwintering period, the animals are energetically arrested and will increase their activities once exposed to warmer temperatures.

When comparing the percent of VtG in the total hemolymph protein at the highest and lowest values for spawned versus non-spawned females, the VtG levels are higher for spawned females with 0.26-3.16% of the total hemolymph protein, whereas non-spawned crabs range from 0.15-1.80%. The spawned females are continually producing VtG to be uptaken by the oocytes which agrees with the higher VtG levels in the hemolymph of the spawned females. The non-spawned females do not appear to be reproductively active in all aspects of vitellogenesis including VtG synthesis, transport, and uptake.

A.



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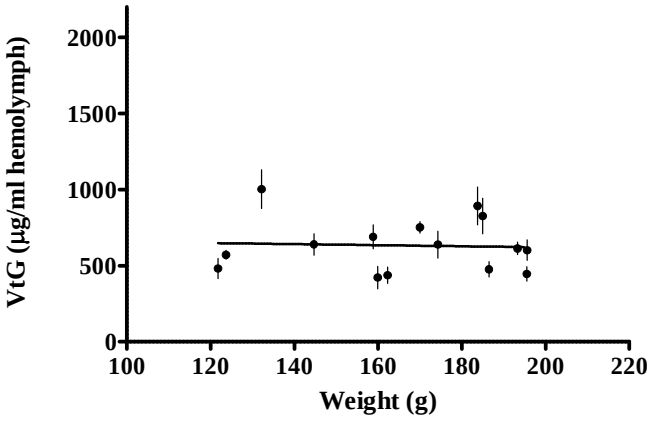

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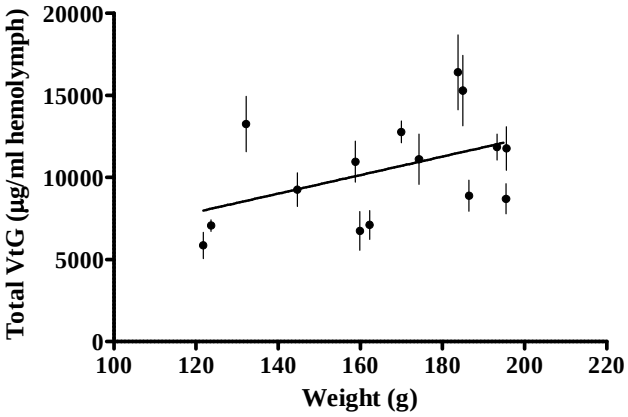


Figure 3.9. Vitellogenin concentrations correlated to the number of larvae hatched and female size. (A) Female VtG levels do not correlate with the number of larvae hatched (r = 0.67). (B) The weight of the female does not correlate with a fraction of the hemolymph VtG (r = 0.05), however, (C) a positive correlation is present when assessing the total hemolymph volume (r = 0.45). VtG concentrations are present in mean ± SEM (n = 15).

Photoperiod

Similar results obtained within the different photoperiod regimes indicate that photoperiod is not as important for the reproductive nature of the female *C. sapidus* as temperature. In both temperature regimes, all photoperiods showed similar VtG levels over the experimental period (Figure 3.7) indicating that temperature has a greater influence on the ovarian development of *C. sapidus* than photoperiod. These results are comparable to the photothermal results of Chapter 2 in which 0L:24D at 21^{o}C was the optimal condition for spawning while 8L:16D resulted in greater production numbers than 16L:8D. In this experiment at 21^{o}C, 0L:8D and 8L:16D had higher VtG levels, which were not significant, than 16L:8D relating to the results of Chapter 2. In 15^{o}C, the 8L:16D also had higher VtG levels than 16L:8D, even though the variation was not significant, which also correspond the findings in Chapter 2 in which shorter photoperiods resulted in higher production. In contrast, *Homarus* sp. spawn when triggered by long photoperiods (16L:8D), but short photoperiods (8L:16D) are needed to initiate primary vitellogenesis (Nelson, 1986; Nelson et al., 1983). *Homarus* sp. are deep water dwelling crustaceans which migrate to shallower depths to spawn; thus, the change between 8L:16D and 16L:8D may simulate the environmental conditions experienced during the migration period. *C. sapidus* also migrates to spawn, but unlike *Homarus* sp., they go from shallow to deeper depths which could explain the variation present between the photoperiods.

Ovarian VtG Regulation

The production of multiple spawns requires a continuous maturation of oocytes. Before the first spawn occurs, the oocytes undergo meiotic division stopping at diakinesis (Charniaux-Cotton, 1985; Meusy and Charniaux-Cotton, 1984). The oocytes will either stay at this phase or grow to mature size by storing free ribosomes, developing the rough endoplasmic reticulum, and synthesizing glycoprotiens. After completing primary vitellogenesis, the oocytes absorb VtG until reaching maturity. The primary oocytes, which are arrested at diakinesis, begin the development of a new batch of oocytes before or during the release of the matured oocytes for spawning (Meusy and Charniaux-Cotton, 1984). This steady maturation of oocytes requires the female to continuously produce VtG for uptake which is seen in this experiment by the high levels of VtG in spawning females. The high levels seen in the 21^{o}C non-spawning crabs, as explained previously, may possibly be due to the spawning of these animals after the experiment was concluded; thus, they were preparing for the spawning event.

Individual Variation

In all experimental animals, individual variation of VtG levels was present which could be due to the genetic variation of the wild population. The mitochondrial DNA of the Chesapeake Bay population is genetically diversified with a haplotype diversity >0.7 resulting in a lack of common haplotypes (Feng, 2009). Ongoing studies of genetic diversity have suggested a yearly genetic restructuring of the population. This yearly restructuring could explain the variations seen in Batches 1 and 3 of the 21^{o}C treatment (Figure 3.6).

CONCLUSION

The current study determined the VtG levels of spawning and non-spawning female *C. sapidus* in captivity. With the females experiencing separate temperature and photoperiod combinations, the environmental influence on vitellogenesis was assessed. The results indicate that the VtG levels decrease prior to or during spawning by preparing the next batch of oocytes for spawning and/or the halting of VtG production in the hepatopancreas. Females in warmer temperatures (21^{o}C) have greater VtG levels then those of colder, inactive temperatures (11^{o}C). In conclusion, vitellogenesis including VtG production, transport, and uptake is a continuous process in which the rate of each step may be differentially regulated causing a constant fluctuation in the hemolymph VtG levels of reproductively active and inactive females.

CHAPTER 4: DISCUSSION

ANIMAL CARE

The handling of animals particularly during the reproductive phase can cause a large amount of physiological stress which can alter their performance. Initially, it was thought that handling stress can hinder spawn production, but this experiment indicates that handling stress does not impede on reproduction. In this experiment, the crabs were handled twice a week for hemolymph sampling where they were removed from the water for ~2 minutes. During this period, a damp towel was placed over the front of the crab to control the chelae, cover the eyes, and calm the crab. With the removal of the crab from the water twice a week and various other times to check for spawning or spawn development, the crabs were capable of producing spawns and in some cases multiple times, thus the handling and sampling stress had little impact the reproductive physiology of *Callinectes sapidus*.

EXPERIMENTAL TANK SYSTEM

Another factor which may have played a significant role in the optimization of spawning of *C. sapidus* was the experimental tank system design. Each tank was fully enclosed allowing different lighting regimes to be tested. The enclosed tanks also allowed for fewer disturbances to occur during daily routine activities in the facility or ambient lighting changes. The tanks were opened during feeding and sampling in which the crabs were also checked for spawns allowing for less stress to the animals. The tank

system was also designed so that the water flowed up through the sand bed instead of directly into the top or middle of the tank. This design seemed to delay the onset of shell disease on the crabs and allowed for easy observation of the crabs while buried or on top of the sand. After one year of holding the crabs in the tank systems, shell disease was minimal, but not overtaking the carapace as seen in the COMB hatchery (personal observation). Overall, the seclusion of the individual tanks and upwelling water flow provided a lower stress environment for the crabs which ultimately benefited their health.

PHOTOTHERMAL MANIPULATIONS

Spawning events for *C. sapidus* increased when exposed to 0L:24D (0 hours of light in a 24 hour period) in 21^{o}C during the current experiment. Prior to the current study, research conducted on *C. sapidus* tested 19-21^{o}C and 15^{o}C temperatures with shorter photoperiods (10.25L:13.75D, 9.75L:14.25D, and 8L:16D) (Sulkin et al., 1976; Zmora et al., 2005; Zohar et al., 2008), but no experiments have been conducted using constant darkness or 11^{o}C. In the wild, warmer temperatures are closely coupled with the reproductive season of *C. sapidus* which occurs from the spring to the fall in the Chesapeake Bay when temperatures range from 15-27^{o}C (MDDNR, 2008b). It was also known that colder temperatures, such as 11^{o}C would inhibit ovarian development and spawning, but it was not known as to how long these crabs could survive in this condition and be reproductively active afterwards. The present study provided insight on the impact of exposure to 6 months of cold temperatures on the ovaries. With 100% of the 11^{o}C females spawning after an increase in temperature (21^{o}C) after the experiment was concluded, the inhibition caused by the cold temperatures was temporary and did not

affect the reproductive ability of these crabs since 43% of these increased temperature females produced two spawns and 29% produced three. The time period to the first spawn (60 days) was shorter then the 110 days calculated in Chpater 2, while subsequent spawns (48 days) were produced at similar intervals as noted before (47 days).

