PHYSIOLOGICAL AND NUTRITIONAL REQUIREMENTS FOR THE

CULTURE OF THE PACIFIC WHITE SHRIMP *Litopenaeus*

vannamei IN LOW SALINITY WATERS

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PHYSIOLOGICAL AND NUTRITIONAL REQUIREMENTS FOR THE CULTURE OF THE PACIFIC WHITE SHRIMP *Litopenaeus vannamei* IN LOW SALINITY WATERS

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A dissertation

Submitted to

the Graduate Faculty of

Auburn University

in Partial Fulfillment of the

Requirements for the

Degree of

Doctorate of Philosophy

Auburn, Alabama
August 7th, 2006

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Luke A. Roy

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VITA

Luke A. Roy, eldest son of Ronald R. Roy and Carla S. Roy, was born on August 25th, 1977 in Forth Worth, Texas. In 1986 he moved to Costa Rica for a year and then to Uruguay for nine years where his parents served as Baptist missionaries for the International Mission Board in both rural and urban locales. Following graduation from the Uruguayan American School in May 1996 he returned to the United States to pursue a Bachelor of Science in Environmental Science and Geographical Information Systems with a minor in Spanish from Samford University in Birmingham, Alabama. Following completion of his degree at Samford he was awarded a graduate research fellowship to attend the University of California in Riverside, California where he studied aquatic ecotoxicology in marine flatfish off the Pacific coast of southern California receiving a Master of Science in Soil and Water Science in August 2002. In the Fall of 2002 he was offered a graduate research fellowship to attend Auburn University in Auburn, Alabama to pursue a Ph.D. in Fisheries and Allied Aquacultures with a focus in aquatic animal nutrition and physiology.

DISSERTATION ABSTRACT

PHYSIOLOGICAL AND NUTRITIONAL REQUIREMENTS FOR THE CULTURE OF THE PACIFIC WHITE SHRIMP *Litopenaeus vannamei* IN LOW SALINITY WATERS

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Doctorate of Philosophy, August 7th, 2006
(M.Sc., University of California, Riverside, 2002)
(B.S., Samford University, 2000)

140 Typed Pages

Directed by D. Allen Davis

The culture of the Pacific white shrimp, *Litopenaeus vannamei*, in inland low salinity well waters (LSWW) is a promising, fledgling industry in Alabama. Despite the success of a small number of farmers in producing *L. vannamei* in LSWW on a commercial scale, a number of problems (high mortalities and poor growth) have arisen that prevent optimum growth and development of the industry. Preliminary investigations revealed water deficient in a number of ions essential for normal growth, survival, and osmoregulatory function, including potassium

(K^+) and magnesium (Mg^{2+}). Experiments were designed to investigate technologies to solve these problems and aid shrimp farmers in west Alabama. Dietary supplementation of cholesterol and phospholipids was evaluated as a potential avenue of improving growth and survival under low salinity conditions. Laboratory and on-farm experiments were conducted to investigate the role of these two dietary supplements in excess of requirement under stressful (i.e. low K^+ and Mg^{2+}) rearing conditions. In both lab and farm trials no benefits from lecithin or cholesterol supplementation in excess of the dietary requirement were observed.

Another potential means to improve growth and survival of *L. vannamei* in LSWW is dietary supplementation of minerals required for normal osmoregulatory function, including K^+, Mg^{2+}, and sodium chloride (NaCl) which may offset deficiencies in the water. Two separate 7-week experiments were conducted in 4.0 ppt (low K^+, Mg^{2+}) artificial low salinity water to evaluate dietary supplementation of these minerals. In trial 1, minerals were added in the form of purified chloride salts, while Trial 2 evaluated the use of a coating agent for the Mg^{2+} and NaCl treatments, while a K^+ amino acid complex was utilized in the K^+ treatments to reduce mineral leaching. Results suggest that chloride salts are no effective supplements while dietary supplementation of a K^+ amino acid complex may help improve growth and survival of *L. vannamei* in low salinity waters.

A series of experiments were carried out to evaluate the effects of several aqueous K^+ and Mg^{2+} concentration/ratios on survival, growth, and respiration in juvenile *L. vannamei*. Four different levels of K^+ (5, 10, 20, and 40 mg L^{-1}) were utilized and a treatment of 4 ppt reconstituted seawater was used as a reference. In order to evaluate the effects of Mg^{2+} , five

concentrations (10, 20, 40, 80, 160 mg L^{-1}) were evaluated over a 6-week period. Results from the 7-week K^+ growth trial indicated significant differences in survival and growth among treatments. Results from the Mg^{2+} trial revealed a difference in survival between the lowest Mg^{2+} treatment (60%) and all other treatments (90-97%). Shrimp respiration in the lowest Mg^{2+} treatment (10 mg L^{-1}) was significantly higher than in the 80 mg L^{-1} treatment, possibly indicating stress.

Upon transfer to high or low salinity, crustaceans must employ a variety of mechanisms to survive, including isosmotic intracellular volume regulation and an anisosmotic extracellular volume regulation. Carbonic anhydrase (CA) is involved in isosmotic extracellular volume regulation by catalyzing the following hydration /dehydration reaction ($CO_2 + H_2O$ <–> $H^+ + HCO_3^-$), which provides the counter ions H^+ and HCO_3^- for exchanging Na^+ and Cl^-, respectively at the gills. Free amino acids can be measured colorimetrically using a total ninhydrin positive substance assay (TNPS) Two experiments were conducted to evaluate branchial CA activity and TNPS (abdominal muscle and hemolymph) upon exposure to low and high salinity. In the CA experiment shrimp were acclimated from 30 ppt to either 45, 15, or 5 ppt. After 7 days, branchial CA activity was highest in the anterior gills at all salinities. In the FAA experiment, shrimp were acclimated from 30 ppt to either 45.0, 15.0, 5.0, or 2.5 ppt. After 14 days, TNPS were measured in both abdominal muscle and hemolymph, revealing an increase in TNPS in the abdominal muscle of shrimp acclimated to 45 ppt and an increase in hemolymph TNPS at the lowest test salinity (2.5 ppt).

ACKNOWLEDGMENTS

This study was funded by the Alabama Agricultural Experiment Foundation and the Department of Fisheries and Allied Aquacultures at Auburn University. First I wish to extend special thanks to my wife, Susan, for her support and help. Susan has been my constant companion at the North Auburn Fisheries Station and/or Swingle Hall at all hours of the day and night, keeping me focused and grounded. I would also like to extend a special thanks to my parents (Ronald and Carla Roy) and grandparents (James and Lucille Winton; Claude and Treva Roy) for their never ceasing encouragement in the pursuit of my dreams. I am deeply indebted to Dr. I. Patrick Saoud for his technical expertise, manuscript editing, and general advice. I would like to extend my gratitude to students from the Aquatic Animal Nutrition lab at Auburn, specifically Tri Nguyen, Herbert Quintero, Elkin Amaya, and Jesus Venero for countless hours of selfless assistance. I wish also to thank Peter Sakaris, Harvey Pine, and Justin Sexton for their technical assistance and advice. I'd like to acknowledge my three academic mentors, Allen Davis, Daniel Schlenk, and Paul Blanchard for their guidance and direction.

Style manual of journal used: Aquaculture

Computer software used: Word Perfect 12, Microsoft Power Point, Microsoft Excel XP, and SAS v. 9.1

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CHAPTER I

INTRODUCTION

The inland culture of Pacific white shrimp (*L. vannamei*) in low salinity well water (LSWW) in Alabama presents several formidable challenges which prevent maximum development of this aquaculture industry. Although some producers have been able to successfully culture marine shrimp inland using LSWW, maximum growth and survival are seldom achieved. Because of the physiological stresses presented by this unique rearing environment, particularly during the early life stages of shrimp, it is necessary to elucidate both the physiological and nutritional requirements that will maximize growth and survival of this species under these conditions. A comprehensive understanding of the physiological pressures this unique environment exerts on *L. vannamei* will provide a foundation of biological knowledge, thereby assisting farmers in development and expansion of the inland shrimp industry in Alabama. The following treatise provides background information necessary for understanding the different experimental approaches detailed in this dissertation.

Pacific White Shrimp

The Pacific White shrimp is a euryhaline species capable of tolerating a wide range (0.5 to 40 ppt) of salinity (Menz and Blake, 1980; Stern et al., 1990; Bray et al., 1994). This species is the most widely cultured shrimp in the western hemisphere and is the obvious choice for culture under low salinity conditions due to its wide range of salinity tolerance (Saoud et al., 2003). Farmers have successfully cultured *L. vannamei* in inland low salinity waters ranging from 0.5 - 28.3 ppt. (Smith and Lawrence, 1990; Samocha et al., 1991). Dall et al. (1990) and Rothlisberg (1998) provide a more comprehensive review of penaeid biology and its relevance to aquaculture.

Low Salinity Culture

Shrimp culture is a worldwide commercial industry with established techniques that are well documented. However, the techniques utilized for the culture of marine shrimp inland are still being developed (Davis et al., 2002). Pacific white shrimp have been successfully cultured in inland LSWW in Thailand, Ecuador, Brazil and the United States (Boyd et al., 2002). Saline water can be found beneath two thirds of the United States, (Feth, 1970) and *L. vannamei* is being cultured in LSWW in Alabama, Arizona, Arkansas, Florida, Indiana, Illinois, and Texas (Samocha et al., 1998; Samocha et al., 2002; Saoud et al., 2003). Despite the success of a number of producers at culturing shrimp in inland LSWW, there are still several problems that impede development of the industry (Davis and Saoud, 2004).

