ACCLIMATION OF PACIFIC WHITE SHRIMP *Litopenaeus vannamei*

TO LOW-SALINITY AQUACULTURE

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I dedicate this work to a place that is very special to me, my home, Hawai‘i.

I do this for my ‘ohana, both immediate and extended families.

Especially for my parents, Charles and Lillian Kuehu,

who never had the opportunity to go to college,

but provided me with everything I needed

so that I could go out into this world

and make a positive difference.

'O wau nō me ka ha‘aha‘a

ame ka ho‘omaika‘i.

Mahalo.

Eō.
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ABSTRACT

Pacific White Shrimp (*Litopenaeus vannamei*) has been developed for intensive breeding and rearing techniques, and is the globally commercially cultured shrimp species. A process for rearing healthy and viable stocks, capable of thriving in low-salinity grow-out systems furthers the development of intensive production of *L. vannamei* in the shrimp industry. This research investigated the development of an integrated model to improve health and survival of *L. vannamei* postlarvae during acclimation to a low-salinity environment by considering four factors, acclimation rate, salinity end-point, ionic and probiotic enhancements. The results showed survival was over 90% with a salinity end-point of 2 ppt at both 2 and 4 day rates. At 0 ppt salinity end-point, survival was 74.4% at 4 days, and 22.2% at 2 days. Probiotic and ionic enhancements did not improve over the non-enhanced, except at the higher 2 ppt salinity end-point at the 4 day rate, indicating potential for long-term benefits.
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2 PPT Mean ± SE

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LIST OF ABBREVIATIONS

Acclimation Rate

2D Accelerated 2 Day Process

4D Gradual 4 Day Process

Salinity End-point

0 ppt Zero parts per thousand

2 ppt Two parts per thousand

32 ppt Thirty two parts per thousand

Enhancements

P Probiotic

I Ionic Composition

PI Combined Probiotic and Ionic Composition

NPI No Probiotic or Ionic Composition

C Control
CHAPTER 1

INTRODUCTION

1.1 Background

Shrimp is the most important commodity by value in the international seafood trade with yearly exports in 2003 exceeding USD$10 billion (Lem, 2006). Worldwide shrimp production including both wild capture and aquaculture grew from 2.4 million tonnes in 1987 to 4.1 million tonnes in 2000, to a record high of 6.5 million tonnes in 2007 (Josupeit, 2010). The world’s population consumes 36 pounds of live weight equivalent of seafood per capita each year. Shrimp ranks number one in the United States (U.S.) per capita consumption of fish and seafood, with annual consumption of 3.7 pounds per person, followed by 3.1 pounds of tuna, and 2.0 pounds of salmon (Damron, 2008). The main shrimp producing country in the world is China with 2.5 million tonnes, followed by Thailand, Indonesia and India, each producing over 500,000 tonnes of shrimp production in 2007 (Josupeit, 2010). The growth in aquaculture production accounts for more than half of the total world shrimp production, overtaking the wild capture production. *Litopenaeus vannamei*, Pacific white shrimp is by far the main species cultured (Damron, 2008; Josupeit, 2010).

Aquaculture is the farming of aquatic organisms, including fish, mollusks, crustaceans, reptiles, and aquatic plants in freshwater, brackish water or saltwater. The opportunities for aquaculture are largely due to the demand for fish and seafood exceeding the capacity for the supply of wild capture. One of the reasons is directly related to the increase in the world population. If seafood supplies are to help maintain
the human population, then alternative sources other than wild capture is needed (Damron, 2008).

1.2 Statement of the Problem

In 2012, consumers in the United States spent an estimated $82.6 billion on seafood, making the U.S. one of the top three seafood markets worldwide, along with Europe and Japan (Josupeit, 2010; Rubin et al., 2014) The U.S. domestic farm gate value of aquaculture products alone approaches $1.3 billion annually, resulting in the U.S. demand relying heavily on international imports. Aquaculture productivity in the U.S. is currently driven by annual Federal investments in research and development providing an input of approximately $100 million. Congress and the Executive Branch passed the National Aquaculture Act in 1980 that stated, “It is, therefore, in the national interest, and it is the national policy, to encourage the development of aquaculture in the United States” (Rubin et al., 2014)

Expansion of the aquaculture industry continues to be a challenge in the United States because production sites need land and water, both of which are decreasing in supply. Marine sites are also guarded by environmental groups fearing unwanted ecological changes, the threats of genetic alterations in the indigenous species, pollution, and ecological systems degradation (Damron, 2008). Greenpeace described shrimp farming as “…an unsustainable industry, migrating from place to place, leaving behind a trail of degraded landscapes stripped of biodiversity, and destitute people.” (http://www.greenpeace.org/politics/wto/shrimp.html) In response to industry pressures, the Global Aquaculture Alliance (GAA) was founded in 1997 to help unite the aquaculture industry by providing proactive guidance toward sustainable aquaculture.
They released the “Codes of Practice for Responsible Shrimp Farming”, which states that “…farms should not be built on ecologically sensitive mangrove areas or other wetlands” (Chamberlain, 2010; GAA, 1999).

Following the development of the Codes of Practice for Responsible Shrimp Farming in 1999, GAA organized the effort to oversee the Best Aquaculture Practices (BAP) certification addressing issues regarding environmental, social, food safety and traceability throughout the aquaculture production chain (Chamberlain, 2010). Adding support for environmental concerns, the Environmental Protection Agency (EPA) in 2004 issued its final rule, 40 CFR Part 451, “Effluent Limitations Guidelines and New Source Performance Standard for the Concentrated Aquatic Animal Production Point Source Category”, establishing the Clean Water Act (CWA) effluent limitations guidelines regulating performance standards for concentrated aquatic animal production facilities (EPA, 2004).

The development of inland culture of shrimp with reduced water exchange enabled shrimp production in low-salinity water. Low-salinity farming techniques are similar to those practices used in coastal areas (Chamberlain, 2010), except the ability to move shrimp production inland reduces the environmental risks to coastal and marine ecosystem degradation. Inland shrimp farming now accounts for a significant share of total production in China, Thailand and other regions (Chamberlain, 2010).