Spawning in constant darkness was not anticipated since this condition was set as a control tank for the photoperiod treatments. The results indicate that temperature is possibly the most important factor; however, within the 21^{o}C conditions, photoperiod also influenced the ovarian development and spawning. Within the 21^{o}C treatment, 0L:24D 86% females spawned while in the opposite conditions, 24L:0D, one female spawned. Since *C.* sapidus is a nocturnal animal, the constant light condition may have been stressful to the crabs resulting in inhibited spawning and low survival. The requirement of a dark period was stated for the first time in the shrimp, *Palaemonetes paludosus* (Paris and Jenner, 1952), and emphasized in later work on crayfish, *Procambarus clarkii* (Castanon-Cervantes et al., 1995). Constant darkness has increased ovarian development in *Cambarus virilis* (Stephens, 1952) and *Orconectes nais* (Armitage et al., 1973), while *Portunus trituberculatus* aquaculture in China exposes the brood stock to 0L:24D conditions to allow for optimal spawning (O. Zmora, personal communication). The current findings of increased ovarian development and spawning during constant darkness at 21^{o}C and the inhibition of ovarian development during cold temperatures are important observations which may be incorporated into *C. sapidus* aquaculture.

LARVAL QUALITY

The optimal conditions for spawning as determined by this experiment, 0L:24D at 21^{o}C, produced several spawns for which a majority were observed after hatching. During the counting of the larvae, the presence of prezoea was higher for these conditions than compared to 8L:16D and 16L:8D. The occurrence of prezoea is due to abnormal development of the embryos (Costlow, 1965). In this experiment, the embryos were not exposed to light except brief red light for observations which may have inhibited the development of some embryos. As for the wild spawned crabs, it is known that the embryos hatch in the lower part of the Bay (Churchill, 1917; Van Engel, 1958), but the location of hatching (shallow or deep water) is unknown. Since the majority of the embryos hatched healthy and viable, light must not have a great influence on their development; therefore, another aspect which may have produced prezoea may have been the hemolymph sampling. The sampling procedure was performed by cleaning the membrane where the leg joins the body with 70% ethanol before and after sampling. During the spawn developmental period, the spawn was exposed to the 70% ethanol during cleaning and seemed to affect the development of an area of the spawn. If the development of these embryos was retarded considerably, the hatched zoea could result in prezoea; however, the fate of these specific embryos was not assessed. The area of the spawn which was noticeably lighter near the sampling area was not large enough to account for the amount of prezoea seen when counting the hatched larvae; therefore, the possible combination of the photoperiod and ethanol exposure may have produced the greater number of prezoea in 0L:24D.

OVARIAN DEVELOPMENT

Studies have been conducted on *C. sapidus* ovarian maturation after mating (Lee and Puppione, 1988; Zmora et al., 2007), but observations through spawning and multiple spawns have not been conducted. *C. sapidus* is different from many species in which vitellogenin (VtG) levels have been analyzed during spawning since they undergo a terminal molt and only mate once in their lifetime. The majority of crustaceans develop oocytes which are arrested at diakinesis. These immature oocytes are held as a continuous supply of oocytes for primary and secondary vitellogenesis. The continuous maturation of oocytes in mature ovaries requires the constant uptake of VtG by the ovaries (Meusy and Charniaux-Cotton, 1984). In work on *Macrobrachium rosenbergii*, the reproductive female VtG levels increased to the highest level (~5 mg/mL) at ~25 days after molting when it decreased to ~1 mg/mL before reproduction (Okumura and Aida, 2001). Similar results were also obtained in *Macrobrachium nipponense* with the highest VtG level of ~9 mg/mL occurring 7 days after molting and then decreasing to ~1 mg/mL 7 days later before spawning (Okumura et al., 1992). The highest VtG concentration (termed lipoprotein II by the authors) seen in *C. sapidus* was ~7 mg/mL hemolymph 60 days after the terminal molt (Lee and Puppione, 1988). In the current experiment, the maximum VtG level observed in the hemolymph was 3990.0 μg/mL, but the majority of the samples remained between 200-1200 μg/mL with the lowest value of 24.1 μg/mL. The variation in VtG levels between experiments might be due to different assay techniques and health of the animals. The high values of lipoprotein II (VtG) obtained by Lee and Puppione (1988) were estimated by a densitometric scan after electrophorsis while the current study used a competitive ELISA combined with VT specific antiserum.

In addition, the crabs used by Lee and Puppione (1988) were collected in Georgia while the current study obtained crabs in the Chesapeake Bay. It has been determined that the crab populations in Georgia and New Jersey differ genetically from each other (McMillen-Jackson and Bert, 2004) which may contribute to different hemolymph VtG concentrations. Overall, VtG levels show great fluctuation over the maturation and spawning period during an active reproductive period with spawning occurring after or during a decrease in hemolymph VtG levels.

PREDICTING SPAWNING

With the determination of the optimal conditions for ovarian development while observing the vitellogenin levels during spawning, the following question is raised: can VtG levels be used to predict the exact time of spawning in environmentally manipulated crabs? The following assessment will use only the results of the current experiment.

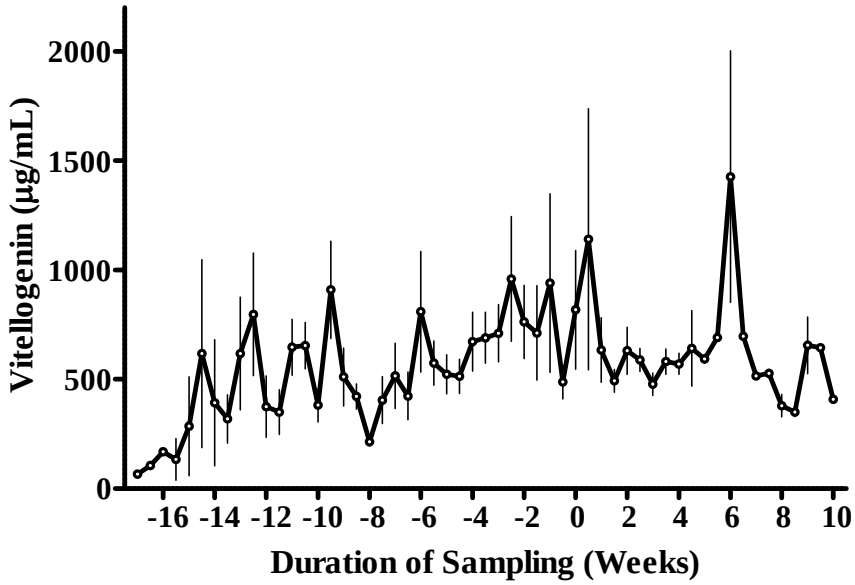
The optimal environmental conditions that have been determined are 21^{o}C with 0L:24D. In these conditions, the averaged spawned female's VtG levels increased to ~1000 μg/mL hemolymph before spawning occurred (Figure 4.1A). The sampling point immediately before spawning indicates a ~450 μg/mL decrease in VtG levels before increasing during the spawning event. In non-spawning females experiencing the same conditions, the levels also peaked above ~1000 μg/mL and decreased dramatically but no spawning occurred (Figure 4.1B). Finally, when assessing separate females, trends are harder to follow and significant decreases do not necessarily mean that a spawning event will transpire which is equivalent to the non-spawning females. Since the VtG levels

fluctuate per spawned or non-spawned female it does not seem that the spawning events can accurately be predicted from hemolymph samples.

On the other hand, the information obtained from the photothermal experiments can allow for an approximate window of spawning between transfer to 21^{o}C and multiple spawning events. Using the average calculated intervals from the 8 years of hatchery and current data, 110 days from movement to spawning and 47 days between spawning, a rough timeline of spawning can be constructed.

Finally, when the crabs are labeled and transferred into the brood stock tanks, hemolymph samples can be drawn to determine the initial level of VtG. If the female's levels are low, then they may not be ready for reproduction until after females whose levels are initially higher. If a crab is not spawning, another hemolymph sample can be drawn and measured to determine the VtG level again to see if the crab was mated. Since the females are assumed to have mated in the wild, the subsequent VtG levels can confirm this notion as seen by Zmora et al. (2007) in which unmated female VtG levels decreased after the 5 week increase while mated crabs remained elevated. As stated before, it does not seem that the crabs will be severely impacted by stress during the sampling event and the results may be an indicator of the potential spawning production for the females.

A.