Much of the poor growth and survival of *L. vannamei* in LSWW can be attributed to

the ionic composition of LSWW, which varies considerably from seawater. The ability of shrimp to carry out normal physiological processes is dependent on the availability of LSWW with certain anions (bicarbonate, carbonate, chloride, sulfate) and cations (calcium, magnesium, potassium, sodium) at physiologically appropriate ratios and concentrations. When concentrations of specific ions are lower than optimal, decreases in shrimp survival and growth can occur (Saoud et al., 2003). The ratios of key ions, such as Na:K or K:Mg, could also potentially influence growth and survival of *L. vannamei* reared in low salinity environments (Saoud et al., 2003; Zhu et al., 2004).

Ionic Requirements

The specific ionic requirements for *L. vannamei* culture depend on the aqueous ionic concentration of the particular rearing environment in which the shrimp is cultured. Waters utilized for the inland culture of shrimp possess different concentrations of ions (Boyd et al., 2002; Boyd and Thunjai, 2003), depending on geographical location and the specific source used for inland water. In Thailand, for example, brine solution derived from seawater evaporation ponds is used to prepare low-salinity water for shrimp culture (Boyd et al., 2002). In this situation, the ionic composition of the low salinity water mirrors more closely the ionic composition of normal seawater. It is perhaps this very reason that inland shrimp culture in Thailand has been so successful. The ions required for effective osmoregulation include Na^+, Cl^-, Ca^{2+}, Mg^{2+}, K^+, SO_4^{2-} (Schmidt-Nielsen, 1990). Both concentrations of these ions as well as the salinities of various groundwater sources vary within the United States (Saoud et al., 2002).

Compared to Thailand, the situation in west Alabama is quite different. In this case, LSWW traditionally used for catfish, a freshwater vertebrate, is also used for shrimp culture in west Alabama. In the continental U.S. there are a number of ancient saltwater aquifers (Feth, 1970) that are available for aquaculture purposes. The LSWW source used by farmers in west Alabama is from one of these aquifers. This LSWW source is much lower in ions that are essential for normal physiological function, including potassium (K^+), magnesium (Mg^{2+}), calcium (Ca^{2+}), and sulfate (SO_4^{2-}). In addition, it is not uncommon to find markedly different ionic concentrations in LSWW sources in close proximity, even from the same aquifer (Saoud et al., 2003). Although the farming of freshwater catfish species using these LSWW sources in west Alabama has been quite successful, growth and survival of shrimp has been variable.

Although it is well established that *L. vannamei* can tolerate a range of salinities, the minimum environmental requirements of essential aqueous ions for culture of *L. vannamei* have not been extensively addressed in the literature (Saoud et al., 2003; Atwood et al., 2003). McGraw et al. (2002) found that survival of post-larval (PL) shrimp (PL_{10}) acclimated down to 0, 1, and 2 ppt salinity was lower than PL survival acclimated to higher salinities (4, 8, 12 ppt). Also, in this study, it was observed that survival of PL_{15} and PL_{20} was reduced only in the 0 ppt salinity treatment, indicating a probable age effect. McGraw and Scarpa (2003) demonstrated that survival of *L. vannamei* in freshwater (0.7 ppt) can be increased with a minimum concentration of 1 ppm K^+ in 24-48 h experiments, while Mg^{2+} and SO_4^{2-} had no effect. In a later study, McGraw and Scarpa (2004) reported that survival of *L. vannamei* acclimated to low salinity waters can be improved by extending the acclimation period from 48 h to 72 h. In

a series of 48 h acclimation bioassays, Saoud et al. (2003) evaluated the survival of PL_{10}, PL_{15}, and PL_{20} shrimp in several different LSWW sources from west Alabama. Results from these bioassays revealed a positive correlation between the aqueous concentrations of K^+ , Mg^{2+} , and SO_4^{2-} and PL survival. Saoud et al. (2003) also reported significant differences in shrimp growth and survival using different LSWW sources over a four week period. In an experiment with four different PL ages (PL_{15}, PL_{19}, PL_{23}, PL_{27}), Davis et al. (2004) reported increased survival and growth with PL age when shrimp were acclimated to inland LSWW.

Although there are several studies evaluating the survival of post-larval shrimp acclimation to low salinity acclimation, few studies have addressed survival and growth over longer periods. Davis et al. (2004) reported increased survival in shrimp when select minerals (K^+, Mg^{2+}) were added to the water as compared to a control without mineral additions over a four week period. McNevin et al. (2004) observed increased shrimp production in low salinity water when raising pond water from 6.2 mg L^{-1} to 40 mg L^{-1} K^+ and 4.6 mg L^{-1} to 20 mg L^{-1} Mg^{2+} using muriate of potash and potassium-magnesium sulfate (Kmag).

Dietary Minerals

In addition to the requirements of K^+, Mg^{2+}, and other ions in the water, nutrition can also influence growth and survival (Davis and Saoud, 2004) of shrimp. Shrimp require certain minerals in order to maximize maintenance and growth (Davis and Lawrence, 1997). In crustaceans, minerals serve as components of hard tissues, soft tissues, metalloproteins, enzymatic cofactors, and enzymatic activators (Davis and Lawrence, 1997). As previously

mentioned, soluble minerals such as calcium, phosphorous, sodium, potassium, and chloride are important in osmoregulatory function. In addition, soluble minerals are important for acid-base balance and maintenance of membrane potentials (Davis and Lawrence, 1997). The importance of dietary mineral supplementation for maximum growth of crustaceans is well substantiated (Davis and Lawrence, 1997; Deshimaru and Kuroki, 1974). Several authors have investigated the dietary potassium and magnesium requirement in prawns, *Penaeus japonicus*. Deshimaru and Yone (1978) and Kanazawa et al. (1984) reported a dietary potassium requirement of 1.0 % and 0.9% diet (g 100 g^{-1}), respectively, for *P. japonicus*. Kanazawa et al. (1984) also reported a dietary requirement of 0.3% (g 100 g^{-1}) diet for magnesium in *P. japonicus*. However, there are few studies evaluating whether or not dietary requirements of key minerals change under low salinity culture conditions. Of special interest are the minerals required for effective osmoregulation that are oftentimes deficient in low salinity waters.

Shiau and Hseih (2001) observed benefits of a dietary source of potassium for *Penaeus monodon* reared in brackish water. According to a study with red drum, it was reported that a dietary addition of NaCl has the potential to provide benefits for euryhaline species (Gatlin et al., 1992). Gong et al. (2004) reported increased growth in shrimp fed a diet containing supplements of KCl, MgO, NaCl, phospholipids, and cholesterol compared to a diet without mineral additions at two different low salinity shrimp farms in the Arizona desert. It is reasonable to surmise that a lack of aqueous ions at the gill-water interface could potentially be mitigated by supplementing ions via dietary sources, increasing absorption of these mineral in the digestive system. One method whereby this can be accomplished is by formulating feeds to

contain suitable concentrations and ratios of ions required for effective growth in low salinity environments. Dietary supplementation of key minerals could prove more cost effective than adding large amounts of agricultural fertilizers such as Kag or muriate of potash to increase the concentrations of K^+ and Mg^{2+} in ponds at commercial shrimp farms operating in inland low salinity waters.

Phospholipids & Cholesterol

The requirements of phospholipids and cholesterol could also potentially shift in organisms reared in low salinity environments (Gong et al., 2004). Phospholipids are polar lipids that make up part of the cell membrane and are essential in maintaining normal cell structure and function (Castell, 1979; Teshima, 1997). Phospholipids also play an important role in the normal function of gill membranes by serving as the lipid bilayer (Teshima, 1997). In addition, phospholipids serve as second messengers in cell signaling and are involved in lipid metabolism, i.e. emulsification (Teshima, 1997). Phospholipids potentially facilitate the incorporation of cholesterol into hemolymph proteins (Teshima, 1997). Shrimp are able to synthesize their own phospholipids, but at levels well below their dietary requirement. An absence of the required amount of dietary phospholipid can have negative impacts on growth in crustaceans. In lobster, the absence of dietary lecithin resulted in molt death syndrome (Conklin et al., 1980; Bowser and Rosemark, 1981).

In crustaceans, cholesterol is utilized as a precursor for steroid and molting hormones (Teshima, 1997). Teshima et al. (1982) reported that dietary cholesterol was essential for

survival of larval prawns, *P. japonicus*. Several authors have postulated that insufficient transport of cholesterol retards weight gain in crustaceans receiving diets deficient in phospholipids (D'Abramo et al., 1985; Teshima et al., 1986). In addition, mortality syndrome has been observed in juvenile lobsters (Conklin et al., 1980; Bowser and Rosemark, 1981) and crayfish (D'Abramo et al., 1985) fed diets lacking dietary sterols. Optimal dietary levels of cholesterol for shrimp depend on the species (Kanazawa et al., 1971; Chen and Jinn, 1991; Chen, 1993; Sheen et al., 1994; Duerr and Walsh, 1996;Teshima et al., 1997; Thongrod and Boonyaratpalin, 1998). In *L. vannamei*, the cholesterol requirement for PLs has been reported to be 0.5% (Emery, 1987), while the requirement for culture in outdoor tanks is 0.23-0.42% (Duerr and Walsh, 1996).

There exists little information on the requirements of phospholipids and cholesterol in relation to salinity. An experiment evaluating two diets at two different shrimp farms in the Arizona desert revealed that *L. vannamei* achieved better growth in the diet containing supplements of cholesterol, phospholipids (lecithin), potassium chloride, magnesium oxide, and sodium chloride (Gong et al., 2004). However, further dietary studies are necessary to specifically evaluate the dietary requirements of cholesterol and phospholipids in the absence of other confounding factors. Gong et al. (2000) surmised that dietary phopholipids and cholesterol probably play an important role in adaptation to low salinity environments due to their role in lipid mobilization and storage in the hepatopancreas.

