HATCHING PERIODICITY, SURVIVAL AND DEVELOPMENT OF THE EARLY LIFE STAGES OF RED DEEPSEA CRAB (*CHACEON QUINQUEDENS*) IN LABORATORY CONDITIONS: EFFECTS OF DIET AND TEMPERATURE

by

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A THESIS

**Submitted in partial fulfillment of the requirements
for the degree of MASTER OF SCIENCE in the
NATURAL RESOURCES Graduate Program
of Delaware State University**

**Dover, Delaware
May 2017**

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DEDICATION

To my parents, Rafael and Nivia Pérez Torres, who always support my never-ending curiosity and positively guided my vivid imagination. And to my brother Rafael E. Pérez Pérez for being my inspiration and muse. They gave me the best of gifts, a loving family with strong values that have being my guidance to become the best strong, secure, and educated woman that I can be.

ACKNOWLEDGMENTS

The completion of this project is an example of how collaboration is the key to support and advance in sciences. I will always be grateful to all those who facilitated me the tools to accomplish my project goals.

First, I would like to thank to my committee members for being involved in every step of the journey; from planning, implementing, and execution of this project and preparing me for the future challenges. Dr. Gulnihal Ozbay for believing in my ideas and supporting me to realize them. For welcoming me into DSU, for seeing my potential and helping me challenge my capabilities to move forward in the realization of my goals. She really went far and beyond to support my research and any aspect that could increase my career and personal growth. Dr. Stacy Smith to accept me in the family of the NOAA-LMRCSC program and give me the tools I needed to perform a successful collaborative research. Dr. Matthew Poach to open the doors of one of the most gratifying and enriching experiences of my life, working in the graduate internship at the NOAA James J. Howard Marine Sciences Laboratory in Sandy Hook, NJ. Dr. Bradley Stevens for his support and advise me as an undergraduate "not to study something that I can't eat", because it changed my research path, which consequently re-united me with him. For treating me as one of his graduate student and always be available for me and my endless questions. To all other faculty members at Delaware State University (DSU) who provided valuable advice and support through the courses of my master degree.

All the NOAA James J. Howard Laboratory personnel and students who each summer kindly welcome me in their families and support me with a smile, in each step of my experiments and during my time in Sandy Hook, NJ. Special thanks to Ehren Habeck, Kristen Habeck,

DeMond Timmons, and Grant Blank for being my saviors in the labs every time I need it to fix, reach, adjust, and find something to setup my systems, for answering all my questions and guide me in the right path with technical advice for the electrical, plumbing, scientific mechanisms, etc. involve in the design, and build of my systems. Ms. Claire L. Steimle and Dr. Cynthia Hong-Wa for hosting me in the NOAA Lionel A. Walford Library and the DSU Claude E. Phillips Herbarium for the longest hours during my reference search and writing process.

Undergraduates students Julianne Murphy, Juliana Tran, and High School students Hayley Ehrlich, Charles Lee, and Remi Goddu for their help in the long sampling hours during weekdays and weekends, looking at larvae survival, preparing diets, feeding, and data collection, without them I would not be able to accomplish my objectives.

Talaysha and the Lingham family, for their warmth and company, because of them I was never by myself in a new country and Talaysha for being a company to me in several long day travels to New Jersey. Johnna Fay and Johnny Moore for taking care of me in times of much stress and difficult situations, and kept me happy, focus in my goals, and true to myself. Natalia López for always being present, recognizes the best of me, celebrates my achievements, and helps me face my challenges as her own.

Lastly but not least, I would like to acknowledge the National Oceanic and Atmospheric Administration (NOAA), the Living Marine Resources Cooperative Science Center (LMRCSC), NOAA Educational Partnership Program (EPP)/Minority Serving Institutions (MSI) Graduate Research and Training Scholarship Program (GRTSP) that believe in me, supported me with an excellent network of professionals and peers, and provided the funding necessary to cover tuition, research, stipend, and travel expenses during the project. Thank you all, teachers, colleagues, friends and family for their support at every step of the way. Without your advice,

support, and friendship my achievements would not be possible. Each of you is part of my motivation to keep me excelled. I will always be very grateful to everyone who has assisted me in my professional growth through my academic journey.

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ABSTRACT

A decline in commercial crustacean species (lobsters, king crab, etc.) has caused an increased interest in the harvest of the red deepsea crab *Chaceon quinquedens*. The red deepsea crab fishery is federally managed by the New England Fishery Management Council-Deep Sea Red Crab Fishery Management Plan (FMP), but little is known about this species' general biology, especially conditions required for larval survival. This multiyear project aimed to answer two main questions about the life history of the red deepsea crab. First, is there a common larval hatching pattern between adult female crabs? Specifically, what is the duration of the hatching process, at what time of the day do larvae hatch, and what is the relationship between female morphometry and the total larvae hatched? Second, what are the factors affecting the survival and development of larval red deepsea crab? In order to answer these research questions, I studied the effects of diet (rotifers, *Artemia sp*., algae, and unfed), temperature (9, 15, and 20°C), and aquaculture systems.

Ovigerous females were obtained from commercial traps and transported to the NOAA James J. Howard Laboratory, Sandy Hook, NJ. They were placed in the Females Husbandry and Hatching Collection System (FHCS) where the larvae hatched. Hatching of adult females was

monitored and measured by volume. A group of them was observed for 3 consecutive days in 4 hours intervals. First stage zoeae were obtained from eggs that hatched in FHCS. The 2014 flow-through system consisted of two temperature tanks (9 and 15°C) containing 10 buckets each. Inside each bucket, three cylindrical containers with capacity for 10 larvae each. That year diets included unfed, rotifers, and algae treatments for each temperature. The 2016 recirculating system had two temperature tanks (15 and 20°C), each containing 12 conical upwellers with capacity for 20 larvae each. Diets for 2016 included unfed, rotifers, *Artemia sp*. and a mixture of the last two, for each temperature. Larvae were fed and counted daily.

A simple linear regression (SLR) was calculated to predict number of larvae hatched based on the measured volumes. This SLR was significant (F = 1196; df = 1, 13; R^2 = 0.9892, P = 3.498e-14) for the relationship. The mean duration of hatching period for red deepsea crab females is 18.1 ± 3.5 days, with a daily lower mean number of larvae hatched at 14:00 hr (mid-day from10 am to 2 pm) than at time 22:00 hr (6 pm to 10 pm) across all crabs, but not different from the other time intervals.

Daily recorded larvae mortality data was analyzed for survival and development hypotheses using non-linear mixed effects models (NLME). In 2014, the model was highly significant, temperature and its interaction with the rotifers diet are different from the other treatments mortality, supporting a longer survival on larvae under these treatments. The fewer larvae that survived longer were also in advanced larval development stages (Zoeae III and IV). During 2016 survival temperature was also the main factor affecting larvae mortality. Temperature, diet, and their interaction affected the capacity of the RDSC larvae to reach advanced stages of development in the years of experiments.

Comparison of both years Larvae Feeding Experimental System (LFS) showed that the type of aquaculture system plays an important role in the mortality of the RDSC larvae with longer survival recorded using the 2014 LFS I. In addition, one of the expected outcomes of these experiments was to answer which feed was the best among rotifers, *Artemia sp.*, and algae for the RDSC larvae in laboratory conditions. I found rotifer and *Artemia sp.* as the best live food option for the RDSC larvae.

My research findings on the optimal survival conditions of the species will facilitate further research in marine aquaculture, to better understand the ecology, fishery, and impacts of climate change on the life history and fishery of red deepsea crab.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

°C	Celsius
ARA	Arachidonic acid
AW	abdominal width
CL	carapace length from the eyes sucked to the back of abdomen
CW	carapace width to the tips of the spines
df	degrees of freedom
DHA	docosahexaenoic acid
DSU	Delaware State University
EPA	eicosapentaenoic acid
FHCS	Females Husbandry and Hatching Collection System
FMP	Fishery Management Plan
h	hours
HUFA	highly unsaturated fatty acid
L	liters
LFS I	Larvae Feeding Experimental System 2014
LFS II	Larvae Feeding Experimental System 2016
LMRCSC	Living Marine Resources Cooperative Science Center
lx	Lux-unit of illuminance and luminous emittance (light intensity)-measure of luminous flux per unit area.
min	minutes
ml	milliliters
NLME	non-linear mixed effects models

NOAA	National Oceanic and Atmospheric Administration
PVC	polyvinyl chloride
RDSC	red deepsea crab
REML	restricted maximum likelihood
sd	standard deviation
SOP	standard operation procedure
t	metric tons (1,000kg)
TAC	total allowable catch
TAL	total allowable landings
UMES	University of Maryland Eastern Shore
USA	United States of America
UVSW	ultraviolet filtered seawater

CHAPTER 1

GENERAL INTRODUCTION

1.1. Aquaculture importance of the red deepsea crab (RDSC) *Chaceon quinquedens*

Worldwide, millions of people depend on fisheries (aquatic organisms which are utilizable by the public as a common property resource) and aquaculture (husbandry and farming of aquatic organism as food resources) as sources of food, income, and livelihoods (FAO 2016).

In 2014, world total production (inland and marine) for capture fisheries and aquaculture was 93.4 and 73.8 million t, respectively; and a new high record (20 kg) of the world per capita fish supply was reached (FAO 2016). These have been made possible by a strong growth in aquaculture (providing half of all fish for human consumption) and improvement of fisheries management strategies in certain fish stocks (FAO 2016). Preference in the ways these fisheries resources are consumed can vary between countries, but most of them can be classified as live, fresh, or chilled, frozen, prepared or preserved, cured, and non-food purposes (Figure 1-1).

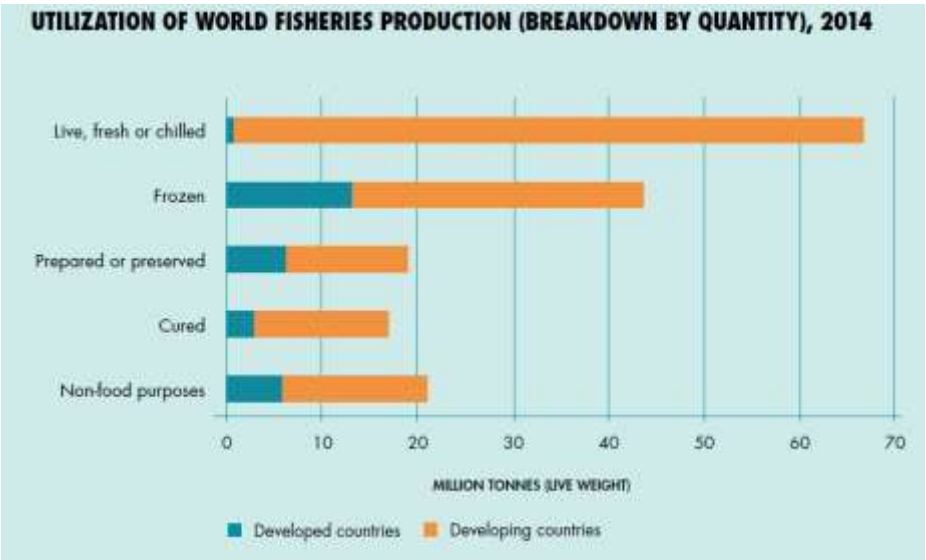


Figure 1-1. —Utilization of world fisheries production (breakdown by quantity), 2014 (FAO 2016).

If we concentrate on crustaceans' production for human consumption in 2014, the total world aquaculture (inland, coastal, and marine) was 6,915,073 t. Asia produced a total of 6,180,178 t, with China as the major producer of 3,993,500 t, whereas the United States of America produced just 65,900 t. However, the volume and value of world aquaculture production are also increasing (Figure 1-2). Recent reports showed that United States of America (USA) is the major importer of fish and fishery products worth $18.8 billion dollars in 2015, down 7% on 2014 (FAO 2016).

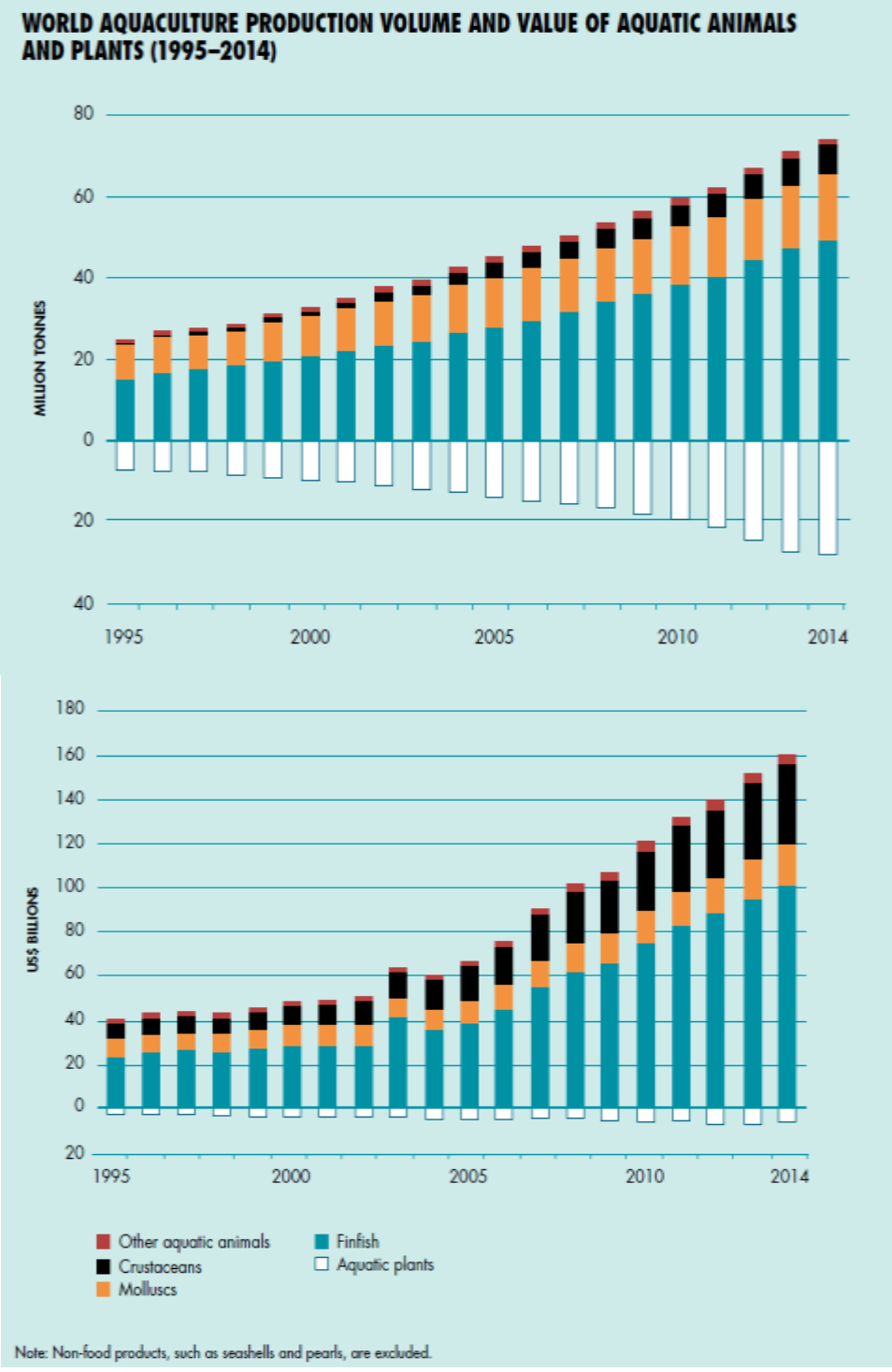


Figure 1-2. —World aquaculture production volume and value of aquatic animals and plants (1995-2014) (FAO 2016).

This supports the USA economic and social interest to investigate species, like the red deepsea crab (RDSC) (*Chaceon quinquedens*), with potential for local aquaculture production and measures to improve harvesting of this species without depleting natural stocks.

Obstacles to aquaculture or rearing of RDSC include the *incomplete knowledge of the developmental and larval biology of the species.* Most of the knowledge on the early life history and development of the red deepsea crabs was gained by hatching the crab eggs in controlled laboratory conditions (Perkins 1973; Rosowski 1979; Sulkin and Van Heukelem 1980; Gerrior 1981; Kelly et al. 1982; Van Heukelem et al. 1983). However, comprehensive and real-time data about the required conditions for optimal larvae survival and development are scarce. Information on essential natural conditions experienced through their life cycle, such as temperature, salinity, pH, and diets, is limited or absent in the literature. In addition, there is a lack of detailed protocol for the cultivation of the larvae in flow-through or recirculation systems of the species. This represents an obstacle to further research on the larval development of red deepsea crabs. The present study will help develop those standard operational procedures and protocols which will facilitate new aquaculture opportunities on deepsea crab species, related organisms and the development of a sustainable fishery. With an increasing need for management actions, and negative factors affecting the wild population, efforts to increase our understanding of the species in order to maintain a sustainable fishery are extremely important.

As the human population grows, demands for food production grow, so it is important to investigate alternative methods to meet the increasing demand such as aquaculture. But many parameters must be considered and investigated in order to increase production without affecting quality, considering the uniqueness of live organisms. Several studies have investigated the optimal developmental conditions and nutritional quality of diets for the different species of

interest, as the blue king crab and spider crab. Studies measuring the effect of diet and temperature on larval survival of blue king crab *Paralithodes platypus* showed that larval survival was significantly different between the diets and temperature treatments, and these also affected the time required for development through larval stages (Stevens et al. 2008a). Rearing conditions of the spider crab *Maja brachydactyla* have also been investigated as an alternative to remediate effects of stock overexploitation (Andrés et al. 2007).

Development of technologies like Biofloc has been suggested as a sustainable method with potential to improve water quality in aquaculture settings, but like every new method it has its pros and cons (Crab et al. 2012). Other parameters to consider in aquaculture include the behavioral tendencies of the species that can affect survival, hatchery efficiency, and financial viability. For example, a study performed with red king crabs (*Paralithodes camtschaticus*) concluded that size grading could be used in hatchery rearing protocols to reduce the effect of cannibalism (characteristic in all their life history stages) by adjusting stocking density of larvae and juveniles (Borisov et al. 2007; Daly et al. 2012).

Red deepsea crab could become the model organism to study climate change effects in deepsea crab (Brachyuran) development. It is a commercially important organism with a representative life cycle of deepsea crabs species. The species life history distribution compared to ocean waters impacted by anthropogenic CO_2 (Feely et al. 2004) suggests the red deepsea crab is affected by ocean acidification during critical life stages (early development, growth, migration, reproduction, egg production, etc.) (Biesiot and Perry 1995; Haefner 1977; Elner et al. 1987). Ocean acidification could have negative impacts on chitin during biomineralization and weaken the crabs' exoskeleton and make them more susceptible to lesions, and infections of

chitinoclastic bacteria and fungi (Young 1989; Rahman and Halfar 2014).

There is a lack of basic life history information about this species. I aimed to characterize the hatching period of the red deepsea crabs, including duration, starting and ending dates, and number of larvae hatched through all periods. Previous studies examined several factors, but did not focus on enhancing culture conditions or combine more than one factor. Perkins (1973) described larval stages after keeping them in temperatures between 18-21°C, which accelerated their development. Previous research where larval RDSC was reared at 12°C, 20°C, and 26° C determined that the coldest temperature contributed to developmental abnormalities, survival to first crab stage was best for the highest temperature, and both developmental stage and temperature affected survival (Rosowski 1979). Van Heukelem et al. (1983) compared different temperature treatments and concluded that 9-15°C was the optimal temperature range for red deepsea crab juvenile development. Larval survival of the red deepsea crab to metamorphosis on diets of rotifer or brine shrimp were not significantly different, but there was a delay in their development (Sulkin and Van Heukelem, 1980).

My goal was to collect baseline information to enhance our understanding of the red deepsea crab life cycle and growth, in order to contribute to the mission and goals of the National Oceanic and Atmospheric Administration. In my research, I planned to determine the optimal conditions for peak survival and development of deep sea red crab larvae under laboratory conditions. These parameters and protocols will open new lines of investigation for this species including those of aquaculture and the effects of environmental changes (climate change, ocean warming, and ocean acidification) upon them. The following sections include objectives and hypotheses in my research project.

1.2. Objectives and Hypotheses

1.2.1. *Part I- Hatching Periodicity of Red Deepsea Crab (RDSC) Chaceon quinquedens*

In this part of the project, I aimed to answer the following specific questions about the hatching process of the RDSC:

Main: Is there a common larval hatching pattern between adult female crabs?

1) Is *duration* of hatching different between adult female crabs held in laboratory conditions?
2) At what time of the day do larvae hatch?
3) Is there a relationship between female morphometry and the total volume of larvae hatched?

The *main objectives* of these experiments are to monitor the laboratory conditions (temperature and salinity) in which the adult female crab's eggs hatched and to observe, characterize, and compare the RDSC hatching diel periodicity.

Specific *objectives* included:

- Maintain live adult females with fertilized eggs in a custom designed Adult Females Husbandry and Hatching Collection System (FHCS).
- Obtain pictures of the live adult female's eggs.
- Count, measure, and transfer hatchlings.
- Produce a linear regression to estimate the number of larvae per volume.
- Describe hatching period (starting and ending date, amount hatched per day, and time of

maximum hatch).

- Describe conditions during hatching events (salinity and temperature).

The *hypotheses* to test these objectives were as follows:

H_{01}: The duration of hatching period is not different between adult female crabs.

H_{A1}: The duration of hatching period is different between adult female crabs.

H_{02}: There is no difference in number of larvae hatched between 4 h time periods of the day.

H_{A2}: There is difference in number of larvae hatched between 4-hr time periods of the day.

H_{03}: There is no relationship between female morphometry and total volume of larvae hatched.

H_{A3}: There is relationship between female morphometry and total volume of larvae hatched.

The same specific objectives were followed to test the same hypotheses from years 1 through 3. However, freshly captured adult females with fertilized eggs were obtained in each year.

1.2.2. *Part II- Survival of Red Deepsea Crab (RDSC) Chaceon quinquedens, Larvae in Cultivation: Effects of Diet and Temperature*

In this part of the project, I aimed to answer the following specific questions about the larval survival of the RDSC:

1) What are the combined effects of diet and temperature on the RDSC larval survival?
2) What are the combined effects of diet and temperature on the RDSC larval development?
3) Which aquaculture setting better promotes larval survival?

The *main objective* of this project was to study the factors affecting the survival of the early life stages of the red deepsea crab in aquaculture settings. A series of experiments were designed to support and complement each other during a multiyear project.

Specific *objectives* included:

- Develop and consequently modify Larvae Feeding Experimental System (LFS) to maintain and improve larvae survival under the desired conditions (temperature, diet, salinity, and light).
- Execute a transfer method of larvae from the adult Females Husbandry and Hatching Collection System (FHCS) to the Larvae Feeding Experimental System (LFS).
- Cultivate live diets to feed the larvae (e.g. rotifers).
- Monitor, record, and compare RDSC larvae survival and development under different combinations of temperatures and diets.
- Quantify feeding conditions (e.g. concentration of diets, density of cultures).

The *hypotheses* to accomplishment these objectives were as follows:

H_{04}: There is no effect on survival of a) diet and b) temperature in RDSC larvae.

H_{A4}: There is an effect on survival of a) diet and b) temperature in RDSC larvae.

H_{05}: There is no effect on development of a) diet and b) temperature in RDSC larvae.

H_{A5}: There is an effect on development of a) diet and b) temperature in RDSC larvae.

H_{06}: There is no effect on survival of the experimental aquaculture systems (LFS) used in different years.

H_{A6}: There is an effect on survival of the experimental aquaculture systems (LFS) used in

different years.

The same specific objectives were followed to test the same hypotheses from years 1 through 3. But years 2 and 3, modifications of experimental parameters were accommodated based on results from year 1.

The proposed project supported NOAA's *Mission of Science, Service, and Stewardship*, by supplying a better understanding of commercial species and educating the public about the species. This project also focuses on NOAA's goal of "*Healthy Oceans: Marine fisheries, habitats, and biodiversity sustained within healthy and productive ecosystems"*. By studying several factors affecting survival and development under controlled conditions in a laboratory, I gained an understanding of the necessary conditions needed in the ocean to enhance the commercial crab resource and its management. Only by learning how to optimally develop red deep sea crab growth can we determine the effect of an anthropogenic change in the ocean. Further this project will help to understand the effect of climate change and provide a baseline of high-quality data to better understand our deep sea red crab and similar living marine resources.

CHAPTER 2

LITERATURE REVIEW:

RED DEEPSEA CRAB (RDSC), *CHACEON* (*GERYON*) *QUINQUEDENS* (SMITH 1879): BIOLOGY AND EARLY LIFE HISTORY

2.1. Distribution

After the discovery of a deep-sea red crab from the genus *Chaceon* in New Zealand waters, and the interest in it as a possible commercial fishery, Dawson and Webber (1991) created a guide for the Family Geryonidae that collected existing information about several crabs species (including *Geryon quinquedens*) in its almost world-wide distribution.

Chaceon (*Geryon*) *quinquedens* (Smith 1879), or red deepsea crab (RDSC) refers to a red colored (Brachyuran) crustacean in the family Geryonidae (Figure 2-1). It is distributed in deep waters throughout the continental shelf edge and slope of the Northwest Atlantic Ocean, Gulf of Maine, and Gulf of Mexico (Steimle et al. 2001; Stevens and Guida 2016) (Figure 2-2).

Figure 2-1. —*Chaceon* (*Geryon*) *quinquedens* (Smith 1879), or red deepsea crab (RDSC).

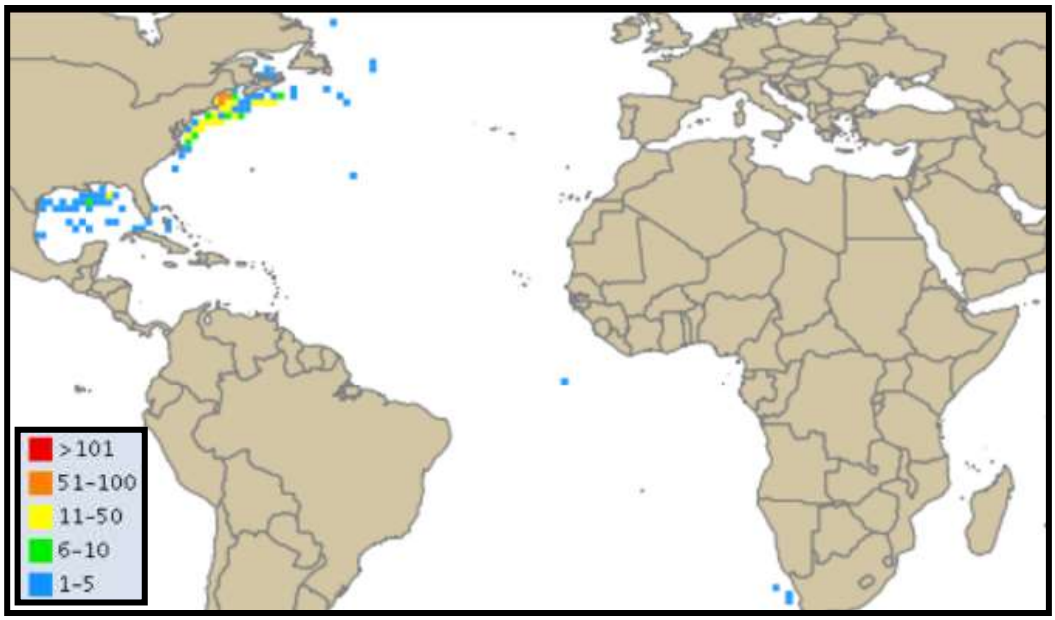


Figure 2-2. —Red deepsea crab distribution reported by April 14, 2016 (http://www.iobis.org/node/213/04/14/2016/3:40pm).

They can be found in depths between 200-1,800 m. Males reach larger sizes than females, are estimated to live up to 15 years, and reach a maximum of 180 mm carapace width (CW), whereas females reach 120 mm CW (Wigley et al. 1975). During a 7-year tag-recapture study of 7,822 trap-caught red crabs (size range of 81 to 154 mm CW) in the continental slope off southern New England, between depths of 275- 1,100 m, Lux et al. (1982) found that most red deepsea crabs moved distances <20 km, with some individuals traveling up to 90 km in and eastern direction and 55 km in a western direction.

Kelly et al. (1982) proposed a recruitment model for *G. quinquedens* and potential larvae dispersal ranges in the Mid-Atlantic Bight, based on larval behavior responses to gravity, hydrostatic pressure, thermoclines, development time, and coastal hydrography. They concluded that temperature is a more significant stimulus for larvae to swim towards the surface than pressure, sinking rates increase as development proceeds, greatest dispersal range is in surface shelf waters during the winter, only larvae hatched at the northeastern end of the adult range develop to megalopa before entering the Gulf stream, and the southwestern end of the range received a stable and persistent level of recruitment from the northeast. This model predicted a continuum of genetic communication between the regional adult populations (Kelly et al. 1982). Meanwhile comparison studies using the mitochondrial 16S rDNA gene and the internal transcribed spacers (ITS) of the nuclear ribosomal, with *Chaceon quinquedens* from southern New England and the Gulf of Mexico, and two congeners *C. fenneri* from the Florida coast and *C. affinis* from the eastern Atlantic species, found the greatest genetic difference among the two geographical groups of *C. quinquedens*. Based on these results, genetic differences between Gulf of Mexico and Atlantic (southern New England) *C. quinquedens* are large enough to be

considered as two different fishery stocks (Weinberg et al. 2003). Genome size of the red deepsea crab was not found reported in the literature.

2.2. Fishery

The red deepsea crab population is a data-poor stock, federally managed by the New England Fishery Management Council-Deep Sea Red Crab Fishery Management Plan (FMP), but little is known about this species' general biology, abundance, reproduction, and development. Information available is insufficient to provide a good understanding of the species population dynamics and measures needed to improve their management. This situation impedes the analysis of the possible linkages between factors affecting the species population abundance, such as predation, diseases, overfishing, and climate change (NEFMC 2013).