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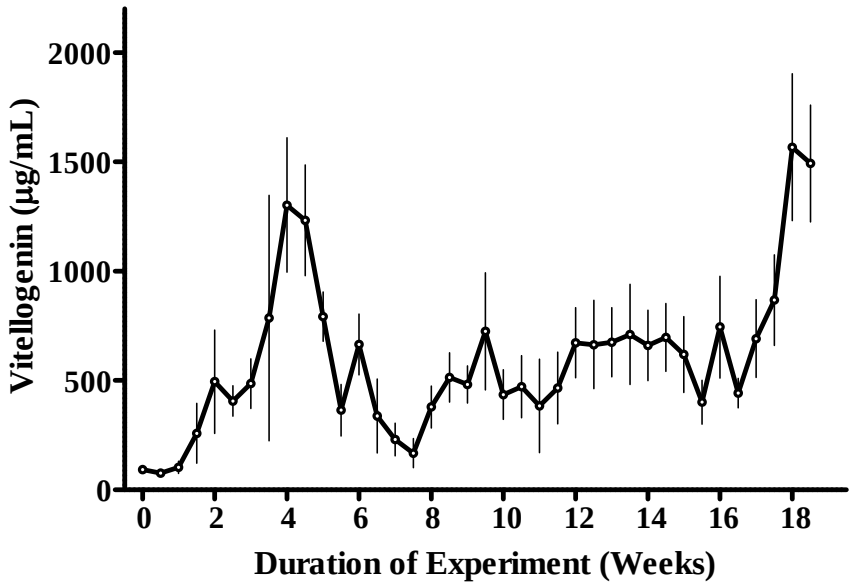

Figure 4.1. Vitellogenin levels of female *C. sapidus* exposed to 0L:24D at 21^{o}C. VtG levels fluctuated during the sampling period of (A) spawned and (B) non-spawned females. (A) Sampling point 0 indicates time of spawning. All results are provided as mean ± SEM.

FUTURE EXPERIMENTS

The current study has provided a further understanding of *C. sapidus* reproductive physiology, nevertheless new questions have developed. The manipulation of the environmental conditions has resulted in the optimization of spawning in *C. sapidus*. Of the crabs held in constant darkness, 86% spawned during the experimental period; however, complete removal of light in an aquaculture facility may not be feasible. The 8L:16D photoperiod also resulted in a high percentage of spawning events (77%); thus, further experimentations with shorter photoperiods, such as 2L:22D or 4L:20D, may be useful.

Light intensity also seems to play an important role during spawning. While 15W light bulbs were used in this experiment, it would be interesting to decrease the lux in the tanks by using shade cloths to filter the 15W light bulbs and produce lower intensities (1-5 lux). The red light bulbs used in this experiment for observation and sampling in the 0L:24D tanks measured 0 lux with an underwater lux meter (Milwaukee, SM700); however, many experiments using red lighting for observation in dark conditions use infrared light since red colored light can be detected by some animals (Cronin and Porter, 2008). Information on the vision of *C. sapidus* was limited prior to the start of the current experiment, but work has since been conducted to determine the visual color spectrum detected by two species of brachyuran crabs. From the sequenced opsin genes of *Portunus pelagicus* and *Hemigrapsus sanguineus*, it has been determined that these species are capable of detecting long wavelengths (blue-green to red) (Cronin and Porter, 2008). Since *P. peglagicus* and *C. sapidus*, are closely related, it can be assumed that the experimental crabs detected the red light used during observations and sampling.

Therefore, the 0L:24D experimental tanks which were observed at 0 lux were not completely dark. The period of exposure to red light over 24 hours was on average 5 minutes for non-sampling days and 15 minutes twice a week during sampling. These brief periods of exposure to visible colored light may not have affected the animals, but further research is needed to determine this speculation.

GENERAL CONCLUSION

The field of crustacean reproductive physiology has been studied for several decades mainly on species of aquaculture or commercial importance. With the declining *C. sapidus* population in the Chesapeake Bay, this species has produced significant commercial aquaculture interest. However, with the current knowledge of the reproductive nature of *C. sapidus* in captivity, optimizations need to occur to increase production for potential commercial scaling up. The current study obtained environmental and physiological information to optimize ovarian development and spawning in captive *C. sapidus*. Mature females exposed to a constant dark (0L:24D) photoperiod at 21^{o}C, increased spawning performance allowing for the determination of spawning intervals. During the ovarian developmental period, vitellogenesis was monitored by measuring the levels of VtG in the hemolymph of the experimental crabs. Both the spawned and non-spawned females showed great fluctuations in hemolymph VtG levels over the experimental period preventing the use of this information alone to predict the exact time of spawning. However, with spawning intervals calculated between move to 21^{o}C and multiple spawns, *C. sapidus* aquaculture can estimate a time frame for spawning. Overall, the understanding of *C. sapidus* reproductive physiology

has been broadened and the optimal environmental conditions for ovarian development and spawning of *C. sapidus* in captivity have been determined.

APPENDIX I: VITELLOGENIN RECEPTOR CLONING

INTRODUCTION

Ovarian development includes the synthesis and uptake of yolk proteins and vitellogenin (VtG) to form the yolk body of the oocytes during vitellogenesis. VtG belongs to the low density lipoprotein (LDL) family along with its receptor (VtGR). The VtGR uptakes the yolk components into the cell through coated pits on the surface of oocytes where they are located by receptor mediated endocytosis which engulfs the receptor and ligand into the cell for processing (Anderson and Kaplan, 1983; Schneider, 1996). This invagination occurs when the internalization signal of the VtG/VtGR complex is recognized by an adaptor which binds to the cytoplamic side of the receptor (Robinson, 1994). The adaptor is bound to a clathrin protein complex and forms a lattice beneath the depressed cell membrane (depression is caused by the exothermic event of the adaptor binding to the receptor) and forms a cluster of receptors in the invaginated cell membrane (Edwards et al., 1996). These ligand-receptor complexes then accept the ligands and the invagination is closed to form a coated vesicle within the cell (Kessell et al., 1989).

VtGR has been studied in the insects *Dermacentor variabilis*, *Locusta migratoria*, *Aedes aegypti*, and *Periplaneta americana* (Mitchell et al., 2007; Rohrkasten and Frerenz, 1989; Sappington et al., 1996; Tufail and Takeda, 2005). However, its existence in crustaceans was first studied by Schade and Shivers (1980) in *Homarus americanus* using electron microscopy to detect horseradish peroxidase tracers in the oocytes. The results indicated that receptor uptake declines with vitellogenesis in the oocytes; newly

synthesized oocytes rapidly uptake vitellogenin (VtG) while older cells decrease in activity. These older cells do not need the VtG; thus, they minimize their active uptake (Schade and Shivers, 1980).

Receptor cells of VtG subunits have only just been molecularly characterized in crustaceans. The VtGR from *Scylla serrata* has been isolated and analyzed to determine a molecular weight of 230 kDa, but the receptor sequence was not determined (Warrier and Subramoniam, 2002). Recently, Tiu et al. (2008) successfully cloned the first crustacean VtGR in the shrimp, *Penaeus monodon*. The cDNA sequence is 6.8 kb with a molecular weight of 211 kDa which is similar to *S. serrata* (Warrier and Subramoniam, 2002). During gene expression in various tissues, the cloned receptor only appeared in the ovary and showed varied quantities with ovarian development (Tiu et al., 2008). During early ovarian development when the hepatopancreas VtG levels were high, ovarian VtGR levels were increasing. In more advanced stage ovaries, the ovarian VtG levels are increased while the VtGR and hepatopancreas VtG abundances decreased. The location of the receptors throughout development within the cells also changed: during early developmental stages the receptors were located throughout the cytoplasm while with maturation the receptors aggregated in the membrane (Tiu et al., 2008). Similar findings were published for the ovarian LDLR in *Marsupenaeus japonicus* which resulted in a 3.5 kb sequence in the ovary (Mekuchi et al., 2008). The cloning of the first VtGR and ovarian LDLR sequences allow for a better understanding of the uptake of the ovarian proteins by the ovaries which will enhance the knowledge of vitellogenesis in crustaceans. The experiment attempts to characterize and clone the VtGR in the blue crab, *Callinectes sapidus*, using PCR with degenerate primers and 5', 3' rapid

amplification of cDNA ends PCR (RACE) and tissue localization to allow for a better understanding of the ovarian maturation process for this species.