Amino Acids

Crustaceans, such as shrimp, require protein (balanced source of essential and nonessential amino acids) for growth, maintenance, and reproduction (Guillaume, 1997). The dietary protein requirement for the culture of shrimp in marine waters is well established (Kanazawa, 1990). However, due to the unique physiological stresses (i.e. energetic costs) imposed by rearing shrimp in low salinity environments, the protein requirements could potentially be different than reported values for marine culture. Hence, dietary protein and the amino acid profile have the potential to influence the culture of shrimp in low salinity environments. These requirements need to be elucidated under low salinity conditions. Dietary protein could potentially affect the ability of shrimp to osmoregulate in low salinity environments such as LSWW. For instance, Shiau et al. (1991) found evidence that optimal dietary protein levels of *Penaeus monodon* were higher when reared in low salinity environments. Robertson et al. (1993) observed an effect of dietary protein on growth in low salinity environments in *L. vannamei*, suggesting that nutritional requirements can vary with culture salinity, and the use of higher protein feeds in hypersaline waters might prove beneficial.

Certain free amino acids (FAA), such as arginine, could also potentially improve growth and survival of shrimp reared in low salinity waters, since at acclimation to low salinity FAA can be used as additional osmolytes during acclimation. Schoffeniels (1970) observed that certain FAA (proline, glycine, alanine) are important for intracellular isosmotic regulation of penaeid shrimp reared in high salinities. Free amino acids might also be used as additional sources of energy to maintain osmotic homeostasis. Arginine is a phosphorylated high energy

derivative with a role in controlling the cell content of ATP. Thus, dietary addition of this FAA could potentially improve adaptation to low salinity environments.

Transferring marine organisms, such as shrimp, from high salinity waters to low salinity waters results in the diffusive loss of salts from the hemolymph to the medium and subsequent uptake of water from the medium (Mantel and Farmer, 1983). As a result of this process tissues and cells take up water, thus increasing their volume (Pequeux, 1995). This alters normal cellular function and can be damaging to the normal physiology of the organism. Most organisms have evolved a cell volume regulatory response to manage abrupt changes in salinity of the medium. A large proportion of the total intracellular osmolality can be attributed to the presence of certain nonessential amino acids and quaternary ammonium compounds (Florkin and Schoeffeniels, 1969) that are dissolved in the cytoplasm of the cells. In response to cell swelling, these compounds are believed to be released into the hemolymph for the purpose of rapidly decreasing intracellular osmotic pressure. Osmotically obligated water also leaves the cell, restoring cell volume to normal conditions (Pierce and Amende, 1981). Gainey and Greenberg (1972) proposed that the degree to which an organism is able to reduce its intracellular FAA pool determines the lower limit of survival with respect to salinity. The total FAA pool can be measured colorimetrically by quantifying the total ninhydrin positive substances (TNPS) (Lee and Takahashi, 1966) in tissues and hemolymph. It is therefore possible to track the FAA pool from intracellular sources to the ambient medium. Applying these techniques will provide further understanding of the underlying mechanisms whereby shrimp control osmotic balance in low salinity environments.

Carbonic Anhydrase

Ionic deficiencies in shrimp lead to a number of physiological stresses that can limit the growth of shrimp and compromise survival. Of specific interest is the deterred ability to effectively osmoregulate. Osmoregulation is an essential function for survival of euryhaline species in waters of shifting salinity and involves the active uptake of various salts, including Na^+ and Cl^-, from the aqueous medium across the gills (Mantel and Farmer, 1983). There are several enzymes whose function is critical for osmoregulation in low salinity conditions, including Na+, K+ ATPase and carbonic anhydrase (Towle, 1984; Henry, 1984; Henry, 1988). Induction of both Na+, K+ ATPase and carbonic anhydrase occur upon exposure to low salinity and both responses are a natural adaptation of organisms that experience a fluctuation in salinity at some point during their life cycle. Carbonic anhydrase is responsible for catalyzing the following reaction: $CO_2 + H_2O \leftrightarrows H^+ + HCO_3^-$, while using zinc as a co-factor (Pequeux, 1995). Carbonic anhydrase produces H^+ to serve as a counterion during the active uptake of Na^+ (Henry, 1996). High activities of carbonic anhydrases have been observed in invertebrate gills, and more specifically, in the individual gills responsible for ion uptake from dilute seawater (Henry, 2001).

The effect of salinity on carbonic anhydrase activity in *L. vannamei* has not yet been adequately addressed in the literature. Palacios et al. (2004) observed no increase in carbonic anhydrase activity from exposure to low salinity in *L. vannamei*. The induction of this enzyme has been widely studied in other Decapod crustaceans, including the blue crab (*Callinectes*

sapidus) and the terrestrial crab, *Gecarcinus lateralis*, among others. (Henry and Cameron, 1983; Henry, 2001). There are, at present, few studies evaluating the effects of low salinity on carbonic anhydrase activity in *L. vannamei* reared in low salinity environments. A more comprehensive examination of the environmental factors governing the induction of this enzyme in *L. vannamei* will provide additional knowledge useful for acclimation and rearing of this shrimp species in LSWW environments.

CHAPTER II

EFFECTS OF LECITHIN AND CHOLESTEROL SUPPLEMENTATION TO PRACTICAL DIETS FOR *Litopenaeus vannamei* REARED IN LOW SALINITY WATERS

Abstract

Inland, low salinity waters are often deficient in key ions necessary for normal physiological function in aquaculture species. In west Alabama, farmers normally remedy ionic deficiencies in the water through addition of fertilizers containing K^+ and Mg^{2+}. It has been suggested that increasing phospholipids (lecithin) and cholesterol in excess of dietary requirement improve osmoregulatory capacity in *Litopenaeus vannamei*, thus leading to better survival and growth under low salinity conditions. Cholesterol is an essential sterol involved in the molting process in shrimp. Phospholipids are important in cholesterol transport, facilitate the storage of lipids in the hepatopancreas, an important energy reserve during the molting process and are an important component of cell membranes. In order to investigate the possibility of improving growth and survival under stressful (i.e. low K^+ and Mg^{2+}) rearing conditions, a series of laboratory and on-farm experiments were conducted. Two, separate 35-day laboratory studies were conducted in reconstituted low salinity (4.0 ppt, low K^+) waters. In both trials, five practical diets were

formulated to contain 36% protein and 8% lipid, and supplemented with varying levels of cholesterol and lecithin. Three of these diets were utilized for an additional experiment carried out on-site at two low salinity shrimp farms in west Alabama. Results from the lab trials indicated no significant differences in survival, growth, or percentage weight gain among treatments. Survival, final weight, and percentage weight gain ranged from 68-77%, 2.70-3.0 g, 415-471%, respectively, in experiment 1, and 56-69%, 2.7-3.2 g, 1572-1913%, respectively, in experiment 2. These results indicate that the shrimp were stressed in both experiments, and there were no apparent benefits to supplementing lecithin and cholesterol in excess of the dietary requirement. Two on-farm trials were conducted in parallel using either a mediated water source (Farm 1) to produce low stress or non-mediated waters (Farm 2) to produce a high stress environment. At Farm 1, survival, final weight, percent weight gain, and FCR ranged from 93.8 – 98.8%, 4.48 – 5.23 g, 4273 -4901 %, and 1.79 - 2.06, respectively. At Farm 2 shrimp had lower survival (37.5 -47.5%), lower final weight (2.65-3.25 g), lower percentage weight gain (2342-3088%), and higher FCRs (6.85- 10.64). No benefits from lecithin and cholesterol supplementation in excess of the dietary requirement were observed when compared to the basal diet under any test conditions. Based on results of the present study, dietary supplementation of cholesterol and phospholipids in excess of the requirement is not warranted for *L. vannamei* reared in low salinity waters.

Introduction

The inland culture of shrimp, particularly the Pacific white shrimp, *Litopenaeus vannamei*, is being undertaken in west Alabama using inland low salinity well waters (LSWW). Depending on the source, LSWW can be of varying salinities, and therefore possess different ionic compositions (Boyd and Thunjai, 2003). Despite the success by some farmers in culturing *L. vannamei* in LSWW, problems still arise as a result of mineral deficiencies in the ionic profiles of pond waters (Saoud et al., 2003; Atwood et al., 2004). The lack of a necessary mix of ions essential for osmoregulation (Castille and Lawrence, 1981; Pequeux, 1996), such as potassium (K^+) and magnesium (Mg^{2+}) has been shown to limit growth and survival of shrimp (Saoud et al., 2003; Davis et al., 2005). Farmers in west Alabama have improved growth and survival of *L. vannamei* in low salinity waters by raising the K^+ and Mg^{2+} levels of their pond waters (McNevin et al., 2004), yet there are still indications or incidences in which the shrimp appear to be stressed.

In a study conducted in Arizona, Gong et al. (2004) suggested incorporating phospholipids (lecithin) and cholesterol in excess of the dietary requirement as a potential means of improving osmoregulatory capacity in *L. vannamei*, thus leading to better survival and growth under low salinity conditions. Gong et al. (2004) observed increased osmoregulatory capacity of shrimp reared in LSWW through dietary addition of K^+, Mg^{2+}, NaCl, lecithin, and cholesterol. However, the influence of each supplement was not individually evaluated. Cholesterol is an essential sterol involved in the molting process in shrimp (Teshima, 1972) and is important in growth and survival of crustaceans (Teshima, 1997). Phospholipids are important in

cholesterol transport, facilitate the storage of lipids in the hepatopancreas, which serves as an important energy reserve during the molting process, and are an important component of cell membranes (Clarke and Wickins, 1980; Teshima et al., 1986; Teshima, 1997). Because shrimp are unable to synthesize cholesterol *de novo* (Teshima, 1997) or synthesize phopholipids in sufficient quantities to meet their dietary requirements, these ingredients are considered essential nutrients for shrimp (Gong et al., 2000).