An increased interest in the commercial harvest of the RDSC, for their legs and attached body section, has been stimulated by the decline in other highly targeted crustacean species (lobsters, king crab, etc.), since the late 1960s-70s (Schroeder 1959; Wigley et al.1975).

The development of the fishery off the east coast of the United States established the need to determine the size and condition of the stock (Serchuk 1977). Wigley et al. (1975) estimated 26761.95 t of commercial-sized male crabs, which at the time were those crabs >114 mm CW. Based on this estimation Wahle et al. 2008 revisited the stock size and conditions; he found the abundance and biomass of large males targeted by the fishery (≥ 114 mm CW), was considerably lower than 1975, and had declined by 42%, being most evident at depths and regions most accessible to the fishing fleet. Chute et al. (2013) tried to reduce the uncertainty around Wigley et al.'s biomass estimates from the 1974 underwater photographic transects, since it has been a key part

for the species assessment. She determined that reduced visibility with distance translates into higher density estimates because more crabs are required to predict the observed data. However, she concluded that the exponential visibility function and area compression effects described in Patil et al. 1979 were possibly taken in consideration by Wigley et al. 1975 conversion from visible number of crabs to estimated crabs.

Directly targeted red deepsea crab fishery has been limited, but there is a part-time catch or bycatch in other major fisheries, for which landings fluctuate by an order of magnitude (Steimle et al. 2001). Suggestions had been made to fishery participants to improve gear selectivity and minimize discard mortality rates, and to managers to include estimates of discard mortality in stock assessments of the species. This was done after studies on targeted male crab (~>105 mm CW) at the continental slope of New England showed lower proportions of small individuals in traps with escape rings, and a ~5% discard mortality was estimated (Tallack 2007).

Landings have been highly variable and a total allowable catch (TAC) was first designated in 2002 (Figure 2-3), under the New England Fishery Management Council FMP (Wigley et al.1975; Wahle et al. 2008). The two main concerns to establish the FMP were the threat of overfishing and the overcapitalization of the red crab fishery (FMP, 2002). The original plan establishes a limited access fishery with a TAC of 2,688 t (5.928 million pounds), 780 days-at-sea allocation, trip limit of 34 t (75,000 pounds), and only male crabs could be retained (FMP, 2002). In recent years landings have been decreasing (NEFMC 2011). Total allowable landing (TAL) specifications for the years 2014-2016 for the Atlantic red deepsea crab fishery was kept at 1,775 t for the male-only directed fishery, as established in the Amendment 3 (76 FR 60379; September 29, 2011) (NEFMC 2013).

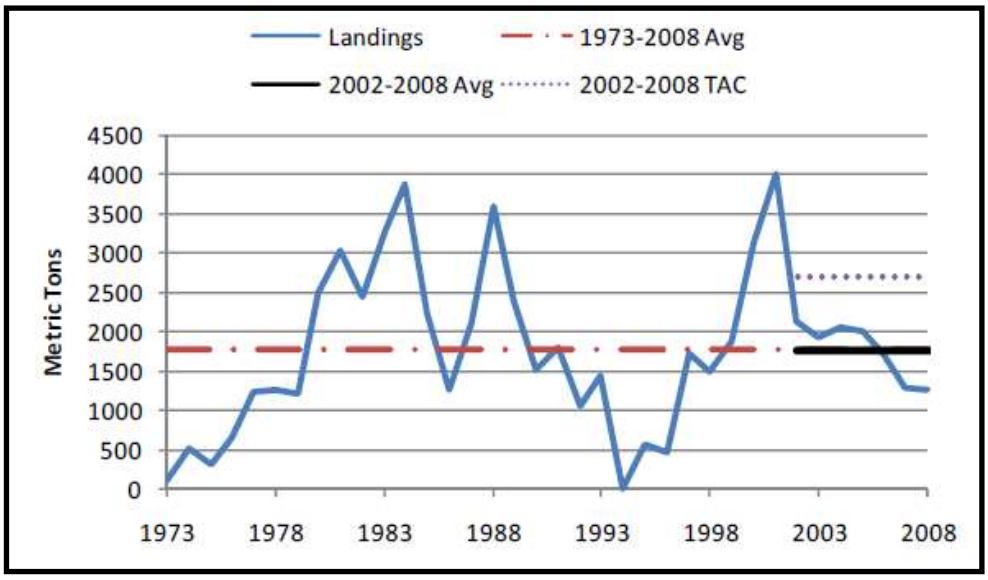


Figure 2-3. —Reported live weight landings of red deepsea crab 1973-2008 showing multi-year average landings and the target total allowable catch that were in effect from 2002- 2008 (NEFMC 2011).

Schroeder (1959) described RDSC as "very good to eat" and made the suggestion of RDSC as a potential commercial fishery with otter trawls for reaching greater depths. Exploratory otter-trawling in 1952 and 1953 showed relative abundance by depths and geographic areas with depths >200 fathom as more productive in most areas surveyed; this stimulated the establishment of a canned meat market for red deepsea crabs. Since it was considered a potential food source, sterol and fatty acid content in the cooked muscle of the species were examined. Cholesterol was the major sterol and 20:5w3 the predominant poly-unsaturated fatty acid (Krzynowek et al. 1982). Analysis of carotenoid content in raw shell showed astaxanthin ester, astaxanthin, astacene, β-doradexanthin ester and zeaxanthin ester, and two additional unidentified carotenoids in the cooked sections (Kuo et al. 1976).

Hastie (1995) summarized a number of previous stock assessments and density estimates for the species to the early 1990s, and described the red deepsea crabs as being a low temperature (3.3-12.7 °C), deep-water (100-2080 m) living species with slow growth (5-6 yrs to enter fishery

at 114 mm CW) and maturation rates, and infrequent recruitment that could present difficulty to maintain high yields as a sustainable fishery.

As discussed by Steimle et al. (2001), the lack of broad-scale, long-term, fishery-independent surveys for the species reduces the information known for relative population abundance and distribution of the species; however, occasionally localized surveys have been performed since the 1950s by Schroeder (1959); McRae (1961); Haefner and Musick (1974); Meade and Gray (1973), Wigley et al. (1975); Haefner (1978); Gerrior (1981); Stone and Bailey (1980); McElman and Elner (1982); Kilgour and Shirley (2008).

The concern of how harvesting may change population size structure of the deep-sea red crab off southern New England was addressed by Weinberg and Keith (2003). He found the proportion of large males in the population in the 350-499 m depth zone was significantly higher in 1974 (0.38) than in 2001 (0.25). A more recent study by Stevens and Guida (2016) compared and analyzed the reported conditions and population characteristics. They compared the previous surveys with more recent information obtained from the surveys performed in two research cruises aboard the NOAA Ship Delaware II and the NOAA Ship Gordon Hunter, during January 2011 and 2012, and July 2013 respectively (Stevens and Guida 2016). As a result, they obtained the first detailed morphometrics and distribution by temperature and shell condition of the species, the extension of the surveys to the southern range of the fisheries, and increased the counts of organisms sampled (Steven and Guida 2016). They concluded that the currently targeted population is different from the one fished decades ago, because of their displacement to southern parts of the Mid-Atlantic Bight, changed abundance, and their consumption by the fishing industry (i.e. processing facilities).

This information stimulated my interest to study this species as a potential sustainable

fishery and aquaculture organism. Potential benefits of determining protocols for the culture of the species will be to produce the species in aquaculture facilities to support the populations and as an alternative for consumption. In addition, the species life history distribution was compared to those locations potentially impacted by anthropogenic CO_2 (Feely et al. 2004) and climate change, indicating that the red deepsea crab could become a model organism to study these anthropogenic effects in deepsea crabs (Brachyuran) development.

2.3. Reproductive Biology and Hatching Periodicity

Several aspects of the reproductive biology and life history of the red deepsea crab (RDSC) *Chaceon quinquedens*, are unknown. The limited information on the reproductive biology of the RDSC is constituted by few published articles. Mature adults generally migrate upslope in the continental shelf for reproduction and females retain their egg clutches until the larvae hatch and become part of the plankton (Hastie 1995).

Hines (1982) compared the allometric relationships of 20 species (including *Geryon quinquedens*) from 7 families of brachyuran crabs from both coasts of North America. Results showed female body size as the main determinant of reproductive output with body weight explaining 95% of the variance in brood weight, 79% of the variance in number of eggs per brood, 63% of the variance in annual brood weight, and 74% of the variance in annual fecundity.

Similar to the brachyuran species, RDSC have large reproductive output (brood mass or clutches), brood weight increases with female body weight, fecundity per brood increases with female body size (approx. 160,000 eggs per brood), brood masses are 22% of body weight, and they have relatively large yolky eggs (730µm diameter) when, all of which could play an important adaptive role for larval survival (Hines 1988).

Ovary development was described through morphology and histology analyses; females reach sexual maturity within the size range of 65-75 mm CL (80-91 mm CW). Abdomen width-carapace length shares an allometric relationship; development and growth of vulvae show a structural pattern related to body size and ovarian development. Evidence of copulation and insemination were determined as blackened vulval margins, but sperm plugs were not found (Haefner 1977). It is believed that red deepsea crabs have a biennial reproductive cycle, which is common in crabs inhabiting low temperatures (Stevens et al. 2008b). The genus *Chaceon* has been described as unusual, since it is the only genus reported with a possible biennial spawning with planktotrophic larvae living at temperatures >6°C (Stevens and Guida 2016). They suggested that the females start developing the ovary during the summer and it takes them approximately 9-11 months to be fully mature. Mating occurs and the females produce a clutch during the second summer of the cycle and after 9-11 months the eggs hatch (Martínez-Rivera unpublished). Hilário and Cunha 2013, observed *Chaceon affinis* display mating behavior indicative of synchrony and reproductive seasonality. They hypothesized a similar biennial female reproductive cycle with molting, mating and oviposition (autumn) during the first year, and spawning during spring of the second year.

I was interested in characterizing the hatching period of the red deepsea crabs under laboratory conditions, including the time required to complete hatching, total volume, and a number of released larvae, as described before for red king crab, *Paralithodes camtschaticus* by Stevens and Swiney (2007). Effects of the water conditions throughout the hatching period including temperature, light, and timing are also of interest in this research adapted from Stevens et al. (2008b). These are very important to enhance our understanding of the RDSC hatching process which hasn't been explored before. Studies performed using blue king crabs

(*Paralithodes platypus*) showed that crabs held longer under laboratory conditions released fewer larvae per day over a longer period of time, suggesting that long-term laboratory holding may impact hatching time due to differences in ambient temperature and other seasonal effects that inhibited their hatching (Herter et al. 2011). The timing of hatching may be critical for the survival of the newly hatch larvae. A delay in their migration towards the surface where they would find favorable conditions for their development (warmer temperatures, prey, etc.) could potentially threaten their survival. It has been shown that a delay on feeding king crab zoeae for 84 h at 6°C reduced their ability to capture prey, if held in temperatures 2 and 4°C, and capacity to capture prey was lost, which resulted in substantial mortality (Paul and Paul 1980). A substantial decrease in total fatty acid content was observed in zoeae V and megalopa stages of mud crab (*Scylla serrata*) after four days of starvation (Holme et al. 2009).

In addition, data collected during my preliminary study allowed me to target maximum hatching times in the middle of the hatching period for hatchlings collection and transfer them to the feeding experimental system. The results obtained in my study contribute to the knowledge about the species reproductive biology and early life stages.

2.4. Life Cycle and Larvae Survival

There is a lack of information on any aspect of early life stages of the RDSC. For commercially harvested species, it is important to understand the essential habitat conditions for its survival and development. Knowing the essential habitat conditions for the species will allow for understanding the impacts of environmental disturbances and developing better management practices, which will help reduce the effects of those pressures on the species stock.

Red deepsea crabs have a typical Brachyuran cold-water crab life cycle, with longer brood incubation time, longer zoeal and total larval period, and larger adult size than species in

warm-water (Hines 1986). Larvae are hatched in spring/summer and occur in relatively shallow waters in comparison with depths (200-1,800 m) occupied by their adult counterparts (Steimle et al. 2001). Larvae develop through six stages: a pre-zoea, four zoeae, and a megalopa (Figure 2-4). The whole early development cycle from pre-zoea to megalopa can be completed in 26 days under temperatures of 18-21°C, which accelerate development under laboratory conditions (Perkins 1973). The benthic settlement of the juvenile crabs occurs near the mid-slope. During their development, they will start migrating upslope to pool with the females for mating. Normally male and juveniles are found in deeper waters than females (Wigley et al. 1975; 43rd SAW 2006). Similar studies in 1978, using otter-trawls on the continental slope off South West Africa, showed that females preferred shallower waters than males, that size is inversely related to depth and that crab survives in extremely low levels of dissolved oxygen and tolerate a temperature range of more than 7ºC (Beyers and Wilke 1980).

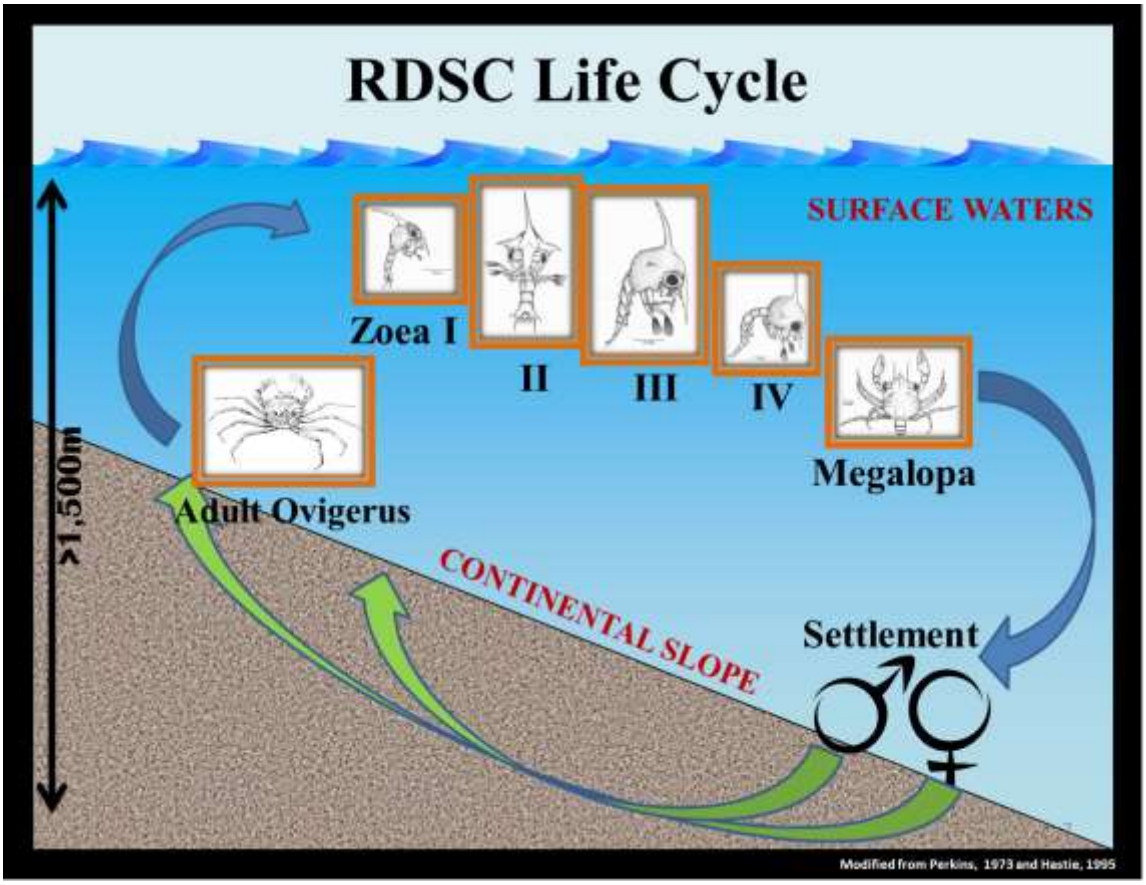


Figure 2-4. —Red deepsea crab (RDSC) *Chaceon quinquedens* life cycle. Modified from Perkins (1973) and Hastie (1995).

Diet is a major component in larval rearing for planktotrophic crab species (Sulkin and Epifanio 1975; Sulkin 1978; Sulkin and Van Heukelem 1980). Studies had shown that lack of suitable prey in specifically sensitive phases in the early life of Brachyuran crabs, cause larvae to reach a no-return-point where they die as a consequence of the initial starvation, even if feeding is resumed (Anger et al. 1981). Even though Hines (1988) explained how the RDSC large yolky eggs could contribute to nutritional flexibility of its larvae, Anger et al. (1981) suggested that zoeal development doesn't use egg reserves until a cue is given by the food consumed. Sulkin and Van Heukelem (1980) contributed to the idea of the larval nutritional flexibility as a tool for

the species to survive in the deepsea habitat and the larvae to survive during their ontogenetic migration.

Finding a cost-effective food source with high survivability for RDSC larvae is essential for aquaculture and future larvae research (Epelbaum and Kovatcheva 2005). Comparisons of larval development and survival between *Chaceon quinquedens* and *Menippe mercenaria* (a marine crab species of shallow water habitats), with rotifer (*Brachionus plicatilis*) and brine shrimp (*Artemia salina*) diets showed no statistical difference on survival rate through zoeal development or mean days to first molt. But mean days to second and fourth moltings showed statistical differences (Sulkin and Van Heukelem 1980). The importance of using these two diets is because the failure of larvae to complete metamorphosis on a rotifer diet alone is considered as evidence of nutritional vulnerability, previously suggested as a primitive reproductive strategy in brachyuran crabs (Sulkin 1975; Sulkin and Epifanio 1975; Sulkin and Van Heukelem 1980). A delay in the larval development of *Geryon quinquedens* was observed under the rotifer diet, but no difference in the survivorship to the megalopa stage was observed for either diet (Sulkin and Van Heukelem 1980).

Another crucial component in the development and survival of the RDSC early life stages is temperature. Larval RDSC were reared at 12°C, 20°C, and 26° C in the study by Rosowski (1979) and coldest conditions contributed to developmental abnormalities. Survivability to the first crab stage was best for the higher temperature (Rosowski 1979). Comparisons made between different temperatures showed that 6°C slows the juvenile crab's development and the range from 9°C and 15°C accelerates the process. This is the optimal temperature range for rapid juvenile crab growth **(**Van Heukelem et al. 1983**)**. In my last year of experiments, temperatures 15°C and 20°C were selected, taking into consideration the temperature data

showed higher in the areas where parental crabs were collected (Stevens and Guida 2016).

Effects of the interactions between different diets and temperatures in the early life stages of the species have not been reported in previous research. My research will provide baseline information and allow future research to advance in crabs' development, maturation, and survivability issues. For example, it is a concern that during the larval development period, they may be more exposed to anthropogenic effects like increased temperatures in surface water or decreased pH, contributors to climate change and ocean acidification. In addition, deep water of the Northwestern Atlantic Ocean where this species lives is affected by anthropogenic CO_2 (Feely et al. 2004). Ocean acidification could affect the strength of chitin in biomineralization, weakening their exoskeleton and making them more susceptible to lesions, and infections of chitinoclastic bacteria and fungi (Young 1989; Rahman and Halfar 2014).

My system design allowed me to systematically contain the red deepsea crab larvae during the feeding experiments and also to measure the effects of diets and temperature. Similar studies measuring the effect of diet and temperature in blue king crab *Paralithodes platypus* showed that larvae survival was significantly different between the diets and temperature and that the time required for development through larvae stages was different (Stevens et al. 2008a).

This summarizes most of the available knowledge about the distribution, fishery, reproductive biology, and early life stages of the red deepsea crab. Through all the documents searched, there are several conflicting results about the early life history of the species and a common prompt for research to help to assess essential knowledge about the species.

CHAPTER 3

RESEARCH METHODOLOGY

3.1. Part I- Hatching Periodicity of Red Deepsea Crab (RDSC) *Chaceon quinquedens*

3.1.1. *Adult Females with Fertilized Eggs*

Red deepsea crab (RDSC) adult females with fertilized eggs were obtained through collaboration with the Atlantic Red Crab Company (Figure 3-1). During their late spring and summer (2014-2016) fishing trips they captured female red deepsea crabs and brought them to the port where the researchers picked them up, and on several occasions researchers were on board during the fishing trips.

Figure 3-1. —Boat Hannah Boden part of the Atlantic Red Crab Company. Picture taken by Stephanie Martínez-Rivera in 2014.

RDSC were captured using approximately 150 round traps/ Japanese beehive pots per line (Figure 3-2) with a total of 4-7 lines per fishing trip. Fishing gear was deployed at depths of >183-610 m

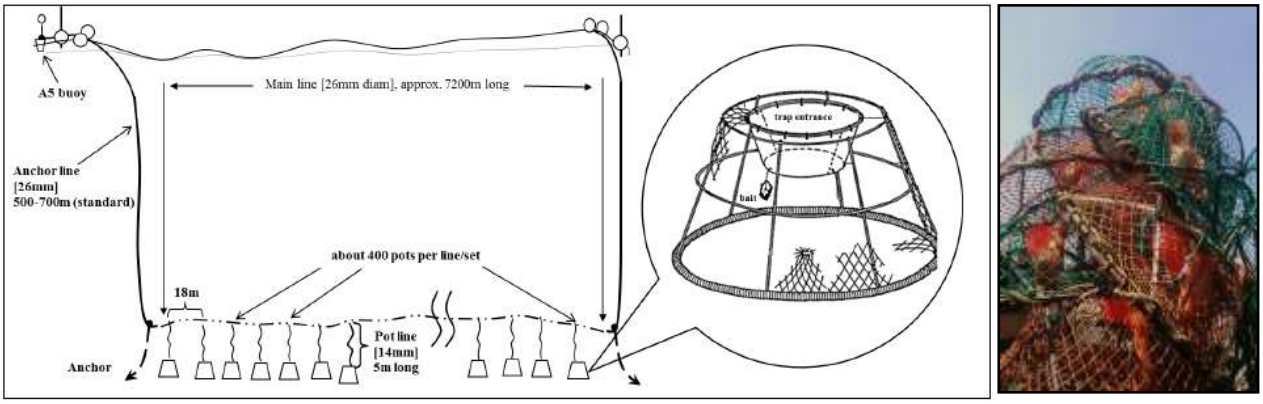


Figure 3-2. —Diagram of deepsea crab fishing gear and illustration of a Japanese beehive pot. Round traps used by the Atlantic Red Crab Company to capture red deepsea crabs (right). Diagram from SEAFO (2015) and picture taken by Stephanie Martínez-Rivera in 2014.

Fishermen commonly used designated fishing/sampling sites in the West Atlantic Ocean (Figure 3-3). All the females obtained from the Atlantic Red Crab Company were captured from sampling sites classified within Site 3-South for the distribution of the red deepsea crab in the USA western Atlantic (NEFMC 2011).

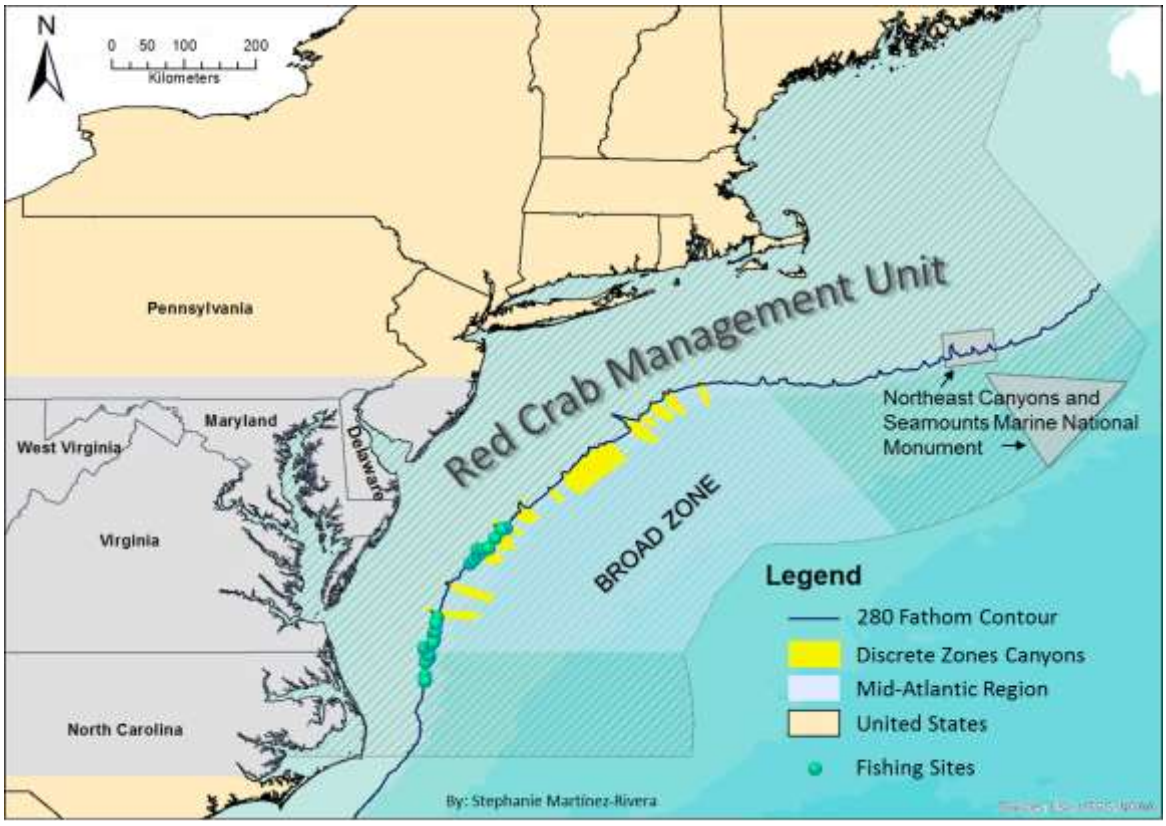


Figure 3-3. —Designated and used fishing/sampling sites at Newport News by the Atlantic Red Crab Company. Map by Stephanie Martínez-Rivera.

The fertilized females were transported in a car, using a cooler with ice packs and wet towels, to the NOAA James J. Howard Sandy Hook lab, New Jersey (Figure3-4). Those that arrived alive were measured, examined (number of legs, shell condition, etc.), and randomly placed in the adult Females Husbandry and Hatching Collection System (FHCS).

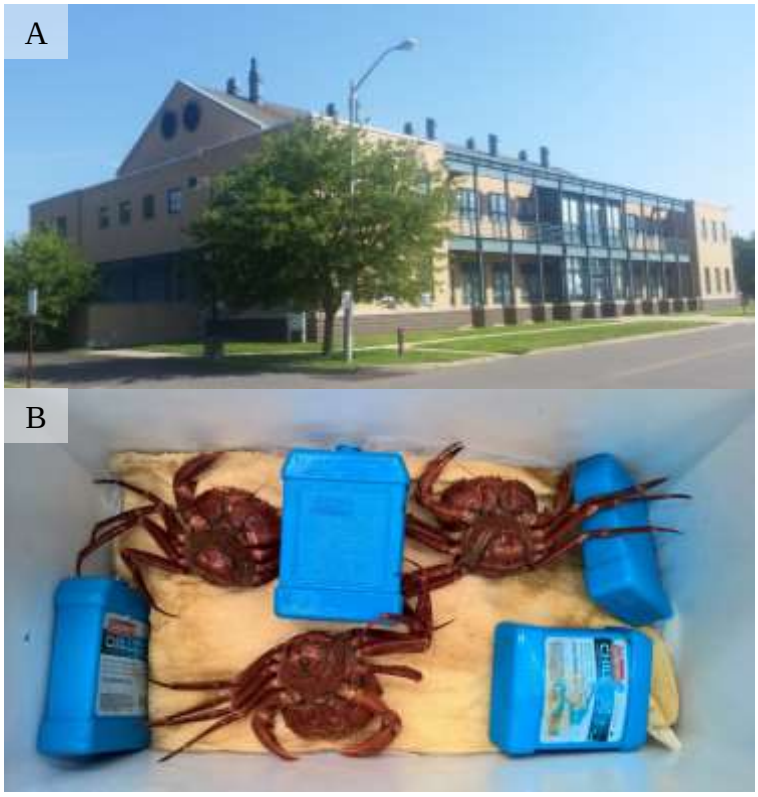


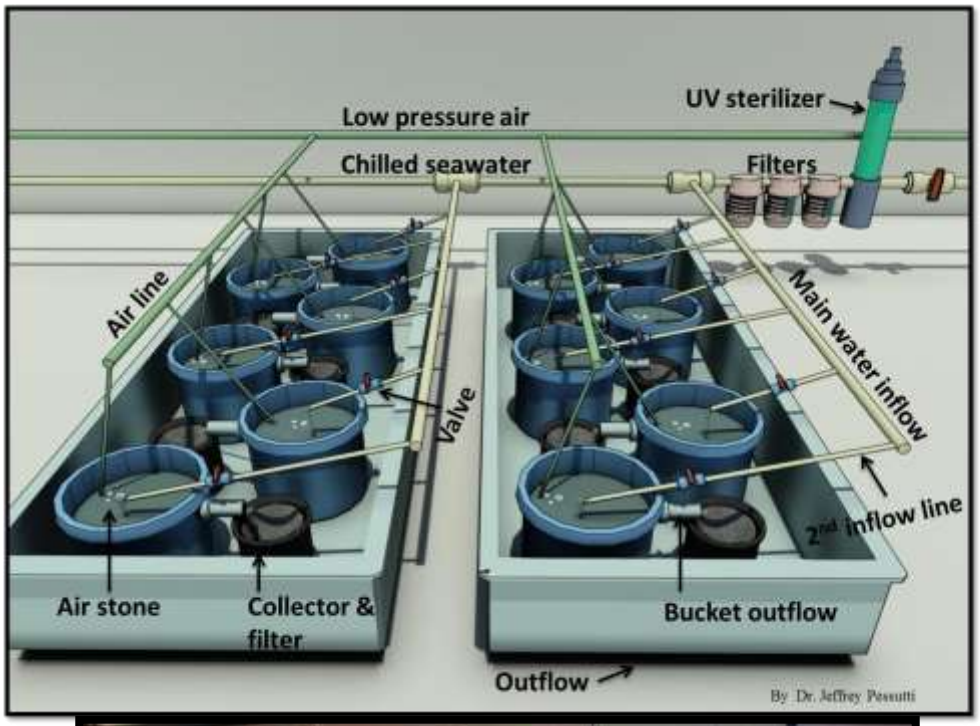
Figure 3-4. —*A.* NOAA James J. Howard Sandy Hook lab, New Jersey. *B.* Transportation method for live red deepsea crab females with fertilized eggs.