MATERIALS AND METHODS

VtGR Cloning

Ovarian RNA

The ovarian cDNA was prepared using *C. sapidus* stage 3 ovaries, determined by size and color, from a hatchery raised crab (Center of Marine Biotechnology Blue Crab Hatchery). RNA was extracted from the ovary using TRIzol reagent (Invitrogen) as per the manufactures' protocol as follows: TRIzol (1 mL) was added to homogenized (Ultra-Turrax T25, Janke and Kunkel IKA Labortechnik) ovarian tissue (50-100 mg). Chloroform (0.2 mL) was then added to the homogenate and shaken by hand for 20 seconds after which it was kept on ice. The mixture was spun at 14,000 RPM (Jouan, Model KR 4i) at 4^{o}C for 15 minutes. The aqueous phase was carefully removed (500 mL) and an equal amount of isopropyl alcohol was added to the RNA mixture. The solution was mixed and put on ice for 10 minutes and then spun for 10 minutes at 14,000 RPM, 4^{o}C to form a RNA pellet. The supernatant was removed and the pellet was washed with 1 mL of 70% ethanol prepared with DEPCwater and centrifuge as before for 3 minutes. The supernatant was removed and the pellet was air dried for 5-10 minutes at room temperature. After drying, 30 μL DEPC water was added and the tube was placed on ice for 15-30 minutes to resuspend the pellet. The RNA was quanitified using a NannoDrop spectrophotometer (ND-1000). The extracted RNA was immediately used for cDNA synthesis and/or stored in -80^{o}C for future use.

First Strand of Ovarian cDNA Synthesis

To eliminate genomic DNA contamination, 1.1 μg of ovarian RNA extracted above was treated with RQ1 RNase-Free DNase (Promega) per the manufactures instructions. Next, the RNA was reverse transcribed using M-MLV Reverse Transcriptase kit (Promega) and random primers by incubated at 37^{o}C for 1 hour. An equal amount of sterilized water was added to the cDNA and the cDNA was stored in -20^{o}C for future use.

Ovarian RACE cDNA

RNA was immediately transcribed into cDNA after extraction. The RACE cDNA was generated using SMART RACE cDNA amplification kit (Clontech Laboratories) per the manufacturer's directions. The RACE cDNA was stored in -20^{o}C until further use.

Cloning

Prior to arriving in the lab, the attempted cloning of the VtGR in *C. sapidus* resulted in the ~3.5 kb sequence (Tsutsui *et al.*, unpublished). Using the partial VtGR sequence and primers generated for the previous cloning, new primers were created for the current cloning process (Table 4.1). Cloning was conducted as described above using two primers and cDNA in a touchdown PCR followed by a nested PCR (using 10x diluted TD PCR product) to obtain a DNA fragment of known size on a 1.5% agarose gel. The PCR products were excised, cloned, and sequenced as above. The sequenced fragment was analyzed using BioEdit Sequence Alignment Editor (http://www.mbio.ncsu.edu/BioEdit/bioedit.html), BLAST

(http://blast.ncbi.nlm.nih.gov/Blast.cgi), translated using ExPASy (http://us.expasy.org/tools/dna.html), and aligned with insect VtGR sequences and the partial *C. sapidus* VtGR using ClustalW (http://align.genome.jp/).

Synthesis of Dig-Labeled Probe

The dig-labeled probe was produced using a 3' RACE product in a plasmid DNA which was amplified with primers VGRSF014 and T7qPR03 (5'-TAATACG ACTCACTATAGGGAGGGCTGCCAGGAT-3'). The resulting PCR product was diluted (20x) and reamplified with *Taq* DNA polymerase kit (Eppendorf), T7 primer, VGRSF014, NTPs, and digoxigenin-11-dUTP at 94^{o}C for 2.5 minutes followed by 50 cycles of 94 ^{o}C for 25 seconds, 50 ^{o}C for 25 seconds, and 72 ^{o}C for 1 minute. The amplified PCR product was filtered using a Chromospin-100 column (Clontech) and then mixed with 16.5mL NorthernMax hybridization buffer (Ambion).

Table A1.1. Primers designed for *C. sapidus* VtGR cloning.

	Primer Name	Primer Sequence	TM
Degenerate	SVgRdF1	TGCATYCCYMWNAWBTGGGT	63.2
	SVgRdF2	TGCGAYGAYGGHWSNGAYGA	66.5
	VTGRD3F1	ATGGTNGARGRNYTNGC	53.6
	VTGRD3F2	TGYWSNCAYTTNTGYTTNYT	54.5
	VTGRD3F3	ATHGGNGSNTTNGCNTAYRA	58.2
	SVgRdR1	CARTCVTCYTCNCCRTCGCA	65.6
	SVgRdR2	GGRCARTTNKCYTCRTCNGA	61.0
	VTGRDR1	CANTSNWVYTCRTCYTCNCC	58.1
	VGRSR009	CACACCCAGTTCAGTGGAA	58.3
	VGRSR010	GCAATTGGCTTCATCTGAGGCAT	62.9
	VGRSR011	GGAAGTACCGGTAGATGACGTAT	59.9
	VGRSR012	CGGGTTGTGAATCGAGTTGTGAAT	62.4
	VGRSR013	CTCTCGTGAGGTGGAGCCTCTT	64.4
	VGRSR014	CAACTGACTGCAGCCACCATTGTA	64.3
	VGRSR015	CAGATGCCAGGCTCCAAACATT	62.4
	VGRSR016	CCACAGGTACCATCGGTTGGCTCATCTT	72.9
	VGRSR017	GTCATTGCCATTGCAGACCAGCTGCAT	74.3
	SB-SR014	GTTACACTCTCGTGAGGTGGAGCCTCT	68.1
	SB-SR015	TAGGTTGTGAATCGGGTTGTGAATCAGT	68.3
	VGRSF009	CGAGAACCCAGAGGAGTGT	59.5
	VGRSF010	GGAGTGTGCTGGAATAGTGTGTA	60.8
	VGRSF011	AGATGAGTTCACTTGTCACAGTGTT	61.3
	VGRSF012	TGGCAGGAATGAATGTGAAAACTA	59.7
	VGRSF013	CACTATTGAGGTGGCAGACTTCAAT	61.8
	VGRSF014	GACTTGTGAGAGTGAAGGCAAT	59.4
	VGRSF015	GAGTGGTGCCGAGAGAAACACCCAACAT	73.3
	VGRSF016	GTTGTGATCTTGAGAGGCCGTGGTAGCAAT	72.7

Determination of mRNA Size of Putative VtGR

RNA Gel

A northern blot analysis was performed using freshly extracted RNA from the ovary, female and male hepatopancreas, and testis. RNA was prepared for the ovary as mentioned above, while the hepatopancreas and testis tissues were extracted using RNeasy mini kit (Qiagen) per the manufacturer's instructions due to lower lipid content in the tissues. The RNA concentrations were quantified as mentioned above. Each freshly extracted RNA (10-15 μg) and the RNA marker (0.3-6.9 kb DIG-labeled, Roche) was mixed with 10x MOPS buffer (2.5 μL), formaldehyde (2.5 μL), and formamide (12.5 μL). The mixture was incubated on a dry block at 68^{o}C for 10 minutes for RNA denaturation and put on ice for 2-3 minutes. Next, loading dye and ethidium bromide (1:1) were mixed in each sample. A 1% agarose gel with 2.2 M formaldehyde and 10x MOPS buffer was prepared by heating in the microwave for 1 minute and set for 1 hour in the fume hood. After the gel set, 1x MOPS buffer was poured over the gel and used to wash the wells of the gel to rid any excess formaldehyde 2-3 times. The prepared RNA samples and marker were loaded onto the gel and run at 80 V for 15-20 minutes until the loading dye completely left the wells. After which, the voltage was increased to 120 V for 1 hour. The gel was then removed from the buffer and photographed (Gel Logic 200, Kodak).

Northern Blot

The gel was placed into 20x SSC and gently shaken at room temperature for 10-20 minutes. The blotting apparatus was assembled as per the manufacturer's directions (TurboBlotter, Schleicher and Schuell Bioscience) using a charged nylon membrane (Immobilon, Millipore) and left overnight at room temperature. After blotting, the gel was soaked in ethidium bromide (10μg/mL) for 1 hour to check the efficiency of the transfer. The transferred RNA was fixed to the membrane using a UV crosslinker (Fisher Scientfic, FB-UVXL-1000) twice on optimum setting.

Hybridization and Detection

The membrane was prehybridized using NorthernMax hybridization buffer (Ambion) to cover the surface (10 mL buffer for 100 cm^2 membrane) and placed in the hybridization oven (Amersham Life Science) at 42^oC for 30 minutes. The membrane was then transferred to the hybridization buffer containing a DIG-labeled DNA probe (30 ng/mL) which was denatured for 5 minutes at 70^oC prior to use. The hybridization buffer with membrane was then incubated overnight at 42^oC.

After the overnight hybridization, the membrane was removed from the DIG-labeled probe and washed twice in 2x SSC and 0.1% SDS at 42^oC, and then washed twice more with 0.2x SSC and 0.1% SDS at 42^oC for 10 minutes each.

The membrane was washed with washing buffer (malic acid and Tween20) twice at room temperature for 5 minutes with gentle shaking. After washing, the membrane

was incubated in blocking reagent (Roche) for 30 minutes at room temperature. The membrane was removed from the blocking buffer and anti-Dig-AP conjugate (20,000 times dilution) was mixed into the buffer. The membrane was placed back into the anti-DIG-AP conjugate blocking buffer and incubated at room temperature for 30 minutes. After incubation, the membrane was washed as before with washing buffer (3 times for 10 minutes each) and soaked in detection buffer for 2-3 minutes, and then CDP-Star (Roche) for 5 minutes. The membrane was then exposed to X-ray film (BioMax XAR film, Kodak) which was developed to determine the presence and size of the VtGR in the ovary, hepatopancreas, and testis.