Mortalities that occur at farms utilizing LSWW during the production period are believed to be associated with the diminished ability of juvenile and subadult shrimp to hyper-osmoregulate in low salinity waters (Saoud et al., 2003; Gong et al., 2004). The inability to effectively maintain adequate hemolymph mineral balance can result in molt-associated mortality (Gong et al., 2004). Gong et al. (2004) also reported low levels of lipid in the hepatopancreas of shrimp reared in low salinity waters, which is a major energy reserve utilized by shrimp during molting (Clarke and Wickins, 1980; Gong et al., 2000).

Dietary supplementation of phospholipids and cholesterol could potentially improve growth and survival of *L. vannamei* raised in low salinity waters. Moreover, such supplementation could prove a more cost-effective strategy when compared to adding large amounts of fertilizers to increase the concentrations of desired ions in ponds at commercial shrimp farms using inland low salinity waters (McNevin et al., 2004). The objective of the present study was to evaluate claims that phospholipid and cholesterol supplementation above the dietary requirement could improve growth and survival *of L. vannamei* in low salinity waters.

Materials & Methods

Indoor Laboratory Trials

Laboratory experiments were conducted at the North Auburn Fisheries Research Station in Auburn, Alabama. Post-larval *L. vannamei* for experiment 1 were obtained from GMSB Shrimp Hatchery (Summerland Key, FL, U.S.A), while shrimp utilized in experiment 2 were obtained from Harlingen Shrimp Farm (Bayview, TX, USA). Post-larvae were acclimated down to low salinity water (4.0 ppt) over a period of 8 hours and maintained in a 220-L polyethylene nursery tank connected to a biological filter. During the first week, PLs were offered a combination of artemia nauplii (200 nauplii per shrimp) and a commercial feed, PL Redi-Reserve (Ziegler Bros. Gardner, Pennsylvania) at 25-50% body weight. Thereafter, shrimp were fed a commercial feed (Rangen 35% protein, Buhl, Idaho) and reared in the nursery system until they were of appropriate size for commencement of growth trials. Both experiments were conducted in a 2400-L recirculating system, containing a series of 60-L aquaria. Artificial low salinity water was prepared 2 weeks before commencement of each experiment by adding 0.5 ppt reconstituted seawater (Crystal Sea Salt, Baltimore, Maryland) and a supplement of calcium ($CaCl_2 \cdot 2H_20$). Salinity was then raised to 4.0 ppt using rock salt (NaCl). Levels of K^+ in the experimental water were below optimal levels for the culture of *L. vannamei* in low salinity water. Light regime was set at 16 hours day and 8 hours night using fluorescent bulbs. Dissolved oxygen (DO), pH, salinity, and temperature were measured daily, whereas ammonia and nitrites were measured twice weekly using methods described by Solorzano (1969) and Parsons et al. (1985), respectively. For laboratory trial 1, dissolved

oxygen (7.27 ± 0.34 mg L^{-1}), temperature (28.6 ± 1.0 °C), pH (8.1 ± 0.1), salinity (4.1 ± 0.04 g L^{-1}), ammonia (0.03 ± 0.02 mg L^{-1}), and nitrites (0.14 ± 0.18 mg L^{-1}) remained within acceptable limits for the culture of this species. Likewise, for laboratory trial 2, dissolved oxygen (6.83 ± 0.44 mg L^{-1}), temperature (28.9 ± 1.3 °C), pH (8.0 ± 0.1), ammonia (0.05 ± 0.12 mg L^{-1}), and nitrites (0.04 ± 0.03 mg L^{-1}) remained within acceptable culture requirements. The experimental water was analyzed for major ions by inductively coupled argon plasma spectrophotometry (Clesceri et al., 1998).

The diets were formulated to contain 35% protein and 8% lipid. Treatments consisted of four diets with varying levels of dietary lecithin and cholesterol (Table 1) and a fifth diet containing no lecithin or cholesterol supplementation. The cholesterol content of the basal diet which did not receive cholesterol supplementation (diet 5) was verified to contain 0.08% cholesterol. Diets were prepared by mixing the ingredients in a mixer (Hobart, Troy, Ohio) for 30 minutes. Subsequently, hot water was added to the mixture until appropriate consistency for pelleting was obtained. Diets were then passed through a meat grinder and a 3 mm die. Pellets were air dried (<50 °C) to a moisture content of less than 10%. Lab trial 2 was a repeat of lab trial 1 using the same diets and an additional commercial diet (Rangen 35, 0) as a commercial reference.

In laboratory trial 1, 20 experimental tanks (5 treatments, 4 replicates) were each stocked with 12 juvenile shrimp (mean individual weight 0.529 g). In lab trial 2, 30

Table 1: Composition (g 100 g^{-1} dry weight) of practical diets designed to contain 35% protein and 8% lipid that were used in the growth trials.

Ingredient	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5
Fish meal[a]	3	3	3	3	3
Poultry meal[b]	15.3	15.3	15.3	15.3	15.3
Soybean meal[c]	33.6	33.6	33.6	33.6	33.6
Menhaden fish oil[d]	4.52	4.52	4.52	4.52	4.52
Wheat starch[e]	9.15	8.95	9.7	8.58	9.98
Whole wheat[e]	19.6	19.6	19.6	19.6	19.6
Trace mineral premix[f]	0.5	0.5	0.5	0.5	0.5
Vitamin premix[g]	1.8	1.8	1.8	1.8	1.8
Stay C[h]	0.1	0.1	0.1	0.1	0.1
Calcium phosphate[e]	2.4	2.4	2.4	2.4	2.4
Cellufil[i]	5	5	3.15	4.87	4.87
Lecithin[j]	0.5	0.5	1	1	0
Cholesterol[j]	0.2	0.4	0.2	0.4	0
Gelatin[i]	4	4	4	4	4

[a] Special Select™, Omega Protein Inc., Hammond, Louisiana, USA

[b] Griffin Industries, Inc. Cold Springs, Kentucky, USA

[c] De-hulled solvent extracted soybean meal, Southern Sates Cooperative Inc., Richmond Virginia, USA.

[d] Omega Protein Inc., Reedville, Virginia, USA.

[e] MP Biochemicals Inc. Aurora, Ohio, USA.

[f] g/100 g premix: cobalt chloride 0.004, cupric sulfate pentahydrate 0.250, ferrous sulfate 4.0, magnesium sulfate heptahydrate 28.398, manganous sulfate monohydrate 0.650, potassium iodide 0.067, sodium selenite 0.010, zinc sulfate heptahydrate 13.193, filler 53.428.

[g] g/kg premix: thiamin HCl 0.5, riboflavin 3.0, pyrodoxine HCl 1.0, DL Ca-Pantothenate 5.0, nicotinic acid 5.0, biotin 0.05, folic acid 0.18, vitamin B12 0.002, choline chloride 100.0, inositol 5.0, menadione 2.0, vitamin A acetate (20,000 IU/g) 5.0, vitamin D3 (400,000 IU/g) 0.002, dl-alpha-tocopherol acetate (250 IU/g) 8.0, Alpha-cellulose 865.266.

[h] 250 mg/kg active C supplied by Stay C® , (L-ascorbyl-2-polyphosphate 25% Active C), Hoffman-La Roche Vitamins Inc., Parsippany, New Jersey, USA.

[i] ICN, Aurora, Ohio, USA

[j] Fisher Scientific, Pittsburgh, Pennsylvania, USA.

experimental tanks (6 treatments, 5 replicates) were each stocked with 12 juvenile shrimp (mean initial weight 0.1 g). In both trials, shrimp were counted weekly and the ration was calculated assuming a 1.75 feed conversion ratio and a doubling in size until individual shrimp weighed one gram. Thereafter, a growth rate of one gram per week was assumed. Feed inputs were adjusted for mortalities on a weekly basis. At the end of a 35-day growth period, shrimp were harvested, counted and group weighed.

Farm Trials

Three of the experimental diets (diets 1,2,3) were utilized for an additional experiment carried out at two low salinity shrimp farms in west Alabama. Flow-through systems consisting of twelve, 600-L tanks (3 dietary treatments, 4 replicates) were set up at these two farms containing waters with different ionic profiles. Twenty shrimp (0.10 g initial weight) were stocked per tank and maintained for 6 weeks. One farm had K^+ supplemented to the low salinity water (1.5 ppt) and is considered a low stress environment, while water at the other farm (3.5 ppt) did not receive K^+ supplementation and is considered a high stress environment. Shrimp were fed assuming a 1.75 feed conversion ratio and a doubling in size until individual shrimp weighed one gram. Thereafter, a growth rate of one gram per week was assumed. At the end of a 42-day growth period, shrimp were harvested, counted and group weighed. Percentage survival and FCR were also assessed. Experimental waters (Table 2) were analyzed for major ions by inductively coupled argon plasma spectrophotometry (Clesceri et al., 1998).

Table 2: Ionic composition (mg L^{-1}) of low salinity waters used to culture *L. vannamei* at North Auburn Research Unit compared to seawater.