The first week after placement was considered as "acclimation time". During this time the females' handling was reduced to a minimum. During the first year, I observed that weak adult females would die during the first three to five days after transportation. The time between placement and first hatching measurements also allowed us to discard eggs and larvae deposited in the collectors as a result of handling in transport. Red deepsea crab are known to scavenge fish and squid and eat them when in aquaria (Gray 1970). After the acclimation period, adult

female crabs were fed every 3 days with defrosted and cut herring fillets, after removing the guts, head, and fins from the fish, in order to maintain the FHCS and hatching containers clean.

3.1.2. *Adult Females Husbandry and Hatching Collection System* (FHCS)

The adult *Females Husbandry and Hatching Collection System* (FHCS) was designed to maintain alive red deepsea crab adult females and also allowed me to collect, count (or measure), and transfer their hatchlings (Figure 3-5).



B

Figure 3-5. —*A.* Diagram of the red deepsea crab adult female hatching system. *B.* Picture of system that was used. Diagram by Dr. Jeffrey Pessutti and picture taken by Tanya Breen in 2014.

I decided to maintain the live females with their egg clutches until hatching, because of previously reported low survival using artificial incubation methods with red deepsea crab ova (Cassels and Krebs 1983). The FHCS is a flow-through system (1.2 L /10 min adjustable) with chilled seawater (6.9°C-11.3°C, salinity 27-30‰) obtained from the main seawater supply of the NOAA James J. Howard Marine Sciences Laboratory at Sandy Hook, New Jersey. The main seawater supply is collected through pumps from the Sandy Hook Bay and distributed around the facility. Chilled seawater was filtered and UV sterilized, before entering the individual female buckets, with a combination of a 25µm and two 5µm or 50µm, 20µm, and 5µm polypropylene cotton filters, depending on the sediment density in the water. Twelve buckets of 15 L each were distributed and placed within two 210 x 60 cm rectangular tanks. Buckets had the capacity to hold one living female red deepsea crab. Each bucket was equipped with an individual airstone, a water inflow hose, and an outflow extended to the exterior of the bucket. The water exiting through the outflow was directed to a filter mesh placed over a collection cup (in combination called a *collector*). Collectors allowed obtaining the hatch larvae (hatchlings) to be counted or measured. The room containing the system was maintained with a 48 lx (light intensity (luminous flow per unit of area)) from 8:30 am (day) to 8:30 pm (night) with a 30 min transition to each (sunrise and sunset).

3.1.3. *Hatchlings Measurements and Observations*

Observations and measurements of hatchlings in collectors of the FHCS were started a day after introduction of adult females. The time in between placement and first measurements allowed us to discard lost eggs and larvae deposited in the collectors as a result of handling. Daily, each mesh filter was removed from its collection cup and replaced with a clean mesh filter, unless no larvae were observed in the filter.

All larvae and eggs collected in the collector were transferred with a squirt bottle containing UV-filtered seawater, and a funnel to a graduated cylinder (25 ml or 50 ml) until they were settled at the bottom (Figure 3-6). The settled larvae volume was recorded and the contents transferred to a petri dish to be completely counted under the dissection microscope with a hand counter. During the first year, eggs and larvae were counted separately, which resulted in three types of counts (just eggs, just larvae, and larvae plus eggs), but in the second and third year volume samples were measured and described as eggs, larvae or both. All samples with larvae were used for statistical analysis. Several volumes (ml) were counted (0.25, 0.5, 1.0, 3.0, 3.5, 5.0, 6.5, 8.0, 10.0, 12.0, 27.0, 22.0, and 33.0 ml) for accurate measurements of larvae. A linear regression was performed with the larvae per ml counts for the different volumes. This regression allowed us to estimate the number of larvae from volume measurements without the need to count each sample individually each time.

Figure 3-6. —A collector with hatched larvae. This method used to gather the larvae and determine larvae/volume relationship to estimate the amount of hatchlings in higher volumes.

Eggs were observed under the dissection microscope to determine their developmental stage (i.e. eyed, not eyed). Hatched larvae were collected every 24 h, as possible, from all females with advanced eggs. Once females started releasing larger volumes of larvae in less than 24 h, larvae were collected from those females at 4 h intervals (200, 600, 1000, 1400, 1800, and 2200 h) for 3 consecutive days. Bucket contents were completely emptied through collectors to count every larva at the specific time. If female volumes decreased substantially after the 3 days, observations would continue every 12 h until hatching was resumed and another set of collection were made at 4 h intervals for 3 days unless volume decreased to less than 1 ml, at which time observation frequency was reduced to 24 h intervals.

3.1.4. *Statistical Analysis*

The statistical analysis described below was used to answer our main question: Is there common larvae hatching pattern between adult female crabs?

In order to obtain the hatched estimation from measured volumes, a simple linear regression with intercept set to zero was used to describe the relationship between number of larvae and volume, and obtain the formula for estimation.

Hypothesis 1: Is *duration* of hatching different between adult female crabs held in laboratory conditions? Larval hatching by individual female crabs was analyzed using a smooth function and those that began and finished hatching (bell-shaped curve distribution of hatching) in the laboratory with at least 20 days of hatch observations were selected. The analyzed data set consisted of the day (or date) of hatching, replicated by the (rounded) number of ml of larvae hatched, e.g. if a total of 50 ml of larvae were released on day 10 of collections, then the data for that day was 50 units of day 10. QQplots and Shapiro–Wilcoxon test were used to tested for normality of distribution of the observed hatch volume units (rounded up to the to the nearest

ml). A logistic regression analysis was conducted to model the proportion of cumulative total larvae hatched by each female on each day of observation. Objective milestones in the hatching period were defined as the first day on which crabs hatched more than 2.5%, 50%, and 97.5% of their eggs.

Hypothesis 2: At what time of the day do larvae hatch? Only female crabs with observations at 4 h intervals were used for the analysis. A log10 transformation was used to normalize the total larvae hatched per observation interval and the variances for all females. Homogeneity of variance was tested using Bartlett's test and QQplot. Linear model and a pairwise t-test were used to test the relationship between log larvae hatched and the observation intervals. Individual differences between females were examined using the non-linear mixed effects models (NLME) with the nlme package in R statistics. These models are similar to the linear model command, but can use a mixture of factors and continuous variables and also include random effects (females). This calculates a mean effect for each crab and then compares the time groups within each female (crab) using standardized data. Three models were considered 1) intercept only, 2) intercept and group effect with CrabID as random, and 3) same as 1 but with Group (time) nested within CrabID. Finally, a single ANOVA was used to compare these models and select the best one based on their AIC values.

Hypothesis 3: Is there a relationship between female morphometry and the total volume of larvae hatched? A simple linear regression between size (carapace width (CW), carapace length (CL), and abdominal segment width (AW)) and total larvae hatched per female crab was performed to assess the relationship between female's morphology and hatch.

3.2. Part II- Survival of Red Deepsea Crab (RDSC) *Chaceon quinquedens*, Larvae in Cultivation: Effects of Diet and Temperature

3.2.1. *System Design*

3.2.1.1. *Larvae Feeding Experimental System 2014 (LFS I)*

The *Larvae Feeding Experimental System 2014* was designed to contain the red deepsea crab larvae during the feeding experiments. A flow-through system (inflow 12 L /5.1 h) that used filtered seawater (salinity 27‰), from the main seawater supply of the NOAA James J. Howard Marine Sciences Laboratory at Sandy Hook, New Jersey. Seawater was UV sterilized and treated through a filtration system of chemically resistant pleated polyester filtration 100% cellulose free cartridges (5µm, 20 µm, and 50 µm) and then distributed into the experimental buckets. Two separate tanks serve as water baths with chillers that kept them at 9°C ± 1°C and 15°C ± 1°C. These temperatures were selected because they were considered as the best range of temperatures for rapid RDSC juvenile growth (Van Heukelem et al. 1983). Each tank contained 10 buckets (19 L each) with lids. Within each bucket 3 small containers (750 ml each) with the capacity to hold 10 living red deepsea crab larvae (density 10 larvae /750 ml). These containers were designed to allow the flow of diets through it, but hold the larvae inside them. Its design has 3 circular holes (diameter 5.5 cm) covered with patches of nitex screen mesh (diameter 6.8 cm) of 150µm and 250µm depending the diet size. Each temperature tank contained 2 buckets per diet (randomly assigned), for a total of 60 larvae per diet treatment by tank (Figure 3-7). The room for the system was maintained with a light cycle of 12 day /12 night hours.

Figure 3-7. —2014 system organization and design for red deepsea crab larvae diet experiments. One tank (230 x 60 cm) for each temperature (9°C and 15°C), ten buckets in each tank, and three small containers within each bucket in each tank. Each of the three diets was randomly assigned to the buckets and each small container randomly labeled 1 through 3. Each small container held 10 larvae, for a total of 60 larvae per diet per temperature tank.

3.2.1.2. *Larvae Feeding Experimental System 2016 (LFS II)*

The main units of this system are custom made upwellers composed of: a clear conical hatcher (capacity 2,400 ml) (Pentair Aquatic Eco-Systems®), a top extension with lid, a base of 4”-3” PVC (10.16-7.62 cm) reducer and a 4” PVC (10.16 cm), a 1 1/2” PVC (3.81 cm) pipe outflow with cap in one end, an elbow in the other, seal with two gaskets, and a rectangular aperture covered with a 250 µm mesh (Figure 3-8). The total capacity of the upweller units after the top extension is 3,150 ml (3.15 L). Within each upweller a fitted cone filter with the capacity to hold 20 living RDSC larvae, allowed access to observe and count the larvae, also avoiding loss of larvae through upwellers inflow at observation times. The recirculation system pumped biofiltered seawater into the bottom of the upwellers with a flow of 0.25 L /min, then upwheller overflow goes through outflow into the reservoir inflow mesh (100 and 25 µm), and finally pump (RESUN-water pump King-2) returns filtered seawater to upwellers. Two individual circular temperature tanks with chillers at 15°C ± 1°C and 20°C ± 1°C (water bath), contained 12 upwellers each (Figure 3-9). Temperatures were selected based on the previous information of rapid larvae and juvenile growth (Rosowski 1979; Van Heukelem et al. 1983) and recent red deepsea crab surveys were reported for the bottom temperatures 14.2°C at 134.8 m (Stevens and Guida 2016). Each temperature tank contained 3 upwellers per diet (randomly assigned) for a total of 60 larvae per diet per temperature tank. The room for the system was maintained with a light cycle of 12 day /12 night hours.

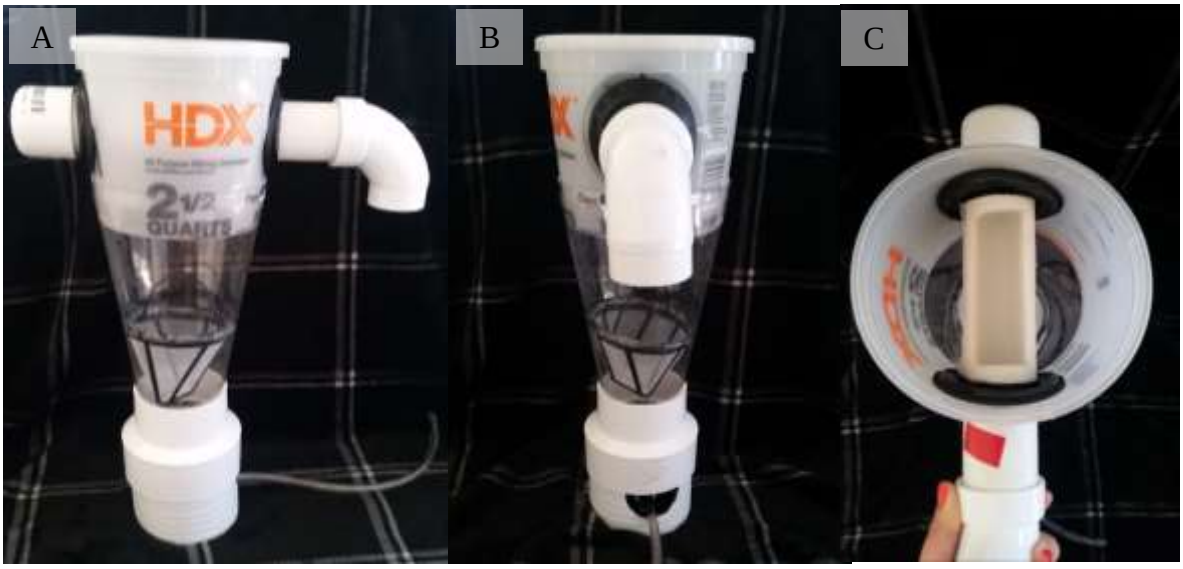


Figure 3-8. —*A.* Custom made upweller side view. *B.* Custom made upweller frontal view. *C.* Outflow aperture turned upwards to show 250 µm mesh. Biofiltered seawater enters through the bottom of upweller and exits from the top through mesh and PVC elbow to 100 and 25 µm mesh into the reservoir. The total capacity of the upweller units after the top extension is 3,150 ml (3.15 L).

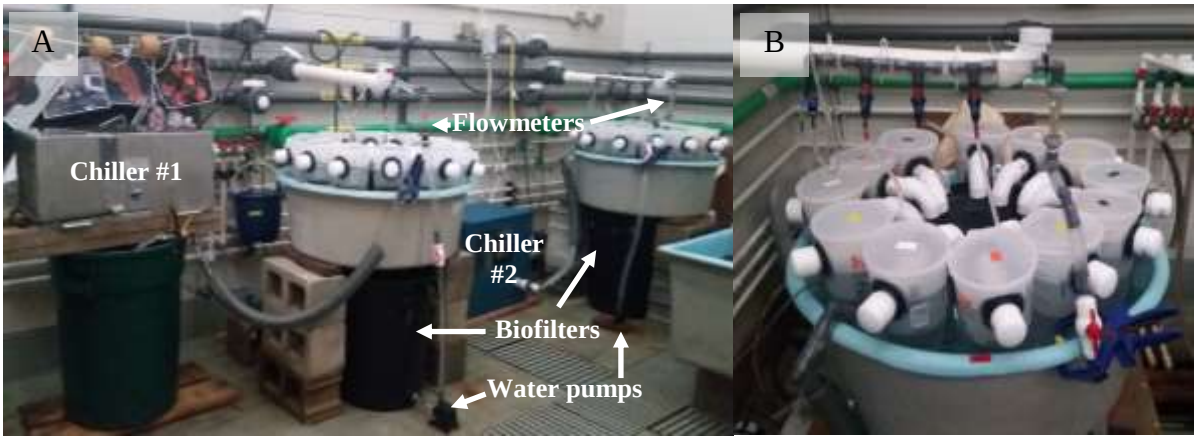


Figure 3-9. —2016 system organization and design for red deepsea crab larvae diet experiments. *A.* Two circular tank one for each temperature (15°C and 20°C). *B.* 12 custom made upwellers in each tank. Each of the four diets was randomly assigned to the upwellers. Each upweller held 20 larvae, for a total of 60 larvae per diet per temperature tank.

3.2.2. *Larvae Processing*

3.2.2.1. *Transfer to Feeding Experiment System*

The adult *Females Husbandry and Hatching Collection System* (FHCS), collectors allowed me to obtain the hatched larvae to be counted and transferred to the *Larvae Feeding Experimental System 2014* and *2016* (LFS I and LFS II). Using the daily counts, I was able to track the hatching patterns and be able to determine the hatching peaks for each female. The *LFS* were started close to those peak hatching days allowing the system to run several days before transferring the larvae.

The mesh collectors with larvae of the FHCS were set in a cup with cold UVSW (temperature similar to the hatching system) to allow all larvae in mesh to be suspended and swimming. Only actively swimming larvae were collected with a plastic pipette and transferred to small beakers with cold filter seawater in groups of ten. Beakers were randomly assigned to temperatures and diets in the *LFS I* and *II*.

During the feeding experiments, larvae were fed once every day. After temperatures were recorded and living larvae were counted, dead larvae and molts were counted, staged, and removed with a plastic pipette for pictures or discarded. During 2014 experiments, lids of the buckets were cleaned daily and the containers wiped at any signal of film in them, to avoid pathogens in the larvae. During the 2016 experiments, the conical upwellers were drained daily to facilitate the observation of the larvae under the microscope. The drained water was filtered through a 25 µm mesh and returned to the water reservoir. Filtering the water was an extra precaution to avoid the mixture of the diet when water recirculated to the reservoir. Interior conical mesh was rinsed daily. Water level in reservoir check and replenish in case of decrease by condensation.

3.2.3. *Feeding Diet Cultures*

Density, temperature, amount fed, and cleaning actions taken for the *feeding diet cultures* (rotifers, *Artemia sp.*, and algae), were recorded daily. Amounts and concentrations of the cultures used to feed the crab larvae were tracked. These actions were taken to quantify and describe the procedures that contributed to larval survival and development in my study. Keeping a detailed description of the procedures before, during, and after feeding the larvae gave us a better understanding of the laboratory conditions needed and requirements for future studies with red deepsea crab larvae. These observations can result in developing standards and protocols to successfully maintain and grow red deepsea crab larvae in laboratory conditions.

Maintaining the live cultures and performing the feeding experiments at the same time was a lot of work to handle by one person. For this reason, local high school students (Marine Academy of Science & Technology (MAST) High School) assisted with the project during the summers. Those students interested in the project were trained in the Standard Operating Procedure (SOP) to maintain and take care of the feeding cultures and larvae feeding and data collection.

The procedures for growing and care of the feeding cultures were followed as described in the SOPs' "Culturing rotifers to be used as larval fish food" SOP (2003) and "Culturing *Artemia* to be used as larval and juvenile fish food" SOP (2015), as established by the NOAA's James J. Howard Marine Sciences Laboratory at Sandy Hook, NJ (see Appendix A-B). My methods differed in the feed-out section because the target species was different. Feed outs of *Rotifers*, *Artemia sp.*, and *Algae* concentration were adjusted as needed to maintain a balance of food availability and health of the larvae. Below is a detailed summary of the methods used with the modifications made to the SOPs; for complete original SOPs see Appendices A-B.

3.2.3.1. *Rotifer Cultures*

Rotifers are commonly used for fish larvae too small to consume *Artemia sp.* and has been used previously as RDSC larvae diet (Sulkin and Van Heukelem 1980; Gerrior 1981; Kelly et al. 1982), therefore I started my experiments using rotifer cultures. During 2014 and 2016, the rotifers *Brachionus plicatilis* also called L-type were used. They have an average lorica length of about 160 µm and tolerate a salinity range of 5-40 ppt (Reed Mariculture, Inc, Campbell, CA).

SOP (2003) included the specific instructions to start rotifer cultures bought from a commercial distributor (see Appendix A), including daily count, density determination, and rotigrow feeding. Even though larvae feed-out was modified, the studied species and system container capacity were different from the ones specified in the SOP.

3.2.3.1.1. Starting cultures

The rotifers were bought from a commercial distributor (Reed Mariculture Inc., Campbell, CA). Hatchers were transported from Delaware State University to the NOAA James J. Howard Sandy Hook laboratory facilities. They are composed of 19 L plastic bottles supported by wooden stands (Figure 3-10).

Figure 3-10. —Hatchers were used in the culture of rotifers. They include a heater, filter, thermometer, and airline.

In order to start up, hatchers are cleaned with a dilute solution of soap (Alconox) and rinsed several times with hot tap water, de-ionized water, and 0.5 µm UV filtered seawater (UVSW). Maintenance cleaning throughout experiments did not include the Alconox solution step.

Hatchers were equipped with an airstone to input air in the culture and promote movement, a submersible heater to maintain a range of temperature between 25-30°C, thermometer, and a suspended filter to capture food residual in the culture (rotifer floss sheet). After cleaning, the hatchers were prepared to be filled with UVSW, when the water reached the temperature required for the rotifers these were transferred.

3.2.3.1.2. Counting

Rotifer cultures need to be counted every day to determine their density, contamination with ciliates, and the needed amount of them to feed out the larvae in the experiment.

The counting process starts collecting around 150 to 200 ml of rotifer sample with a 250 ml beaker from each hatcher. Rotifers were evenly mixed in the beakers and using a pipette 1 ml of the sample was collected and placed in a Segwick Rafter Chamber (SRC), label “water”, and cover by a glass slide. SRC over a 4x10 units counting grid is placed under the microscope. At a 6x magnification swim speed was categorized as good (most individuals actively moving) or bad (most individuals not moving). At the same time, the % female with eggs was determined by counting 10 randomly selected individuals and multiplying the fraction carrying eggs by a hundred. Possible contaminations in the culture were tracked counting the number of ciliates in 6 grids (0.15 ml) and multiplying it by 5 (0.75 ml). Then, the SRC was emptied and rinsed with de-ionized water and dried with a towel.

After observing the organism alive, a new 1 ml sample in combination with 3 drops of bleach was placed in the SRC labeled “bleach”, follow by carefully dragging the slide from one end of the SRC and placed it by leaving all air bubbles out. Place SRC in counting grid under a 12x magnification in the microscope using the hand counter obtain an accurate count of rotifers. The number of rotifers in rows 1 and 3 of the grid were counted. I repeated the “bleach” SRC steps with a new 1 ml sample, counted rows 2 and 4 and calculated the average of the two counts.

The total number of rotifers was calculated by multiplying the average count by 1000 (total rotifer per Liter) and multiply it by the total number of liters in the hatcher. I repeated these methods for each hatcher’s sample daily.

3.2.3.1.3. Feeding

Rotifers are feed daily after counting with 5 ml of a commercial mix of supplemented algae called RotiGrow® (Reed Mariculture Inc.) diluted in 80 ml UVSW. During 2015-2016 experiments, larval food was changed to RotiGrow® Nanno instead of regular RotiGrow, because it kept the hatchers cleaner for longer. RotiGrow® Nanno (*Nannochloropsis)* is a single species culture that provides high eicosapentaenoic acid (EPA) and arachidonic acid (ARA) pre-enrichment for protocols needing high docosahexaenoic acid (DHA) enrichment for the larvae.

3.2.3.2. *Artemia sp. Cultures and Protocol*

Artemia sp. or brine shrimp is an aquatic crustacean commonly used to feed juvenile and small aquatic organisms. Previous studies had used *Artemia sp.* as a diet for RDSC larvae (Perkins 1973; Rosowski 1979; Sulkin and Van Heukelem 1980; Gerrior 1981; Kelly et al. 1982). *Artemia sp.* being approximately 450µm at hatching with a non-feeding first larvae stage

that last around 24 hours at 20°C (see Appendix B). *Artemia* Cysts (Inve Aquaculture 801-876-2002) were commercially purchased and used as a diet in the experiments.

3.2.3.2.1. Preparation of Sodium Hydroxide solutions

Enough stock solutions need to be made before starting the decapsulation process. An initial 40%NaOH solution was prepared. 400g of NAOH pellets were measured under the fume hood (NAOH is caustic and the reaction to be made is exothermic). On a stirring plate inside the fume hood, a cold 1,000 ml Erlenmeyer flask was placed with a stirring magnet in an ice bath. 600 ml of deionized water was added to the flask. The pellets were slowly added to the water, waiting it to be dissolved before adding more. The second 6.6%NaOH solution was prepared adding 66 ml of the 40%NaOH solution to 934 ml of UVSW into a cold 1,000 ml Erlenmeyer flask. During the solution preparation personnel involved in this part of the research was required to use the protective equipment (laboratory coat, gloves, and goggles).

3.2.3.2.2. Hydration

The amount of cysts needed for the next 3-7 days was determined. Then I weighed this amount in a plastic jar, without exceeding 100g of cysts per jar. Hydration was done by adding deionized water to around 7/8 of the jars total volume and aerating for about 45-90 minutes.

3.2.3.2.3. Decapsulation

Jars with hydrated cysts were individually poured into a 125 µm- 6" diameter PVC (15 cm) sieve, drained and rinsed with deionized water. The rinsed hydrated cysts were transferred with a spatula to a 2 L beaker. In a separate beaker, bleach was measured, depending on the concentration of sodium hypochlorite in it: 6.00% sodium hypochlorite is equal to 8.75 ml of bleach per gram of dry cysts or 5.25% sodium hypochlorite is equal to 10 ml of bleach per gram

of dry cysts. Cold 6.6%NaOH solution was measured and added, using 5 ml of NaOH solution per gram of dry cysts. Slowly the measured bleach was incorporated into the cysts solution using a thermometer to stir continuously and track the solution temperature stays under 30°C. After the solution turned white and foamy, I waited for the first signal of a rust-orange color, kept stirring for 45-60 seconds more and then pour the cysts solution in a 125 µm-6" diameter PVC (15 cm) sieve. Cysts were rinsed with deionized water until the smell of bleach was undetectable. The cysts were kept in plastic container labelled with their weight in grams before and after decapsulation (dry and wet) and date of decapsulation, in the refrigerator.

3.2.3.2.4. Hatching and Transfer

A hatcher (as the ones used for the rotifer cultures) was filled with 12 L of UVSW with a salinity of 27 ppt, at a temperature range of 25-30°C (heater was used for keeping the temperature), and constant aeration. Decapsulated cysts were added to the hatcher and the time and date were recorded. The number of grams used varied based on how many were needed, it was 80g (wet weight) per hatcher at highest production during experiments. In 2014 *Artemia sp.* cultures were grown for 48 h before being fed out to the crab larvae. During 2016 the crab larvae were fed with 24 h hatched *Artemia* sp. nauplii, for a smaller prey size. After hatching time *Artemia sp.* cultures were ready to transfer process.

3.2.3.2.5. Enrichment

DHA Selco enrichment media in 500 ml of UVSW was vigorously shaken to mix for 1 minute. The amount of DHA Selco added was 7.2 g of selco per 12 L hatcher of *Artemia sp.* hatched. The amount of mix necessary for enrichment was added to the culture an hour before

the culture would be used to feed out to the crab larvae. The enrichment time and date were recorded.

3.2.3.2.6. Feed Out of Crab Larvae

One of the expected outcomes of these experiments was to answer the following question. How much and which concentrations of the diets is optimal to maintain red deepsea crab larvae in laboratory condition? The feed out concentrations of *rotifers, Artemia sp.,* and *algae* were adjusted as needed to maintain available a balance diet for healthy larvae.

3.2.3.3. *Algae Cultures*

The algae culture used during the experiments depended on the availability of cultures at the NEFSC Milford Laboratory, Connecticut. Previously collaborations with the institution's scientist made it possible to obtain the algae cultures from them. *T. isocrysis* was used during the 2014 experiments.

3.2.4. *Diet Treatments*

To determine the best diet for red deepsea crabs I used rotifers, *Artemia sp*., algae, and combinations of them. Diet treatments in the 2014 experiments included two rotifer treatments: one group of larvae were fed exclusively using rotifers (R) throughout the experiment, and another group was fed with rotifers plus *Artemia sp*. (A+R). In addition, two *Artemia sp*. treatments were used: one group of larvae was fed exclusively using *Artemia sp*. (A) throughout the experiment and another group was fed with *Artemia sp.* plus algae (A+AL). Finally, a control treatment of unfed (U) larvae was incorporated for starvation comparisons (Anger et al. 1981). These initial diet treatments were modified after *Artemia sp.* nauplii of >48 h old were fed to crab larvae; these were big for the red crab larvae, and contributed to their death within 48

h. In addition, *Artemia sp.* nauplii concentration was too high for the crab larvae container volume. As a result, the remaining diets in the experiment were continued (unfed and rotifer) and the *Artemia sp.* diet containers were substituted by an additional rotifer and two algae treatments (for a total of 120 larvae in each rotifer and algae treatment) (Table 3-1). During the summer of 2016, I used rotifer, *Artemia sp*., a combination of both, and unfed treatments. Table 3-1 summarizes the different diet treatment combinations per temperature, for both years. The diets were distributed to each larval container with an automatic pipette individually; the diet concentration and volume added to the containers was recorded.

Table 3-1. —Diet treatment combinations for both experimental years (2014 and 2016) with the number of larvae per temperature treatment and amount female broods used to obtain larvae.

Experiment	*Diet	# of larvae	# of broods
2014 LFS I Flow Through	U	60	1
	R	60	1
	A	60	1
	A+R	60	1
	A+AL	60	1
	U	60	1
	R	120	1
	AL	120	1
2016 LFS II Recirculating	U	60	7
	R	60	7
	A	60	7
	A+R	60	7

*Unfed (U), Rotifers *Brachionus plicatilis* (R), *Artemia sp.* (A), and Algae *T. isocrysis* (AL)

Previous studies indicated that survival of red deepsea crab larvae to metamorphosis in rotifer and brine shrimp diets were not different, but there was a delay in their development

(Sulkin and Van Heukelem 1980**)**. Comparing the effects of different temperatures in juvenile crabs' development, 9-15°C is the optimal temperature range for juvenile survival **(**Van Heukelem et al. 1983), but combined effects of diet and temperature have not been measured until now**.**

In my last year of experiments temperatures 15°C and 20°C were selected, taking into consideration areas with higher temperatures where the adult crabs were being collected (Stevens and Guida 2016).