RESULTS

Cloning

The cloning of the full length VtGR was unsuccessful in *C. sapidus*; however the previously generated fragment of the receptor can be used for future work on this experiment. By generating new primers from the partial fragment of the VtGR sequence, the N- and C-terminuses were unable to be cloned and the fragments which were obtained showed similar sequence fragments as the insect VtGRs present in the BLAST database, but were determined to be repeated segments of the partial *C. sapidus* VtGR. When the partial fragment receptor sequence was aligned with the *P. monodon* VtGR (Tiu et al., 2008), a large segment of the sequence was absent from the middle. When attempting to clone this segment, it was unsuccessful.

Tissue Localization

From the northern blot, it was determined that the VtGR in the ovary was ~7.5 kb, but a similar sized band was also present in the testis. Smaller bands were present in the ovary, hepatopancreases, and testis (~5 kb), but the bands were not as strong as the ~7.5 kb band in the ovary (Figure A1.1).

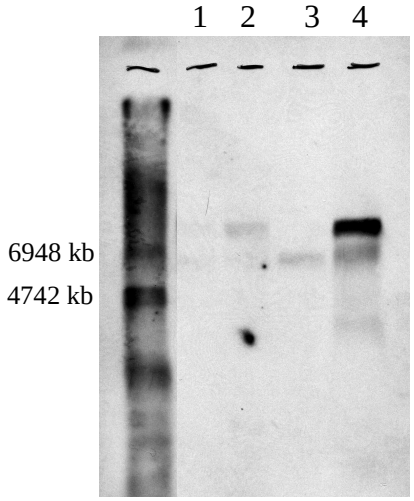

Figure A1.1. Northern blot to assess tissue localization of the vitellogenin receptor. RNA extracted from female ovaries (lane 4) and hepatopancreas (lane 1) and male testis (lane 2) and hepatopancreas (lane 3) were analyzed with a dig-labeled probe to determine the size and location of VtGR. Two distinct bands are present: ~7.5 kb in the ovary and testis and ~5 kb in all tissues.

DISCUSSION/CONCLUSION

The characterization of *C. sapidus* VtGR will enhance the understanding of the ovarian development and reproduction of *C. sapidus*. The VtGR was localized in the ovary with an expected size of ~7-8 kb. These results correspond to the findings in *P. monodon* (Tiu et al., 2008) in which a 7 kb band is present in the ovary. In addition, the ~7.5 kb band in the testis and the ~5 kb band seen in the *C. sapidus* ovaries, hepatopancreas, and testis suggest that the region used for DIG-labeling was not specific

to the VtGR, but possibly to another receptor belonging to the LDLR family. Interestingly, the ovarian LDLR cloned in *M. japonicus* is much smaller then in *P. monodon* with a size of 3.5 kb (Mekuchi et al., 2008). However, the size determination of ~7.5 kb from the northern blot was similar to the findings in *P. monodon* (7 kb) (Tiu et al., 2008) which justifies further characterization of the *C. sapidus* VtGR.

With the hepatopancreas synthesizing VtG and releasing two subunits into the hemolymph which are taken into the ovaries and broken down from two subunits in the hemolymph to three subunits in the ovaries (Zmora et al., 2007), the understanding of how the subunits of VtG are taken into the ovaries and processed will allow for a more complete story of ovarian development to unfold. Since the location of the second degradation of the protein is unknown, many questions remain unanswered: does the VtGR recognize two subunits?; if so, are there more then one VtGR present on the ovaries to recognize the two subunits or does one receptor recognize both?; and does the breakdown from two to three subunits occur inside the ovary or during the VtGR endocytosis? Several questions are present from this study; thus, further experiments need to be conducted to obtain the full VtGR clone in *C. sapidus*.

APPENDIX II: COMB HATCHERY DATA FROM 2001-2006

2001

Order	Female	Spawning Tank	Spawn Number	Spawn Date	Hatch Date	Number of Zoea Released	Moved to Summer	Number of Days after Move until Spawning	Number of Days Between Spawns	Photoperiod	Temperature (°C)
1	9/0/31		1	20-Dec-00			20-Oct-00	60		14:10	21
2	9/0/49		1	3-Jan-01			29-Sep-00	73		10:14	21
3	9/0/28		1	5-Jan-01			29-Sep-00	75		14:10	21
4	R01	Raceway	1	8-Jan-01			20-Oct-00	78		ambient	21
5	R25	Raceway	1	8-Jan-01			20-Oct-00	78		ambient	21
6	R01	Raceway	2	4-Feb-01					27	ambient	21
7	R01	Raceway	3	20-Feb-01					16	ambient	21
8	R01	Raceway	4	27-Feb-01					7	ambient	21
9	9/0/25		1	30-Mar-01	15-Apr-01	500 000	29-Sep-00	160		14:10	21
10	9/0/13	6-7	1	5-Apr-01	25-Apr-01	1 200 000	29-Sep-00	165		14:10	21
11	9/0/38	6-8	1	15-Apr-01			20-Oct-00	175		10:14	21
12	9/0/23	6-7	1	17-May-01			29-Sep-00	207		14:10	21
13	9/0/43	6-7	1	17-May-01			29-Sep-00	207		14:10	21
14	9/0/41	6-7	1	24-May-01	4-Jun-01	40 000	29-Sep-00	214		14:10	21
15	9/0/39	6-7	1	8-Jun-01	17-Jun-01	100 000	29-Sep-00	228		14:10	21
16	9/0/44	6-7	1	8-Jun-01			29-Sep-00	228		14:10	21
17	9/0/43	6-7	2	6-Jul-01					49	14:10	21
18	9/0/25		2	17-Jul-01					107	14:10	21
19	9/0/05	6-7	1	20-Jul-01			29-Sep-00	270		14:10	21
20	9/0/52	6-7	1	20-Jul-01			20-Oct-00	270		14:10	21
21	A2.1	Wild	1	20-Jul-01	24-Jul-01	60 000	12-Jul-01	8		14:10	21
22	A2.2	Wild	1	20-Jul-01	21-Jul-01	96 000	12-Jul-01	8		14:10	21
23	BS1	Wild	1	20-Jul-01	27-Jul-01	500 000					
24	A3.10	Wild	1	23-Jul-01			23-Jul-01				
25	A3.12	Wild	1	23-Jul-01			23-Jul-01				
26	A3.13	Wild	1	23-Jul-01			23-Jul-01				

Order	Female	Spawning Tank	Spawn Number	Spawn Date	Hatch Date	Number of Zoea Released	Moved to Summer	Number of Days after Move until Spawning	Number of Days Between Spawns	Photoperiod	Temperature (°C)
27	A3.14	Wild	1	23-Jul-01			23-Jul-01				
28	A3.15	Wild	1	23-Jul-01			23-Jul-01				
29	9/0/03	6-7	1	31-Jul-01			21-Jun-01	40		14:10	21
30	None	6-7	1	31-Jul-01			23-Jul-01	8		14:10	21
31	9/0/18	6-8	1	5-Aug-01			29-Sep-00	285		10:14	21
32	9/0/38	6-8	2	5-Aug-01					110	10:14	21
33	A3.10	6-13	2	5-Aug-01					12	14:10	21
34	A3.3	6-13	1	5-Aug-01			23-Jul-01	12		14:10	21
35	A3.5	6-13	1	5-Aug-01			23-Jul-01	12		14:10	21
36	BS1	6-14	2	5-Aug-01	Dropped				15	10:14	21
37	9/0/05	6-7	2	15-Aug-01					25	14:10	21
38	B05	Wild	1	15-Aug-01			15-Aug-01				
39	B31	Wild	1	15-Aug-01			15-Aug-01				
40	B05	6-14	2	5-Sep-01	Dropped				21	14:10	21
41	B31	6-14	2	5-Sep-01					20	14:10	21
42	9/0/43	6-7	3	11-Sep-01					65	14:10	21
43	C1	Wild	1	21-Sep-01			21-Sep-01				
44	C10	Wild	1	21-Sep-01			21-Sep-01				
45	C4	Wild	1	21-Sep-01			21-Sep-01				
46	C5	Wild	1	21-Sep-01			21-Sep-01				
47	C7	Wild	1	21-Sep-01			21-Sep-01				
48	C1	6-8	2	5-Oct-01	Dropped		21-Sep-01		14	14:10	21
49	C4	6-8	2	5-Oct-01					14	14:10	21
50	C5	6-8	2	5-Oct-01					14	14:10	21
51	C7	6-8	2	5-Oct-01					14	14:10	21
52	9/0/06	6-8	1	15-Oct-01			29-Sep-00	355		10:14	21
53	C10	6-8	2	29-Oct-01					38	14:10	21