Minerals (mg L^{-1})	Laboratory Trial 1	Laboratory Trial 2	Farm 1	Farm 2	Seawater*
Sodium	1415	1763	367.4	1187.5	10500
Potassium	7.7	7.6	8.3	7.5	380
Magnesium	25.3	25.2	4.6	13.1	1350
Calcium	70.2	93.2	21.8	56.2	400
Phosphorous	1.2	5.6	0.1	<0.1	-
Zinc	<0.1	<0.1	<0.1	<0.1	0.005 - 0.014
Iron	<0.1	<0.1	<0.1	<0.1	0.002 - 0.02
Copper	<0.1	<0.1	<0.1	<0.1	0.001 - 0.09
Manganese	<0.1	<0.1	<0.1	<0.1	0.001
Salinity (ppt)	4	4	1.4	3	34.5
Ratios					
Na:K	183.8:1	232:1	44.3:1	158.3:1	28:1
Ca:K	9.1:1	7.3:1	2.6:1	7.5:1	1:0.95
Mg:Ca	0.36:1	0.27:1	0.21:1	0.23:1	3.4:1

* (Goldberg 1963)

Throughout the experiment, dissolved oxygen (DO), pH, salinity, and temperature were measured daily, whereas ammonia(Solorzano, 1969) was measured weekly. At Farm 1 dissolved oxygen (10.40 ± 3.9 mg L^{-1}), temperature (29.6 ± 2.1 °C), pH (8.7 ± 0.3), salinity (1.4 ± 0.11 g L^{-1}), and ammonia (0.72 ± 0.23 mg L^{-1}) remained within acceptable limits for the culture of *L. vannamei*. At Farm 2, dissolved oxygen (10.58 ± 3.7 mg L^{-1}), temperature (28.9 ± 3.0 °C), pH (8.2 ± 0.2), salinity (3.0 ± 0.15 g L^{-1}) ammonia (0.64 ± 0.47 mg L^{-1}), also remained within acceptable limits.

Statistical Analysis

Statistical analyses were performed using SAS (version 9.2, SAS Institute, Cary, North Carolina). Data from both experiments were analyzed using one-way analysis of variance to determine if significant differences ($P \leq 0.05$) existed among treatment means. Student-Newman-Keuls multiple comparison test (Steel and Torrie, 1980) was utilized to determine differences among treatment means.

Results

Indoor Laboratory Trials

In trial 1, there were no significant differences in survival, mean individual weight, and percent weight gain among treatments (Table 3), although growth trends in the data were apparent. Mean individual weight ranged from 2.70 - 3.03 g. Survivals in this trial ranged from 68.4 - 76.8% with diet 4 and diet 3 resulting in lowest and highest survivals, respectively. Shrimp

Table 3: Response of *L. vannamei* reared in artificial low salinity waters in separate laboratory trials (initial weight trial 1: 0.52 g; initial weight trial 2: 0.1 g) fed experimental diets supplemented with different levels of lecithin and cholesterol. Values represent means ± standard deviation.

Trial 1	Cholesterol	Lecithin	Final Mean Weight (g)	Weight Gain (%)	Survival (%)
Diet 1	0.2	0.5	2.94 ± 0.24	461.9 ± 62.9	73.4 ± 12.5
Diet 2	0.4	0.5	2.70 ± 0.18	416.2 ± 55.4	75.0 ± 5.7
Diet 3	0.2	1	2.86 ± 0.30	440.8 ± 48.1	76.8 ± 10.8
Diet 4	0.4	1	3.03 ± 0.24	471.4 ± 56.6	68.4 ± 7.1
Diet 5	0	0	2.80 ± 0.23	415.3 ± 42.6	71.6 ± 15.3
PSE *			0.108	24	0.049
P-value			0.28	0.36	0.78
Trial 2					
Diet 1	0.2	0.5	2.91 ± 0.26	1814.0 ± 212.5	63.4 ± 16.1
Diet 2	0.4	0.5	2.96 ± 0.35	1805.7 ± 227.2	58.4 ± 11.8
Diet 3	0.2	1	3.05 ± 0.11	1887.3 ± 93.8	65.0 ± 14.8
Diet 4	0.4	1	3.20 ± 0.36	1912.9 ± 215.8	58.4 ± 13.1
Diet 5	0	0	2.68 ± 0.42	1572.8 ± 364.1	56.6 ± 7.1
Commercial Reference Diet*	0	0	2.79 ± 0.40	1663.1 ± 317.3	68.8 ± 14.1
PSE **			0.15	114.2	0.06
P-value			0.21	0.71	0.28

* 35% Protein, Rangen 35,0 (Buhl, Idaho)

** Pooled Standard Error

reared using the diet containing no lecithin and cholesterol supplementation (diet 5) displayed the least percentage weight gain (415.3 %) while shrimp offered the diet containing the highest amount of lecithin and cholesterol supplementation (diet 4) displayed the highest percentage weight gain (471.4%). Trial 2 yielded similar results to Trial 1, with no significant differences in mean individual weight, percent weight gain, or survival among treatments. Mean individual weights ranged from 2.68 - 3.20 g for shrimp offered the commercial 35% protein shrimp feed as a control. The diet containing no lecithin and cholesterol supplementation (diet 5) yielded the lowest mean individual weights and percent weight gains. Survivals were slightly lower than in trial 1, ranging from 58.4 - 68.8%.

Farm Trials

Overall, shrimp performed better at the low stress farm in which the water received K^+ supplementation, but there was no additional benefit incurred from dietary lecithin and cholesterol supplementation above dietary requirement. Results from the on-site trials at farm 1 (low stress environment) revealed no significant differences in mean final individual weight (4.48 - 4.77 g), percent weight gain (4272.8 - 4639.7%), and survival (93.4 - 97.5%), or FCR (1.99 - 2.06) among treatments (Table 4). The on-site trial conducted at farm 2 (high stress environment) which receives no K^+ supplementation to the water also revealed no significant differences in mean final individual weight (2.65 - 3.25), percentage weight gain (2342.2 - 3087.7%), and survival (37.5 - 43.8%) or FCR (7.26 - 10.64).

Table 4: Response of *L. vannamei* reared in artificial low salinity waters in separate farm trials fed experimental diets supplemented with different levels of lecithin and cholesterol.

Farm 1 (Low Stress)	Final Indiv. Wt. (g)	Percent Wt. Gain (%)	Survival (%)	FCR
Diet 1 (basal)	4.74	4639.7	93.4	2.06
Diet 2	4.77	4660.5	95	1.99
Diet 3	4.48	4272.8	97.5	2.03
PSE *	0.246	265.2	4.23	0.13
P-value	0.67	0.53	0.82	0.94
Farm 2 (High Stress)				
Diet 1 (basal)	3.25	3087.7	41.3	7.26
Diet 2	2.65	2342.2	37.5	10.64
Diet 3	2.77	2508.4	43.8	8.38
PSE *	0.24	274.1	4.89	0.97
P-value	0.23	0.19	0.67	0.091

* Pooled Standard Error

Discussion

Farmers that utilize inland LSWW for commercial production of shrimp may not have optimal water available for maximum growth and survival when compared to coastal waters (Saoud et al., 2003). In addition to having low salinities, ionic profiles of LSWW sources are deficient in key ions essential for osmotic and ionic regulation. Thus, mineral amendments in the form of fertilizers rich in K^+ and Mg^{2+} dietary supplementation of minerals essential for osmoregulation (K^+, Mg^{2+} , and NaCl), dietary supplementation of amino acids, and dietary supplementation of phospholipids and cholesterol have all been suggested as potential avenues whereby the osmoregulatory capacity of shrimp cultured in low salinity waters might be improved (Gong et al., 2004; McGraw and Scarpa, 2003; McNevin et al., 2004; Saoud and Davis, 2005; Roy et al., unpublished data). In theory, improved osmoregulatory capacity would result in less expenditure of energy directed towards regulation of hemolymph osmolality, therefore better survival and growth.

Reported cholesterol requirements for *L. vannamei* range from 0.23-0.42% for *L. vannamei* reared in outdoor tanks (Duerr and Walsh, 1996) to 0.5% for postlarval *L. vannamei* (Emery, 1987). Gong et al. (2000) reported that cholesterol requirement for *L. vannamei* in the absence of supplemental phospholipids was 0.35%. However, supplementation of 1.5% and 3% phospholipid reduced dietary cholesterol requirement to 0.14% and 0.13%, respectively (Gong et al., 2000). In the present study, increasing the dietary supplementation of cholesterol and lecithin from 0.2-0.4% and 0.5-1.0% did not improve growth or survival of *L. vannamei* in either laboratory or farm trials. In another study

conducted in low salinity waters, Gong et al. (2004) supplemented 0.1% cholesterol and 1.5% lecithin to an experimental diet also supplemented with 0.5% potassium chloride, 0.8% magnesium oxide, and 0.5% sodium chloride. In addition, different diets were utilized on different farms, further confounding their results. They compared their diet to a control diet without supplementation of any minerals or additional cholesterol and lecithin. Gong et al. (2004) concluded that the experimental diet resulted in improved osmoregulatory capacity and larger shrimp at harvest. However, it is unclear which supplemented ingredient, combination of ingredients, or site specific conditions (i.e. different farm locations) were responsible for the observed effects. Based on our results from both laboratory and farm trials, dietary supplementation of phospholipids and cholesterol in excess of requirement did not provide any advantages in terms of survival and growth of *L. vannamei* reared in low salinity waters.

In the present work, K^+ levels in reconstituted low salinity waters were intentionally kept low (high Na:K ratio) to stress the shrimp and observe whether dietary lecithin and cholesterol in excess of requirement would provide an additional advantage in the absence of adequate K^+. The unsuitable water composition (inadequate Na:K ratio) in which the shrimp were reared was most likely responsible for the poor growth and survival observed in both laboratory trials and farm trial 2. The Na:K ratio in natural seawater is approximately 28:1, and inadequate Na:K ratios have been attributed to poor growth and survival of both marine fish and crustaceans reared in saline water. Zhu et al. (2004) observed poor survival at high Na:K ratios (187.3:1) in L. vannamei reared at 30 ppt. Optimal Na:K ratio at 30 ppt ranged between 40-43:1, while suboptimal ratios resulted in additional energetic costs for the shrimp

(Zhu et al., 2004). Forsberg et al. (1996) reported that survival in red drum cultured in inland saline water was correlated to Na:K and Cl:K ratios. Na:K ratios in lab trial 1, lab trial 2, and farm trial 2 ranged from 158:1 - 232:1. At farm 1, where best growth and survival at the lowest rearing salinity (1.4 ppt) were observed, the Na:K ratio (approximately 44:1) was closer to what is found in natural seawater (Table 2, Table 4). However, as in the laboratory trials, no additional advantage was observed in shrimp offered diets supplemented with lecithin and cholesterol in excess of dietary requirement.