1.3 Purpose and Importance of the Research

The purpose of this research is to develop an integrated process for acclimation of L. vannamei postlarvae to low-salinity for aquaculture. The importance of this research
is to further the knowledge of the acclimation process by experimenting with treatments that affect survival rates of *L. vannamei* postlarvae. Increasing the understanding of this process will help to support inland shrimp farming which requires a supply of seed stock, postlarvae and juveniles acclimated to low-salinity water conditions, preparing them for the grow out environment where they will be raised for food to supply the market (Damron, 2008). To contribute toward the call for action in support of the U.S. national policy on the development of aquaculture, and to further the knowledge regarding shrimp production, this research is aligned with Strategic Goals 1, 4 and 7 of the nine strategic goals identified in the National Strategic Plan for Federal Aquaculture Research (2014-2019) (Rubin *et al.*, 2014):

Strategic Goal 1: Advance Understanding of the Interactions of Aquaculture and the Environment

Strategic Goal 2: Employ Genetics to Increase Productivity and Protect Natural Populations

Strategic Goal 3: Counter Disease in Aquatic Organisms and Improving Biosecurity

Strategic Goal 4: Improve Production Efficiency and Well-Being

Strategic Goal 5: Improve Nutrition and Develop Novel Feeds

Strategic Goal 6: Increase the Supply of Nutritious, Safe, High-Quality Domestic Seafood

Strategic Goal 7: Improve Performance of Production Systems

Strategic Goal 8: Create a Skilled Workforce and Enhance Technology Transfer
Strategic Goal 9: Develop and Use Socioeconomic and Business Research to
Advance Domestic Aquaculture

1.4 Research Questions and Hypotheses

This research will contribute to the literature furthering the development of
acclimation to inland shrimp culture conditions by determining the acclimation rate of
salinity reduction, the salinity end-point, and the effects of probiotic and ionic
eenhancements with interactions for optimum survival of the *L. vannamei* shrimp
postlarvae. This investigative effort will focus on three research questions and
hypotheses:

Research Questions:

1. What rate of reducing salinity of natural sea water is optimal for the survival of *L.
   vannamei* postlarvae during acclimation between an accelerated rate and a
   gradual rate?
2. What salinity end-point after reducing salinity of natural sea water is optimal for
   survival of *L. vannamei* postlarvae after acclimation between fresh water and
   brackish water?
3. What are the effects on *L. vannamei* postlarvae survival when probiotic, ionic and
   a combination of probiotic and ionic enhancements are applied during low-
   salinity acclimation?

Hypotheses:

1. *L. vannamei* postlarvae acclimated to low-salinity has a higher survival rate when
   processed at a gradual rate.
2. *L. vannamei* postlarvae acclimated to brackish water has a higher survival rate over freshwater at a higher salinity end-point.

3. *L. vannamei* postlarvae acclimated to low-salinity has improved survival rates with probiotic, ionic, and a combination of probiotic and ionic enhancements.

1.5 Organization of the Thesis

Chapter 1 of this thesis presents the background of the research, the statement of the problem, the purpose and importance of the research, and the research questions and hypotheses. Chapter 2 presents the literature review, and a conceptual framework guiding the research, and validity and reliability measures. Chapter 3 presents the methodology including the research design, a description of the setting, sample, instrumentation, data collection procedures, and the research assumptions. Chapter 4 presents the findings of this research, the data and statistical analysis of the results. Chapter 5 discusses and summarizes the findings, research limitations, implications of the research, and provides recommendations and conclusions. Finally, the references conclude this thesis.
CHAPTER 2

LITERATURE REVIEW

2.1 Review of the Literature

This chapter describes the focus areas of this research related to four factors affecting *L. vannamei* shrimp postlarvae survival undergoing low-salinity acclimation: acclimation rate, salinity end-point, and two enhancements (probiotic, ionic, and a combination of probiotic and ionic). In order to provide adequate knowledge to comprehend the nature of this study’s three research questions in which hypotheses can be constructed, the taxonomy, anatomy, life cycle, and physiology will be reviewed to develop a conceptual framework for understanding the biological aspects of *L. vannamei* affected during the acclimation procedure. Scientific literature review will provide support for the selection and utility of the factors investigated with consideration of research assumptions and limitations.

2.2 Conceptual Framework

Taxonomy

A conceptual framework based on the biological aspects of *L. vannamei*, helps to provide a basis for the choices made in the experimental design in order to obtain the data necessary in this investigation. The taxonomic classification of the genus and species *Litopenaeus vannamei*, originates from the Domain Eukaryota, Kingdom Animalia, Phylum Arthropoda, Class Malacostraca, Order Decapoda and Family Penaeidae (Fransen *et al.*, 2015). The Phylum Arthropoda includes insects, spiders, lobsters, shrimp, crabs, etc. The physical characteristics common to the arthropods are:
bilateral symmetry, segmented body, hard exoskeleton, jointed legs, and many pairs of limbs. Bilateral symmetry \((bi = \text{two}, \ latus = \text{side})\) means the right half of the animal is a mirror image of its left half. The segmented body is divided into sets of internal and external series of segments that are grouped into larger units that often have slight variations such as the abdomen and cephalothorax. The hard exoskeletons are made of chitin, which is like a form-fitting suit, produced by the “skin” and then hardened into an outer covering, providing protection and preventing water loss, which is shed periodically and regrown as they outgrow them. All arthropods \((arthro = \text{joint}, \ pod = \text{foot})\) have jointed limbs, where the joints are softer and bendable, allowing movement of a hard exoskeleton. Arthropods have many pairs of limbs, evolving from one pair of limbs on each segment, into new shapes and specialized for different functions such as limbs for swimming, walking, feeding, defense and sensory (Fig. 1).

Figure 1. Phylum Arthropoda, Subphylum Crustacea taxonomic classification for \textit{L. vannamei} (Understanding Evolution, 2016)
Anatomy

*L. vannamei* belongs to the Family Penaeid (Fig. 2). This family includes the most valuable marine commercial species of shrimp production (Tavares, 2003). The names “shrimp” and “prawn” are sometimes used interchangeably, thereby adding some confusion, but because different regions in the world use the terms differently, it is not consistent. In general, the global common use of the term “prawn” is used for the larger, more laterally compressed forms with a well developed rostrum, while “shrimp” is more commonly used for the smaller forms with a dorsoventrally depressed form, and a poorly developed rostrum. In North America, the term “prawn” is nearly obsolete, and so the term “shrimp” is more generally used to describe these invertebrate animals (Tavares, 2003; Holthius, 1980).

Shrimps constitute a large group of crustaceans varying in size from microscopic to about 35 cm long. Their body is almost always laterally compressed, the rostrum usually compressed and toothed, and the abdomen long, longer than the carapace or head. The antennules, or first pair of feelers, in most species bear a small scale or spine, the stylocerite, at their bases. The antennal scales of the second pair of feelers, the antennae, are generally large and plate-like. The pereiopods or legs are usually slender, but in some, a single leg or pair of legs may be stout, and some pereiopods end in pincers. The pleopods or abdominal appendages used for swimming, are well developed and, most often present on all five anterior abdominal segments (Tavares, 2003).
Life Cycle

Aquaculture life cycles are generally segmented based on the stages of the life cycle of the animal (Fig. 3). The hatchery stage is responsible for maintaining the broodstock, spawning the mature adults, then hatching the fertilized eggs to produce larvae, nurturing them until they are moved to the nursery stage. In the nursery stage, the postlarvae are raised to juveniles, then released to be stocked in a production unit for the growout stage (Damron, 2008).