2001

Tank Conditions

Summer, Short	21°C	10L:14D	30 ppt
Summer, Long	21°C	14L:10D	30 ppt

Harvest Locations and Dates

Batch 9/0/__: Rhode River, MD – September 29, 2000
Batch A: Assateauque Island, MD – July 12, 2001
Batch B: York River, VA – August 15, 2001
Batch C: Rhode River, MD – September 21, 2001

2002

Order	Female	Spawning Tank	Spawn Number	Spawn Date	Hatch Date	Number of Zoea Released	Moved to Summer	Number of Days after Move until Spawning	Number of Days Between Spawns	Photoperiod	Temperature (°C)
1	G34	6-8	1	28-Dec-01	15-Jan-02		30-Oct-01	58		10:14	23
2	G46	6-8	1	28-Dec-01			30-Oct-01	58		10:14	23
3	G37	6-8	1	7-Jan-02			30-Oct-01	67		10:14	23
4	G7	6-7	1	31-Jan-02	16-Feb-02		30-Oct-01	90		14:10	23
5	G56	6-13	1	18-Feb-02			30-Oct-01	108		14:10	18
6	G68	6-13	1	18-Feb-02			30-Oct-01	108		14:10	18
7	G15	6-7	1	22-Feb-02			30-Oct-01	112		14:10	23
8	G56	6-13	2	24-Apr-02			21-May-02			14:10	18
9	Jorge's	wild		10-May-02	13-May-02	1 914 000	10-May-02				
10	G102	6-7	1	30-May-02	17-Jun-02	1 044 000	30-Oct-01	210		14:10	20
11	G80	6-13	1	8-Jun-02	30-Jun-02	435 000	30-Oct-01	218		14:10	18
12	G80	6-7	2	31-Jul-02					53	14:10	20
13	G73	6-13	1	15-Aug-02			30-Oct-01	285		14:10	18
14	G80	6-7	3	4-Sep-02					34	10:14	18
15	#7	6-7	1	3-Dec-02			2-Nov-02	31		14:10	23
16	G75	6-13	1	3-Dec-02			30-Oct-01	393		14:10	18

Tank Conditions

6-7	Summer, Long	23°C	14L:10D	30 ppt
6-8	Summer, Short	23°C	10L:14D	30 ppt
6-13	Cool, Long	18°C	14L:10D	30 ppt
6-14	Cool, Short	18°C	10L:14D	30 ppt

Harvest Location and Date

Batch G: Near Thomas Point, MD – October 30, 2001

2003

Order	Female	Spawning Tank	Spawn Number	Spawn Date	Hatch Date	Number of Zoea Released	Moved to Summer	Number of Days after Move until Spawning	Number of Days Between Spawns	Photoperiod	Temperature (°C)
1	Female	QOval1	1	29-Jan-03			29-Jan-03			8:16	15
2	Y28	6-13	1	25-Feb-03	12-Mar-03	20 000	4-Feb-03			16:08	21
3	Y24	90-7	1	27-Feb-03	16-Mar-03		24-Feb-03	3		16:08	21
4	#15	6-14	1	11-Mar-03			20-Nov-02	111		16:08	21
5	X20	90-7	1	11-Mar-03	31-Mar-03	1 186 940	24-Feb-03	17		16:08	21
6	Y20	6-13	1	16-Mar-03	5-Apr-03	512 320				16:08	21
7	X19	90-7	1	26-Mar-03	14-Apr-03	1 500 000	24-Feb-03	32		16:08	21
8	V1	8-6	1	28-Mar-03	18-Apr-03	81 600	3-Apr-03			8:16	15
9	Y16	90-7	1	28-Mar-03	16-Apr-03	404 570	24-Feb-03	34		16:08	21
10	Z23	90-7	1	1-Apr-03	19-Apr-03	1 938 000				16:08	21
11	X15	6-14	1	3-Apr-03	24-Apr-03	1 600 000	8-Apr-03			16:08	21
12	X21	6-14	1	13-Apr-03	1-May-03		13-Apr-03			16:08	21
13	Y2	90-7	1	19-Apr-03	9-May-03		18-Apr-03	1		16:08	21
14	#9	6-7	1	21-Apr-03	9-Jun-03		24-Oct-02			8:16	15
15	V21	8-6	1	23-Apr-03			29-Apr-03			8:16	11.5
16	V31	8-6	1	23-Apr-03			29-Apr-03			8:16	11.5
17	V41	8-6	1	23-Apr-03	11-May-03	1 600 000	29-Apr-03			8:16	11.5
18	X22	90-7	1	28-Apr-03	18-May-03	1 000 000	24-Feb-03	64		16:08	21
19	V11	8-6	1	29-Apr-03	18-May-03	800 000	29-Apr-03			8:16	11.5
20	V13	90-7	1	29-Apr-03			19-Apr-03	10		16:08	21
21	X13	90-7	1	30-Apr-03			19-Apr-03	11		16:08	21
22	X16	90-7	1	1-May-03			19-Apr-03	12		16:08	21
23	Y16	6-14	2	6-May-03					38	16:08	21
24	X12	90-7	1	14-May-03	2-Jun-03		19-Apr-03	25		16:08	21
25	Y20	6-13	2	20-May-03				64		16:08	21
26	V3	6-14	1	21-May-03			21-May-03			16:08	21
27	Y19	6-14	1	21-May-03			21-May-03			16:08	21
28	X24 or R24		1	23-May-03	13-Jun-03						

Order	Female	Spawning Tank	Spawn Number	Spawn Date	Hatch Date	Number of Zoea Released	Moved to Summer	Number of Days after Move until Spawning	Number of Days Between Spawns	Photoperiod	Temperature (°C)
29	V41	6-14	2	26-May-03	13-Jun-03	500 000			33	16:08	21
30	Z23	6-8	2	27-May-03	14-Jun-03					8:16	15
31	Y2	R-1.3	2	29-May-03	11-Jun-03	800 000			40	16:08	21
32	V31	6-14	2	3-Jun-03	13-Jun-03	500 000			40	16:08	21
33	V3	90-7	2	11-Jun-03	30-Jun-03				20	16:08	21
34	Y16	6-14	3	17-Jun-03	25-Jun-03				41	16:08	21
35	V2	90-7	1	26-Jun-03	9-Jul-03		17-Jun-03	9		16:08	21
36	X12	6-14	2	26-Jun-03					42	16:08	21
37	V5	90-7	1	30-Jun-03	14-Jul-03		21-May-03	39		16:08	21
38	II	6-8	1	3-Jul-03	21-Jul-03	20 000				8:16	15
39	G8	90-7	1	4-Jul-03	18-Jul-03		4-May-03	60		16:08	21
40	V21	R-1.4	2	5-Jul-03	17-Jul-03				72	16:08	21
41	V31	6-14	3	7-Jul-03	14-Aug-03				34	16:08	21
42	III	90-5	1	10-Jul-03	28-Jul-03	56 000	11-Jul-03			8:16	15
43	Z23	90-5	3	17-Jul-03						8:16	15
44	Y16	90-7	4	20-Jul-03	4-Aug-03		24-Feb-03	146		16:08	21
45	X12	90-7	3	30-Jul-03	15-Aug-03				34	16:08	21
46	V7	90-7	1	4-Aug-03	20-Aug-03		21-May-03	73		16:08	21
47	A1	6-14	1	5-Aug-03	21-Aug-03	700 000				16:08	21
48	II	90-7	2	9-Aug-03	25-Aug-03	747 000			35	16:08	21
49	Unknown	90-7		19-Aug-03	4-Sep-03	see Z23				16:08	21
50	Z23	90-7	4	20-Aug-03	4-Sep-03	1 000 000				16:08	21
51	Unknown 2	90-5		27-Aug-03						8:16	15
52	II	90-7	3	8-Sep-03	24-Sep-03				29	16:08	21
53	III	90-7	2	22-Sep-03	12-Oct-03	1 240 000			72	16:08	21
54	V31	90-7	4	30-Sep-03					41	16:08	21
55	U1	90-7	1	6-Oct-03	27-Oct-03	1 000 000				16:08	21

2003

Tank Conditions

6-7/8	Winter	15°C	8L:16D	30ppt
90-5	Winter	15°C	8L:16D	30ppt
6-13/14	Summer	21°C	16L:8D	30ppt
90-7	Summer	21°C	16L:8D	30ppt
8-6	Winter	11.5°C	8L:16D	30ppt