Survival of crab larvae and developmental stage at death was recorded daily for each temperature and diet treatment. These observations allowed determination of *survival rates* (the percentage of larvae alive at a specific time) and development through time.

3.2.5. *Statistical Analysis*

Because all experiments were conducted until all larvae in the treatment had died, data used for this analysis were the number of days that larvae survived in each treatment, weighted by the number of deaths. To create this data set, the dates (or day numbers) on which larvae died (the independent variable) were replicated by the number of mortalities on that date (the dependent variable). For example, if 6 larvae died on day 10, the values for that date were 6 units of 10. To test the hypotheses for each question, statistical analyses were conducted as described below:

Hypothesis 4: What are the effects of the two main factors, a.) diet and b.) temperature, as well as zoeal stage, on the survival of RDSC larvae? Daily recorded larvae mortality data was analyzed using non-linear mixed effects models (NLME) with the nlme package in R statistics. Homogeneity of variance was tested with Bartlett's test, histogram, and QQplot for the survival time. After square-root transformation was performed, normality of the data was improved but

not achieved. However, the data were analyzed by ANOVA, because it is robust to non-normality and allowed the use of mixed effects models. Data were then standardized using the scale command in R to convert days into standard deviations from the mean. NLME models are similar to the linear models command, but can use a mixture of factors (e.g. diet) and continuous variables (e.g. temperature). Also, because my experimental design used 60 larvae in 3 containers in each of 2 tanks or 3 upwellers in 2016, the data are nested. The nesting variables (i.e. which tank or container larvae is in) might affect survival, but their labels have no real meaning or value (i.e. tank 1 could easily have been labeled tank 2, etc.), so these were considered to be random factors. Four different model variations were created with or without interactions and with different nesting levels, and were fit by maximum likelihood. All models were compared using a single ANOVA statement by comparing the AIC values. The model with the lowest AIC was then rerun with a slightly different method, i.e. it was fit by restricted maximum likelihood (REML).

Hypothesis 5: What are the effects of the two main factors, a.) diet and b.) temperature, as well as zoeal stage, on the development of RDSC larvae? Similar to the analysis in the survival hypothesis, non-linear mixed effects models (NLME) with the nlme package in R statistics were used to analyze the relationship of larvae developmental stage at death. Three additional model variations were fit by the maximum likelihood method with or without interactions (between diets and temperatures), adding larval stage as a factor, and with different nesting levels. All models were compared using a single ANOVA statement by comparing the AIC values. The best model (lowest AIC) was rerun using REML.

Hypothesis 6: Which aquaculture system better promotes larval survival? Larval mortality data of 2014 and 2016 for unfed and rotifers diets at 15 °C was combined and analyzed

using non-linear mixed effects models (NLME) with the nlme package in R statistics. Homogeneity of variance was tested with Bartlett's test, histogram, and QQplot for the survival time. A square-root transformation was performed. Data was then standardized using the scale command to convert days into standard deviations from the mean. A NLME fit by maximum likelihood was run using diet as a factor and that year's aquaculture system as continuous variables. The model was then rerun fit by REML.

CHAPTER 4

RESEARCH FINDINGS

4.1. Part I-Hatching Periodicity of Red Deepsea Crab (RDSC) *Chaceon quinquedens*

During the spring and summer of 2014, 2015, and 2016, a total of 74 females were obtained from several fishing trips by the Atlantic Red Crab Company and transported from Newport News, VA to the NOAA James J. Howard Sandy Hook Laboratory, NJ. Females were placed and monitored in the Females Husbandry and Hatching Collection System (FHCS). The mean water temperature was 8.2 ± 0.83°C in 2014 and 7.6 ± 2.1°C in 2016 with a salinity range of 27-30‰. During the first week after arrival the adult female red deepsea crabs were individually measured and their egg clutches were described and photographed. Appendix C contains all the morphological measurements (carapace length and width, and abdominal segment length and width) and egg observations obtained from the females (pictures not included) during those 3 years of investigation.

Not all of these females survived or hatched in the laboratory, as shown in Table 4-1. A total of 36 females died (48.6% of total females transported to the laboratory). Based on initial egg descriptions as "Eyed (dark brown-purple (pre hatching)) or Hatching", 37 females were expected to hatch, but 31 actually hatched larvae. A total of 41 females in the process of hatching were monitored and 10 carried newly fertilized egg clutches that did not hatch in the lab; their eggs were used to describe embryo development and morphometry through time using image analysis, but will not be discussed in my thesis because of the lack of complete temporal data. Twenty-one females had already begun hatching before arrival to the laboratory, and just

10 started hatching in the lab. Among the last 31 females, 17 were used to perform 4 h hatching observations for 3 consecutive days, and three showed a typical bell-shaped curve for their hatching distribution during their time in the laboratory.

Table 4-1. —Adult females record from transport to observations in the Females Husbandry and Hatching Collection System (FHCS).

		Dead			Measurements		
Year	Total	Transport	"Acclimation time"	Total	Morphometry	Hatch	Every 4hrs
2014	20	4	7	11	13	9	0
2015	19	2	10	12	9	7	2
2016	35	0	*7	13	27	25	15
Total	74	6	24	36	49	**41	17
%		8.1	32.4	48.6			
mean	24.67	2.00	8.00	12.00	16.33	13.67	5.67
SD	8.96	2.00	1.73	1.00	9.45	9.87	8.14

*Additional 6 dead were documented because of electric problems increased the water temperature
**10 females carried newly fertilized egg clutches that did not hatch in the laboratory

During the first year, eggs and larvae were counted separately, which resulted in three types of counts (just eggs, just larvae, and larvae plus eggs), but during the second and third years, volume samples were measured and described as eggs, larvae or both. Only samples that had larvae or both were considered for the statistical analysis. Even when these samples were mostly larvae, they also included a smaller fraction of eggs which allowed me to use the same regression throughout the hatching period.

A simple linear regression (SLR) with intercept set at zero was calculated to predict number of larvae hatched based on the measured volumes. A significant regression equation was found (F = 1196; df = 1, 13; R^2 = 0.9892, P = <0.0001). Predicted number of larvae hatched is equal to 838.20*(volume) (Figure 4-1).

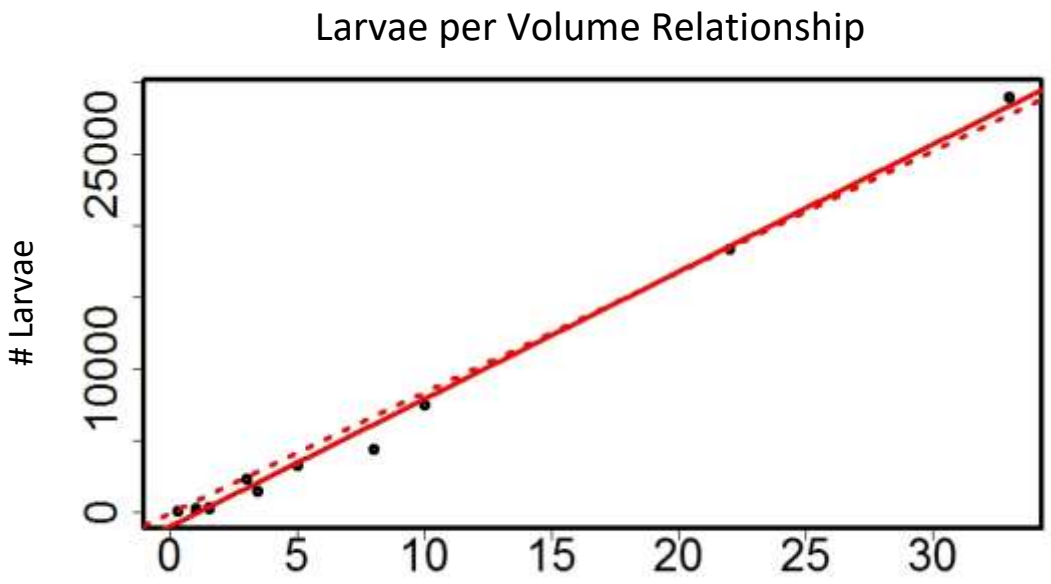


Figure 4-1. —Simple linear regression (SLR) with best fit line (solid line) and set intercept on zero line (pointed line). This latter was used to describe the relationship between number of larvae and volume.

Following sections are organized according to my research questions:

Hypothesis 1: Is *duration* of hatching different between adult female crabs held in laboratory conditions? Temperature data of the FHSC was monitored during the 3-year research timeline, allowing me to monitor and make adjustments several times because of increases in the temperature due to electric or structural malfunctions of the main water system in the laboratory. During the 3 years of experiments, the laboratory suffered moments of electric malfunctions due to weather, maintenance, and infrastructure. These sporadic events altered temperature drastically during the experiments. The FHSC was maintained within the suggested temperature range of 5-8°C (Haefner and Musick 1974; Wigley et al. 1975; Steimle et al. 2001; Wahle et al. 2008), with the obtained mean temperature of 8.2 ± 0.83°C in 2014 and 7.6 ± 2.1°C in 2016.

In 2015, due to weather conditions, the Atlantic Red Crab Company fishing trips were limited during the spring and early summer. This situation resulted in the acquisition of just 19 females of which 12 died during the first week after transportation to the laboratory or "acclimation time". Of the other 7 females, 1 had immature eggs and 6 had small or no egg clutches, indicating that the latter female's larvae had recently hatched (also explaining the presence of larvae during the first days in the laboratory but not for long).

As shown in Table 4-1, during the 3 years of observation a total of 41 females were kept in Females Husbandry and Hatching Collection System (FHCS) and 31 hatched larvae observed in the laboratory. Female 17_16 was excluded from the analysis because I only observed it for 2 days. A scatterplot showing the larvae hatched through time with a smooth function was performed for each individual female crab. After analyzing all the hatched data, just 3 female crabs (Female ID: VIII14 (crab 1), 8_16 (crab 2), and 14_16 (crab 3)) began and finished

hatching (bell-shaped curve distribution of hatching) in the laboratory with at least 20 days of hatch observations (Figure 4-2). Female VIII14 showed some hatching during the first days after arrival in the laboratory but this initial loss of larvae could be due to handling.

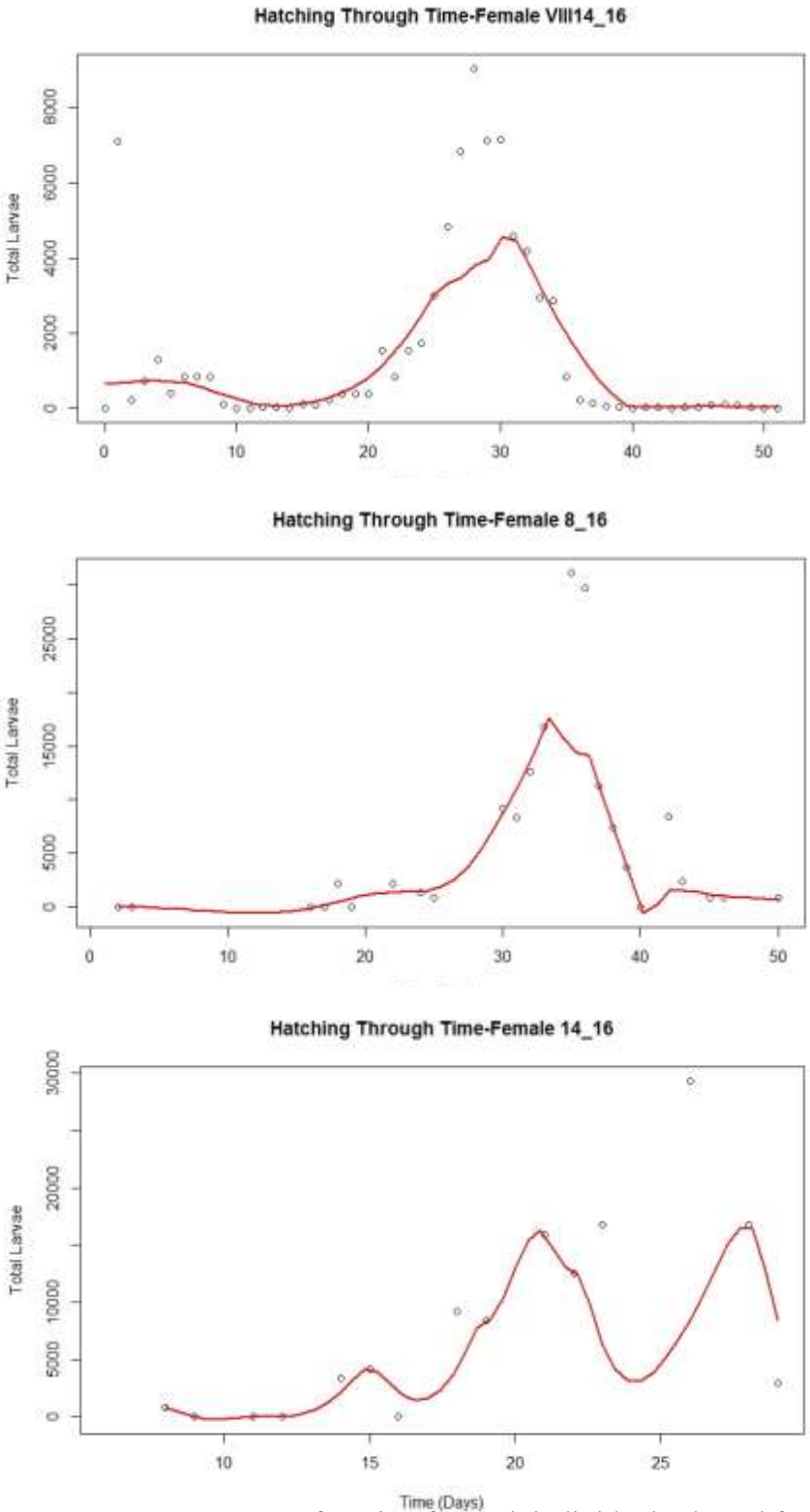


Figure 4-2. —Scatter plot with smooth function for each individual selected female crab's (VIII14, 8_16, and 14-16) hatches through time.

The data set consisted of observed hatch volume (rounded up to the nearest ml) replicated as many times as the day of hatching for each crab (as described earlier). The Shapiro–Wilcoxon test for normality of distribution was statistically significant: crab 1 (Shapiro-Wilk: W = 0.9709, *P* = 0.04286), crab 2 (W = 0.89995, *P* = < 0.0001), and crab 3 (W = 0.93278, *P* = < 0.0001). QQplots showed a normal distribution (Figure 4-3 to 4-5). Owing to these results *F* statistics assuming homogeneity of variance were calculated.

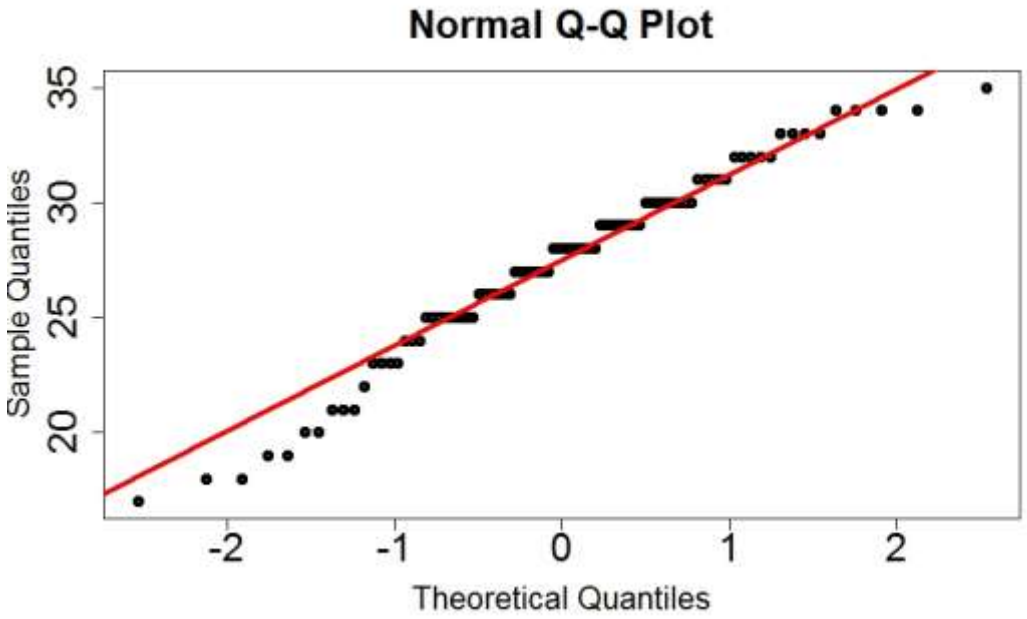


Figure 4-3. —QQplot for observed hatch volume unit (ml) for crab 1.

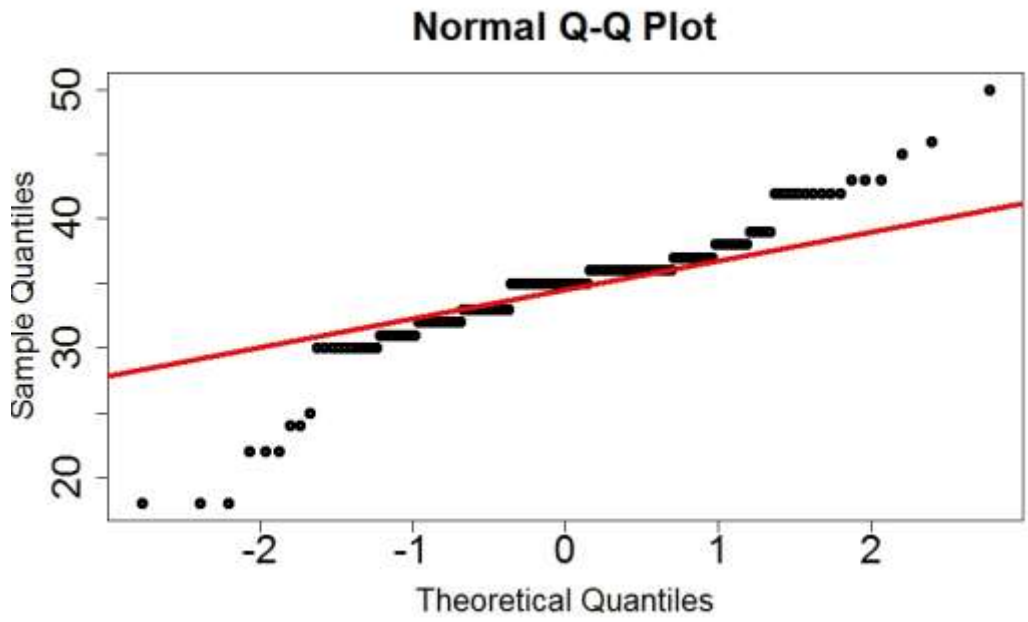


Figure 4-4. —QQplot for observed hatch volume unit (ml) for crab 2.

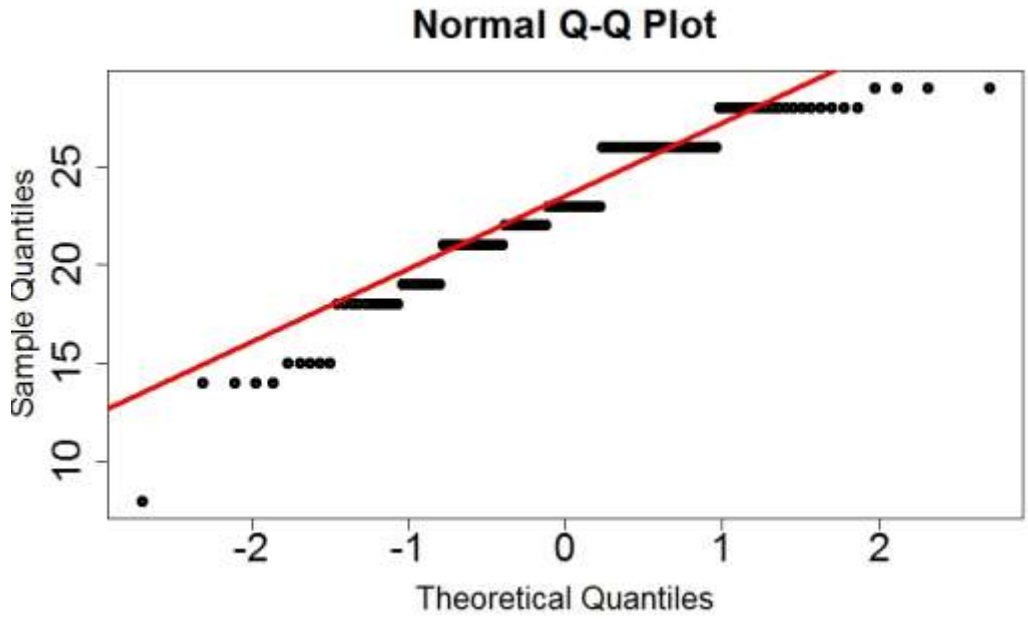


Figure 4-5. —QQplot for observed hatch volume unit (ml) for crab 3.

Comparison of the volume (ml) of larvae hatched by three females (crab 1-3) through time showed different hatch totals, which could be related to variable female size or fecundity (Figure 4-6). A logistic regression analysis was conducted to model the cumulative proportion of total larvae hatched by each female on each day (Figure 4-7). The model was statistically significant, indicating that the predictors described the progression through time of the hatching for the females: crab 1 ($z = 24.73$; $df = 1, 49$; $P = <0.0001$), crab 2 ($z = 37.72$; $df = 1, 49$; $P = <0.0001$), and crab 3 ($z = 32.43$; $df = 1, 49$; $P = <0.0001$).

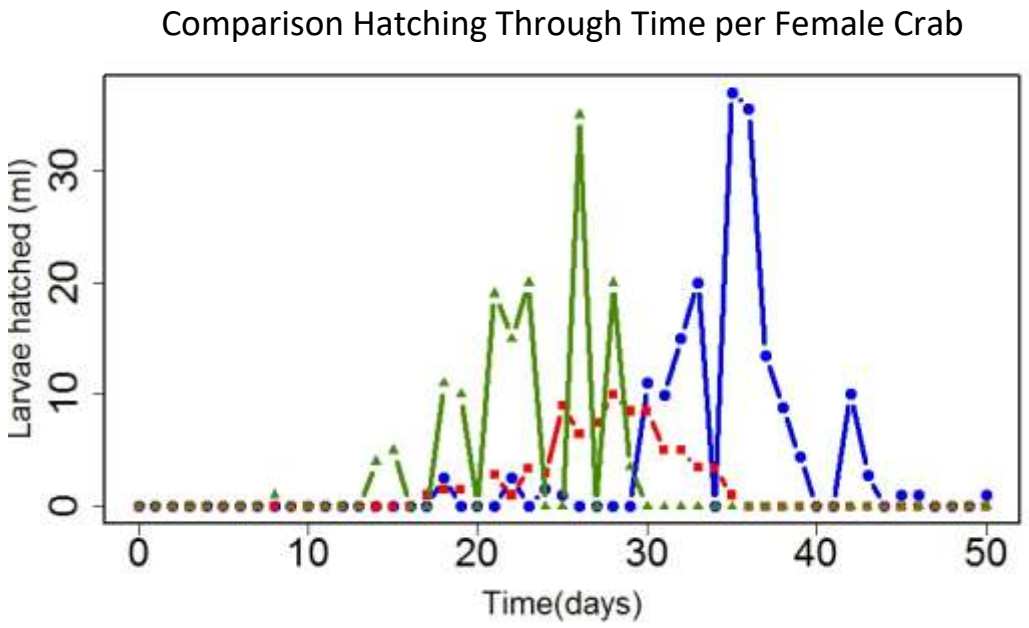


Figure 4-6. —Comparison larvae hatched (ml) through time per female. Crab 1 (red/squares), crab 2 (blue/circles), and crab 3 (green/triangles).

Because crabs started hatching at different times, objective milestones in the hatching period were defined in order to model hatching relative to these points. Milestones were set the first day crabs hatched more than 2.5%, 50%, and 97.5% of their eggs (Figure 4-7). Table 4-2 shows the dates that each female crab reached each milestone (2.5%, 50%, and 97.5%).

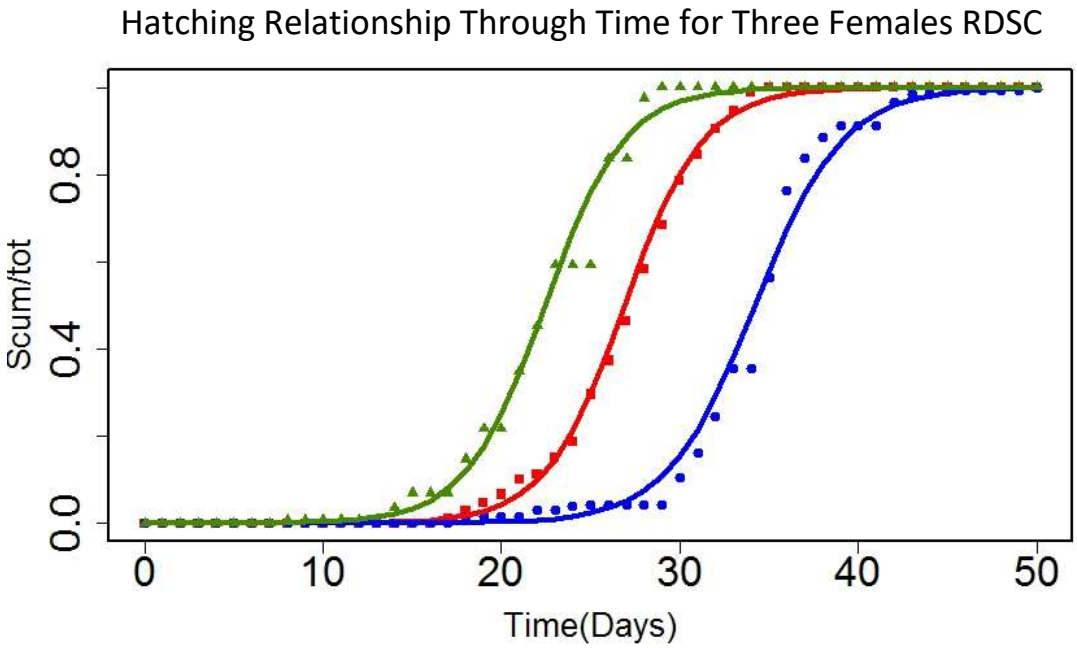


Figure 4-7. —Comparison of cumulative sums of larvae hatched for each day and predicted proportions hatched each day for the three crabs. Crab 1 (red/squares), crab 2 (blue/circles), and crab 3 (green/triangles).

The hatching data were aligned using their median day of hatching (the day on which 50% of cumulative larvae were hatched) (Figure 4-8).

Table 4-2. —Female crabs hatching duration quantiles (milestones) and total duration.

		Quantile				
Crab ID	SM50	mean	SD	2.5%	97.5%	Duration
1	26.9	27.3	4.1	18.2	34.0	16.8
2	34.2	34.7	4.5	22.0	43.0	22.0
3	22.4	22.9	4.1	14.0	28.4	15.4
Mean	27.8	28.3	4.2	18.1	35.1	18.1
SD	6.0	6.0	0.2	4.0	7.4	3.5

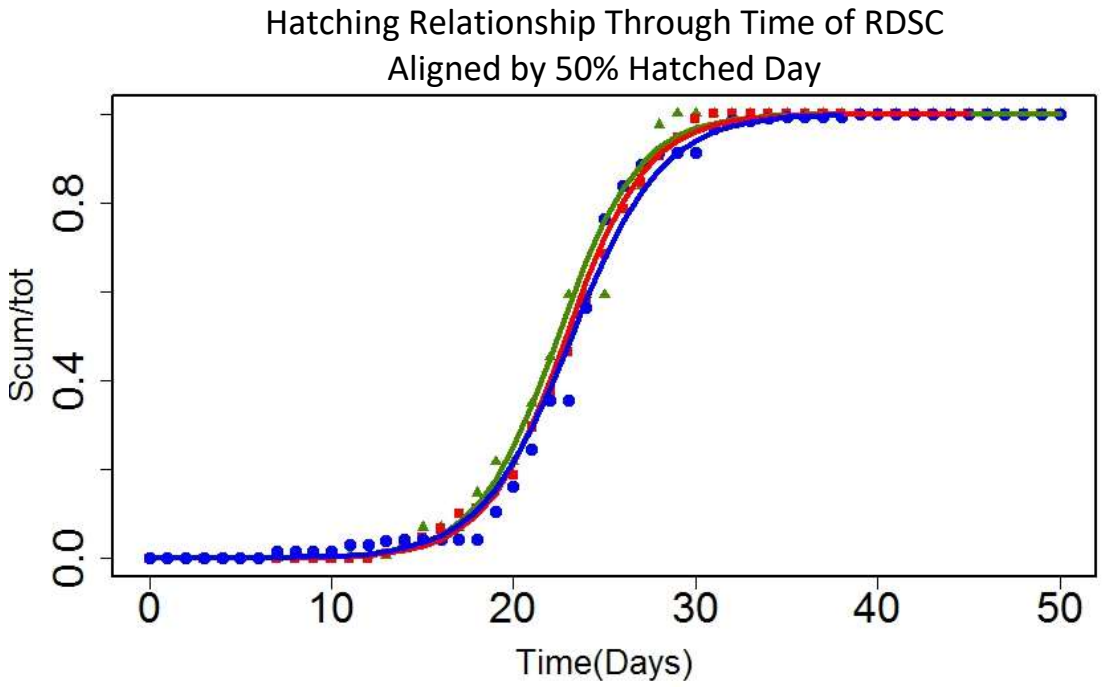


Figure 4-8. —Comparison of cumulative sums of larvae hatched for each day and predicted proportions hatched each day for the three female crabs, aligned at each female 50% hatch day. Crab 1 (red/squares), crab 2 (blue/circles), and crab 3 (green/triangles).