In the natural life cycle of *L. vannamei*, the juvenile and young adult shrimp migrate offshore to a more stable ocean environment where they complete maturity, mate and spawn eggs. Within twenty four hours of successful mating, spawning occurs as the female releases her eggs into the water, fertilized as they pass through
spermatophores deposited by the male petasma, placed near the thelycum, the female genital duct during mating. Hatching occurs around sixteen hours post fertilization, then the larvae go through three different phases of metamorphosis and biological development by molting: five stages as nauplii, three stages as protozoa, and three stages of mysis, until they complete their last metamorphosis into a postlarvae with a complete adult body plan. Molting or ecdysis is the shedding of the exoskeleton, making way for new growth. During the early postlarvae stage, they are planktonic and are carried toward the shore by tidal currents. About five days after molting into postlarvae, they become benthic and spend their juvenile, adolescent and sub-adult stages in coastal estuaries, lagoons or mangroves (Boone, 1931). *L. vannamei* is found in waters with a wide salinity range between 1 to 40 parts per thousand (ppt) (Davis, 2004). It is the postlarvae stage that this research is based, after metamorphosis has been completed.

![Life Cycle of Penaeid Shrimp](image.png)

Figure 3. Penaeid Shrimp Life Cycle (Bauer, 2006).
Physiology

The Pacific white shrimp is a euryhaline species that can tolerate a wide range of salinities (0.5-45 g L\(^{-1}\)) (Menz & Blake 1980; Bray *et al*. 1994; Roy *et al*. 2010). There are even some indications that it is capable of growing in waters of less than 0.5g L\(^{-1}\) (Araneda *et al*. 2008; Cuvin-Aralar *et al*. 2009; Roy *et al*. 2010). Euryhaline species are commonly found in habitats such as estuaries and tide pools where salinity changes regularly. However, some organisms are euryhaline because their life cycle involves migration between freshwater and marine environments, as is the case with salmon, eels and marine invertebrates. They must be able to handle salt and fresh water conditions. Euryhaline intertidal species employ a variety of different strategies to tolerate mixed salinity waters (Wheatley, 1988). In contrast, stenohaline species can tolerate only a relatively narrow range of salinity (Boundless, 2016).

Osmoregulation is the active process by which an organism maintains its level of water content. It is the relationship between solute to solvent concentrations of internal body fluids. The osmotic pressure in the body is homostatically regulated in such a manner that it keeps the organism’s fluids from becoming too diluted or too concentrated. Osmotic pressure is a measure of the tendency of water to move into one solution from another by osmosis, the diffusion of water through a semipermeable membrane. The metabolic rate of some crustaceans is affected by the osmotic pressure of their environment.

Two major types of osmoregulation are osmoconformers and osmoregulators. Osmoconformers match their body osmolarity to their environment actively or passively. *L. vannamei*, like most marine invertebrates are osmoconformers, although their ionic
composition may be different from that of seawater. Osmoconformers are marine organisms that maintain an internal environment that is isosmotic to their external environment. This means that the osmotic pressure, or osmolarity, of the organism's cells is equal to the osmotic pressure of their surrounding environment. By minimizing the osmotic gradient, this subsequently minimizes the net influx and efflux of water into and out of cells. Even though osmoconformers have an internal environment that's isosmotic to their external environment the types of ions in the two environments differ greatly in order to allow critical biological functions to occur.

Osmoregulators can control their internal osmolarity independent of their environment, because their body fluids are not isosmotic with their surroundings. A benefit to osmoconformators is that organisms don't need to expend as much energy as osmoregulators in order to regulate ion gradients. However, to ensure the correct types of ions are in their desired location, a small amount of energy is expended on ion transport. A disadvantage to osmoconformators is that organisms are subject to changes in the osmolarity of their environment. Ion gradients are crucial to many major biological functions on a cellular level. Consequently, the ionic composition of an organism's internal environment is highly regulated with respect to its external environment. Osmoconformers have adapted so that they utilize the ionic composition of their external environment, which is typically seawater, in order to support important biological functions. For instance, seawater has a high concentration of sodium ions, which helps support muscle contraction and neuronal signaling when paired with high internal concentrations of potassium ions.
Osmoconformers generally tolerate changes in the concentration of body fluids although a slow time course for osmotic equilibration can assist survival in rapidly changing tidal salinities (e.g., Diehl and Lawrence, 1984). Species which actively osmoregulate exhibit a variety of different ionoregulatory mechanisms (Wheatley, 1988).

Crustacea are predominantly aquatic arthropods, and their aquatic respiratory organs are gills formed by epipodites of one or more thoracic appendages. These thin-walled, well-vascularized epipodites are usually assumed to function as gills. Also, the inner surface of the carapace may also have respiratory epithelium which is like that of the gills and supplements the later, or constitutes the main or sole respiratory organ (Waterman, 1960) (Fig. 4 & Fig. 5).

Marine invertebrates are generally isosmotic in 100% salt water (SW) and hyperregulate upon external dilution. In crustaceans this is achieved by actively taking up ions at the gills and eliminating the water load via the antennal gland (kidney analogue). Since the latter can only produce an isosmotic urine it is less than ideal since it constitutes a significant route for ion loss. It is important to emphasize that, while
certain salinities may not be lethal, they may affect successful survival by affecting life-sustaining processes such as molting in crustaceans (Boundless, 2016).

2.3 Factors for Acclimation Treatments

Marine shrimp are traditionally cultured in coastal or estuarine waters. The Pacific white shrimp, *Litopenaeus vannamei* is found in waters with a wide salinity range between one to forty parts per thousand (ppt). The high tolerance of *L. vannamei* to low salinity makes this species an excellent candidate for inland farming (Davis, 2004). Although penaeid species exhibit tolerance to a wide range of salinities, this does not imply that animals can grow well at all the salinities. In order to determine optimal conditions for rearing *L. vannamei* in low salinity waters, it is also necessary to understand its salinity tolerance limits and effect of age on low-salinity survival. Moreover, ionic composition of water, and particularly calcium, potassium and magnesium ions have been shown to be more important than salinity itself in low-salinity aquaculture (Jayasankar *et al.*, 2009). Phosphorous (P) is a mineral required by penaeid shrimp because of its limited availability under rearing conditions. Phosphorous is mainly associated with calcium (Ca) in the exoskeleton. Studies have suggested that dietary Ca/P ratio should be considered, as excessive dietary Ca may result in increased P requirements of shrimp (Li *et al.*, 1986, Cheng *et al.*, 2006).

Optimal conditions for the tanks requires supplying each with dissolved oxygen at a rate of 5 mg/l and kept at ambient temperature ranging between 28°C to 30°C (Whetstone, *et al.* 2002; Davis *et al.*, 2004).
Acclimation Rate

Low-salinity rearing of *L. vannamei* requires the transfer of postlarvae (PL) from high salinity hatchery systems to low salinity conditions. In previous research, in order to determine the effects of rate of salinity reduction, an accelerated acclimation to lower salinity was conducted in a single step from 30 ppt to 5 ppt and 1 ppt, respectively. Fifty fifteen-day-old postlarvae (PL15) were subjected to fast acclimation by being placed in 1 L of artificial sea water (ASW) at 30 ppt, then 29 L of fresh water was added to the tank in a single step to obtain an end-point salinity of 1 ppt. Another fast acclimation to lower salinity was conducted in a single step from 30 ppt to 5 ppt by placing the fifty PL15 in 4.25 L of ASW at 30 ppt, then adding 20.8 L of freshwater to obtain an end-point salinity of 5 ppt (Jayasankar et al., 2009).