Harvest Locations and Dates

Females 1-12: Rhode River, MD – October 24, 2002

Females 18-20: York River, VA – November 28, 2002

Batches X, Y, and Z: York River, VA – February 24, 2003

2004

Order	Female	Spawning Tank	Spawn Number	Spawn Date	Hatch Date	Number of Zoea Released	Moved to Summer	Number of Days after Move until Spawning	Number of Days Between Spawns	Photoperiod	Temperature (°C)
1	C10	90-7	1	28-Mar-04	14-Apr-04	3 300 000	28-Jan-04	60		16:08	22
2	C11	90-7	1	2-May-04	19-May-05	2 400 000	28-Jan-04	94		16:08	22
3	C9	90-7	1	9-May-04	27-May-05		28-Jan-04	101		16:08	22
4	C5	90-7	1	27-May-04			28-Jan-04	119		16:08	22
5	D8	90-7	1	6-Jun-04	24-Jun-04	500 000	2-Mar-04	94		16:08	22
6	C13	6-7	1	15-Jun-04	30-Jun-04	719 000	28-Jan-04	137		16:08	22
7	D7	90-7	1	15-Jun-04	1-Jul-04	770 000	2-Mar-04	103		16:08	22
8	C3	90-7	1	16-Jun-04	6-Jul-04	3 000 000	28-Jan-04	138		16:08	22
9	C8	90-7	1	20-Jun-04	6-Jul-04		28-Jan-04	142		16:08	22
10	C10	90-7	2	28-Jun-04	16-Jul-04	2 400 000			90	16:08	22
11	C16	6-8	1	12-Jul-04			28-Jan-04	164		16:08	22
12	D7	90-7	2	19-Jul-04	5-Aug-04	1 200 000			34	16:08	22
13	D9	90-7	1	20-Jul-04	7-Aug-04	200 000	2-Mar-04	138		16:08	22
14	C4	90-7	1	31-Jul-04	20-Aug-04	900 000	28-Jan-04	183		16:08	22
15	C10	90-7	3	4-Aug-04	23-Aug-04	1 100 000			36	16:08	22
16	C9	90-7	2	7-Aug-04	26-Aug-04	1 250 000			88	16:08	22
17	D7	90-7	3	16-Aug-04	2-Sep-04				164	16:08	22
18	D9	90-7	2	23-Aug-04	9-Sep-04	1 000 000			33	16:08	22
19	C10	90-7	4	9-Sep-04	25-Sep-04	1 200 000			35	16:08	22
20	C17	90-7.4	1	11-Sep-04	29-Sep-04	750 000	8-Sep-04	3		16:08	22
21	C18	90-7.4	1	17-Sep-04	1-Oct-04	680 000	8-Sep-04	9		16:08	22
22	C9	90-7	3	17-Sep-04	5-Oct-04	2 000 000			40	16:08	22
23	C11	90-7	2	23-Sep-04	12-Oct-04				141	16:08	22
24	C12	90-7	1	4-Oct-04	22-Oct-04		28-Jan-04	246		16:08	22
25	D7	90-7	4	14-Oct-04	30-Oct-04	800 000			58	16:08	22
26	C17	90-7	2	19-Oct-04	7-Nov-04	3 900 000			38	16:08	22
27	C18	90-7	2	19-Oct-04	7-Nov-04	see C17			32	16:08	22
28	C10	90-7	5	23-Oct-04	9-Nov-04	1 375 000			44	16:08	22
29	D5	90-7.6	1	26-Oct-04	13-Nov-04	600 000	25-Oct-04	1		16:08	22

Order	Female	Spawning Tank	Spawn Number	Spawn Date	Hatch Date	Number of Zoea Released	Moved to Summer	Number of Days after Move until Spawning	Number of Days Between Spawns	Photoperiod	Temperature (°C)
30	B31	90-7.2	1	30-Oct-04	17-Nov-04		25-Oct-04	5		16:08	22
31	B16	90-7.3	1	14-Nov-04	4-Dec-04	1 500 000	8-Sep-04	66		16:08	22
32	B18	90-7.3	1	23-Nov-04	13-Dec-04		19-Oct-04	34		16:08	22
33	A15	90-7.6	1	4-Dec-04	21-Dec-04	1 600 000	25-Oct-04	39		16:08	22
34	C18	90-7	3	7-Dec-04	29-Dec-04	3 000 000			48	16:08	22
35	C17	90-7	3	9-Dec-04	30-Dec-04	1 150 000			50	16:08	22
36	COMB1	90-7		24-Dec-04			3-Dec-04			16:08	22

Tank Conditions

6-7/8	Summer	22°C	16L:8D	30 ppt
6-13/14	Winter	15°C	8L:16D	30 ppt
90-7	Summer	22°C	16L:8D	30 ppt

Harvest Locations and Dates

Batch A: Mid-Bay near Kent Island, MD – October 31, 2003

Batch B, C, and D: Mid-Bay near Kent Island, MD – October 31, 2003 and November 10, 2003

2005

Order	Female	Spawning Tank	Spawn Number	Spawn Date	Hatch Date	Number of Zoea Released	Moved to Summer	Number of Days after Move until Spawning	Number of Days between Spawns	Photoperiod	Temperature (°C)
1	B31	90G 2004	2	15-Jan-05	2-Feb-05				75	16:08	21
2	C18	90G 2004	4	28-Jan-05	17-Feb-05				51	16:08	21
3	B19	90G 2004	2	30-Jan-05					67	16:08	21
4	F35	6-8	1	12-Mar-05	30-Mar-05	160 000	3-Dec-04	99		15:09	21
5	F46	90G	1	25-Mar-05	12-Apr-05		23-Feb-05	32		16:08	21
6	F32	90G	1	17-Apr-05	1-May-05	440 000	23-Feb-06	54		16:08	21
7	F27	90G	1	26-Apr-05	13-May-05	1 600 000	23-Feb-05	63		16:08	21
8	F37	90G	1	1-May-05	20-May-05	4 800 000	23-Feb-05	68		16:08	21
9	F46	90G	2	6-May-05					41	16:08	21
10	F40	90G	1	13-May-05	29-May-05		23-Feb-05	80		16:08	21
11	F30	90G	1	19-May-05	2-Jun-05	1 200 000	23-Feb-05	86		16:08	21
12	F43	90G	1	19-May-05	3-Jun-05	7 500	23-Feb-05	86		16:08	21
13	F50	90G	1	20-May-05	6-Jun-05	216 000	7-Dec-04	163		16:08	21
15	F32	90G	2	24-May-05	8-Jun-05	1 950 000			37	16:08	21
14	F48	90G	1	24-May-05	11-Jun-05	1 700 000	23-Feb-05	91		16:08	21
16	F44	90G	1	29-May-05	13-Jun-05		26-May-05	3		16:08	21
17	F36	90G	1	7-Jun-05	22-Jun-05	480 000	23-Feb-05	104		16:08	21
18	F29	90G	1	21-Jun-05	9-Jul-05		17-Jun-05	4		16:08	21
19	F53	90G	1	26-Jun-05	12-Jul-05		23-Feb-05	123		16:08	21
20	F38	90G	1	12-Jul-05	28-Jul-05	1 200 000	3-Dec-04	219		16:08	21
21	F36	90G	2	18-Jul-05	3-Aug-05	760 000			41	16:08	21
22	F10	90G	1	3-Aug-05	21-Aug-05	400 000	16-Mar-05	137		16:08	21
23	F12	6-13	1	4-Aug-05			31-Aug-05			8:16	14
24	F32	90G	3	10-Aug-05					76	16:08	21
25	F42	90G	1	13-Aug-05			3-Dec-04	250		16:08	21
26	F22	90G	1	11-Sep-05			30-Aug-05	11		16:08	21
27	F57	90G	1	11-Sep-05	28-Sep-05		31-Aug-05	11		16:08	21
28	F7	90G	1	11-Sep-05	30-Sep-05	380 000	31-Aug-05	11		16:08	21
29	F35	90G	2	16-Sep-05					184	16:08	21

Order	Female	Spawning Tank	Spawn Number	Spawn Date	Hatch Date	Number of Zoea Released	Moved to Summer	Number of Days after Move until Spawning	Number of Days between Spawns	Photoperiod	Temperature (°C)
30	F42	90G	2	16-Sep-05	4-Oct-05	960 000			33	16:08	21
31	F9	90G	1	21-Sep-05			31-Aug-05	21		16:08	21
32	F54	90G	1	22-Sep-05			11-Aug-05	41		16:08	21
33	F54	90G	2	7-Nov-05					45	16:08	21
34	F13	90G	1	9-Nov-05			16-Mar-05	233		16:08	21

Tank Conditions

6-7/8	Summer	21°C	16L:8D	30 ppt
6-13/14	Winter	14°C	8L:16D	30 ppt
90G	Summer	21°C	16L:8D	30 ppt