Based on my statistical analysis, I accepted the null hypothesis that duration of hatching period is not different between adult female crabs. Results indicated that all females follow a hatching period that fits a logistic regression. The mean duration for red deepsea crab hatching period is 18.1 ± 3.5 days.

Hypothesis 2: At what time of the day do larvae hatch? Female crabs for which observations were made at 4 h intervals were used for the daily hatching peak time analysis (Table 4-1). Hatching measurements were made for 3 consecutive days for a total of 17 adult females throughout summers 2015-2016. These were female ID: VI15 (F6), VII15 (F7), 3_16 (F3), 12_16 (F12), 15_16 (F15), 16_16 (F16), 18_16 (F18), 19_16 (F19), 20_16 (F20), 21_16 (F21), 22_16 (F22), 23_16 (F23), 24_16 (F24), 25_16 (F25), 26_16 (F26), 27_16 (F27), and 28_16 (F28). The mean total larvae hatched per time observation interval for all females were calculated (Figure 4-9 and Figure 4-10).

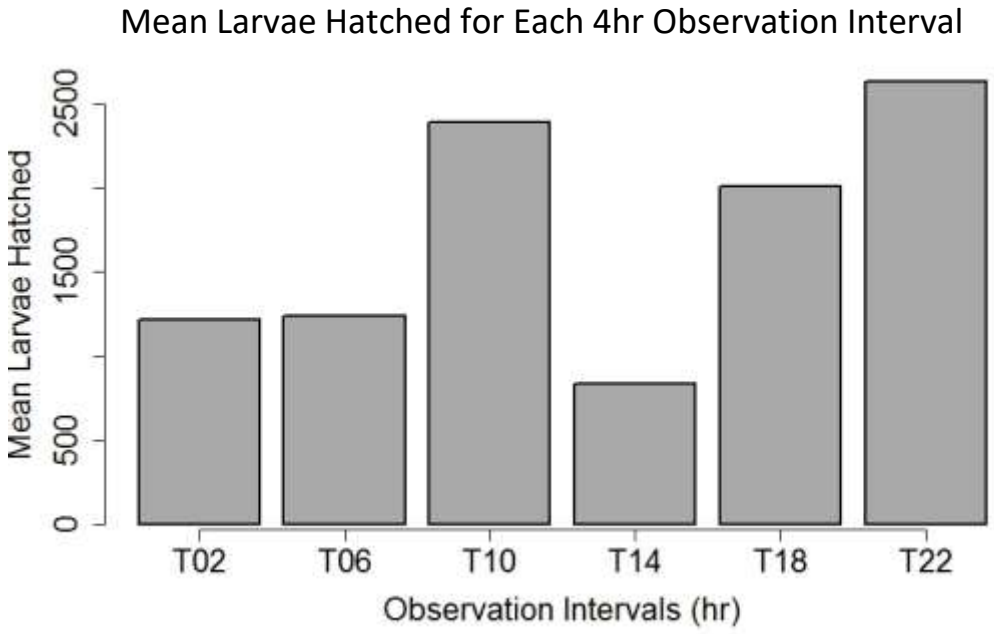


Figure 4-9. —Comparison of mean larvae hatched in each time interval for all females.

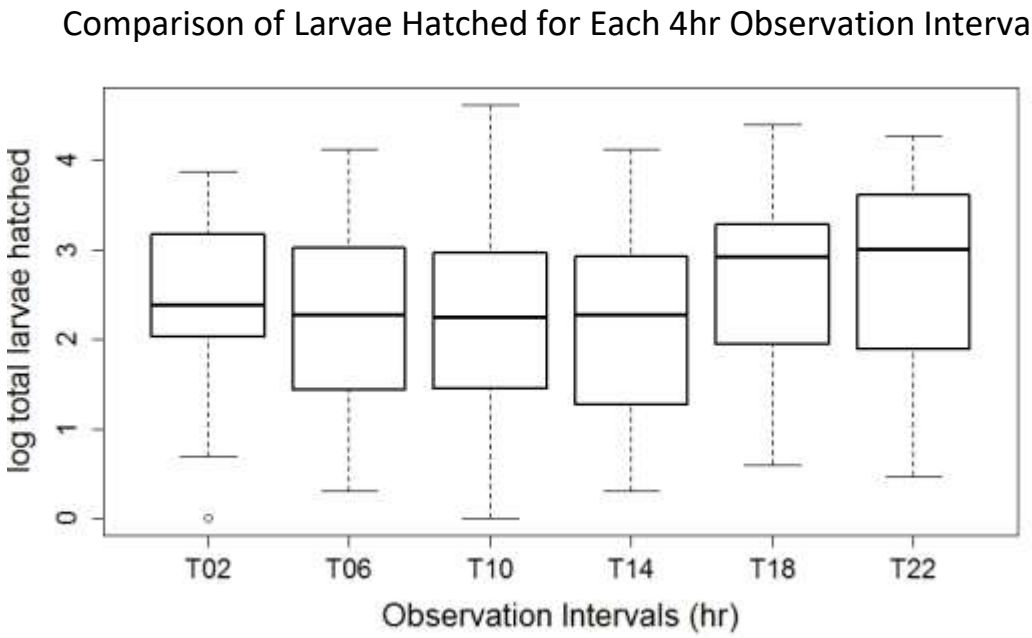


Figure 4-10. —Comparison of total larvae hatched distributions (log transformed) in each time interval for all females.

A log10 transformation was used to normalize the data and variances. Variances were found to be homogenous using Bartlett's test (K-squared = 1.9373; df = 5; p-value = 0.8577) and QQplot (Figure 4-11).

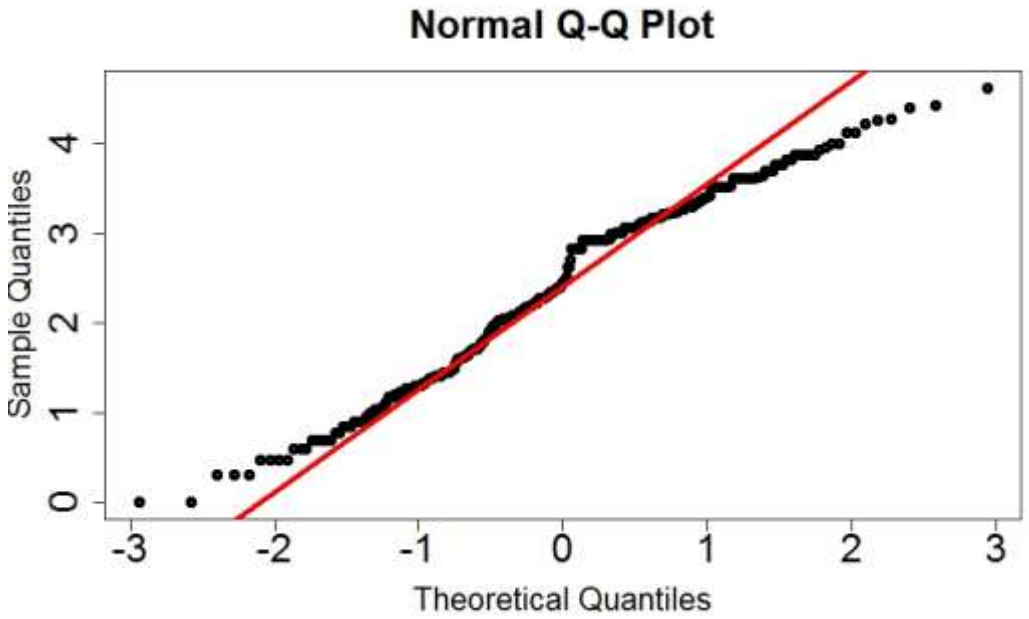


Figure 4-11. —QQplot of total larvae hatched (log transformed) for all females.

Linear model (F = 3.3367; df = 5, 300; *P* = 0.005996) and a pairwise t-test were used to test the relationship between log larvae hatched and the observation intervals. It showed that time interval T14 was significantly different from T22 (*P* = 0.019), and all others are similar. The NLME analysis showed that model 1 (intercept only) was the best, as it had the lowest AIC value (F = 4.100405; AIC= 831.5; df = 5,284; *P* = 0.0013). In other words, there was no effect of female or nesting of time intervals within females.

The results showed that the mean number of larvae hatched (after log transformation) was lower at T14 (mid-day from10 am to 2 pm) than at time T22 (6 pm to 10 pm) across all crabs, but does not differ from the other time intervals. Similar patterns have been shown in other crabs (Stevens and Swiney 2007) that showed higher hatching at dusk. I rejected my null hypothesis of no difference in number of larvae hatched between 4 h time periods of the day, as female red deepsea crab showed different hatching amounts during the day.

Hypothesis 3: Is there a relationship between female morphometry and the total volume of larvae hatched? Simple linear regression (SLR) for hatching adult female sizes (carapace width (CW), length (CL), or abdominal segment width (AW)) and total hatched larvae in the laboratory were calculated to determine if the number of hatched larvae was related to the measured adult female's morphology. No significant relationships were found between total hatched larvae and CW (mm) (F = 2.6; df = 1, 28; R^2 = 0.0523; P = 0.1181) (Figure 4-12), CL (mm) (F = 1.813; df = 1, 28; R^2 = 0.02726; P = 0.189) (Figure 4-13), or AW (mm) (F = 0.07565; df = 1, 28; R^2 = -0.03292; P = 0.7853) (Figure 4-14).

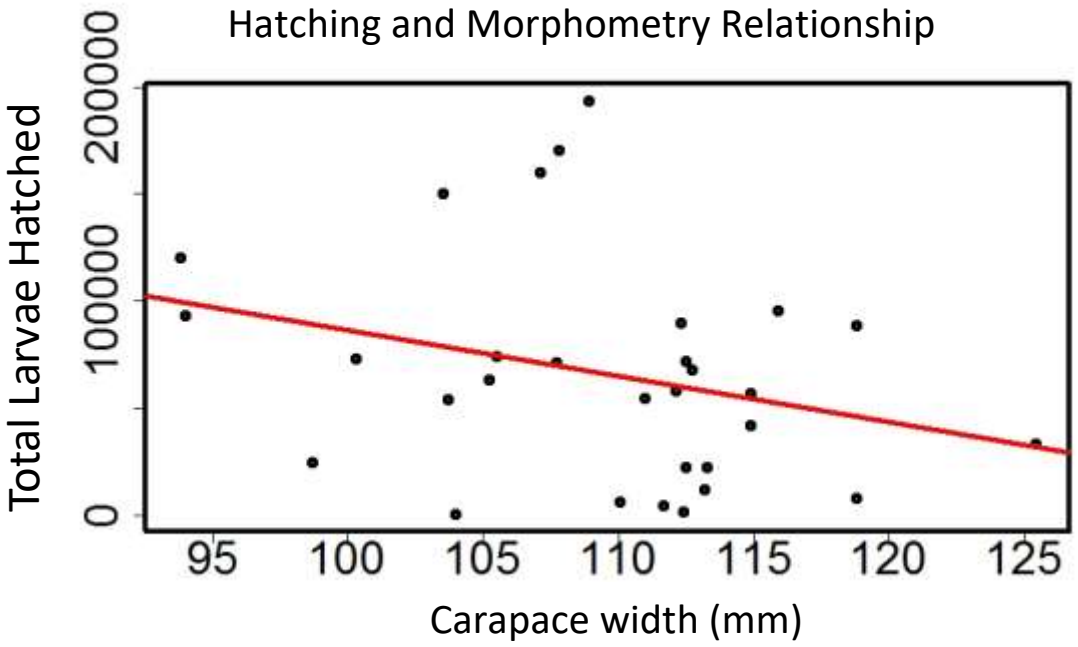


Figure 4-12. —Total hatched larvae vs. adult female's carapace width (mm).

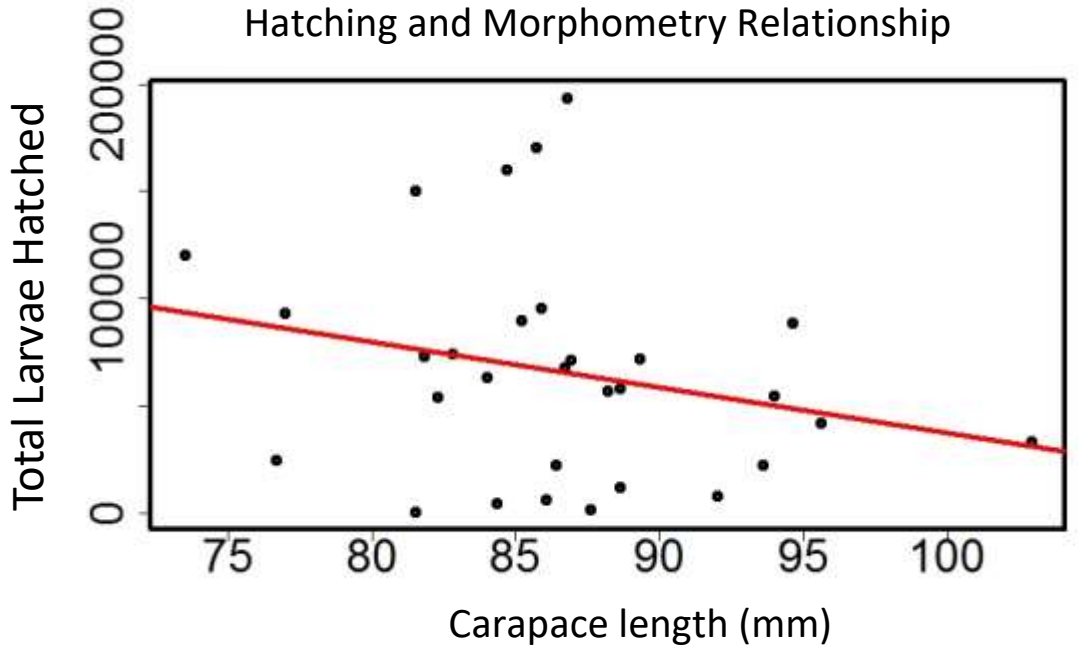


Figure 4-13. —Total hatched larvae vs. adult female's carapace length (mm).

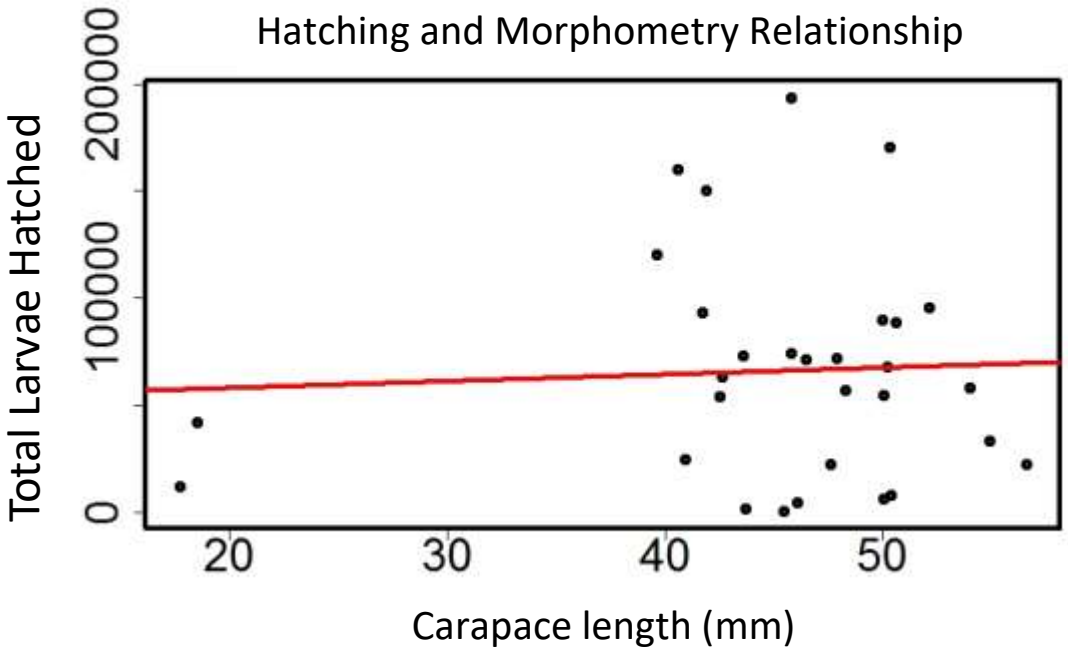


Figure 4-14. —Total hatched larvae vs. adult female's abdominal width (mm).

Based on my statistical result, I accepted my null hypothesis that no relationship exists between female morphometry and total volume of larvae hatched. Statistical results indicated that I may have insufficient data. The lack of starting day for hatching and complete hatching period for most females could be affecting the evaluation of this relationship.

4.2. Part II-Survival of Red Deepsea Crab (RDSC) *Chaceon quinquedens*, Larvae in Cultivation: Effects of Diet and Temperature

These studies investigated the factors affecting the survival and development of the early life stages of the red deepsea crab in aquaculture settings during a multiyear project.

4.2.1. *2014 Survival and Development Analysis*

Larvae Feeding Experimental System 2014 (LFS I) was set up using transferred larvae from one brood hatched (Female ID: VIII14) in the Females Husbandry and Hatching Collection System (FHCS) and ran for the larvae survival experiments. The water temperature was consistently maintained at 9°C ± 1°C and 15°C ± 1°C. Live rotifers diets were successfully cultured and adjusted daily to the desired concentrations of live diet organisms by volume in LFS containers (Table 4-3). Algae cultures (*T. isocrysis*) were obtained from the NEFSC Milford Laboratory, CT and diluted for crab larvae feeding. Concentrations were modified depending on their availability for the crab larvae.

Table 4-3. —Diets concentrations and volume fed to the larvae per container ranges for both year experiments.

Year	*Diet	Concentration range (Diet/ml)	Fed per container range (ml)
2014 LFS I Flow Through	U	0	0
	R	5-3	40-7
	**A	5	20
	**A+R	2.5 + 5	20 + 40-25
	**A+AL	2.5 + 10000000	20 + 50-20
	U	0	0
	R	5-3	40-7
	AL	10000000	30-10
2016 LFS II Recirculating	U	0	0
	R	7-4	18-2
	A	0.7-0.5	7-3
	A+R	0.4-0.3 + 3.5-2.5	4-1.5 + 9-1

*Unfeed (U), Rotifers *B. plicatilis* (R), *A. salina* (A), and Algae *T. isocrysis* (AL)
**Substituted by an additional rotifer and two algae treatments. Final diet treatments for 2014 showed below.

Salinity was 27‰ throughout the experiments. I was not able to set up the automatic light cycle of 12 day /12 night h in the room, although experimental tanks were covered with an opaque lining to reduce the intrusion of light.

Hypothesis 4: What are the effects of diet and temperature on the survival of RDSC larvae? Daily recorded larvae mortality data was analyzed using non-linear mixed effects models (NLME) with the nlme package in R statistics. Homogeneity of variance was not found after testing with Bartlett's test (K-squared = 135.35; df = 5; P = < 0.0001), histogram (Figure 4-15), and QQplot (Figure 4-16) for survival time. A square-root transformation was performed to improve ANOVA robustness to non-normality (Figure 4-17).

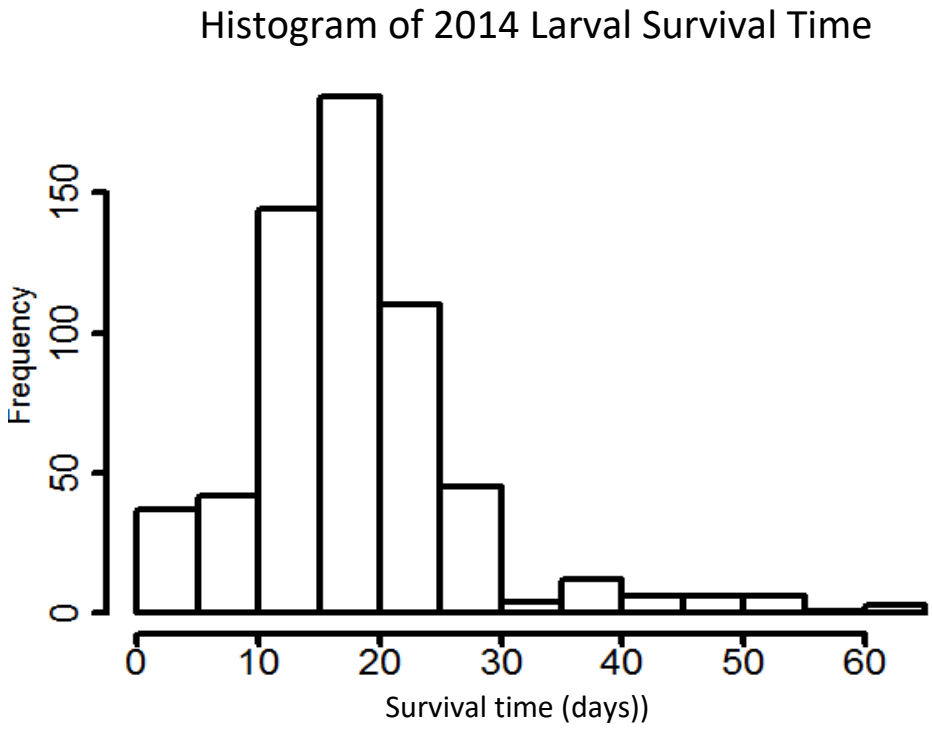


Figure 4-15. —Histogram of survival times for larval experiments in 2014.

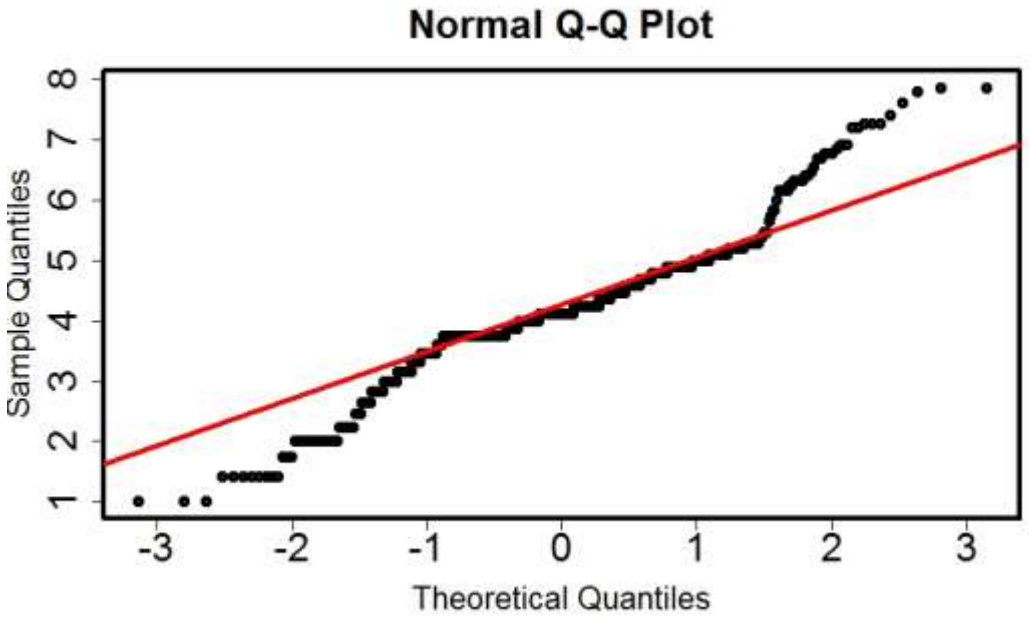


Figure 4-16. —QQplot of square-root transformed survival times for larval experiments in 2014.

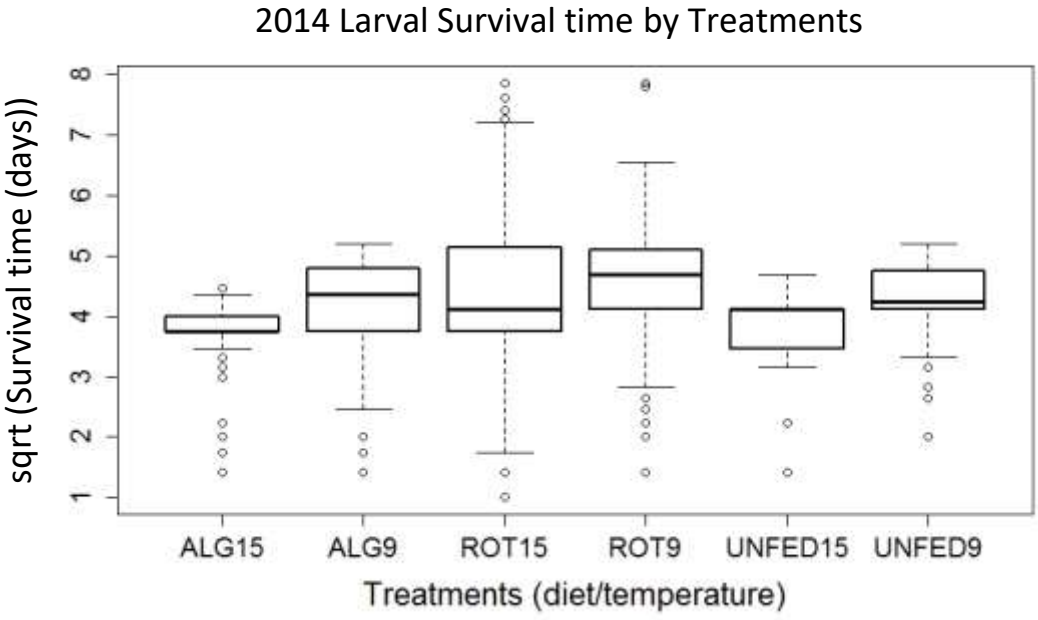


Figure 4-17. —Boxplot of square-root transformed survival times for diet and temperature in 2014.

Data was then standardized using the scale command to convert days into standard deviations from the mean. Table 4-4 shows the variables used in each model. Model 1 was run using a standard ANOVA (R procedure lm), whereas other models were run using nlme.

Table 4-4. —Models of larval survival tested using NLME. Response variables are: Temperature (T) and Diet (D).

Model	Response	Ind. Variables	Interaction	Random	Nested
lme.1	Days	T+D	No	Tanks	
lme.2	Days	T+D	No	Tanks	Containers
lme.3	Days	T x D	Yes	Tanks	Containers
lme.4	Days	T+D	No	Containers	

A statistically significant base linear model was found using a 2-way ANOVA, with significant effects for: temperature (F = 14.5064; df = 1,594; P = 0.0001), diets (F = 23.8472; df = 2,594; P = < 0.0001), and interaction (F = 3.5027; df = 2,594; P = 0.0307395). Four different model variations fit by maximum likelihood were created with or without interactions and with different nesting levels.

The best nlme model using only diet and temperature was model 3, which had significant effects for both diet and temperature, and their interaction, with tanks as random variables and containers nested within Tanks (Table 4-5). The REML version of this analysis is shown in Table 4-6. This model is interpreted as follows for each diet:

Algae : Survival (in √d) = 4.9127 + (-0.08365) (°C)

Rotifers : Survival (in √d) = (4.9127-0.2989) + (-0.08365+0.0758)(°C) = 4.6138-0.0078 (°C)

Unfed: Survival (in √d) = (4.9127+0.0400) + (-0.08365+0.0080)(°C) = 4.2716-0.0757 (°C)

When calculated using the experimental temperatures (9 or 15°C), these equations predict the values shown in the interaction plot (Figure 4-18). Survival was greatest for the Rotifer diet, and temperature had a significant negative impact on survival of larvae in the Unfed and Algae

treatments, but not the Rotifer treatment (Figure 4-19).

Table 4-5. —Comparison of NLME models tested, listed in order of decreasing AIC value. Test indicates a comparison between two adjacent models.

Model	df	AIC	BIC	logLik	Test	L.Ratio	p-value
lme.2	7	1715.536	1746.314	-850.768			
lme.1	6	1713.536	1739.918	-850.768	1 vs 2	0.000	0.9997
lme.4	6	1713.231	1739.612	-850.615			
lme.3	9	1712.501	1752.074	-847.251	3 vs 4	6.729	0.081

Table 4-6. —REML version of nlme model lme.3. Standard error of estimate (SE); degrees of freedom (DF).

	Value	SE	DF	t-value	p-value
(Intercept)	4.913	0.266	589	18.492	0.000
Temps	-0.084	0.021	589	-3.895	0.000
DietsRotifers	-0.299	0.376	589	-0.796	0.427
DietsUnfeed	0.040	0.460	589	0.087	0.931
Temps:DietsRotifers	0.076	0.030	589	2.496	0.013
Temps:DietsUnfeed	0.008	0.037	589	0.214	0.831

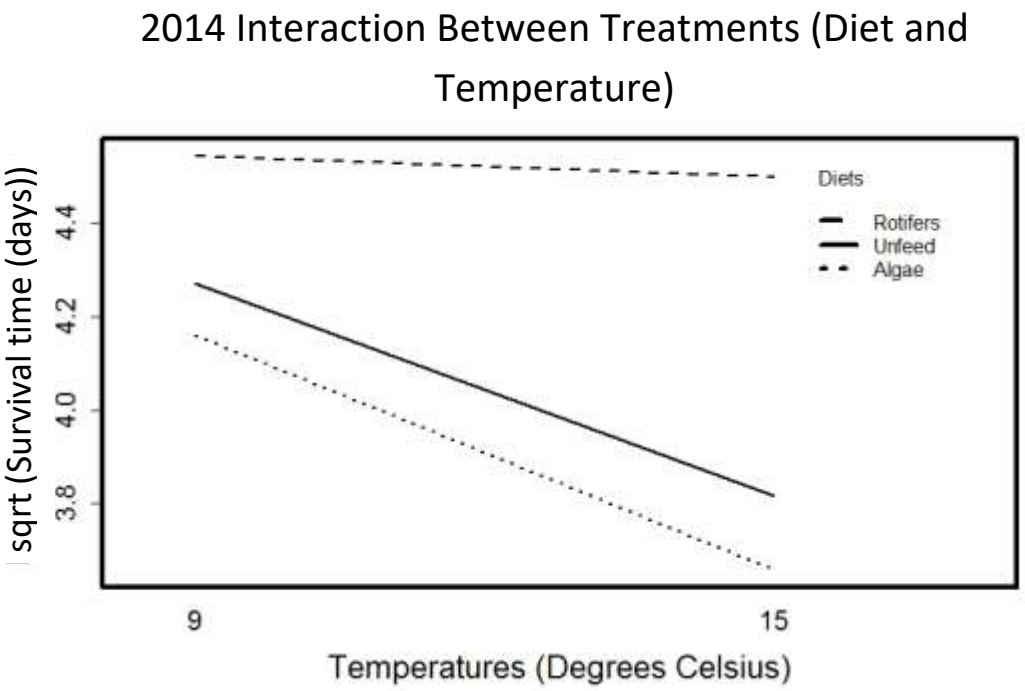


Figure 4-18. —Interaction plot of mean survival time (square-root of days) for each combination of diet (Rotifers, Algae, or Unfed) and temperature (9 or 15 °C).