A gradual salinity reduction procedure over a period of 1, 3 and 7 days was conducted. One hundred PL15 were placed in 1.71 L of ASW at 30 ppt, then equal volumes of fresh water were added 3 to 4 times per day, until the final volume of 50 L was reached, reducing the salinity to 1 ppt. For the gradual acclimation to 5 ppt, one hundred PL15 were placed in 8.3 L of ASW at 30 ppt, then equal volumes of fresh water were added 3 to 4 times per day, until the final volume of 50 L was reached, reducing the salinity to 5 ppt. Higher survival rates were obtained with gradual acclimation than with the single-step acclimation (Jayasankar, et al., 2009).

Salinity End-Point

To determine the lowest levels of salinity that postlarvae could tolerate without acclimation, postlarvae (PL20) and juvenile age shrimp were directly transferred from 30
ppt to artificial seawater with 0, 0.5, 0.75, 1.5, 2 and 5 ppt salinities, respectively. Salinity tolerance has been shown to vary with age of shrimp in a number of penaeid species including *L. vannamei, M. japonicas, L. setiferus*, and *L.stylirostris*. Salinity tolerance increases with postlarvae age (Jayasankar et al., 2008). Animals were maintained at 28°C with gentle aeration for 10 days. Surviving animals in each treatment were counted at the end of the experiment and survival rates were compared. Survival of PL20 and juvenile acclimated to salinities of 1.5 ppt and above had higher survival rates than those of 0.75 ppt and below (Jayasankar, et.al., 2009).

Enhancements

Probiotic Enhancement

Probiotic is a relatively new term which is used to name microorganisms that are associated with the beneficial effects for the host. The term “probiotic” comes from Greek *pro* and *bios* meaning “prolife”. The need for increased disease resistance, growth of aquatic organisms, and feed efficiency has brought about the use of probiotics in aquaculture practices. The first application of probiotics occurred in 1986, to test their ability to increase growth of hydrobionts (organisms that live in water). Later, probiotics were used to improve water quality and control of bacterial infections. Recent studies indicate that probiotics can improve the digestibility of nutrients, increase tolerance to stress, and encourage reproduction (Cruz et al., 2012). Mixed cultures of bacteria and yeast were used to enhance nonspecific immune paremeters of tilapia, *Oreochromis niloticus* (Taoka et al., 2006). Abalones supplemented with probiotics had a higher survival rate of 62% to the pathogenic bacterium *Vibrio anguillarum* compared to 25% survival of untreated animals (Macey and Coyne, 2005). Increased survival and growth
of white shrimp (*Litopenaeus vannamei*) was reported in Ecuadorian shrimp hatcheries, increasing production by 35% using probiotics (Gomez *et al.*, 2007). Currently, there are commercial probiotic products prepared from various bacterial species and yeast.

In penaeid shrimps, *Vibrio spp.* is the main cause of bacterial diseases, such as *V. parahaemolyticus, V. alginolyticus, V. harveyi* (Garriques and Arevalo, 1995) and *V. penaeicida* (Aguirre-Guzman and Asencio-Valle, 2001). Pathogens like *Vibrio spp.*, which cause detachment of the epithelium in the midgut trunk, can affect high mortality in shrimp by eliminating 2 layers that protect the shrimp from infections: the epithelium and the peritrophic membrane it secretes. In addition, loss of the epithelium may affect the regulation of water and ion outtake into the body (Mykles 1977, Neufeld and Cameron 1994; Luis-Villasenor *et al.*, 2012).

Previous studies show that inoculation with a probiotic strain during cultivation of whiteleg shrimp *Litopenaeus vannamei* larvae (nauplii stage V) prevents colonization by a pathogenic strain, because the probiotic succeeds in colonizing the gut of the larvae (Gomez-Gil *et al.*, 2000; Zherdmant *et al.*, 1997). Previous applications of probiotic organisms have shown beneficial host effects, including improved growth, survival and health (Moriarty, 1998; Skjermo and Vadstein, 1999).

Ionic Enhancement

Of the commercial species currently cultured in the Western hemisphere, *L. vannamei* is probably one of the best adapted species for culture in waters of low salinity. Information on essential environmental ions and minimum concentrations necessary for survival and growth of this shrimp is lacking (McGraw *et al.*, 2002). The
concentrations of the major ions involved in shrimp osmoregulation are: Na\(^+\), Ca\(^{2+}\), Mg\(^{2+}\), K\(^+\), Cl\(^-\), SO\(_4^{2-}\), (Schmidt-Nielsen 1990), but total salinities of ground waters vary widely by geography (McGraw et al., 2003). Modification strategies to alter the low salinity water rearing medium to make it more acceptable for production might theoretically improve osmoregulatory capacity of shrimp (Roy et al., 2010).

The ionic composition of water appears to be more important than salinity. It has been demonstrated that single salt solutions of sodium chloride are not suitable for shrimp culture at any salinity, even though in seawater the ions most important in osmoregulation are chloride and sodium. Current research suggests that if the salinity is adequate, calcium (Ca), potassium (K) and magnesium (Mg) are the most important ions for shrimp survival (Davis et al., 2004).

Among the minerals required by penaeid shrimp, phosphorus (P) is crucial because of its limited availability under rearing conditions. P is mainly associated with Ca in the exoskeleton. Also P is related to alkaline phosphatase (AP), which responds to acclimation salinity and is associated with osmoregulation in crustaceans (Lovett et al., 1994; Pinoni and Lopez Mananes, 2004). Other studies have suggested that dietary Ca:P ratio should be considered as well as individual dietary levels of the minerals (Li et al., 1986). Excessive dietary Ca may result in increased P requirements of shrimp. Optimal Ca:P ratio for species varies, although poor correlation of dietary Ca:P ratio was found for *L. vannamei* reared in seawater, the dietary P requirement was dependent on the Ca content (Davis et al., 1993)(Cheng et al., 2006).
Probiotic and Ionic Enhancement

After extensive searching, no peer reviewed publications were found regarding a combination of probiotic and ionic enhancements applied to low-salinity *L. vannamei* production. Therefore, this aspect of the research is considered a novel investigation into the potential interactions of probiotic and ionic enhancement treatments applied together. It is gleaned from the literature review that there could be a potential for a synergistic effect when applied together.

2.4 Validity and Reliability Measures

In aquaculture, accurate sampling and counting procedures are essential to determine precisely the number of animals in culture, to develop feedings regimes and water exchange rates, and to calculate the survival rates. For precise direct counting of fish and shrimp larvae, we suggest selecting sample volumes yielding 30 to 80 animals per sample. Using this relatively large number of animals per sample allows using less replicates to achieve the desired standard error (Nagel & Gomez-Humaran, 1998).
CHAPTER 3

RESEARCH METHODOLOGY

3.1 Methodology

The purpose of this research was to develop an integrated process for acclimation of *L. vannamei* postlarvae to low-salinity for aquaculture, and this study examined the effects of four factors, including acclimation rate, salinity end-point, probiotic and ionic enhancements on postlarvae survival.