Conclusions

Results from the present study confirm that growth and survival of juvenile shrimp are suppressed in LSWW with inadequate Na:K ratios. Irregardless of the Na:K ratio, dietary supplementation of phospholipid and cholesterol in excess of the dietary requirement did not improve growth or survival and is not warranted. Based on current information, farmers with inadequate levels of K in their water should continue to supplement their pond waters with agricultural fertilizers containing sources of both potassium and magnesium. Further studies are necessary to evaluate the effects of other nutritional supplements, such as minerals and non-essential free amino acids, that could improve growth and survival of *L. vannamei* reared in inland LSWW.

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CHAPTER 3

SUPPLEMENTATION OF POTASSIUM, MAGNESIUM, AND SODIUM CHLORIDE IN PRACTICAL DIETS FOR THE PACIFIC WHITE SHRIMP *Litopenaeus vannamei*, REARED IN LOW SALINITY WATERS

Abstract

The culture of *Litopenaeus vannamei* in inland low salinity waters is currently being practiced in various countries around the world. These environments are deficient in key ions essential for normal physiological function, including potassium (K^+) and magnesium (Mg^{2+}). Farmers have sometimes been able to counteract ionic deficiencies in the water profile by adding mineral salts containing sources of K^+ and Mg^{2+}. The purpose of this study was to explore the possibility of correcting deficiencies of K^+ and Mg^{2+} in the water profile with dietary supplementation of these minerals. Two 7-week experiments were conducted in 4.0 ppt artificial low salinity water to evaluate the effects of mineral supplements (K^+, Mg^{2+}, and NaCl) to diets of *L. vannamei* reared in low salinity waters. In Trial 1, seven diets were formulated (1% NaCl, 2% NaCl, 150 ppm Mg^{2+}, 300 ppm Mg^{2+}, 0.5% K^+, 1.0% K^+, and a basal diet to serve as a control). Minerals were added in the form of purified potassium chloride (KCl) , magnesium chloride ($MgCl_2.6H_20$), and NaCl. Trial 2 evaluated the use of a coating agent for the Mg^{2+} and NaCl treatments, while a K^+

amino acid complex was utilized in the K^+ treatments to reduce mineral leaching. Trial 2 was performed using similar treatment levels as Trial 1. Shrimp survival and growth were assessed in both experiments. Results from trial 1 indicated no significant differences in survival, growth, or percent weight gain. Results from trial 2 revealed no significant differences in survival and growth in the NaCl and Mg^{2+} treatments. However, significant differences in growth ($P < 0.05$) were observed when using the 1% K^+ treatment, suggesting that dietary supplementation of a K^+ amino acid complex may help improve growth of the species in low salinity waters.

Introduction

The inland culture of shrimp, particularly the Pacific white shrimp, *Litopenaeus vannamei*, is becoming more widespread throughout the world. Depending on their source, inland waters available for shrimp culture can be of different salinities, and therefore possess different ionic compositions (Boyd and Thunjai, 2003). Consequently, although *L. vannamei* can tolerate a wide range of salinities (0.5 - 60 ppt) deficiencies in the ionic profiles of pond waters may still limit shrimp performance (Saoud et al., 2003; Atwood et al., 2004). The lack of an optimal mix of essential ions, such as K^+ and Mg^{2+} has been shown to limit growth and survival of shrimp postlarvae (PL) at acclimation (Saoud et al., 2003) as well as during growout (Davis et al., 2005).

Alabama has several saltwater aquifers (Feth 1970) that are being utilized as sources of low salinity water for aquaculture (Davis et al., 2002). Farmers in west Alabama have been successful in raising *L. vannamei* in inland low salinity waters by raising the K^+ and Mg^{2+} concentration of their pond waters to more ideal levels. McNevin *et al.* (2004) observed increased shrimp production in Alabama low salinity waters by raising the levels of K^+ (6.2 mg L^{-1}) and Mg^{2+} (4.6 mg L^{-1}) to 40 mg L^{-1} and 20 mg L^{-1}, respectively. Such water treatment using muriate of potash and potassium-magnesium sulfate modified ionic compositions to levels similar to dilute seawater. While the usefulness of supplementing minerals to inland low salinity well-waters to improve growth and survival has been substantiated, there still exists little information on whether or not dietary supplements of these minerals could also play a role in improving growth and survival of *L. vannamei*.

Shiau and Hseih (2001) observed benefits of dietary supplements of K^+ for *Penaeus monodon* reared in brackish water. In Arizona, Gong et al. (2004) observed increased production through dietary addition of K^+, Mg^{2+}, NaCl, phospholipids, and cholesterol to a diet formulated for shrimp cultured in low salinity water. A dietary addition of NaCl has also been reported to provide benefits to euryhaline fish reared at low salinities (Gatlin et al., 1992).

Dietary supplementation of key minerals could potentially prove more cost-effective than adding large amounts of fertilizers to improve ionic profile of inland low salinity waters at inland shrimp farms. The primary objective of the present study was to explore the possibility of remedying ionic deficiencies in the water profile through dietary supplementation of these minerals. Two separate studies were conducted evaluating the supplementation of K^+, Mg^{2+}, and NaCl in practical diets of *L. vannamei* reared in low salinity waters.

Materials & Methods

Culture Conditions

The study was conducted at the North Auburn Fisheries Research Station in Auburn, Alabama. Post-larval *L. vannamei* for experiment 1 were obtained from GMSB Shrimp Hatchery (Summerland Key, Florida), while shrimp utilized in experiment 2 were obtained from Harlingen shrimp farm (Bayview, Texas). Postlarvae were acclimated to low salinity water (4.0 ppt) over a period of 8 hours and maintained in a 220-l polyethylene nursery tank connected to a biological filter. During the first week, PL were offered a combination of artemia nauplii (200 nauplii per shrimp) and a commercial feed, PL Redi-Reserve (Ziegler Bros. Gardner, Pennsylvania, USA) at

25-50% body weight. Thereafter, shrimp were offered a commercial feed (Rangen 35% protein, Buhl, Idaho USA) and reared in the nursery system until they were of appropriate size for commencement of growth trials. Both experiments were conducted in 60-L aquaria within a 2400-L recirculating system. Low salinity water comparable in ionic profile to well waters in West Alabama was prepared 2 weeks before the commencement of each experiment by adding calcium ($CaCl_2*2H_20$) to 0.5 ppt reconstituted seawater (Crystal Sea Salt, Baltimore, Maryland, USA). Salinity was then raised to 4.0 ppt using rock salt (NaCl). The water reconstituted in the present experiment mimicked the composition of one of the waters in west Alabama where a shrimp aquaculturist was experiencing high mortality. The experimental water was analyzed for major ions by inductively coupled argon plasma spectrophotometry (Table 1)(Clesceri et al., 1998). In both experiments, temperature was maintained at approximately 27.0 ˚C. Light regime was set at 16 hours day and 8 hours night. Dissolved oxygen (DO), pH, salinity, and temperature were measured daily, whereas ammonia (Solorzano, 1969) and nitrites (Parsons et al., 1985) were measured twice weekly. Water quality parameters were maintained within acceptable limits for *L. vannamei* (Table 2) throughout the experiments.

Trial 1

The basal diet and six test diets (Table 3) were formulated by substituting an equal weight of cellufil with selected ACS grade mineral supplements. Seven diets were formulated to contain 36% protein and 8% lipid. Treatment diets contained one of the

Table 1: Ionic composition (mg L^{-1}) of artificial low salinity waters (4.0 g L^{-1}) used to culture *L. vannamei* at North Auburn Research Station. Composition of seawater is added for comparison.

Minerals (mg L^{-1})	Trial 1	Trial 2	Seawater (34.0 g L^{-1})*
Na	832	1407	10500
K	17	35	380
Mg	21	36	1350
Ca	41	80	400
P	3.5	5.3	-
Zn	0	0	0.005 - 0.014
Fe	0	0	0.002 - 0.02
Cu	0	0	0.001 - 0.09
Mn	0	0	0.001
Ratios			
Na:K	49:1	40:1	28:1
Ca:K	2.4:1	2.3:1	1:0.95
Mg:Ca	0.5	0.5	3.4:1

* (Goldberg 1963)

Table 2: Water quality parameters for growth trials with juvenile *L. vannamei* reared in low salinity waters. Values represent the mean ± standard deviation.

Parameter	Trial 1	Trial 2
Dissolved O_2 (mg L^{-1})	6.8 ± 0.3	7.1 ± 0.5
Temperature (C)	28.8 ± 1.0	28.2 ± 1.0
Salinity (g L^{-1})	4.1 ± 0.7	4.3 ± 0.1
pH	7.7 ± 0.4	8.1 ± 0.1
TAN (mg L^{-1})	0.03 ± 0.04	0.14 ± 0.08
NO_2 (mg L^{-1})	0.06 ± 0.01	0.13 ± 0.13

Table 3: Composition (g 100 g^{-1} dry weight) of the basal diets formulated to contain 35% protein and 8% lipids and used in the two growth trials.