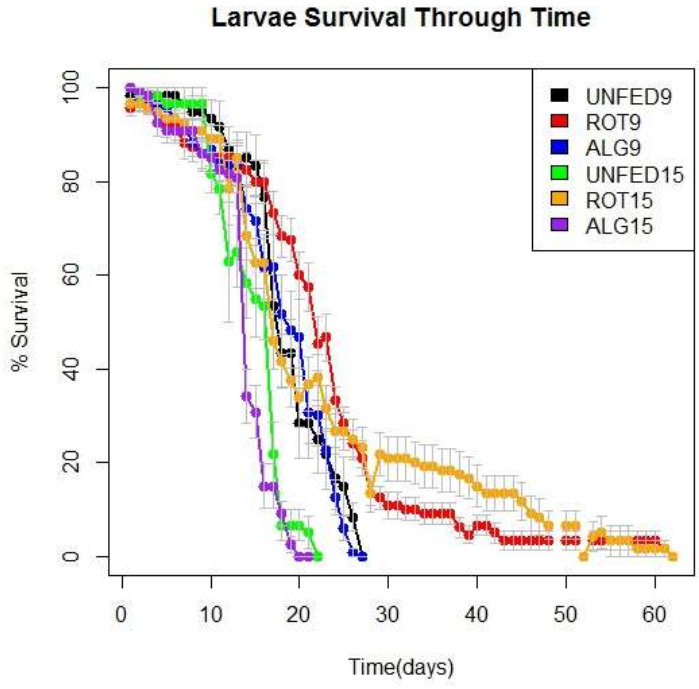


Figure 4-19. —2014 Larvae survival through time for all treatments of diet-temperature combinations.

Hypothesis 5: What are the effects of diet and temperature on development rate of RDSC larvae? Development stages at which red deepsea crab larvae died in 2014 were compared by treatment (diet and temperature) (Figure 4-20). As mentioned, a total of 120 larvae were assigned to each rotifer and algae treatment and 60 larvae to each unfed treatment (Table 3-1).

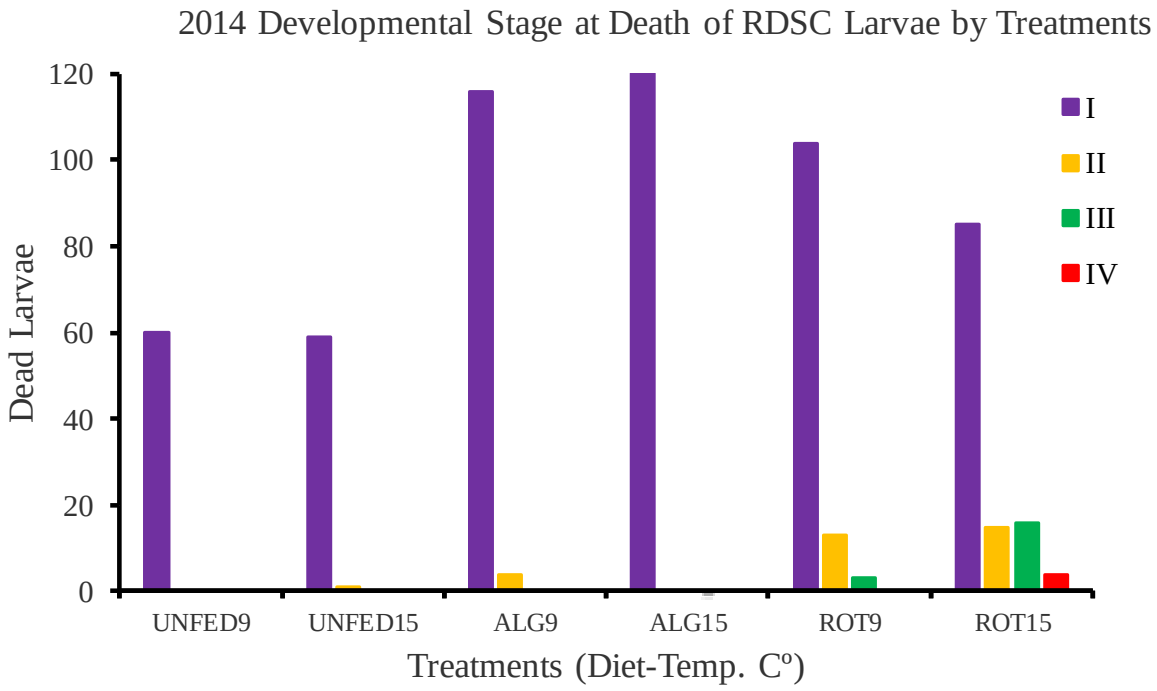


Figure 4-20. —2014 Red deepsea crab larval developmental stage at death by treatment (diet and temperature).

Variances were found to be not homogenous using Bartlett's test (K-squared = 13.726; df = 3; *P* = < 0.003303), histogram (Figure 4-21), and QQplot (Figure 4-22). Survival time by developmental stage is shown in Figure 4-23.

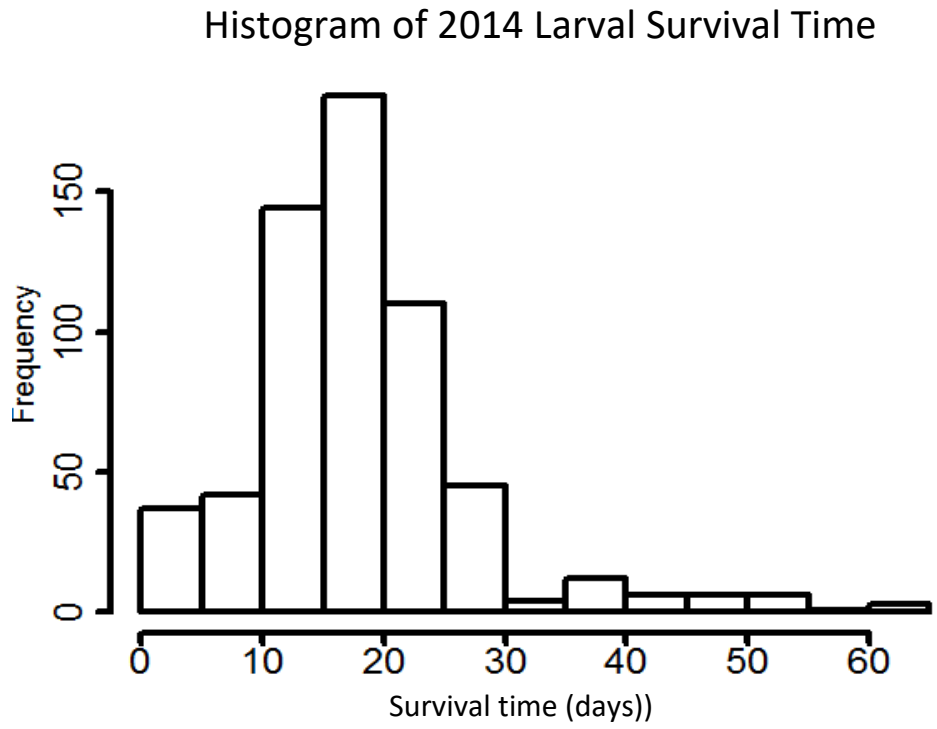


Figure 4-21. —Histogram of survival times for larval experiments in 2014.

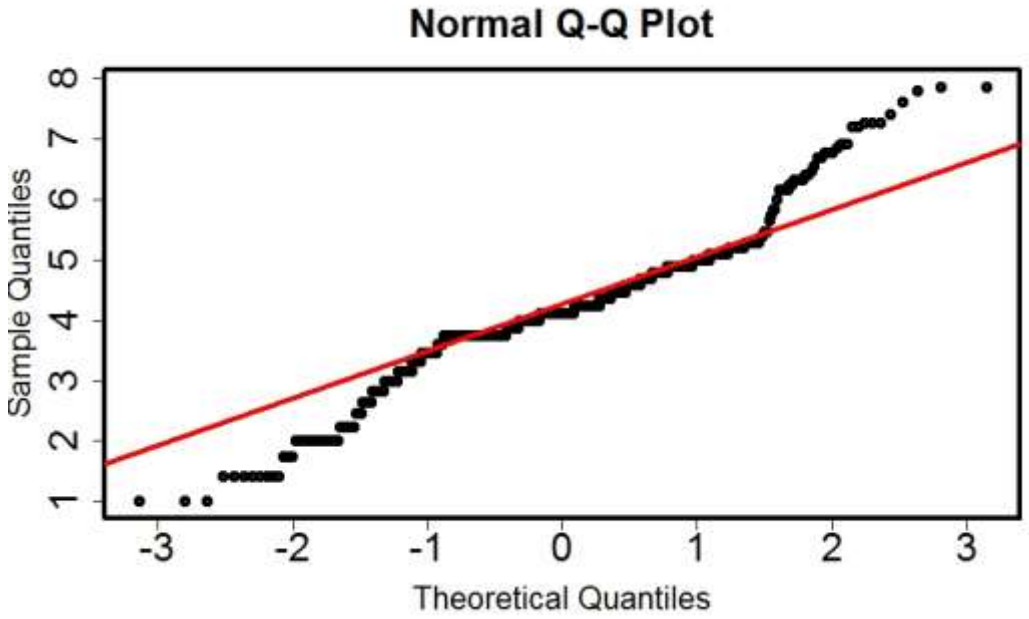


Figure 4-22. —QQplot of square-root transformed survival times for larval experiments in 2014.

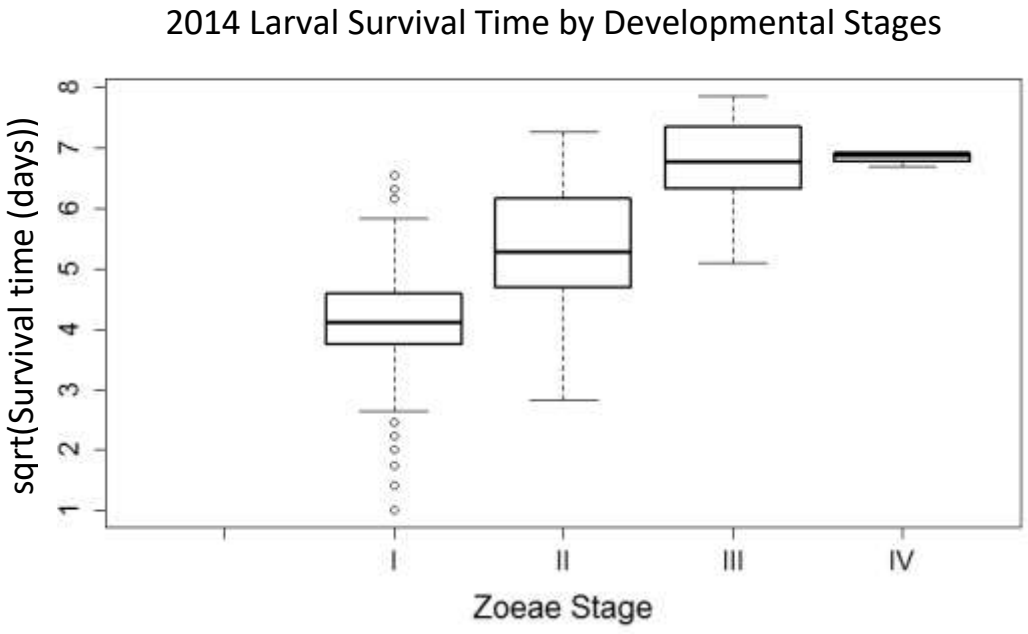


Figure 4-23. —Boxplot of survival time by developmental stage for 2014.

Data of larvae developmental stage at death was analyzed using non-linear mixed effects models (NLME) with the nlme package in R statistics. The model describe before were run with the addition of developmental stage as a factor (Table 4-7). The REML version of this analysis is shown in Table 4-8. This results for this model show that mean survival for stage ZI fed the Algae diet was 23.8 d minus 0.67(°C), survival to stage ZII differed from stage ZI by 10.4 d (t = 8.630519; df = 588; *P* = 0.014), ZIII by 30 d (t = 19.047994; df = 588; *P* = 0.001), and ZIV by 31 d (t = 9.403595; df = 588; *P* = 0.001). The intercepts for the Rotifer and Unfed diets differed by +2.33 and +0.97, respectively, although the latter was not significant. However, since there was no interaction, the temperature coefficient did not change with diet or stage. I rejected both parts of the hypothesis of no effect on development of a) diet and b) temperature in RDSC larvae. When stage was accounted for survival time for larvae was 2 days greater in the rotifer diet, but did not differed between algae and unfed diets. Larvae starved and died before they could progress to advanced developmental stages.

Table 4-7. —Comparison of NLME models tested with the addition of does with developmental stage as factor, listed in order of decreasing AIC value. Test indicates a comparison between two adjacent models.

Model	df	AIC	BIC	logLik	Test	L.Ratio	p-value
lme.0	3	1766.625	1779.816	-880.313			
lme.2	7	1715.536	1746.314	-850.768	1 vs 2	59.089	<.0001
lme.1	6	1713.536	1739.918	-850.768	2 vs 3	0.000	0.9997
lme.4	6	1713.231	1739.612	-850.615			
lme.3	9	1712.501	1752.074	-847.251	4 vs 5	6.729	0.081
lme.6	12	1504.799	1557.563	-740.400	5 vs 6	213.702	<.0001
lme.5	10	1500.801	1544.771	-740.401	6 vs 7	0.002	0.999

Table 4-8. —REML version of nlme model lme.5. Standard error of estimate (SE); degrees of freedom (DF).

	Value	SE	DF	t-value	p-value
(Intercept)	4.828	0.148	588	32.546	0.000
Temps	-0.078	0.012	588	-6.785	0.000
DietsRotifers	0.228	0.080	588	2.834	0.005
DietsUnfeed	0.145	0.093	588	1.549	0.122
ZoeaII	1.101	0.154	588	7.128	0.000
ZoeaIII	2.824	0.203	588	13.930	0.000
ZoeaIV	2.971	0.424	588	7.008	0.000

4.2.2. *2016 Survival and Development Analysis*

As I mentioned earlier females obtained in 2015 had immature eggs that didn't hatch timely to perform the larval survival experiments or had already hatched in capture prior. The new feeding experimental system was designed and built. The Larvae Feeding Experimental System 2016 was named after the year when the larvae survival and developmental data were collected and ran with a new group of adult females.

Larvae Feeding Experimental System 2016 (LFS II) was set up with the transferred larvae from seven broods or clutches (Female ID: 19_16, 21_16, 22_16, 24_16, 25_16, 27_16, and 28_16) hatched in the FHCS. It is important to mention that during transfer and count of larvae from collectors in FHCS to the LFSII, the laboratory building was suffering electric problems resulted in water temperature to increase. The situation was remediated using ice packs and ice to keep larvae and females in cold water long as possible. Females 19_16, 21_16, 22_16, 27_16, and 28_16 were observed dead the same day larvae system was set up.

During survival and development experiments the water temperature in LFS II was maintained consistent at 15°C ± 1°C and 20°C ± 1°C. Live diets treatments were modified. As observed in the 2014 larvae survival and development experiments with algae *T. iso* diet didn't show any difference from the unfed diet and rotifer was the most successful diet. It was decided to eliminate the algae, keep the rotifer, and incorporate an *Artemia sp.* diet. Rotifers and *Artemia sp.* live diets were successfully cultured and daily prepared to concentrations (i.e. 7 rotifers/ml, 0.7 *Artemia sp.*/ml, etc.) (Table 4-3). Salinity was 26-30‰ throughout the experiments. Finally, I was able to set up an automatic light cycle of 12 day /12 night h in the room and experimental tanks were maintained under 48 lx of light intensity.

Hypothesis 4: What are the effects of diet and temperature on the survival of RDSC larvae survival? Daily recorded larvae mortality data was analyzed using non-linear mixed effects models (NLME) with the nlme package in R statistics. After square-root transformation, variances were found to be homogeneous with Bartlett's test (K-squared = 6.0278; df = 7; P = 0.5365), histogram (Figure 4-24), and QQplot (Figure 4-25) for time to survival (Figure 4-26).

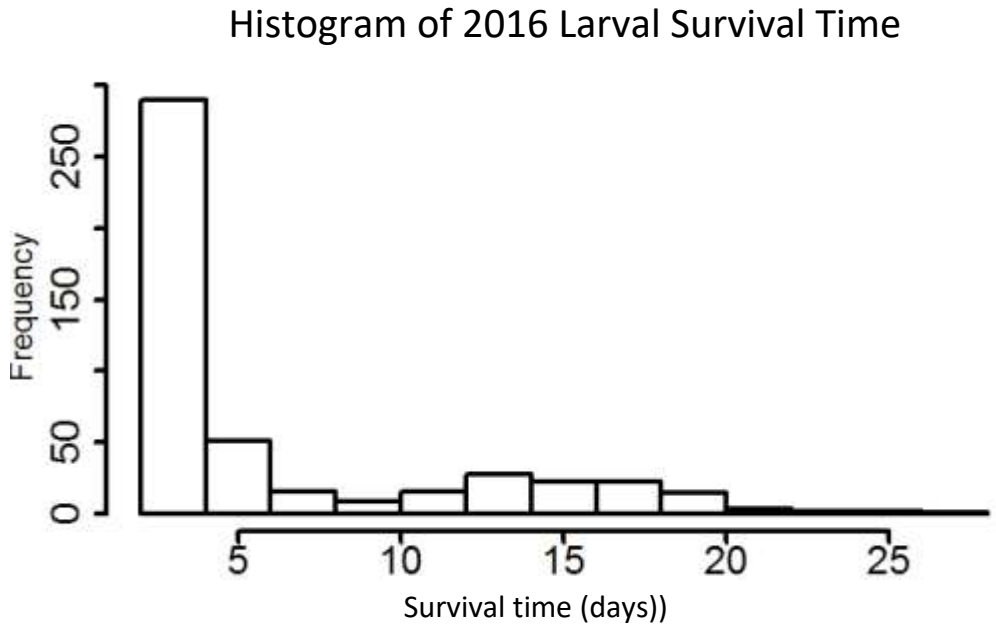


Figure 4-24. —Histogram of survival times for larval experiments in 2016.

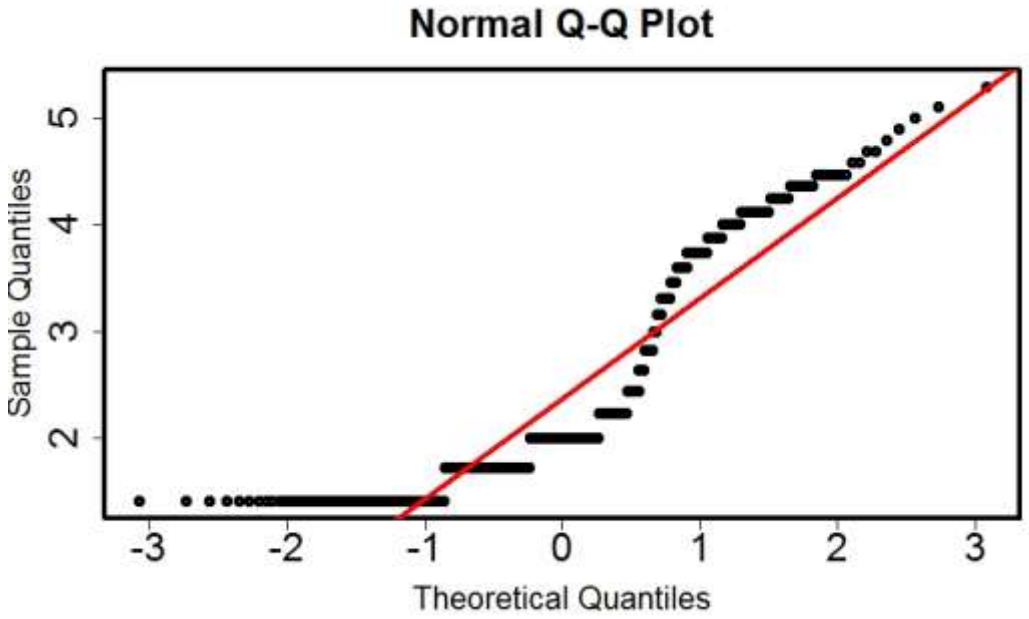


Figure 4-25. —QQplot of square-root transformed survival times for larval experiments in 2016.

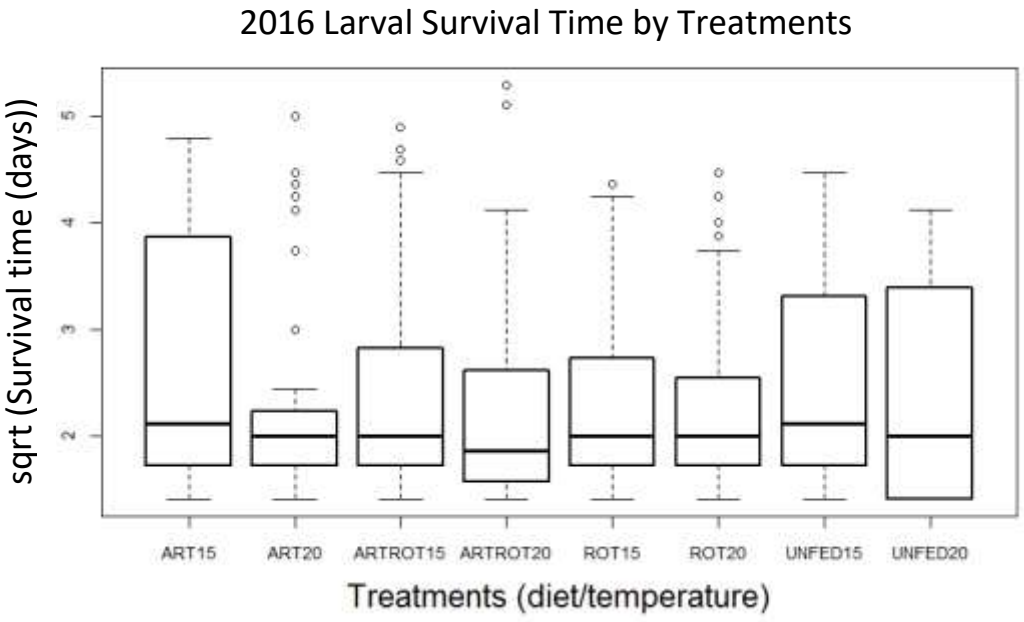


Figure 4-26. —Boxplot of square-root transformed survival time for diet and temperature in 2016.

Data was then standardized using the scale command to convert days into standard deviations from the mean. A 2-way ANOVA comparing survival days between temperatures and diets showed a significant effect only for temperature (F = 4.5488; df = 1,472; P = 0.03346), but not for diet or the interaction between temperature and diet. Two different model variations fit by maximum likelihood were created with or without interactions and with upwellers as random variables. Table 4-9 shows the variables used in each model.

Table 4-9.—Models of larval survival tested using NLME. Response variables are: Temperature (T) and Diet (D).

Model	Response	Ind. Variables	Interaction	Random
lme.1	Days	T+D	No	Upweller
lme.2	Days	T x D	Yes	Upweller

Both models were compared using a single ANOVA statement by comparing the AIC values (Table 4-10). The model with the lowest AIC was then rerun with a slightly different method fit by restricted maximum likelihood (REML) (Table 4-11).

Table 4-10. —Comparison of NLME models tested, listed in order of decreasing AIC value. Test indicates a comparison between two adjacent models.

Model	df	AIC	BIC	logLik	Test	L.Ratio	p-value
lme.2	10	1347.466	1389.204	-663.733			
lme.1	7	1346.556	1375.773	-666.278	1 vs 2	5.090	0.165

Table 4-11. —REML version of nlme model lme.1. Standard error of estimate (SE); degrees of freedom (DF).

	Value	SE	DF	t-value	p-value
(Intercept)	3.132	0.338	473	9.273	0.000
Temps	-0.038	0.018	473	-2.152	0.032
DietsArtemiaRotifer	-0.111	0.125	473	-0.889	0.374
DietsRotifers	-0.174	0.125	473	-1.389	0.166
DietsUnfeed	-0.074	0.125	473	-0.589	0.556

The best model was the one without interaction and random upwellers, and was significant only for the temperature effect (t = -2.151990; df = 473; $P = 0.0319$). As a result, the hypothesis of no effect on larval survival of diet was accepted, but the hypothesis of no effect of temperature was rejected. My interpretation of these results is that temperature is the main factor driving larval mortality. Larval survival through time for all treatments of diet-temperature combinations was similar (Figure 4-27 and 4-28).

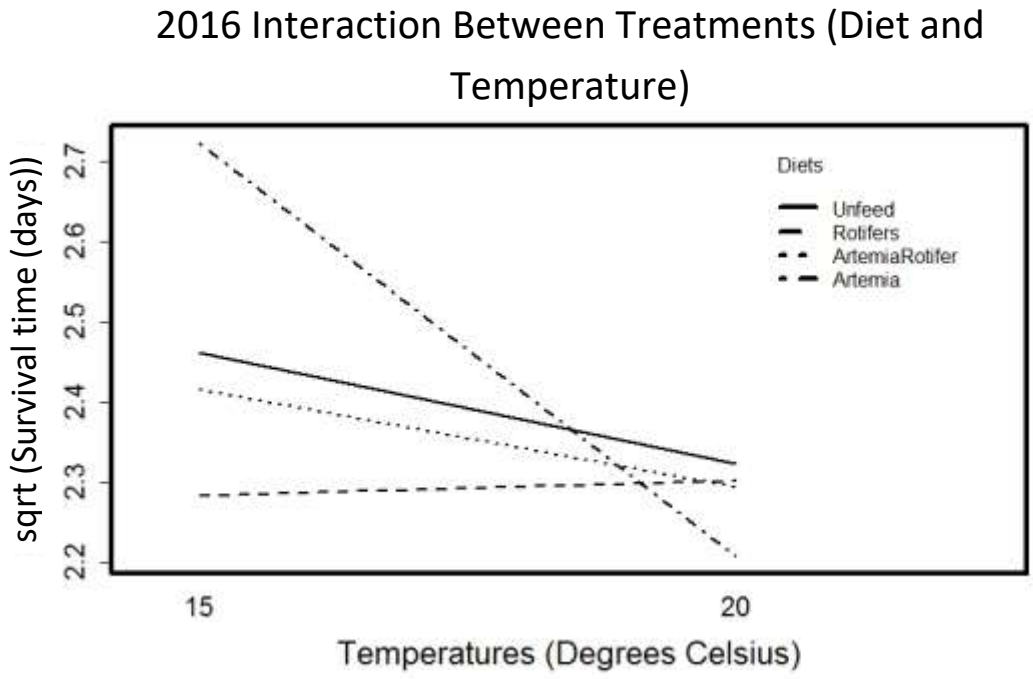


Figure 4-27. —Interaction plot of mean survival time (square-root of days) for each combination of diet (Rotifers, Algae, or Unfed) and temperature (15 or 20 °C).

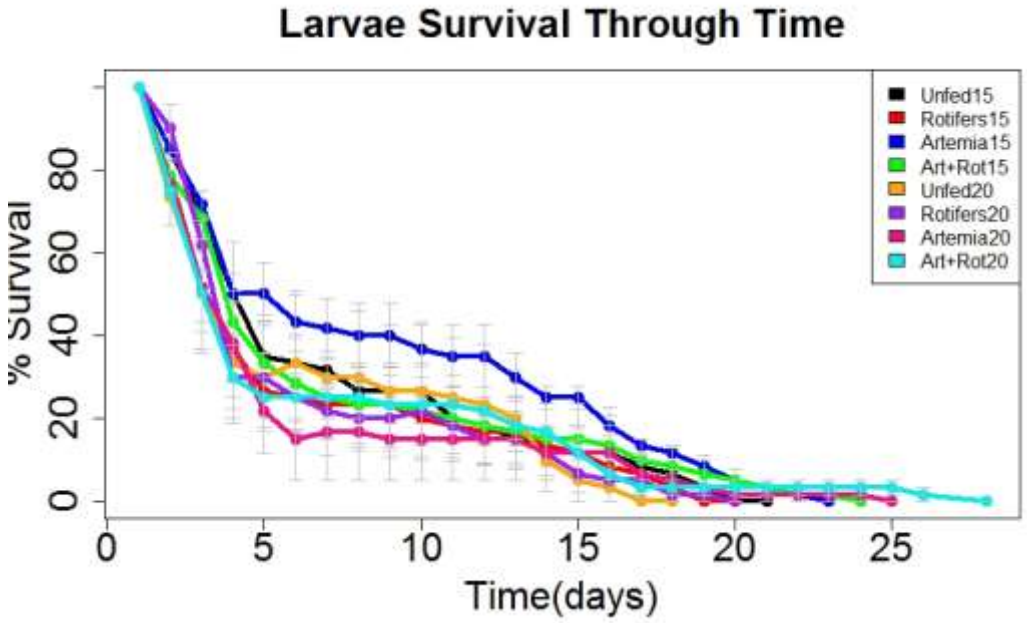


Figure 4-28. —2016 Larval survival through time for all treatments of diet-temperature combinations.