3.2 Research Design

The experimental design structure was a full factorial experiment to study the effect of each factor on the response variable, as well as effects of interactions between factors on the response variable. The four factorial treatments were: 1) Acclimation Rate, 2) Salinity End-point, 3) Probiotic, 4) Ionic composition, and further, a combined probiotic and enhancement treatment. These factors were chosen as treatments because each has a direct effect on the survival of the postlarvae as it is being acclimated from normal seawater salinity to lower-salinity water. The control was maintained at full natural seawater of 32 ppt over the entire period (Fig. 6).

The experimental unit was the tank, and there were a replication of three tanks for each treatment for a total of 48 treatment tanks, plus 6 control tanks that did not receive any treatment. There were two different acclimation rates, accelerated 2 days, and gradual 4 days, two different salinity end-points 0 ppt and 2ppt, probiotic enhancement, ionic enhancement and a combination of probiotic with ionic enhancements.
3.3 Sample Population

The sample population was the Pacific white shrimp, *L. vannamei*, at the postlarvae stage after the final metamorphosis, with morphological development completed to the full adult body plan. For this research, the experimental animals, specific pathogen-free (SPF) *L. vannamei* postlarvae (PL), were obtained from a commercial hatchery, Oceanic Institute in Hawaii at the PL16 stage. They were held in holding tanks for 48 hours in full natural seawater (NSW) of 32 ppt before acclimation began. The NSW was obtained from the Waikiki Aquarium, part of the University of Hawaii. The PL's were individually counted and stocked thirty each to six liter tanks, and they were fed once a day with crumbled commercial shrimp feed (Rangen, USA; PROD.40 >40% protein) throughout the duration of the experiment.
3.3 Experimental Setting

The research was conducted on the University of Hawai‘i at Mānoa campus, at the Solar Radiation Outdoor Facility. The tanks were 6 liter of which there were 48 used in treatments and 6 control tanks for a total of 54 tanks. Each tank was equipped with an aeration stone to supply dissolved oxygen at the rate of 5 mg/l.

1) Acclimation Rate & Salinity End-point

In this research, one factor was to determine the effects of rate of salinity reduction at two different rates, accelerated and gradual. Another factor was to determine the effects of two different salinity end-points. Nineteen-day-old postlarvae (PL19) were subject to an accelerated acclimation process over a period of 2 days, and a gradual acclimation process over a period of 4 days, starting from 32 ppt salinity and ending at two different points of 2 ppt and 0 ppt. Changing the salinity of water was accomplished by removal of the natural sea water (NSW) from the tank water where the postlarvae were stocked, and replacing it with an equal volume of fresh water. The fraction of the water being removed and replaced to bring about the desired salinity change was calculated from the following formula: (Van Wyk, 1999)

\[
Fraction\ of\ New\ Water\ Needed = 1 - \frac{P_{new} - P_{final}}{P_{new} - P_{initial}}
\]

where,

\[ P_{new} \] = salinity (temperature) of the water added

\[ P_{initial} \] = initial salinity (temperature) of tank water

\[ P_{final} \] = desired final salinity (temperature)
In the accelerated acclimation tanks, thirty PL19 were stocked in 3 L of NSW at 32 ppt with water changes conducted four times throughout each day by removing and replacing the calculated amount of water to reduce the salinity by 4 ppt each time, until reaching the end-point salinity of 2 ppt and 0 ppt. This was accomplished over a period of two days.

In the gradual acclimation tanks, thirty PL19 were placed in 3 L of NSW at 32 ppt with water changes conducted four times throughout day by removing and replacing the calculated amount of water to reduce the salinity by 2 ppt each time, until reaching the end-point salinity of 2 ppt and 0 ppt were. This was accomplished over a period of four days.

2) Probiotic Enhancement

In this research, one factor was to determine the effects of probiotic enhancement on PL survival during acclimation. In this trial, thirty, nineteen-day-old postlarvae (PL19) were stocked in 6 L tanks which underwent the low-salinity acclimation process, both accelerated and gradual, and to salinity end-points of 2 ppt and 0 ppt as described above. A commercial probiotic, EPICIN-G2 treatment obtained from Epicore BioNetworks, Inc. of New Jersey, USA was used as the inoculant to enhance the water environment undergoing low-salinity acclimation. The total aerobic count was reported at 4.0E+09 cfu/gm, (colony forming units per gram). The inoculant was provided as a surface application at the rate of 1.5 ml at each water change interval as described above.

3) Ionic Enhancement
In this research, one factor was to determine the effects of ionic composition enhancement on PL survival during acclimation. Determining an appropriate amount of ionic composition enhancement as a treatment during the acclimation process to low-salinity for aquaculture is not clearly defined in the literature. Table 1 is a list of mineral salts used in aquaculture. It provides a number of agricultural products that can be used as an application to adjust ionic profiles of inland, low-salinity well water, often very different than marine brackish water, making the acclimation process more stressful. To calculate the dose rate of mineral salt for a desired concentration of any one of the variables listed in the last column, the following equation is used:

$$\text{Dose (g/m}^3\text{)} = \frac{\text{Desired concentration of variable (mg/L)}}{\text{Percentage variable in salt/100}}$$

For example, to use muriate of potash to increase potassium concentration by 25 mg/L:

$$\text{Dose of muriate of potash} = \frac{25 \text{ mg K/L}}{50\% \text{ K/100}} = 50 \text{ mg/L} \ (\text{Davis et al., 2004})$$

<table>
<thead>
<tr>
<th>Mineral salt</th>
<th>Formula</th>
<th>Common or trade name</th>
<th>Typical composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium sulfate</td>
<td>CaSO$_4$•2H$_2$O</td>
<td>Gypsum</td>
<td>22% Ca; 53% SO$_4$; 55% hardness</td>
</tr>
<tr>
<td>Potassium chloride</td>
<td>KCl</td>
<td>Muriate of potash</td>
<td>50% K; 45% Cl</td>
</tr>
<tr>
<td>Potassium magnesium sulfate</td>
<td>K$_2$SO$_4$•2MgSO$_4$</td>
<td>K-mag</td>
<td>17.8% K; 10.5% Mg; 63.6% SO$_4$</td>
</tr>
<tr>
<td>Potassium sulfate</td>
<td>K$_2$SO$_4$</td>
<td>—</td>
<td>41.5% K; 50.9% SO$_4$</td>
</tr>
<tr>
<td>Magnesium sulfate heptahydrate</td>
<td>MgSO$_4$•7H$_2$O</td>
<td>Epsom salt</td>
<td>10% Mg; 39% SO$_4$</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>NaCl</td>
<td>Rock salt, minerun salt</td>
<td>39% Na; 61% Cl; 98% salinity</td>
</tr>
</tbody>
</table>

(Davis et al., 2004)
Table 2 is a list of stock laboratory chemicals that were obtained for their mineral salt content in order to prepare a treatment mixture to replace the ionic composition of NSW during the acclimation process to low-salinity water. The dosage rate calculation recommended by Davis et al., 2004 was used to determine the proper replacement amount of each mineral salt during the acclimation process to low-salinity. An ionic enhancement was added each time a water change occurred, in an effort to match the desired concentrations as listed in Table 3, water characteristics for shrimp culture.