Harvest Location and Date

Batch F: Mid-Bay near Kent Island, MD – November 12, 2004

2006

Order	Female	Spawning Tank	Spawn Number	Spawn Date	Hatch Date	Number of Zoea Released	Moved to Summer	Number of Days after Move until Spawning	Number of Days between Spawns	Photoperiod	Temperature (°C)
1	G2	90-5.1	1	19-Feb-06	9-Mar-06		21-Nov-05	88		16:08	21
2	H6	90-7.4	1	20-Feb-06	7-Mar-06	970 000	12-Dec-05	68		16:08	21
3	H10	90-7.5	1	5-Mar-06	24-Mar-06	672 000	12-Dec-05	87		16:08	21
4	H6	90-7.4	2	26-Mar-06	13-Apr-06	2 560 000			36	16:08	21
5	J1	6-7	1	29-Mar-06	16-Apr-06		31-Mar-06			8:16	15
6	J18	6-7	1	29-Mar-06	16-Apr-06	880 000	31-Mar-06			8:16	15
7	J16	6-14	1	1-Apr-06	9-May-06		3-May-06			8:16	15
8	J9	6-7	1	10-Apr-06			12-Apr-06			8:16	15
9	H10	90-7.5	2	17-Apr-06	4-May-06	800 000			38	16:08	21
10	H25	6-14	1	19-Apr-06	16-May-06	1 050 000	3-May-06			8:16	15
11	J17	6-14	1	19-Apr-06	14-May-06	1 500 000	3-May-06			8:16	15
12	H4	90-7.2	1	22-Apr-06	11-May-06	500 000	12-Dec-05	130		16:08	21
13	H49	90-5.5	1	22-Apr-06			12-Jan-06	100		16:08	21
14	H6	90-7.4	3	27-Apr-06	15-May-06	2 400 000			31	16:08	21
15	G11	90-5.6	1	29-Apr-06	18-May-06	684	21-Nov-05	158		16:08	21
16	H8	90-7.3	1	30-Apr-06	17-May-06	1 700 000	12-Dec-05	138		16:08	21
17	J14	6-8	1	30-Apr-06						8:16	15
18	J8	6-7	1	30-Apr-06	18-May-06	1 710	3-May-06			8:16	15
19	K7	6-7	1	1-May-06	18-May-06	41 040	3-May-06			8:16	15
20	K1	6-7	1	4-May-06			20-Jul-06			8:16	15
21	H35	6-14	1	5-May-06						8:16	15
22	G20	90-5.2	1	7-May-06	22-May-06	1 000	12-Jan-06	115		16:08	21
23	G4	90-5.2	1	7-May-06	22-May-06	300	21-Dec-05	136		16:08	21
24	J18	90-5.4	2	7-May-06					38	16:08	21

Order	Female	Spawning Tank	Spawn Number	Spawn Date	Hatch Date	Number of Zoea Released	Moved to Summer	Number of Days after Move until Spawning	Number of Days between Spawns	Photoperiod	Temperature (°C)
25	K11	6-14	1	7-May-06	16-Jun-06	700 000	9-Jun-06			8:16	15
26	G17	90-5.2	1	15-May-06	27-May-06		12-Jan-06	123		16:08	21
27	H2	90-7.1	1	15-May-06	31-May-06		12-Dec-05	153		16:08	21
28	H13	6-13	1	16-May-06			11-Jul-06			8:16	15
29	J20	6-13	1	17-May-06						8:16	15
30	Naoaki's	R-1		17-May-06	31-May-06	1 200 000	10-Apr-06			16:08	21
31	H49	90-5.5	2	21-May-06	2-Jun-06				29	16:08	21
32	K2	6-7	1	31-May-06						8:16	15
33	Sook's	R-1	1	31-May-06			9-Mar-06			16:08	21
34	G20	90-5.2	2	4-Jun-06					27	16:08	21
35	G4	90-5.2	2	6-Jun-06					29	16:08	21
36	H51	6-14	1	6-Jun-06	22-Jul-06	1 520 000	12-Jan-07	144		8:16	15
37	H9	90-7.5	1	7-Jun-06	24-Jun-06	780 000	12-Dec-05	175		16:08	21
38	H10	90-7.5	3	11-Jun-06	21-Jun-06	22 500			54	16:08	21
39	J16	90-5.1	2	14-Jun-06	24-Jun-06	see H9			73	16:08	21
40	K7	90-7.1	2	14-Jun-06	24-Jun-06	see H9			53	16:08	21
41	K5	6-8	1	24-Jun-06			29-Jul-06			8:16	15
42	H25	90-7.6	2	27-Jun-06	27-Jun-06	900 000			68	16:08	21
43	K11	90-7.6	2	27-Jun-06			12-Dec-05	196		16:08	21
44	H7	90-7.3	1	28-Jun-06					24	16:08	21
45	H18	6-13	1	1-Jul-06	29-Jul-06		18-Jul-06			8:16	15
46	H32	6-13	1	1-Jul-06	30-Jul-07		18-Jul-06			8:16	15
47	H37	90-5.6	1	2-Jul-06			26-Jan-06	156		16:08	21
48	H17	90-5.3	1	8-Jul-06			15-Feb-06	142		16:08	21
49	H19	6-13	1	20-Jul-06	4-Aug-06	1 080 000	23-Jul-06			8:16	15

Order	Female	Spawning Tank	Spawn Number	Spawn Date	Hatch Date	Number of Zoea Released	Moved to Summer	Number of Days after Move until Spawning	Number of Days between Spawns	Photoperiod	Temperature (°C)
50	J3	6-7	1	20-Jul-06			23-Jul-06			8:16	15
51	K11	90-7.3	3	27-Jul-06	10-Aug-06	960 000			71	16:08	21
52	J8	R-2	2	30-Jul-06	2-Aug-06	500 000			90	16:08	21
53	L2	8-6	1	5-Aug-06			21-Jul-06	14		16:08	21
54	M2	8-6	1	8-Aug-06			23-Jul-06	15		16:08	21
55	unknown	8-6	1	8-Aug-06			22-Jul-06	16		16:08	21
56	J10	6-14	1	31-Aug-06						8:16	15
57	Naoaki's	R-1		31-Aug-06	7-Sep-06					16:08	21
58	Naoaki-2	R-1		1-Sep-06	8-Sep-06					16:08	21
59	K1	90-7.4	2	12-Sep-06					128	16:08	21
60	H2	90-7.4	2	14-Sep-06	29-Sep-06	922 000			119	16:08	21
61	H51	90-7.2	2	18-Sep-06	30-Sep-06	3 400 000			102	16:08	21
62	H32	90-7.1	2	19-Sep-06	30-Sep-06	see H51			77	16:08	21
63	H19	90-7.3	2	21-Sep-06	6-Oct-06	2 330 000			61	16:08	21
64	H13	90-7.2	2	25-Sep-06	6-Oct-06	see H19			128	16:08	21
65	H43	90-7.3	1	25-Sep-06	5-Oct-06	406 000	19-Sep-06	6		16:08	21
66	K7	90-7.5	3	25-Sep-06					108	16:08	21
67	J5	6-14	1	11-Oct-06			14-Oct-06			8:16	15
68	J20	6-13	2	13-Oct-06					139	8:16	15
69	K3	6-13	1	13-Oct-06	1-Nov-06	500 000	14-Oct-06			8:16	15
70	H32	90-7.1	3	20-Oct-06	8-Nov-06				31	16:08	21
71	H51	90-7.2	3	30-Oct-06					42	16:08	21
72	J12	R-2.1	1	9-Nov-06			3-Sep-06	66		16:08	21
73	H40	R-2.1	1	10-Nov-06			29-Jul-06	101		16:08	21
74	H45	R-2.2	1	10-Nov-06	29-Nov-06	600 000	29-Jul-06	101		16:08	21

Order	Female	Spawning Tank	Spawn Number	Spawn Date	Hatch Date	Number of Zoea Released	Moved to Summer	Number of Days after Move until Spawning	Number of Days between Spawns	Photoperiod	Temperature (°C)
75	H23	90-5	1	16-Nov-06			15-Nov-06	1		16:08	21
76	H26	90-5	1	16-Nov-06			15-Nov-06	1		16:08	21
77	H32	90-5	4	17-Nov-06	7-Dec-06	see H46			27	16:08	21
78	J21	90-5	2	17-Nov-06					180	16:08	21
79	H46	R-2.2	1	29-Nov-06	8-Dec-06	1 000 000	29-Jul-06	120		16:08	21

Tank Conditions

6-7/8	Winter	15°C	8L:16D	30ppt
6-13/14	Winter	15°C	8L:16D	30ppt
90-5	Summer	21°C	16L:8D	30ppt
90-7	Summer	21°C	16L:8D	30ppt
R-1	Summer	21°C	16L:8D	30ppt
R-2	Summer	21°C	16L:8D	30ppt
8-6	Summer	21□C	16L:8D	30ppt

Harvest Locations and Dates

Batch G: Mid-Bay near Kent Island, MD – November 9, 2005
Batch H: Mid-Bay near Kent Island, MD – November 23, 2005
Batch J: York River, VA – January 19, 2006
Batch K: York River, VA – March 1, 2006
Batch L: Mid-Bay near Kent Island, MD – July 19, 2006
Batch M: Lower-Bay near Hoopers Island, MD – July 19, 2006
Batch N: Lower-Bay near Hoopers Island, MD – August 19, 2006

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