Ingredient	Trial 1	Trial 2
Fish meal [a]	3	3
Poultry meal [b]	15.3	15.3
Soybean meal [c]	33.6	33.6
Menhaden fish oil [d]	4.52	4.52
Wheat starch [e]	13.78	13.78
Whole wheat [e]	19.6	19.6
Trace mineral premix [f]	0.5	0.5
Vitamin premix [g]	1.8	1.8
Stay C [h]	0.07	0.07
Calcium phosphate [e]	0.8	0.8
Cellufil [i]	2	5
Gelatin [i]	4	4

[a] Special Select™, Omega Protein Inc., Hammond, Louisiana, USA

[b] Griffin Industries, Inc. Cold Springs, Kentucky, USA

[c] De-hulled solvent extracted soybean meal, Southern Sates Cooperative Inc., Richmond Virginia, USA.

[d] Omega Protein Inc., Reedville, Virginia, USA.

[e] MP Biochemicals Inc. Aurora, Ohio, USA.

[f] g/100 g premix: cobalt chloride 0.004, cupric sulfate pentahydrate 0.250, ferrous sulfate 4.0, magnesium sulfate heptahydrate 28.398, manganous sulfate monohydrate 0.650, potassium iodide 0.067, sodium selenite 0.010, zinc sulfate heptahydrate 13.193, filler 53.428.

[g] g/kg premix: thiamin HCl 0.5, riboflavin 3.0, pyrodoxine HCl 1.0, DL Ca-Pantothenate 5.0, nicotinic acid 5.0, biotin 0.05, folic acid 0.18, vitamin B_{12} 0.002, choline chloride 100.0, inositol 5.0, menadione 2.0, vitamin A acetate (20,000 IU/g) 5.0, vitamin D3 (400,000 IU/g) 0.002, dl-alpha-tocopheryl acetate (250 IU/g) 8.0, Alpha-cellulose 865.266.

[h] 250 mg/kg active C supplied by Stay C® , (L-ascorbyl-2-polyphosphate 25% Active C), Roche Vitamins Inc., Parsippany, New Jersey, USA.

[i] ICN, Aurora, Ohio, USA

following minerals: 1% NaCl, 2% NaCl, 150 ppm Mg^{2+} (0.06% $MgCl_2.6H_20$), 300 ppm Mg^{2+} (0.12% $MgCl_2.6H_20$), 0.5% K^+(0.96% KCl) and 1.0% K^+ (1.92% KCl). Each diet was prepared by mixing the dry ingredients in a mixer (Hobart, Troy, OH, USA) for 30 minutes. Subsequently, hot water (~40% by weight) was blended into the mixture until appropriate consistency for pelleting was obtained. The mash was passed through a 3-mm die, and pellets were dried at 40°C in a forced air kiln to a moisture content of approximately 8%. Diets were stored at -20 °C until commencement of experimental trials, when they were mechanically crumbled and sieved to desired size. Each diet was tested in four replicate tanks with 12 juvenile shrimp (mean individual weight 0.5 g) per tank. Shrimp were counted weekly, and ration was calculated assuming a 1.75 feed conversion ratio and a doubling in size until individual shrimp weighed one gram. Thereafter, a growth rate of one gram per week was assumed. At the end of a 44-day growth period, shrimp were harvested, counted and group-weighed. Hemolymph osmolality, Cl^-, Na^+, K^+ levels were measured using a composite sample from several shrimp per tank. Shrimp and hemolymph samples were then stored at -70 ˚C pending further analysis.

Trial 2

The second trial also tested effects of seven practical diets containing 36% protein and 8% lipid (Table 3), with similar treatment levels as the first experiment. The same purified mineral sources were utilized for NaCl and Mg^{2+} as in experiment 1, however, a coating agent (CA), (Xtra-Dry, Uniscope Inc. Johnston, Colorado, USA) was combined at a 5% CA level (by weight) with each mineral in an attempt to reduce leaching. Furthermore, a chelated amino acid complex (Chelated

Minerals Corporation, Salt Lake City, Utah, USA) was used as the dietary K^+ source. The following inclusion levels were used replacing cellufil; 1% NaCl (1.05%: NaCl + 5% CA); 2% NaCl (2.1%: NaCl + 5% CA), 140 ppm Mg^{2+} (140 ppm from trace mineral premix); 300 ppm (0.13 %: $MgCl_2$ x $6H_20$ + 5%CA). Each diet was tested on five replicate tanks with 12 juvenile shrimp (mean individual weight 0.28 g) per tank. Shrimp were counted weekly and the ration was calculated assuming a 1.75 feed conversion ratio and a doubling in size until individual shrimp weighed one gram. Thereafter, a growth rate of one gram per week was assumed. At the end of a 49-day growth period, shrimp were harvested, counted and group-weighed. Hemolymph was collected using a syringe, and all samples were pooled in a 1.5 ml Eppendorf tube. The sample was used to determine hemolymph osmolality, chloride, K^+, and Na^+ levels. Shrimp and hemolymph samples were then stored at -70 ˚C pending further analysis.

Hemolymph osmotic and ionic concentrations

In order to determine hemolymph osmotic and ionic concentrations, stored samples were thawed on ice and then sonicated (25 W, 30 sec, Heat Systems Microson) to disrupt the clot (Henry et al., 2003). Samples were then centrifuged (10,000 g for 60 sec) to separate the clot from serum. Total osmolality was then determined using a vapor pressure osmometer (Wescor 5100C, Logan, Utah). Hemolymph chloride ion concentration was determined by Ag titration (Labconco chloridometer, Kansas City, Missouri). Finally, hemolymph Na^+ and K^+ concentrations were determined using flame photometry (Cole Parmer digital flame photometer, Vernon Hills, Illinois).

Hepatopancreas mineralization

Whole shrimp were thawed and the hepatopancreas of five intermolt shrimp from each tank were thawed and dissected. Hepatopancreas samples from shrimp in each dietary treatment were pooled into four composite samples from trial 1 and five composite samples from trial 2. Samples were then oven dried and wet ashed as described in "Association of Official Analytical Chemists" (1984). Subsequently, K^+, Na^+, Mg^{2+}, Ca^{2+} were analyzed by inductively coupled argon plasma spectrophotometry according to standard protocols (Clesceri et al., 1998).

Leaching

Mineral leaching from diets in trial 2 was evaluated in order to assess efficacity of the coating agent used. Two diets (300 ppm Mg^{2+} without a coating agent; 2% NaCl without a coating agent) that were prepared concurrently with diets from trial 2, but not used in the growth study were used as controls. A sample of each of the 9 diets was dried overnight at 95 °C. Two subsamples (approximately 5 grams each) of each dried diet were placed in 50 ml of distilled water for 30 minutes. The water was then passed through a dried and preweighed glass filter and analyzed using ICAP (Clesceri et al., 1998). The filter was dried to constant weight at 95 °C and weight of leachate determined. The amount of K^+, Na^+, and Mg^{2+} that leached out of each diet was then calculated. There are no standard methods for determining leaching rates of minerals, hence, we chose to simply place intact feed in distilled water to provide a relative value of what could leach under the most extreme conditions.

Statistical Analysis

Statistical analyses were performed using SAS (version 8.2, SAS Institute, Cary, North Carolina). Data from both experiments were analyzed using one-way analysis of variance to determine if significant differences ($P \leq 0.05$) existed among treatment means. Student-Newman-Keuls multiple comparison test (Steel and Torrie, 1980) was used to determine differences among treatment means.

Results

Trial 1

There were no significant differences in survival and growth among treatments (Table 4). Survival among treatments ranged from 79-92%, being lowest in the 300 ppm Mg^{2+} treatment and highest in the 2% NaCl treatment. Shrimp reared using the basal diet displayed the lowest weight gain (784.7 %) while shrimp from the 1% NaCl(954.3 %) and 1% K^{+} (917.1 %) treatments had the largest weight gain. Shrimp offered the basal diet exhibited the smallest mean individual weight (4.49 g), although values were not statistically different from other treatments ($P>0.05$).

Trial 2

There were no significant differences in percentage weight gain or survival among treatments in Trial 2 (Table 4). However, there were significant differences in mean individual weights of shrimp at harvest ($P = 0.035$). The final individual weights of shrimp offered the 1.0%

Table 4: Weight gain and survival of *L. vannamei* reared in artificial low salinity water fed two experimental diets supplemented with K^+, Mg^{2+}, and NaCl. Shrimp from Trial 1 had an initial individual weight of 0.5 g, while shrimp from Trial 2 had an initial weight of 0.28 g. Values are mean of four replicates in Trial 1 and five replicates in Trial 2. Values with different superscript are significantly different ($P \leq 0.05$).

Trial 1	Mean Weight (g)	Weight. Gain (%)	Survival (%)
Basal	4.49 [a]	784.7 [a]	81 [a]
1% NaCl	4.94 [a]	949.3 [a]	84 [a]
2% NaCl	4.77 [a]	886.7 [a]	92 [a]
150 ppm Mg	4.71 [a]	876.6 [a]	86 [a]
300 ppm Mg	4.91 [a]	901.6 [a]	79 [a]
0.5% K	4.95 [a]	865.2 [a]	85 [a]
1.0% K	4.92 [a]	917.1 [a]	83 [a]
PSE [1]	0.206	40.68	0.4
Trial 2			
Basal	4.54 [b]	1515.1 [a]	78 [a]
1% NaCl	4.89 [ab]	1605.9 [a]	82 [a]
2% NaCl	4.73 [ab]	1548.7 [a]	75 [a]
140 ppm Mg	4.53 [b]	1487.6 [a]	80 [a]
300 ppm Mg	4.86 [ab]	1596.6 [a]	80 [a]
0.5% K	4.91 [ab]	1617.0 [a]	80 [a]
1.0% K	5.34 [a]	1787.9 [a]	78 [a]
PSE [1]	0.169	72.6	0.56

[1] Pooled Standard Error

K^+ (5.34 g) diet were significantly higher than shrimp offered the basal (4.54 g) and 140 ppm Mg^{2+} (4.53 g) diets.