<table>
<thead>
<tr>
<th>Mineral Salt</th>
<th>Formula</th>
<th>Ionic Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium Phosphate</td>
<td>Ca(H₂PO₄)₂</td>
<td>39% Ca; 20% P; 41% O</td>
</tr>
<tr>
<td>Magnesium Sulfate</td>
<td>MgSO₄</td>
<td>10% Mg; 39% SO₄</td>
</tr>
<tr>
<td>Potassium Chloride</td>
<td>KCl</td>
<td>50% K; 45% Cl</td>
</tr>
</tbody>
</table>

(Kuehu, 2015 Research Experimental Design Method)

4) Probiotic & Ionic Enhancement

In this research, one factor was to determine the combined effects of probiotic and ionic enhancement on PL survival during acclimation. There is a definite lack of information regarding a combination of probiotic and ionic enhancements which separately have been found to have positive effects on PL survivability.

The process of preparing for the probiotic and ionic enhancement treatments were the same as described in the previous sections, except instead of applying separately, the probiotic and ionic enhancements were applied in combination with each water change interval, and specific to the volume of water change occurring.
Table 3. Water characteristics for shrimp culture. (All parameters in ppm [mg/L] unless noted otherwise).

<table>
<thead>
<tr>
<th>Variable</th>
<th>Form in water</th>
<th>Desired concentration</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Boron</td>
<td>Borate (H₂BO₃. H₂BO₃⁻)</td>
<td>0.05 - 1</td>
<td>See ¹</td>
</tr>
<tr>
<td>Cadmium (Cd)</td>
<td>&lt;0.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Calcium</td>
<td>Calcium ion (Ca²⁺)</td>
<td>100 - 500</td>
<td></td>
</tr>
<tr>
<td>Carbon dioxide</td>
<td>Dissolved CO₂ Gas</td>
<td>1 - 10</td>
<td></td>
</tr>
<tr>
<td>Chloride</td>
<td>Chloride ion (Cl⁻)</td>
<td>2,000 - 20,000</td>
<td></td>
</tr>
<tr>
<td>Copper</td>
<td>Copper ion (Cu²⁺)</td>
<td>&lt;0.0005</td>
<td>See ¹</td>
</tr>
<tr>
<td></td>
<td>Total Copper</td>
<td>0.0005 - 0.01</td>
<td></td>
</tr>
<tr>
<td>Iron</td>
<td>Ferrous iron (Fe²⁺)</td>
<td>0</td>
<td>See ¹</td>
</tr>
<tr>
<td></td>
<td>Ferric iron (Fe³⁺)</td>
<td>Trace</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Total iron</td>
<td>0.05 - 0.5</td>
<td></td>
</tr>
<tr>
<td>Magnesium</td>
<td>Magnesium ion (Mg²⁺)</td>
<td>100 - 1,500</td>
<td></td>
</tr>
<tr>
<td>Manganese</td>
<td>Manganese ion (Mn²⁺)</td>
<td>0</td>
<td>Trace</td>
</tr>
<tr>
<td></td>
<td>Manganese dioxide (MnO₂)</td>
<td>Trace</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Total manganese</td>
<td>0.05 - 0.2</td>
<td></td>
</tr>
<tr>
<td>Molybdenum</td>
<td>Molybdate (MoO₃)</td>
<td>Trace</td>
<td></td>
</tr>
<tr>
<td>Nitrogen</td>
<td>Dissolved N₂ Gas</td>
<td>5 - 15</td>
<td>See ²</td>
</tr>
<tr>
<td></td>
<td>Molecular nitrogen (N₂)</td>
<td>pH 7 - 9</td>
<td>See ³</td>
</tr>
<tr>
<td></td>
<td>Ammonium (NH₄⁺)</td>
<td>0.2 - 2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ammonia (NH₃)</td>
<td>&lt;0.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Nitrite (NO₃⁻)</td>
<td>0.2 - 10</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Nitrate (NO₂⁻)</td>
<td>&lt;0.23</td>
<td></td>
</tr>
<tr>
<td>Oxygen</td>
<td>Dissolved O₂ Gas</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH</td>
<td>H⁺-[log(H⁺)=pH]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Potassium</td>
<td>Potassium ion (K⁺)</td>
<td>100 - 400</td>
<td></td>
</tr>
<tr>
<td>Salinity</td>
<td>5,000 - 35,000</td>
<td></td>
<td>See ⁴</td>
</tr>
<tr>
<td>Sodium</td>
<td>Sodium (Na⁺)</td>
<td>2,000 - 11,000</td>
<td></td>
</tr>
<tr>
<td>Sulfur</td>
<td>Sulfate (SO₄²⁻)</td>
<td>500 - 3,000</td>
<td>&lt;0.02 (preferably not detectable)</td>
</tr>
<tr>
<td>Suspended Solids</td>
<td>Hydrogen Sulfide</td>
<td>&lt;100</td>
<td></td>
</tr>
<tr>
<td>Temperature</td>
<td>26-29 °C (78.8-84.2 °F)</td>
<td></td>
<td>See ⁵</td>
</tr>
<tr>
<td>Turbidity</td>
<td></td>
<td></td>
<td>See ⁶</td>
</tr>
<tr>
<td>Zinc</td>
<td>Zinc ion (Zn²⁺)</td>
<td>&lt;0.01</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Total zinc</td>
<td>0.01 - 0.05</td>
<td></td>
</tr>
</tbody>
</table>

Notes:

1 The desirable ranges for these substances are poorly understood. The values listed as the desired concentrations are actually the usual concentrations of these trace metals in surface waters.

2 O₂ for growth, 2-3 ppm minimum.

3 pH directly influences shrimp (pH of 4 - acid death point; 4-5 - no reproduction; 4-6 - slow growth; 6-9 - best growth; 9-11 - slow growth; 11 - alkaline death point).

4 Salinity is normally referred to in parts per thousand or ppt (5,000 - 35,000 ppm = 5 - 35 ppt). 35 ppt is generally considered a normal salinity for open ocean water. Some shrimp can grow in salinities outside these ranges.

5 Temperature for tropical shrimp. For growth, 23-25 °C (73.4-77 °F) minimum, and 33-34 °C (91.4-90.2 °F) maximum.

6 Turbidity (Goal is Secchi disk reading of 25-40 cm (10-16 in.) and water color of yellowish-brown).

(Whetstone, et al. 2002)
3.5 Instrumentation

A Milwaukee MA885% Brix digital refractometer instrument was used to measure the refractive index of a solution, providing a measurement of water salinity. The UNESCO Practical Salinity Scale of 1978 (PSS78) defines salinity in terms of a conductivity ratio, which is dimensionless. Salinity was formerly expressed in terms of parts per thousand (ppt) or by weight (parts per thousand or 0/00), which meant that a salinity of 32ppt meant 32 pounds of salt per 1,000 pounds of seawater. Open ocean salinity is generally in the range from 32 to 37 (NASA Science). The MA885 refractometer specifications measure a range of 0 to 50 %Brix with a 0.1 resolution, and an accuracy of ±0.2. For accurate usage, a minimum sample volume of 100 µL placed on the glass prism is measured after a simple calibration with deionized or distilled water (Milwaukeeinst, 2011).