Hypothesis 5: What are the effects of diet and temperature on development rate of RDSC larvae? Development stages at which red deepsea crab larvae died in 2016 were compared by treatment (diet and temperature) (Figure 4-29). As mentioned earlier, a total of 60 larvae were assigned to each diet treatment (Table 3-1).

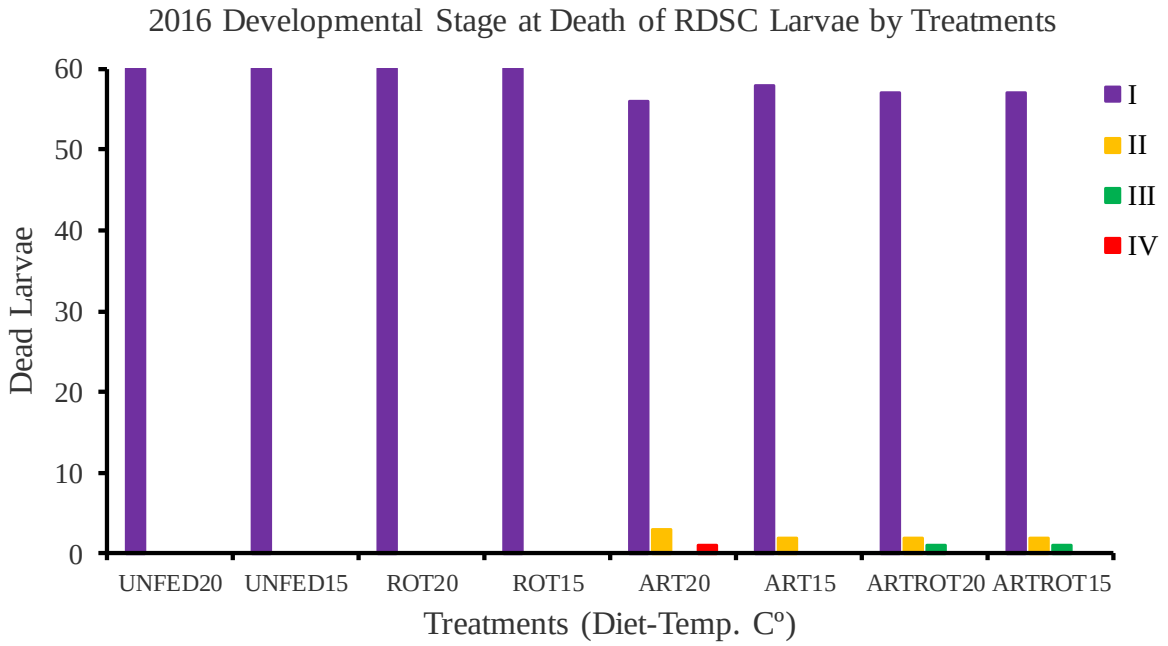


Figure 4-29. —2016 Red deepsea crab larval developmental stage at death by treatment (diet and temperature).

Variances were found to be homogenous using Levene's test (F = 1.7238; df = 3, 476; P = 0.1612), histogram (Figure 4-30), and QQplot (Figure 4-31). Survival time by developmental stage is shown in figure 4-32.

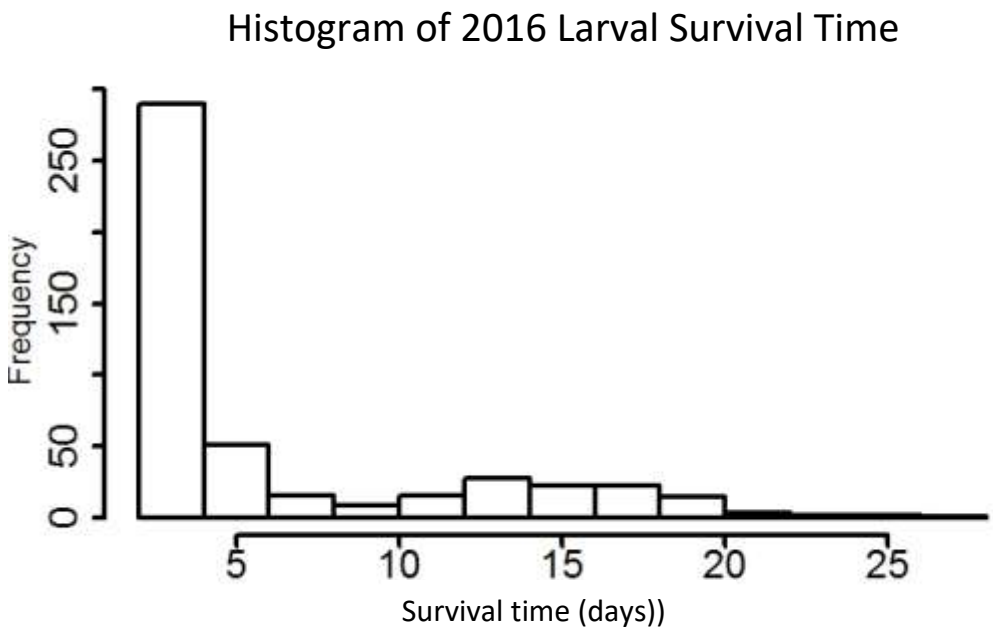


Figure 4-30. —Histogram of survival times for larval experiments in 2016.

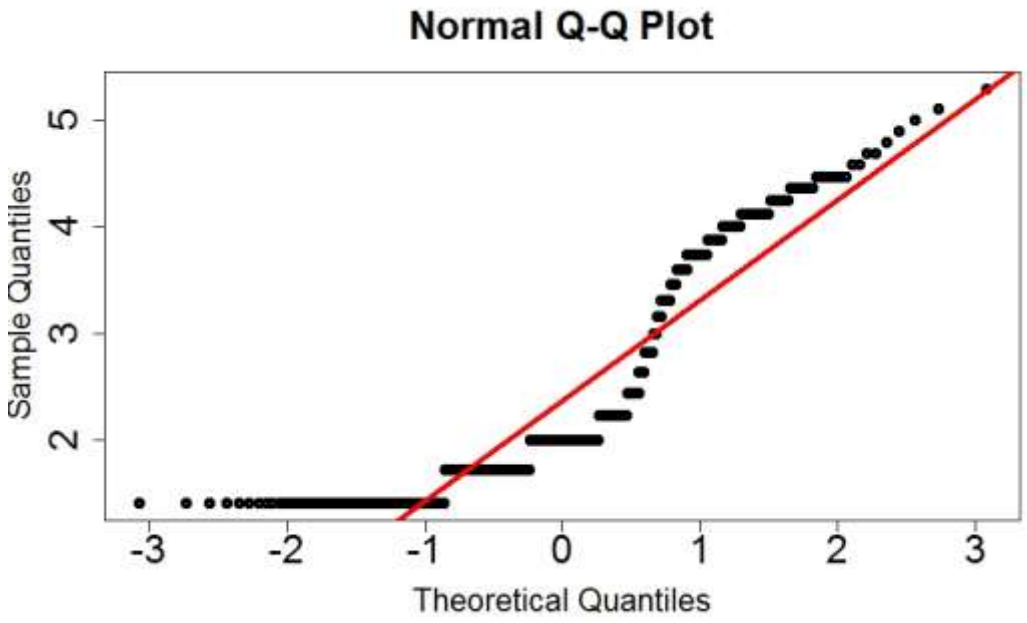


Figure 4-31. —QQplot of square-root transformed survival times for larval experiments in 2016.

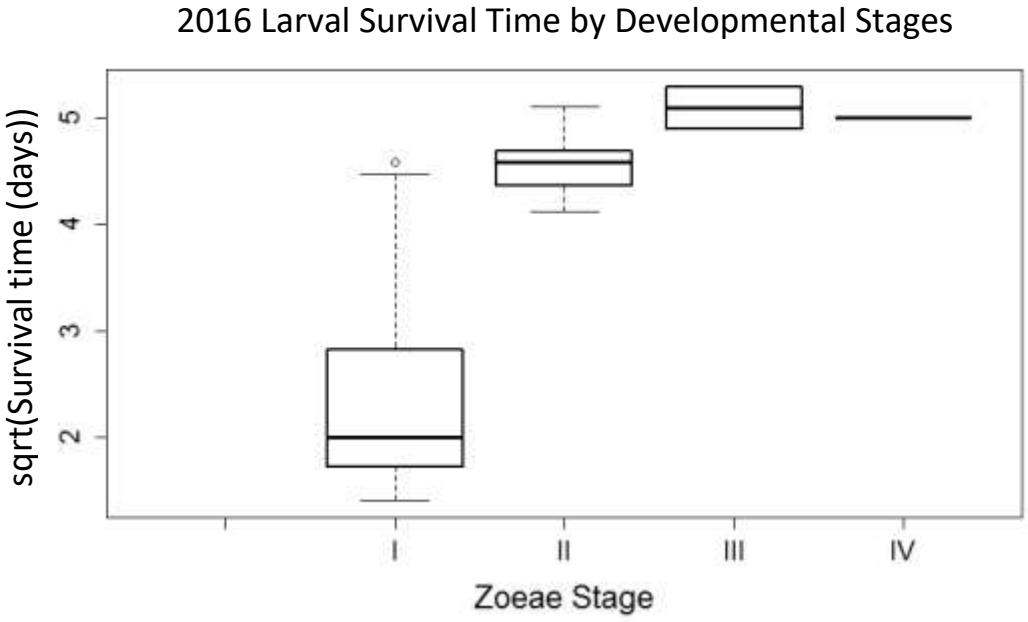


Figure 4-32. —Boxplot of survival time by developmental stage for 2016.

Data of larvae developmental stage at death was analyzed using non-linear mixed effects models (NLME). The model describe before were run with the addition of developmental stage as a factor (Table 4-12). The REML version of this analysis is shown in Table 4-13. A statistically significant base linear model was obtained using a 2-way ANOVA for temperature (F = 4.0529; df = 1,472; P = 0.04466). Five different model variations fit by the maximum likelihood were created of this with or without interactions (between diets and temperatures) adding larval stage as a factor. All models were compared using a single ANOVA statement by comparing the AIC values (Table 4-12). The model including developmental stage as a factor with interaction accounted for more variance, and provides a lower AIC value, resulting in a better model (lme(data = morts, Days~Zoea+Temps+Diets + (Temps*Diets), random = ~ 1|Upweller, method="ML"). The REML version of this analysis is shown in Table 4-13. Resulted in zoea II differs from diet unfed (t = 5.696945; df = 467; P = <0.0001), zoea III differs from unfed (t = 8.488411; df = 467; P = <0.0001), and zoea IV differ from unfed (t = 5.598944;

df = 467; *P* = <0.0001). I accepted part *a* of my hypothesis for no effect on development of a) diet and b) temperature in RDSC larvae. Part *b* and the interaction between diet and temperature were rejected. These results showed that temperature and its interaction with diet had an effect in capacity of the larvae to reach advance stages of development.

Table 4-12. —Comparison of NLME models tested with the addition of does with developmental stage as factor, listed in order of decreasing AIC value. Test indicates a comparison between two adjacent models.

Model	df	AIC	BIC	logLik	Test	L.Ratio	p-value
lme.2	10	1347.466	1389.204	-663.733			
lme.1	7	1346.556	1375.773	-666.278	1 vs 2	5.090	0.165
lme.0	3	1345.219	1357.741	-669.610	2 vs 3	6.663	0.155
lme.3	10	1278.845	1320.583	-629.423	3 vs 4	80.374	<.0001
lme.4	13	1276.654	1330.913	-625.327	4 vs 5	8.191	0.042

Table 4-13. —REML version of nlme model lme.4. Standard error of estimate (SE); degrees of freedom (DF).

	Value	SE	DF	t-value	p-value
(Intercept)	4.455	0.585	467	7.610	0.000
ZoeaII	2.235	0.306	467	7.300	0.000
ZoeaIII	2.860	0.642	467	4.457	0.000
ZoeaIV	2.840	0.906	467	3.134	0.002
Temps	-0.120	0.033	467	-3.659	0.000
DietsArtemiaRotifer	-1.793	0.820	467	-2.187	0.029
DietsRotifers	-2.224	0.820	467	-2.713	0.007
DietsUnfeed	-1.578	0.820	467	-1.924	0.055
Temps:DietsArtemiaRotifer	0.096	0.046	467	2.064	0.040
Temps:DietsRotifers	0.124	0.046	467	2.668	0.008
Temps:DietsUnfeed	0.093	0.046	467	1.995	0.047

Hypothesis 6: Which aquaculture system better promotes larval survival? For the two-year study, I used the same temperature (15 ºC) and two equal diet (unfed and rotifers) treatments, but different aquaculture systems. Larvae Feeding Experimental System 2014 (LFS

I) (Figure 3-7) and Larvae Feeding Experimental System 2016 (LFS II) (Figure 3-9). Larvae mortality data sets of both years were combined and analyzed using linear models in R statistics. Even after square-root transformation, variances were found to be not homogeneous Bartlett's test (K-squared = 59.671; df = 3; P = < 0.0001), histogram (Figure 4-31), and QQplot (Figure 4-32).

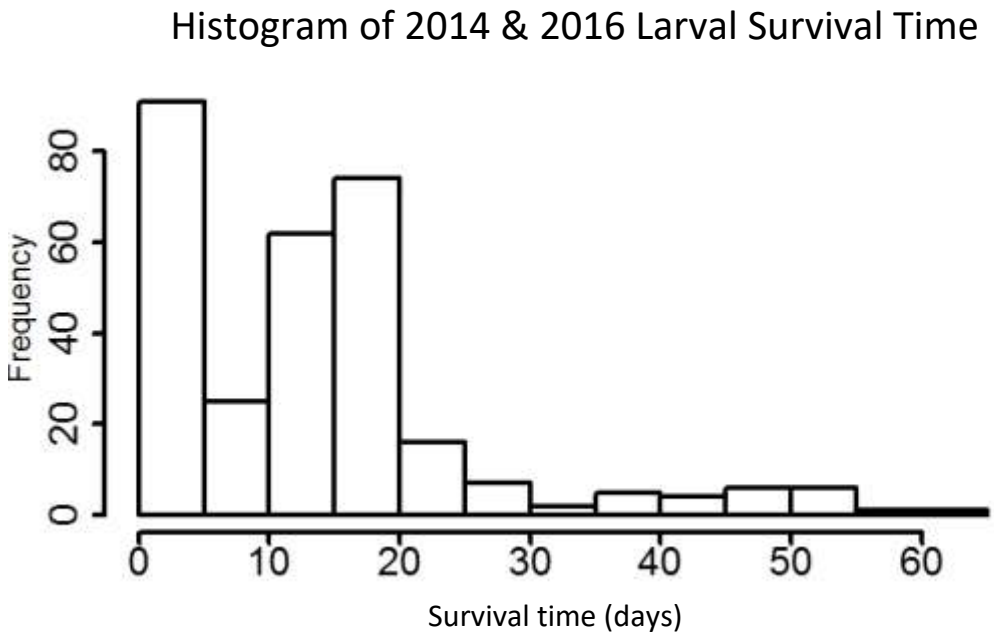


Figure 4-33. —Histogram of larval survival time for diets (rotifers and unfed) and aquaculture systems in 2014 and 2016.

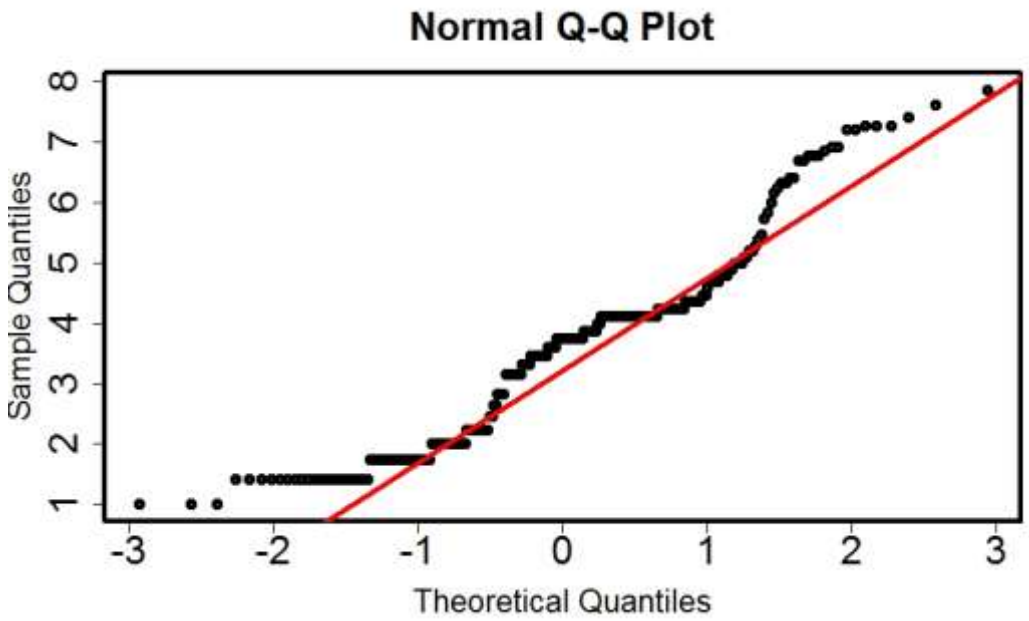


Figure 4-34. —QQplot of larval survival time (square-root transformation) for diets (rotifers and unfed) and aquaculture systems in 2014 and 2016.

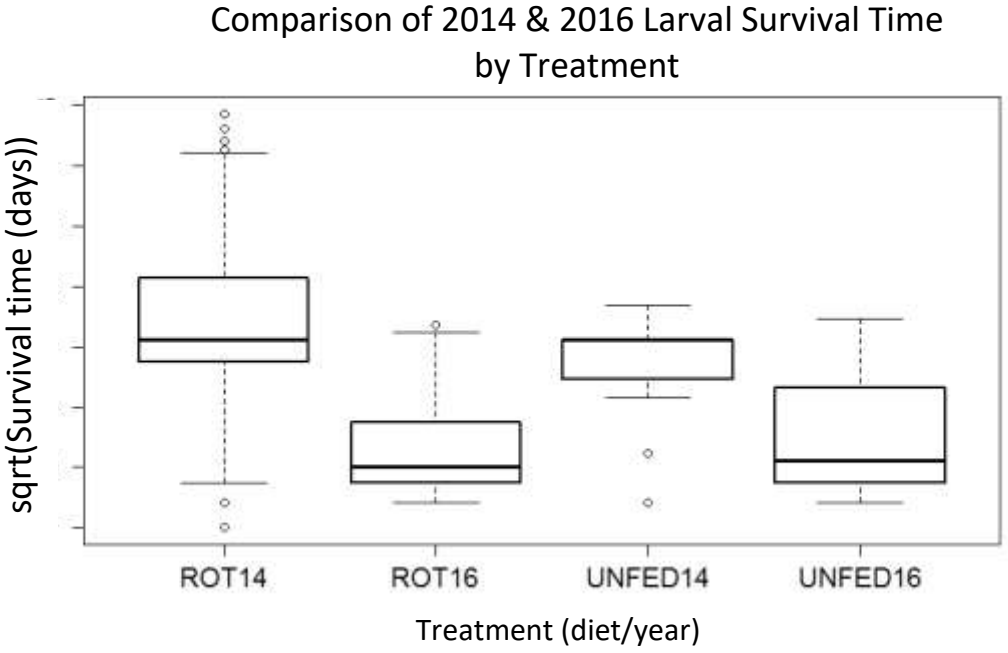


Figure 4-35. —Boxplot of square-root transformed survival time for diet and year in 2014 and 2016.

Data was then standardized using the scale command to convert days into standard deviations from the mean. A linear model ANOVA was run using diets (unfeed and rotifers) and aquaculture system (2014 LFS I and 2016 LFS II) as factors. Significant effects were found for year (F = 205.4649; df = 1, 296; P = <0.0001), diets (F = 5.3794; df = 1, 296; P = 0.021056) and their interaction (F = 9.9680; df = 1, 296; P = 0.001757). The overall model was highly significant (F = 73.6; df = 3, 296; R^2 = 0.4215; P = <0.0001). I rejected my hypothesis of no effect on survival of the experimental aquaculture systems (LFS) used in different years. The type of aquaculture system plays an important role in the mortality of the larvae. Each year's experiments used a different aquaculture system and larvae survival times were significantly different between them, which indicates that larvae responded differently to the different systems. Finally, the larvae in the unfed diet lived the longest before dying, but these observations don't take in consideration their developmental stage (which could be delayed in comparison with the rotifer diet). Differences in the unfed diet can also reflect the duration of their survival which could be shorter in comparison with their counterparts.

CHAPTER 5

CONCLUSIONS, DISCUSSION, AND FUTURE RECOMMENDATIONS

The long-term goal of this project was to study the factors affecting the early larval stages of the red deepsea crab (*Chaceon quinquedens*). Two series of studies were designed to investigate the hatching periodicity, survival, and development of red deepsea crab larvae: effects of diet and temperature; complementing each other's during a multiyear project.

A total of 74 red deepsea crab (RDSC) adult females with fertilized eggs were obtained from several fishing trips performed by the Atlantic Red Crab Company during the spring and summer of 2014-2016. Thirty-six females died, most of them during "acclimation time".

First set of experiments were conducted to answer three hatching questions: duration, maximum hatching time, and morphometry relationship. A total of 31 females showed hatching either starting before or after coming into the adult Females Husbandry and Hatching Collection System (FHCS) in the NOAA James J. Howard Sandy Hook Laboratory, NJ. After both experimental years, mean temperature was 8.2 ± 0.83°C in 2014 and 7.6 ± 2.1°C in 2016. From these hatching females, only 3 showed a typical bell-shaped curve hatching distribution in the laboratory and 17 were used to perform hatching observations at 4 h intervals for 3 consecutive days.

A simple linear regression with intercept set at zero (F = 1196; df = 1, 13; R^2 = 0.9892; P = < 0.0001) was calculated to predict number of larvae hatched by measured volume (# larvae hatched=838.20*(volume)).

A linear model and pairwise t-test showed statistical significance in the relationship between larvae hatched at the time interval T14 and T22, when all other intervals were similar.

Simple linear regressions did not show significant relationship between numbers of the red deepsea crab larvae hatched and morphometry parameters (CW, CL and AW).

The second set of experiments was conducted using larvae hatched in the FHCS during 2014 and 2016. These experiments were conducted to answer questions about the effects of diet, temperature, and aquaculture systems in the survival, and development of larval RDSC.

The Larvae Feeding Experimental System 2014 (LFS I) was maintained in a constant water temperature of 9°C ± 1°C and 15°C ± 1°C. The initial diet treatments included an unfed control, rotifers, *Artemia sp.*, *Artemia sp* +rotifers, and *Artemia sp* + algae (*T. iso*). *Artemia sp* treatments resulted in crab larval dead after 48 h and were no longer used for the remainder of the experiment. Final treatment for 2014 included the unfed control, two rotifers, and two algae treatments (for a total of 120 larvae in each rotifer and algae treatment). All diet concentrations were modified through time depending on their needed availability for the crab.

Finally, the Larvae Feeding Experimental System 2016 (LFS II) was consistently maintained 15°C ± 1°C and at the newly selected temperature of 20°C ± 1°C. Live diets treatments were modified from the previous year. The diet treatments included an unfed control, rotifers, *Artemia sp.* and a mix of both diets. For both years, daily recorded larvae survival by diet and temperature was tested for homogeneity of variance with a Bartlett's test, histograms, and QQplots. Non-linear mixed effects models (NLME) with interaction term for the three continuous variables (diet, temperature, and year) were used to model the survival time and statistically significant models were obtained for both years individually and compared with each other.

5.1. Discussion

The hatching data of the 3 females that began and finished hatching in the laboratory followed a normal distribution in my research. Hatching period was expected to follow a normal distribution as eggs developed and hatched increasingly with time until hatching peaks (50% of total hatched) and then progressively decreases. Total number of hatched larvae from these three females was different, which could be attributed to different female sizes. It was necessary to use the proportion of larvae hatched per day for each female to model the red deepsea crab hatching period.

All females captured from the wild and brought to the laboratory have unknown egg extrusion days and just the ones that started hatching in the laboratory after a period of time after arrival (i.e. female 8_16 hatched 24 days after arrival) have hatching starting point was known. The use of milestones for the individual hatching periods, when 2.5, 50, and 97.5% of total hatching was complete, provided a standardize measure of time. The resulting hatching mean duration for females red deepsea crab is 18.1 ± 3.5 days. I was fortunate to obtain three females with the complete hatching period. Most of the other females release their eggs because of handling stress or when they were already in the middle to the end of their hatching period, their eggs were lost or hatching delayed after arriving to the laboratory. This result improved our understanding of the hatching process of red deepsea crab and serves as guidelines to future experiments using wild capture females with fertilized eggs.

Now it is possible for researchers to determine how advance wild caught females are in their hatching period in the laboratory, based on duration and size of the female (fecundity relationship). This hatching duration estimation of 18.1 days implicates a small window of time when the larvae are released into the water column, during the spring-summer. Changes in their

environment, increase in temperature, lack of suitable, and others, during this period can potentially affect this process, delay or interrupt it.

Understanding where and when females primarily spawn has been mentioned as research need for the species discussed by Steimle (2001). My study addresses the daily aspect of when females red deepsea crab spawn (hatch). The mean number of larvae hatched was lower at T14 (mid-day from10 am to 2 pm) than at time T22 (6 pm to 10 pm) across all crabs, but it was not different from other time intervals. The artificial set-up of the automatic light cycle of 12 day /12 night h in the laboratory, coincides with T22 being the first observation time after sunset. Similar patterns have been shown in other crabs with higher hatching at dusk. For example the red king crab *Paralithodes camtschaticus* released most of their larvae (91-95%) in the 4-hour period between 18:00 and 22:00 h, while 50% were released in the first half-hour of darkness (Stevens and Swiney 2007). These findings could facilitate future aquaculture operational procedure and protocols, concentrating larvae collection efforts and reducing disturbances over the system in the first 4 hours after sunset.

The information available in my study did not show any relationship between red deepsea crab morphometry and total hatch collected from the females. This could be due to insufficient total hatching data for most females and a clustered female size range. There was a lack of hatching (starting day and completion period) and fecundity (total larvae hatched) data for most females. Previously, fecundity of female red deepsea crab has been estimated between 36×10^3 to 226×10^3 eggs per female (similar to our total hatched for the 3 selected females for hatching duration). This was mainly due to female body size (Hines 1988).

Perkins (1973) found hatching from April to June in laboratory study, which would explain why females received in the laboratory during late summer gave low or no hatchlings

(immature eggs) during my studies. After the systems were set up with larvae hatched in the FHCS, I observed their survivals until all larvae died. Variance of the mortality data in both years of experiments was homegenous having fewer mortality at the beginning and end of the experiments. The 2014 experiment using the LFS I, showed very similar behavior in survival through the study timeline as it was described by Casper (2013). Both studies show strong relationship between survival and temperature/ diet. In 2014, individual factors (diet and temperature) and interaction between diet and temperature were significant. It was interesting to obtain a significant interaction between temperature and rotifers diet. Confirms that fewer larvae survived in this treatment, but the ones died, did so at a later time. This result supports a longer survival of larvae under this treatment and that these fewer larvae were also in advanced larval development stages (Zoeae II, III, and IV). Rotifers were the best diet for the RDSC larvae during my 2014 experiment.

During 2016**,** temperature was the main factor driving larvae mortality, no difference was found between diets. Larval mortality for all treatments of diet-temperature combinations behaved similar. During 2014, larval development differed from the unfed diet, reaffirming the inability of the larvae to advance in their development under starvation conditions. Mortality of larvae unfed, rotifer, artemia+rotifers diet and their interaction with temperature differed at developmental stages. Diet affects the developmental stage of the RDSC larvae in my study. Similar mortality rate was also observed between treatments. Rotifers and artemia+rotifers were the best diet for the RDSC larvae during my experiment.

The contrasting results between 2014 and 2016 survival could have been produced by the three possible causes that influenced the 2016 experiments: higher temperature exposure at transfer, unidentified system defect, and handling of larvae. First, as I mentioned earlier, the

2016 larvae transfer day was affected by the electricity failure in the building. This issue caused water temperature to increase during transfer and exposed larvae to drastic changes in temperature even when remediation measures were taken during collection and count of the larvae. It was expected under this condition that I would lose the last possible hatching females of the season (which actually happened later that day), so I decided to continue with the larvae transfer even if it was projected to take longer than expected. A second cause could have been an unidentified system defect in the cone mesh. Most of these meshes got lose after days of saltwater and handling. The research observation team didn't notice the larvae were getting trapped in the mesh during their observations. The third cause may be due to the inexperience of the research observation team at the beginning of the experiments. This could have affected discarding trap larvae, flushing them through the system, rough handling of larvae at counts, or misidentifications of them as dead. All these factors could have contributed to the rapid mortality of the larvae and difference in data outcome.

Similar studies by Casper (2013) examined the nutritional value of phytoplankton consumption to larval brachyuran crabs, feeding crab larvae *Lophopanopeus bellus*, *Metacarcinus* (*Cancer*) *magister*, and *Hemigrapsus nudus* with combination treatments of the alga *Isochrysis galbana*, and zooplanters *Artemia sp*. and rotifers *Brachionus plicatilis*. Author concluded that mean Point-of-No-Return values for unfed and algal fed were not different, and *I. galbana* has no significant effect on the larvae survival or development rate (Casper 2013). These results match my results with *T. isocrysis* diets in 2014, where I found no difference betweeen the unfed diet at either temperature (9 and 15 ºC).

Suprayudi et al. (2004) identified two kinds of mortality patterns due to rotifer diet quality in mud crab *S. serrata* larvae; the first pattern observed when using rotifers with low

level of omega-3 HUFA resulted in a gradual decrease in survival rate during the developmental period, and the second with excess omega-3 HUFA that produced a high frequency of incomplete molting to the megalopa stage and low survival rate at the first crab stage. The first pattern resembles my results in the 2014 (Figure 4-18) and the second the ones resembles my results in 2016 (Figure 4-26) for the rotifer diets. Gerrior (1981) performed experiments with similar set-up and results for larval survival than my 2014 experiment. In 2014, a gradual decrease in survival rate during development was observed in the RDSC larvae rearing under rotifer diets. During this experiment, diet concentrations were prepared, which involved changing the seawater on the culture sample diet, but they were not re-enriched with RotiGrow® (rotifers) or DHA Selco (*Artemia sp*.). My 2016 results resemble the second pattern described, which produced a low survival rate at the first crab stage. On the other hand, during 2016 all diets were re-enriched before adding them to the larvae system. RotiGrow® Nanno contains protein (58.6%), lipid (14.5%), carbohydrate (20.0%), and ash (5.9%), more specificly DHA: EPA: ARA (0: 1.0: 0.12), EPA+DHA (30 mg/g d.w. (Biomass)), total HUFAs, 20:3n3 (35 mg/g d.w. (Biomass)), and total fatty acids (105 mg/g d.w. (Biomass)) (http://reedmariculture.com/product_rotigrow_nanno.php#tab_tech/3/17/2017/3:18pm). This enrichment measure could had supply good nutrient sources and enhanced the survival.

Possible contributors to the mortality of RDSC larvae to developmental stages past the Z4 stage are the lipid composition and specific requirements in their diet. Anger et al. (1981) discussed how early starvation can affect intermolt time and survival throughout the whole zoeal development of branchyuran crabs, with a common prolongation of sZ3 and Z4 stages, and megalopa delay; this results from lacking biochemical cues from initial food taken with sterols, precursors of ecdysterone molting hormone. Related aquaculture studies in the commercially

important mud crab *Scylla serrata* (Brachyura: Porturidea) had concluded that mass mortality of larvae can be caused by an omega-3 highly unsaturated fatty acid (omega-3 HUFA) deficiency in rotifers diet (Suprayudi et al. 2002 and Hamasaki et al. 2002). In addition to this highly unsaturated fatty acids, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) deficiencies are identified as a cause of low survival, longer intermolt period, and narrower carapace in *S. serrata* (Suprayudi et al. 2004). A study by Suprayudi et al. (2004) showed that diet could not be completely substituted by the artificial foods. Alternatively, the use of suitable vegetable oil supplements, to reduce feed cost, can only be used as a partial replacement (50%) of cod liver oil without compromising growth or survival (Unnikrishnan et al. 2010). Based on this evidence the *Artemia sp*. culture diets used in the second year of experiments were enriched with DHA Selco. This product formula is rich in HUFA. It also contains lipids (min. 67.0%), ash (max 2.0%), DHA/EPA (1), sum 3 HUFA (min. 200 mg/g dwt), vitamin A, D3, E, C (min. 1.500.000 IU/kg, 150.000 IU/kg, 3.600 mg/kg, 800 mg/kg), emulsion droplets (0.5-5 um), and antioxidant (ethoxyquin) needed by crustaceans (http://www.artemia-international.com/default.asp?contentID=582#selco/3/17/2017/2:58pm). Improvement in the 2016 *Artemia sp*. could have be driven by the resolution of this larval deficiency. Even though it was already established that failure of the 2014 *Artemia sp*. diets was because of unsuitable prey size for crab larvae and high concentration of the diet per container volume. Efficiency of the *Artemia sp*. in comparison to the rotifers diet could be enhanced by this enrichment method. However, future studies in RDSC larvae should investigate their fatty acid requirements and highly unsaturated fatty acid synthesis of enzyme systems, hormonal systems, and the effects of substitute and alternative diets and supplements.

Starvation events in the natural marine environment affect larvae development and

survival (Anger et al. 1981). Biesiot and Perry (1995) investigate the biochemical composition of RDSC embryos during development, depletion of their yolk reserves and adult variability of biochemical constituents. They concluded that even when eggs were morphologically (diameter) and developmentally similar, they differ biochemically between individual clutches, showing different patterns of lipids, protein, and carbohydrates loss during embryogenesis. Authors suggested that biochemical variability of embryos is important to their migration as Z1 larvae, which would use yolk reserves if their migration is not timed with spring plankton bloom or suitable plankton patches. As mentioned by Anger et al. (1981), this requires the need for suitable prey for larvae as a starting biochemical cue to begin zoeal development. Biochemical studies in RDSC larvae are crucial to understand specific enzymatic and hormonal systems controlling molting. This will allow us to convey better supplemented diets and timing to offer for larval development, in ways to increase their health and survival through development stages.

Based on both studies in 2014 and 2016, temperature affected the survival of the red deepsea crab larvae. But effects in the development stage did support this result even after I observed higher temperature producing faster development. This may be due to a number of unexpected changes during the experiment, or the lack of representation of each larval stage in most treatments. Even though Perkins (1973) explained how higher temperature prevents the growth of harmful pathogens that could interfere with larvae growth and development. Van Heukelem et al. (1983) compared the effects of different temperatures in juvenile crabs' development and found that 9-15°C as the optimal temperature range for juvenile survival. Optimal temperature range for larval red deepsea crab could vary as they migrate and their development progress. This could present a challenge for further research in aquaculture for the species, but further investigations are needed to determine theses critical values.

NOAA's *Mission of Science, Service, and Stewardship*, is supported by this project supplying a better understanding of commercial species and educating the public about the species. The James J. Howard Laboratory at Sandy Hook, NJ is highly committed to community education and outreach, and it hosts a diverse group of visitors every year. The project had the potential to connect and influence the public; it was something new, interesting, and people can relate to this important fisheries resource, several outreach activities were conducted on the RDSC research. Word spread about the new species being held in the laboratory facilities and captured the interest of the local media. As a result, the local newspaper published an article about the red deepsea crab and also made an informative video, with the participation of students, focusing on the impact studies of climate change and ocean acidification in marine species. The link to this video can be found at:
http://www.app.com/story/news/education/2014/07/22/crab-research-sandyhook/13028411/

A better understanding about the hatching periodicity duration and peak of hatch was gained. Contributions were made obtaining data on the effects of temperature and diet in the survival and development of larvae red deepsea crab. These discoveries will help future studies in aquaculture, reproductive biology, and management of the species. It represents a baseline to new research exploring the effect of climate change in the deep sea red crab population and associated living marine resources.

5.2. Future recommendations

Efficient and constant communication with the fishermen is crucial to organize the experiments depending on their schedules. The fisherman collaboration is crucial to obtain the crabs on time to perform the experiments, also provide important input about the conditions where the adult crabs are captured. During 2014 experimental period, crab arrival was at the

beginning of July which extended the experimental season longer than expected. In addition, just two females arrived with viable eggs that hatched in the laboratory most female's egg were immature, since their capture was at the end of the hatching season. Similar happened in July of 2015, but none of the females came with viable eggs and all were females without eggs or immature that year. In 2016, we captured females from May and late July (missing June peak of hatching because of weather conditions prevented fishing trips), this time we obtained a much better range of eggs development stages. If it possible I will suggest obtaining females around their reproductive maturity size 65-75 mm CL (80-91 mm CW) to target the first extruded eggs by the young females. The lack of genotypical information in the species does not allow to currently explore the option of phenotypical trades selection (as harder shell, faster molting or swimming) in the larvae to improve their survival. By using genotypical markers selection this can be develop in laboratory studies.

During 2016 experimental year, the adult Females Husbandry and Hatching Collection System (FHCS) was subjected to a lot electrical and water malfunctions. The main water intake from the Sandy Hook Bay to the laboratory was being changed and in the process water quality (metal, substrates, etc.) was affected. Even with the filtration system it was challenging to avoid clogging and maintaining the buckets containing the females and the collector clean; which impeded the measurement of larvae volumes and affected female's health causing stress or even dead.

Another inconvenience with the system was the continuous problems with the electric system. Weather inclemency would shut down main chillers of the building and increase temperature in the female's tanks. It is important in future studies to secure the water quality and

temperature of the adult females placing them in a recirculating system with individual chillers since temperature changes can drastically affect the survival of this organism.

Biesiot and Perry (1995) mentioned that clutches of crabs were infested with nematodes and were stripped off by the female in the normal process of grooming the clutches. Other clutches were stripped off for unknown reasons after 6-10 weeks, even when the embryos look normal. The females kept in the FHCS exhibited similar behavior grooming and venting clutches. In addition to nematodes some fungi were observed on the eggs, some dispersed in the whole clutch avoiding any further embryo development. Further investigations should be done to include antibiotics that avoid the proliferation of these pathogens in the egg clutches during husbandry.

Larvae in the collectors' mesh of the FHCS were combine into one mesh and set in a cup with cold filtered seawater, during transfer to allow larvae to be suspended and swim. Suspended larvae should be sorted with a plastic pipette and transfer to small beakers with cold filtered seawater in groups of 10 or 20 larvae. Maintaining a desirable temperature for the larvae at this time is very important. During 2016 transfer, the electric system was not working in the laboratory. Temperature in FHCS increase and also during the counting process, ice was used to maintain a cold temperature for the larvae. This initial drastic temperature change could affect the whole development and survival of the larvae. Further studies should prevent handling of larvae in unstable temperature. The use of a reliable cold seawater source or cold room should be secured before performing the larval transfer.

The change in aquaculture system from the 2014 LFS I to 2016 LFS II was driven by the necessity of modification to improve the larvae survival and daily maintenance and handling of the larvae in the system. The 2014 LFS I provided a continuous input of UV-filtrated seawater

from an additional filtering system to the main in the laboratory. Also, system container removal, and maintenance, and larvae handling was easy for the daily bases. Even though the inflow rate (12 L /5.1 h) was too slow to remove diet residuals or keep larvae in suspension, this cause problems with pathogens growing in the larvae as they stayed in the flat bottom of the containers. Also, the light cycle was not automatic in the laboratory where it was set up. The 2016 LFS II provided a higher inflow rate (0.25 L /min), automatic light cycle set up, and a conical shape that minimize the accumulation of debris and facilitated handling and larvae observation. On the other hand, the recirculating system was not working to its full potential and water changes were required, the conical mesh for larvae collection had small crevices that trap the larvae, and the circular water bath tanks minimize the maneuver space around the upwellers. I suggest rectangular water bath tanks, a more establish recirculation system, and a change in the conical mesh for the 2016 LFS II. It could be the optimal aquaculture system prototype for the study of crab larvae and other aquatic species larvae. Constant and careful cleaning and maintenance of the LFS I and LFS II were crucial for the success of the experiments. Even when it is done as a precaution to prevent growth of pathogens, wiping containers, draining upwellers and cleaning meshes are a risk of touching or losing larvae. It may be advisable to facilitate maintenance adding antibiotics or improving filtration system.

My studies will help jump start further research studies about this species. It provides important tools and information to run aquaculture operations for the production of the red deepsea crab larvae. These operations could supply larvae to investigators who would develop research to answer questions about the species general biology, increasing the knowledge about the species and related organisms that share the unique characteristics of the species (deep water, biennial cycle, planktotrophic larvae). My research can also serve as basis for the development

of future stock enhancement management strategies for the species. Growing larvae when they are more vulnerable and then introducing them again as juveniles in nature will supplement the wild population. The possibilities for use of this information are vast, and I'm proud of contributing to the world with my grain of sand.

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APPENDICES

Appendix A

Culturing rotifers to be used as larval fish food

OVERVIEW:

Rotifers are to be used as the primary food for larval windowpane and cunner up until the time that the fish are large enough to consume *Artemia*. Rotifer populations will be grown on a 3 or 4 day schedule with Day 0 being the take down (transfer) day. The feed-out level of rotifers will be at 5 rotifers per ml in the larval rearing containers. Approximately 320,000 rotifers will be added to each rearing container each day, or 7.8 million per day for a 24-container rearing experiment. Algae is to be used as last stage of enrichment of rotifers while rotifers are condensed prior to feed-out. Algae may also have a additional benefit to the larval fish rearing system.

ITEMS NEEDED:

1. Algae culture starting stock
2. Rotifer starting stock
3. 18-L algae carboys
4. 120-L algae Kalwells (fiberglass cylinders)
5. 120-L rotifer Kalwells
6. Algae formula (F/2 parts A and B)
7. Rotifer formula (Selco 3000)
8. Fluorescent lamps and light timer
9. Air pumps
10. Air stones
11. Air tubing
12. Blender
13. 100 ml, 1-L, and 2-L beakers
14. Rotifer sampling ladle
15. Segwick-Rafter 1 ml counting chamber
16. Dissecting microscope (12X)
17. Hand counter
18. 53-µm Mesh plankton collector
19. Filtration System with UV sterilizer
20. Filtered and sterilized seawater

PROTOCOL:

ROTIFER CULTURE

1. Obtain starter stock of approximately 10 million *Brachionus* as available in house or from colleagues in the North East Fisheries Science Center (NEFSC).

2. Fill 120-L Kalwell with 60 L of UV-sterilized and 0.5 -µm filtered seawater. Blend Selco 3000 into UV-sterilized and 0.5-µm filtered seawater in the following amounts:

Day 0 – 0.48 g per million rotifers into 1,200 ml of seawater, Days 1, 2, and 3 – 0.4 g per million rotifers into 1,000 ml of seawater

3. Blend Selco and seawater in blender for 5 minutes. Use a 200 ml aliquot of the blended solution for each of the 5 daily feedings (0630 to 1830) with the exception of Day 0 when an additional 200 ml serves as a background feeding into the new Kalwell an hour before the addition of the rotifers.

4. Count rotifer density in duplicate before first feeding. The average of the two, 1 ml counts will be used to identify the weight of Selco 3000 to be used for the day and to determine the volume of rotifers to be harvested for feed-out. Rotifers will be slowed or killed after sampling from Kalwell but prior to count by adding a drop of Chlorox to the Sedgwick-Rafter counting chamber.

5. After each feed out of 200 ml, cover the remaining solution and place it in the refrigerator. Stir the solution before each use.

6. On transfer day, the residual rotifers not harvested will be collected in a 53-µm mesh bag, rinsed with UV-sterilized and 0.5 -µm filtered seawater, and transferred to a new Kalwell.

7. For purposes of feed-out to larval rearing containers, the volume to be condensed in the 53-µm mesh bag or sieve will be determined by the daily counts and will be condensed into a bath of *Isochrysis* harvested without concentration immediately prior to condensing of the rotifers. The volume of algae will be > 200 ml but exceed 500 ml per feed-out tank and will be chosen so as to simplify the division of the volume by the number of rearing containers requiring feeding (e.g., 24 rearing containers each will receive 250 ml so an initial volume of 6 L of algae will be harvested).

8. The rotifers and algae will be placed in 2 to 3-L beakers, aerated, and brought to the temperature of the larval rearing containers by floating the beaker in the same water bath used to control temperatures of the larval rearing containers.

ALGAE CULTURE

1. Obtain starter stock of *Isochrysis* (var. Tahitian) from NMFS Laboratory at Milford, CT.

2. Prepare seawater media for algae by adding F/2 parts A and B to UV-sterilized and 0.5 -µm filtered seawater in the three culture containers (two groups in small volume – 14 L in 18-L carboy – and one group in a large volume – 72 L in 120-L Kalwell) in concentrations prescribed on containers.

3. Split starter stock into two groups (one half stocks the two small volume containers and the other half stocks the one large volume container)

4. Harvest algae from the large volume container as needed to provide transport volume for rotifers at feed-out

5. Restart small and large algae culture containers every 2 weeks or sooner if more volume is needed (Kalwell culture can be re-upped to starter volume after each draw down for feed-out).

Appendix B

Culturing *Artemia* to be used as larval and juvenile fish food

OVERVIEW:

Brine shrimp, *Artemia* sp., are to be used as the primary food for late larval and juvenile windowpane and cunner. The time at transition from rotifers as prey to *Artemia* will be based on an assessment of the sizes of fish (per treatment combination) with respect to their mouth size and ability to capture and consume *Artemia*. *Artemia* are approximately 450 µm at hatching. The first instar is non-feeding and lasts approximately 24 h at 20 ºC. At 46 h after addition of cysts (and 2 h before feed out) the *Artemia* are enriched with DHA Selco in order to improve their nutritional quality as a food source for larval and juvenile fish. The *Artemia* process is a two-part protocol. Although the hatch out and enrichment of the *Artemia* is fundamental to their use as a food source, the first step in the process concerns the decapsulation of the *Artemia* cysts in order to remove the non-digestible capsule surrounding the *Artemia* embryo.

ITEMS NEEDED:

1. *Artemia* decapsulated cysts (Inve Aquaculture - 801 876-2002)
2. *Artemia* hatcher(s) (2)
3. Carboy, topless w/ lid (2)
4. Immersion heater(s)
5. Squirt bottle
6. 0.5-µm filtered, UV-sterilized seawater (UVSW)
7. UVSW wet/dry scoops
8. DHA Selco enrichment media or like product (Inve Aquaculture)
9. Aeration pump, vinyl and rigid tubing
10. 50-70-µm mesh bag
11. 125-µm 6”-diameter sieve
12. Deionized water
13. Stem thermometer (2)
14. Spigot bucket
15. 2-L plastic beaker (1)
16. Plastic dish pan
17. 100-µm mesh bag with clips
18. 2000 ml *Artemia* scoop
19. UVSW hose

PROTOCOL:

HATCHING:

1. Fill Plexiglas *Artemia* hatcher with 12L of 0.5-µm filtered, UV-sterilized seawater at approximate salinity 27 ppt and 28°C (heat with immersion heater to obtain and maintain this temperature: always keep it under 30 C). Use rigid tube securely fixed to very bottom of hatcher to aerate vigorously. Add decapsulated cysts into hatcher. Add sticker to hatcher stating day of week and AM/PM.

2. Periodically wash down cysts from interior hatcher walls with UVSW squirt bottle.

3. Hatching is complete in approximately 24 h. **Unplug heater and remove air** after 24 h to start transfer process.

4. Hatchers are started every morning.

TRANSFER PROCESS:

1. Pour 12 L of UV seawater into carboy A from 'Assigned' *Artemia* UV seawater buckets. Leave carboy B empty. Set both carboys on carboy shelf for later use. Have air lines and lids ready for carboys.

2. Clip 100-µm mesh bag to inside of spigot bucket. (Spigot bucket is located under hatchers, 100-µm mesh bag is by the sink and the rubber tube is on shelf next to hatchers). 100-µm mesh bag has 'Artemia' written on it.

3. Attach rubber tube to end of nosel on *Artemia* hatcher. Clip other end of rubber tube into 100-µm mesh bag. Tube should be pointed down so *Artemia* will be captured in mesh bag.

4. Turn handle on hatcher to allow the *Artemia* to drain through rubber tube into mesh bag. Use a UV seawater squirt bottle to wash down *Artemia* from interior hatcher walls. When the hatcher completely drains, pour 1 L of UV seawater into hatcher to flush remaining *Artemia* in rubber tube.

5. Remove rubber tube from mesh bag and unclip. Briefly lift mesh bag with *Artemia* to drain water. Quickly move mesh bag over carboy A and invert underwater to allow *Artemia* to distribute into carboy. A UV seawater squirt bottle may be needed to remove *Artemia* from mesh bag seams. Once all *Artemia* are in carboy A, place lid on carboy and place aeration tube through lid and secure so the tube is pointing to the lowest part of the carboy. Prop carboy up on upside down cups behind carboy to prevent buildup of *Artemia* on the bottom.

6. Clean and rinse hatching tank, heater, and airline.

7. Begin to prepare SELCO enrichment for carboy A.

***ARTEMIA* ENRICHMENT**

1. Mix DHA SELCO enrichment media (kept tightly capped in refrigerator) with 500 ml of UV seawater. Add 0.6g DHA Selco per L of *Artemia* culture being fed (i.e., 12 liters of *Artemia* culture in a carboy will receive 3.6g of DHA SELCO). Mix vigorously for 1 minute.

2. Add proper volume to carboy A. Add sticker with day and AM/PM to carboy next to 'Enriched' sticker. SELCO is done the night before carboy is used for feed out. (In the event you need carboy the same day, wait 2 hours after SELCO before feed out).

FEED OUT

1. The density of *Artemia* to be fed to fish is required. First collect a small sample (approximately 40 ml) with marked *Artemia* beaker (located next to *Rotifer* counting station) from SELCO enriched carboy, heat in microwave (20 seconds maximum), take to lab bench, swirl, and draw off 1 ml subsample for counting. Place subsample in a Sedgwick-Rafter chamber and count the number of *Artemia* per ml using microscope. *Artemia* should be counted twice and averaged to get density.

2. Adjust density and feed out volume on designated *Artemia* feed out sheet. Also adjust feed out sheet from background check sheets for basins/tubs as needed.

3. Reference *Artemia* feed out sheet to see what volume is needed from carboy A. Pour proper volume of contents of carboy through 125-µm 6"-diameter sieve that is labeled for *Artemia* only with proper tray underneath in the sink. Rinse Artemia with DI while still in the sieve.

3. Transfer contents of sieve to appropriate beaker using DI squirt bottle. Add appropriate 'ppt' water to bring up volume as allocated on *Artemia* feed out sheet for larval/juvenile rearing containers. Sieve any large particles of SELCO from the beaker using the small blue sieve.

4. Feed *Artemia* to fish once or twice daily as allocated on *Artemia* feed out sheet.

ABBREVIATED ARTEMIA HATCHING/FEEDOUT LIST

1. Each morning,
    A. **Start a new hatcher**.
        1) Add 12L UVSW to empty hatcher.
        2) Plug in heater and add air line.
        3) Add Artemia cysts.
        4) Add label to hatcher indicating when it was started.

    B. **Transfer 24hr old hatcher**.
        1) **Unplug heater**.
        2) Remove air.
        3) Set up mesh bag in bucket with hose connected to the hatcher nozzle with the other end in the mesh bag.
        4) Open hatcher spigot and drain hatcher. (They should always be in water.)
        5) Meanwhile, fill clean carboy with 12L UVSW.
        6) Drain mesh bag and flip Artemia contents into the carboy. Use squirt bottle to get excess Artemia off into carboy.
        7) Add lid with air tube through it to the carboy pointing downward and prop carboy up on cup. Add sticker next to 'Transferred' indicating when it was moved.
        8) Clean hatcher, air tube and heater. Remember to shut the nozzle.

C. Feedout

1) Count density of enriched carboy (enriched the night before).
2) Update feedout sheet on the computer adding density, changing date, changing feedout densities is necessary and print.
3) Pour designated volume ('total Artemia volume needed (L)') into the blue strainer over the sieve. Make sure the sieve is in the pan and not touching the bottom of the sink.
4) Drain sieve and rinse Artemia with DI water.
5) Transfer condensed Artemia from sieve to 2L plastic beaker. Use DI squirt bottle.
6) Add proper 'ppt' water to beaker to bring it up to the amount shown on sheet in the 'Selco-Artemia condensed and placed into:' box.
7) Feedout using the amounts designated on the sheet under 'feed out volume per container (ml).'

2. Each evening before leaving,

A. Enriching

1) Only enrich carboys that have spent 24 hours in the hatcher with heat, and another 12 hours in the carboy. Enriching will take place the PM before AM feedout. (Total 48 hours). Enrich the hatcher that was transferred the same AM.
2) Weigh out 3.6g of Selco, located in the fridge in L115 using designated spoon and beaker (above sink in L115). Add 400-500 ml UVSW and stir vigorously for 1 minute.
3) Add mixture to carboy and add sticker indicated when enrichment took place.
4) Rinse beaker and spoon.

Appendix C

Morphological measurements obtained from adult females transported to the James J. Howard Sandy Hook Laboratory, NJ and legend

			Date			Carapace (mm)		Abdominal Segment (mm)			
ID	**Year**	**Female ID**	**Transported**	**Measured**	**Shell**	**Length**	**Width**	**Length**	**Width**	**Eggs**	**Egg/Color**
I	2014	I14	7/9/14	7/10/14	4	73.24	90.08		41.95	3	O-Br
II	2014	II14	7/9/14	7/10/14	3	93.24	114.44		52.1	3	O-Br
III	2014	III14	7/9/14	7/10/14	3	92.4	113.94		52.94	3	O-Br
IV	2014	IV14	7/9/14	7/10/14	3	114.07	140.18		66.62	3	O-R
V	2014	V14	7/9/14	7/10/14	4	94.76	119.16		55.58	3	RBR
VI	2014	VI14	7/9/14	7/10/14	4	86.48	104.85		50.62	3	RBR
VII	2014	VII14	7/9/14	7/10/14	3	87.04	111.48		50.48	2	O-R
VIII	2014	VIII14	7/9/14	7/10/14	4	82.81	105.48		45.82	4	DBR
IX	2014	IX14	7/9/14	7/10/14	2	92.22	116.59		52.82	4	RBR
X	2014	X14	7/9/14	7/10/14	4	82.26	105.2		47.84	3	O-R
XI	2014	XI14	7/9/14	7/10/14	4	93.98	110.98		50.05	4	DBR
XII	2014	XII14	7/9/14	7/10/14	4	101.08	122.66		60.47	5	DBR
XIII	2014	XIII14	7/9/14	7/10/14	3	92.86	113.71		59.73	3	O-R
I	2015	I15	7/8/15	7/10/15	2	80.68	101.5	17.32	41.56	5	DBR
II	2015	II15	7/8/15	7/10/15	3	84.32	111.65	13.34	46.09	5	DBR
III	2015	III15	7/8/15	7/10/15	3	95.22	115.47	18.71	53.54	5	DBR
IV	2015	IV15	7/8/15	7/10/15	2	92.01	118.77	19.04	50.37	5	DBR
V	2015	V15	7/8/15	7/10/15	3	86.05	110.04	16.52	50.04	5	DBR
VI	2015	VI15	7/8/15	7/10/15	2	85.89	115.89	19.54	52.14	5	DBR
VII	2015	VII15	7/8/15	7/10/15	3	93.57	113.29	19.45	56.59	5	DBR
VIII	2015	VIII15	7/8/15	7/10/15	2	81.51	103.98	13.67	45.46	5	DBR

IX	2015	IX15	7/8/15	7/10/15	2	84.89	106.01	16.75	42	2	DBR
1	2016	1_16	4/27/16	5/4/16	4	85.7	107.8	14.7	50.3	4 & 5	DBR
2	2016	2_16	4/27/16	5/4/16	3	91.3	120.2	16.4	51.9	4	DBR
3	2016	3_16	4/27/16	5/4/16	4	88.6	112.1	16.9	54	4	DBR
4	2016	4_16	4/27/16	5/4/16	4	82.8	107.4	14.1	43.1	4	DBR
5	2016	5_16	4/27/16	5/4/16	3	76.7	98.7	12.9	40.9	4 & 5	DBR
6	2016	6_16	4/27/16	5/4/16	3	77	94	13	41.7	4	DBR
7	2016	7_16	4/27/16	5/4/16	3	84.7	107.1	15.4	40.6	4	DBR
8	2016	8_16	4/27/16	5/4/16	3	81.5	103.5	14.5	41.9	4	DBR
9	2016	9_16	4/27/16	5/3/16	3	82.3	99.6	13.6	39.1	4	DBR
10	2016	10_16	4/27/16	5/3/16	2	94.3	109.5	18.2	52.6	4	DBR
12	2016	12_16	5/24/16	5/31/16	3	86.8	108.9	15.9	45.8	4 & 5	DBR
13	2016	13_16	5/24/16	5/31/16	3	86.4	112.5	16.4	47.6	4 & 5	DBR
14	2016	14_16	5/24/16	5/31/16	3	73.5	93.8	14.1	39.6	4	DBR
15	2016	15_16	5/24/16	5/31/16	3	86.7	112.7	16	50.2	4 & 5	DBR
16	2016	16_16	5/24/16	5/31/16	3	89.3	112.5	16.2	47.9	5	DBR
17	2016	17_16	5/24/16	5/31/16	3	79.5	102.6	14.1	42.3	3	DBR
18	2016	18_16	5/24/16	5/31/16	4	86.9	107.7	15.8	46.5	3	O-R
19	2016	19_16	7/21/16	7/27/16	3	81.8	100.3	15	43.6	5	DBR
20	2016	20_16	7/21/16	7/27/16	3	84	105.2	15.1	42.6	5	DBR
21	2016	21_16	7/21/16	7/27/16	3	88.2	114.9	17	48.3	5	DBR
22	2016	22_16	7/21/16	7/27/16	3	102.9	125.4	21	54.9	5	DBR
23	2016	23_16	7/21/16	7/27/16	2	94.6	118.8	16.3	50.6	5	DBR
24	2016	24_16	7/21/16	7/27/16	3	85.2	112.3	17	50	5	DBR
25	2016	25_16	7/21/16	7/27/16	3	95.6	114.9	51.8	18.5	5	DBR
26	2016	26_16	7/21/16	7/27/16	3	87.6	112.4	16.1	43.7	5	DBR
27	2016	27_16	7/21/16	7/27/16	4	88.6	113.2	48.2	17.7	5	DBR
28	2016	28_16	7/21/16	7/27/16	2	82.3	103.7	14.5	42.5	5	DBR

Legend

Codes	Shell	Eggs
0		Absent
1	Soft	New, bright orange, pre blast
2	New, clean	Embryo stage, orange
3	Hard, some scratches	Eyed, late develop, dark orange
4	Old, dark	Eyed, dark brown-purple (pre hatching)
5		Hatching
6		Empty egg cases

Egg/Color	
W	White
B	Beige
Y	Yellow
O	Orange
O-BR	Orange-Brown
BR	Brown
O-R	Orange-Red
RBR	Red-Brown
DBR	Dark Brown
NF	not Found