A YSI 550A Dissolved Oxygen Meter was used to measure temperature and dissolved oxygen simultaneously using highly accurate thermistors and polagraphic technology. The YSI 550A DO meter specifications has a measurement range of 0 to 500% air saturation, or 0 to 50 mg/L, and temperature range between 5 to 45 °C (YSI Environmental 2006).

A Hannah HI98106 meter was used to measure pH and has a measurement range of 0.0 to 14.0 pH. The pH resolution is ± 0.1 pH, and pH accuracy is ± 0.2 pH. The pH meter can operate under environmental conditions of 0 to 50 °C, and relative humidity maximum of 95%.
3.7 Research Assumptions

The success of the operation is dependent upon the quality of the postlarvae stock used in the research investigation. Strong, healthy postlarvae placed into a healthy environment will grow well and give good survival. Finding a hatchery that can supply Specific pathogen free (SPF) or high health postlarvae is important (Van Wyk, 1999). The postlarvae for this research was obtained from HPU Oceanic Institute of Waimanalo, Oahu, Hawaii, considered to be a biosecured facility supplying SPF postlarvae.

3.8 Data Collection and Quantitative Analysis Procedures

Surviving shrimp in each tank were hand counted 48 hours after completion of acclimation. The raw data was prepared using Excel, and the percentage of survival rate for each treatment was calculated. The data were biological variable and was not normally distributed. The control group was not included in the analysis, however it was used to check the viability of the shrimp and the effectiveness of the tank systems. A nominal logistic regression using a fit model to determine significant differences in survival rates among treatments was done using JMP software (SAS Institute, Version 12, 2015).
CHAPTER 4

RESULTS

4.1 Findings of the Research

The results and analysis are organized into two sections. The first section of this chapter presents the findings of the research and provides the results as a percentage of survival. All data is presented as the mean ± SE (Table 4). The second section presents the statistical data analysis and provides the calculated probability of effects of the factors and interactions expressed as $P$-Values (Table 5).

<table>
<thead>
<tr>
<th>End-Point (ppt)</th>
<th>Rate (days)</th>
<th>Ion</th>
<th>Probiotic</th>
<th>Mean Survival (%)</th>
<th>±SE</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>2</td>
<td>-</td>
<td>-</td>
<td>22.2</td>
<td>2.4</td>
<td>3</td>
</tr>
<tr>
<td>0</td>
<td>2</td>
<td>+</td>
<td>-</td>
<td>11.1</td>
<td>0.7</td>
<td>3</td>
</tr>
<tr>
<td>0</td>
<td>2</td>
<td>-</td>
<td>+</td>
<td>8.8</td>
<td>0.9</td>
<td>3</td>
</tr>
<tr>
<td>0</td>
<td>2</td>
<td>+</td>
<td>+</td>
<td>3.3</td>
<td>0.0</td>
<td>3</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>-</td>
<td>-</td>
<td>92.2</td>
<td>0.3</td>
<td>3</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>+</td>
<td>-</td>
<td>68.8</td>
<td>2.7</td>
<td>3</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>-</td>
<td>+</td>
<td>88.8</td>
<td>2.0</td>
<td>3</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>+</td>
<td>+</td>
<td>40.0</td>
<td>5.5</td>
<td>3</td>
</tr>
<tr>
<td>0</td>
<td>4</td>
<td>-</td>
<td>-</td>
<td>74.4</td>
<td>3.9</td>
<td>3</td>
</tr>
<tr>
<td>0</td>
<td>4</td>
<td>+</td>
<td>-</td>
<td>34.4</td>
<td>2.6</td>
<td>3</td>
</tr>
<tr>
<td>0</td>
<td>4</td>
<td>-</td>
<td>+</td>
<td>65.5</td>
<td>1.9</td>
<td>3</td>
</tr>
<tr>
<td>0</td>
<td>4</td>
<td>+</td>
<td>+</td>
<td>52.2</td>
<td>2.4</td>
<td>3</td>
</tr>
<tr>
<td>2</td>
<td>4</td>
<td>-</td>
<td>-</td>
<td>93.3</td>
<td>1.0</td>
<td>3</td>
</tr>
<tr>
<td>2</td>
<td>4</td>
<td>+</td>
<td>-</td>
<td>97.7</td>
<td>0.7</td>
<td>3</td>
</tr>
<tr>
<td>2</td>
<td>4</td>
<td>-</td>
<td>+</td>
<td>95.5</td>
<td>1.3</td>
<td>3</td>
</tr>
<tr>
<td>2</td>
<td>4</td>
<td>+</td>
<td>+</td>
<td>85.5</td>
<td>2.4</td>
<td>3</td>
</tr>
<tr>
<td>32</td>
<td>n/a</td>
<td>-</td>
<td>-</td>
<td>100</td>
<td>0</td>
<td>6</td>
</tr>
</tbody>
</table>

Table 4. shows the mean survival (%) at the final count after 48 hours post-treatment. The data is grouped by treatment factors, Salinity End-point (0 ppt or 2 ppt), Acclimation Rate (2 Days or 4 Days), Enhancement Treatments of Ion and Probiotic ( - is without or + is with). Each group had three tank replicates (n=3).
Survival rates of *L. vannamei* postlarvae (PL 19) acclimated to salinity end-point of 0 ppt using an accelerated 2 day acclimation process. All data are presented as the mean ± SE.

Survival rate for the group without enhancement was 22.2%, 11.1% for the group with ion enhancement, 8.8% for the group with probiotic enhancement, and 3.3% for the group with a combination of ion and probiotic enhancement.
Figure 8. Survival rates of *L. vannamei* postlarvae (PL 19) acclimated to salinity end-point of 0 ppt using a gradual 4 day acclimation process. All data are presented as the mean ± SE.

Survival rate for the group without enhancement was 74.4%, 34.4% for the group with ion enhancement, 65.5% for the group with probiotic enhancement, and 52.2% for the group with a combination of ion and probiotic enhancement.
Figure 9. Survival rates of *L. vannamei* postlarvae (PL 19) acclimated to salinity end-point of 2 ppt using an accelerated 2 day acclimation process. All data are presented as the mean ± SE.

Survival rate for the group without enhancement was 92.2%, 68.8% for the group with ion enhancement, 88.8% for the group with probiotic enhancement, and 40.0% for the group with a combination of ion and probiotic enhancement.
Survival rate for the group without enhancement was 93.3%, 97.7% for the group with ion enhancement, 95.5% for the group with probiotic enhancement, and 85.5% for the group with a combination of ion and probiotic enhancement.
4.2 Statistical Data Analysis

<table>
<thead>
<tr>
<th>Factorials and Interactions</th>
<th>( P )-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Salinity End-Point</td>
<td>&lt;0.00001</td>
</tr>
<tr>
<td>Acclimation Rate</td>
<td>&lt;0.00001</td>
</tr>
<tr>
<td>Ion</td>
<td>&lt;0.00001</td>
</tr>
<tr>
<td>Probiotic</td>
<td>0.0003</td>
</tr>
<tr>
<td>Acclimation Rate<em>Salinity End-Point</em>Ion</td>
<td>0.0037</td>
</tr>
<tr>
<td>Salinity End-Point<em>Ion</em>Probiotic</td>
<td>0.0053</td>
</tr>
<tr>
<td>Acclimation Rate*Ion</td>
<td>0.014</td>
</tr>
<tr>
<td>Acclimation Rate*Salinity End-Point</td>
<td>0.014</td>
</tr>
<tr>
<td>Acclimation Rate<em>Salinity End-Point</em>Ion*Probiotic</td>
<td>0.040</td>
</tr>
<tr>
<td>Acclimation Rate<em>Salinity End-Point</em>Probiotic</td>
<td>0.072</td>
</tr>
<tr>
<td>Acclimation Rate*Probiotic</td>
<td>0.079</td>
</tr>
<tr>
<td>Ion*Probiotic</td>
<td>0.119</td>
</tr>
<tr>
<td>Salinity End-Point*Probiotic</td>
<td>0.458</td>
</tr>
<tr>
<td>Acclimation Rate<em>Ion</em>Probiotic</td>
<td>0.865</td>
</tr>
<tr>
<td>Salinity End-Point*Ion</td>
<td>0.909</td>
</tr>
</tbody>
</table>

Table 5. Nominal Logistic Regression Analysis and Calculated Probability

Table 5. shows all the factorials and interactions in the order of significance based on the calculated probability (\( P \)-Value).

Because the significant interaction (\( P = .040 \)) of all factors, the focus is on individual treatment combinations rather than the main effects (Table 5). Figure 11 shows the effect of treatment combinations. Salinity end-point, acclimation rate, ion and probiotic enhancements had a \( P \)-value \( \leq 0.001 \). Multiple factorial interactions involving acclimation rate with salinity end-point and ion, or salinity end-point with ion and probiotic had a \( P \leq 0.01 \). Multiple factorial interactions involving acclimation rate and ion, or acclimation rate and salinity end-point, or acclimation rate with salinity end-point and probiotic had a \( P \leq 0.05 \). The remaining interactions show a trend leading toward significance, or of no significance.
Figure 11. shows the effect of treatment combinations with the results of the 0 ppt salinity end-point on the left side of the chart and the 2 ppt salinity end-point on the right side of the chart. The solid bars represent no enhancements, the right-side diagonal represents ionic, the left-side diagonal represents probiotic, and the diamond cross represents the combination of probiotic and ionic enhancements.
5.1 Discussion

This chapter will include a discussion of the research outcomes, research limitations, implications of the research, and future research. The major results of this research were consistent with the theoretical framework introduced in the literature review. A conclusion will be provided with correlation to the literature reviewed.

Previous research cited regarding acclimation rate indicated that survival rates were higher following gradual acclimation compared to single-step acclimation (Jayasankar et al. 2009). The summary of findings in this research concurs with the cited literature, where a gradual acclimation period of 4 days resulted in survival rates of 74% at 0 ppt salinity end-point, and 93% at 2 ppt salinity end-point.

Previous research cited regarding acclimation rate indicated that acclimation to very low salinities can be achieved, but requires longer times than does acclimation to moderately low salinities (Jayasankar et al. 2009). The summary of findings in this research concurs with the cited literature, where the salinity end-point of 2 ppt resulted in survival rates of 92% over a 2 day accelerated period and 93% over a 4 day gradual period.

A study of the effect of probiotics isolated from L. vannamei, two commercial probiotics, and a commercial antibiotic were evaluated on survival and development of shrimp larvae. Nevertheless, studies of probiotics to improve growth or survival in crustacean larvae are scarce (Luis-Villasenor, et al., 2011).
Previous research cited regarding the benefits of probiotics report documented evidence of increased tolerance to stress (Cruz et al., 2010). Water modification strategies that improve low salinity waters used for production by adding potassium and magnesium, might improve osmoregulatory capacity of *L. vannamei* (Roy et al., 2010). In low-salinity water with low calcium content, mineral requirements of shrimp would be different from those in full strength seawater, and studies have suggested that dietary Ca/P ratio should be considered (Cheng et al., 2006). The summary of findings in this research concurs with the cited literature, where a gradual acclimation period of 4 days and a higher salinity end-point of 2 ppt results in the highest survival rates. The postlarvae survival rates were for the probiotic enhancement of 96%, the ionic composition enhancement of 98%, and the combined probiotic and ionic composition enhancement of 86% , respectively. There was one notable exception for high survival rate in the 2 day accelerated process ending in a salinity end-point of 2 ppt which was 89% for the probiotic enhancement treatment.

5.2 Research Limitations

This research was specifically limited to the factors affecting the acclimation process of *L. vannamei* postlarvae to low-salinity conditions. Therefore, the research is limited to a specific time period of a few days within the entire life cycle of the species. There was also an opportunity limitation where only one set of environmental conditions was tested, the enhancement or non-enhancement of ionic and probiotic treatments, which did not allow for a comparison of different concentration ratios.
Limitations and voids in the literature warrant further research in ionic regulation in low-salinity environments, and the interaction effects of the probiotic and ionic enhancements on *L. vannamei* postlarvae survival and development.

5.2 Implications of the Research

The importance of this research is to support inland shrimp farming in low-salinity water which requires a supply of postlarvae and juveniles acclimated to the environmental conditions of the final grow out phase to adults. Improving postlarvae survivability directly impacts the final yield potentials in a shrimp farming operation. In addition, by managing low-salinity environmental conditions for *L. vannamei*, successfully, the potential for introduction of other animal and plant species tolerant to the same conditions can improve the prospects of polycultures, with an impact on the final yields of the overall farming operation. This research provides a process that is capable of producing a supply of postlarvae at a high survivability rate at 2 ppt.

5.3 Future Research

The future research is in the long-term effect of low-salinity environmental conditions on the survivability, development and growth of *L. vannamei*. A better understanding of the animal physiology would improve the use of probiotics and ionic enhancements to modify low-salinity conditions, which could indicate long term benefits for shrimp production and yields. In addition to environmental modifications, research in dietary feed can have an important role in overcoming the deficiencies a low-salinity environment may present.
5.4 Conclusions

In conclusion, this research experience has provided good evidence of *L. vannamei* osmoregulatory capabilities in low-salinity conditions. An integrated process of acclimation rate, salinity end-point and enhancements consisting of probiotic and ionic compositions in some proportion is attainable, but must continue to be refined. There appears to be evidence of the benefits of enhancements at the higher salinity end-point and over a gradual processing rate, however, it may be that those enhancements would be better applied at a later time after acclimation to low-salinity has been completed. To some degree, this delicate balance will be site specific, directly related to the local environmental conditions, while remaining a global challenge.
REFERENCES


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