Hemolymph osmotic and ionic concentrations

There were no significant differences observed among treatments with regards to hemolymph osmolality or chloride concentration in both experimental trials (Table 5). Mean hemolymph osmolalities (± pooled standard error) were 618.47 ± 25.56 mmol kg^{-1} and 637.46 ± 26.3 mmol kg^{-1} for trials 1 and 2, respectively. Mean hemolymph chloride concentrations were 252.03 ± 10.8 mEq L^{-1} and 251.1 ± 12.6 mEq L^{-1} for Trials 1 and 2, respectively. Finally, mean hemolymph K^+ levels were 6.67 ± 0.26 mEq L^{-1} and 7.80 ± 0.37 mEq L^{-1} for trials 1 and 2, respectively, while Na^+ concentrations in both trials 1 and 2 were 302.86 ± 12.37 and 294.15 ± 12.6 mEq L^{-1} , respectively.

Hepatopancreas mineralization

There were no significant differences observed in hepatopancreas mineralization among minerals examined (Table 6). In both trials, dietary supplementation of K^+, Mg^{2+}, and NaCl did not affect the levels of K^+, Na^+ , Mg^{2+}, and Ca^{2+} in the hepatopancreas. The use of a chelated K^+ resulted in higher hepatopancreas K^+ levels in trial 2 (3.885-4.127 mg g^{-1}) compared to trial 1 (2.628-2.791 mg g^{-1}) where KCl was utilized as the K^+ source.

Table 5: Hemolymph osmolality and ionic concentrations in shrimp fed diets with mineral supplements of K^+, Mg^{2+}, and NaCl. Values represent the mean of four replicates in Trial 1 and five replicates in Trial 2.

Trial 1	Osmolality (mmol Kg^{-1})	Cl (mEq L^{-1})	Na (mEq L^{-1})	K (mEq L^{-1})
Basal	652.6	247.7	310.5	6.47
1% NaCl	611	248.6	304	7.1
2% NaCl	633.5	270.5	312.6	6.55
150 ppm Mg	595.6	245	293.8	7.02
300 ppm Mg	630.8	247.5	305.6	6.93
0.5% K	618.9	261.7	300.8	6.78
1.0% K	586.9	243.2	292.7	6.34
PSE [1]	25.56	10.8	9	0.26

Trial 2	Osmolality (mmol Kg^{-1})	Cl (mEq L^{-1})	Na (mEq L^{-1})	K (mEq L^{-1})
Basal	632.5	244.8	294.2	7.57
1% NaCl	600.7	232.3	283	8.54
2% NaCl	643.3	253.4	298.9	7.7
140 ppm Mg	633.8	249.5	298	8.12
300 ppm Mg	674.2	279.1	307.1	7.64
0.5% K	648.3	256.8	284.3	7.42
1.0% K	629.5	241.8	293.7	7.6
PSE [*]	26.3	12.6	12.37	0.37

[*] Pooled Standard Error

Table 6: Selected mineral content (mg g^{-1}) of the hepatopancreas for shrimp fed diets with mineral supplements of K^{+}, Mg^{2+}, and NaCl. Values represent the mean of four replicates. No significant differences were observed among treatments.

Trial 1

Diets	Na	K	Mg	Ca
Basal	7.977	3.453	0.654	2.828
1 % NaCl	6.919	2.684	0.584	2.491
2 % NaCl	7.602	3.147	0.639	3.145
150 ppm Mg	7.313	3.182	0.593	2.69
300 ppm Mg	7.048	3.105	0.64	2.674
0.5 % K	6.541	2.628	0.584	2.961
1.0 % K	6.778	2.791	0.557	2.261
PSE *	0.421	0.272	0.052	0.417
P-value	0.261	0.322	0.811	0.805

Trial 2

Diets	Na	K	Mg	Ca
Basal	8.733	3.749	0.636	3.776
1 % NaCl	9.524	3.926	0.684	3.348
2 % NaCl	9.4	3.988	0.621	3.376
140 ppm Mg	8.802	3.783	0.619	3.274
300 ppm Mg	10.04	4.12	0.656	3.838
0.5 %K(chelated)	9.009	3.885	0.637	2.778
1.0 %K(chelated)	9.603	4.127	0.663	3.252
PSE *	0.769	0.395	0.048	0.561
P-value	0.884	0.989	0.96	0.872

* Pooled Standard Error

Leaching

Ions quantified as leachates from diets used in trial 2 were calcium, potassium, magnesium, phosphorous, copper, iron, manganese, sodium, and zinc (Table 7). In the 0.5% K^+ and 1.0% K^+ treatment, 35.7% and 38.3% of the K^+ leached out of the diet, respectively. In the case of Na^+, when a coating agent was utilized, approximately 34.5% and 41% of the Na^+ in the feed leached out of the 1% NaCl and 2% NaCl treatments, respectively. In the 2% NaCl treatment without coating agent, a slightly higher amount of Na^+ leached from the diet (44.3%). When placed in distilled water for 30 minutes, all of the Mg^{2+} leached from diets irrespective of treatment with or without a coating agent.

Discussion

As the production of shrimp in inland low salinity waters continues to expand, so does the need for cost-effective methods for increasing the availability of essential ions to the organism in order to ensure proper growth and survival. Traditional practices, such as the application of fertilizers (Kmag and muriate of potash) directly to the pond water, have been proven effective at improving growth and survival (McNevin et al., 2004). However, the use of dietary supplements may either allow reductions in the level of supplements added to the water or provide additional performance enhancements in these marginal environments. It is well accepted that there is an interaction between dietary mineral requirements and the level of minerals in the water, hence, the supplementation of minerals such as K^+, Mg^{2+} and NaCl to the diet may help to reduce osmotic stress associated with these unique environments.

Table 7: Levels of ions leached (g kg^{-1}) from feed pellets (trial 2) submerged for 30 minutes in distilled water.

Diet	Ca	K	Mg	P	Cu	Fe	Mn	Na	Zn
Basal	0.145	3.788	0.288	0.82	0	0	0	0.448	0.02
1% NaCl (CA [a])	0.148	4.163	0.296	0.677	0	0	0	1.833	0.02
2% NaCl (CA [a])	0.163	4.88	0.329	0.701	0	0	0	3.698	0.02
140 ppm Mg	0.141	3.826	0.3	0.801	0	0	0	0.431	0.02
300 ppm Mg (CA [a])	0.144	4.079	0.316	0.852	0	0	0	0.595	0.02
0.5% K	0.136	6.028	0.334	2.063	0.02	0	0	0.464	0.02
1.0% K	0.137	8.074	0.354	3.712	0.02	0	0.02	0.489	0.01
300 ppm Mg (w/o CA [a])	0.113	3.77	0.291	0.745	0	0	0	0.427	0.02
2% NaCl (w/o CA [a])	0.148	5.196	0.341	0.751	0	0	0	3.963	0.02
DI water	0	0	0	0	0	0	0	0	0

[a] CA= Xtra Dry coating agent

Experiments in the present study were conducted at a salinity of 4.0 ppt, which is comparable to the salinity utilized by commercial shrimp farms in West Alabama. However, K^+ concentration in various waters in west Alabama varies by site, and consequently, K^+ requirement for *L. vannamei* reared in these well waters varies by site. In addition to salinity of the medium, concentrations of individual elements as well as ratios of various ions necessary for normal osmoregulatory function vary from farm to farm (Saoud et al., 2003). Saoud et al. (2003) reported that inland well waters utilized for shrimp culture in West Alabama were highly variable in terms of ionic profile. Thus, the concentrations of K^+, Mg^{2+} and other ions in the culture water can be markedly different even among farms in the same vicinity drawing water from the same aquifer.

Maintenance of potassium balance within the body is necessary for proper functioning of membrane potentials and all life systems. The lack of an adequate supply of K^+ in the water has been shown to negatively impact survival and growth of *L. vannamei* (McGraw and Scarpa, 2003, 2004; Saoud et al., 2003). Furthermore, while shrimp reared in full strength seawater and offered a K^+ free diet grew normally, Mg^{2+} storage in the carapace decreased noticeably (Davis et al., 1992). It is apparent that K^+ levels in the diet affect the physiology of shrimp. In Trial 2, shrimp offered the diet containing the 1.0% K^+ amino acid complex yielded significantly greater growth than shrimp fed the basal diet, thus demonstrating benefits of supplementation of chelated K^+ to diets formulated for shrimp cultured in low salinity waters. These results are supported by Shiau and Hseih (2001), who concluded that there is a K^+ requirement for *Penaeus monodon* that cannot be met solely by the available K^+ in brackish water containing 360 ppm K^+. Additionally, Gong et al. (2004) reported increased growth in *L. vannamei* fed a diet containing KCl, MgO,

and NaCl compared to a diet without any of these mineral additions at two different low salinity shrimp farms in Arizona. In a field trial conducted in low salinity waters at a shrimp farm in west Alabama, supplementation of a chelated source of K^+ to the feed increased growth rate of shrimp (unpublished data). Potassium supplementation to feed was also demonstrated to be beneficial to teleosts. Shearer (1988) reported that juvenile chinook salmon benefitted from dietary supplementation of K^+ when reared in fresh water.

Magnesium is an essential mineral required by crustaceans for normal growth and development (Davis and Lawrence, 1997). Magnesium serves as a cofactor in many enzymatic reactions important for normal function, and it is involved in osmoregulation, protein synthesis, and growth (Furriel et al., 2000; Guillaume et al., 2003). A lack of dietary Mg^{2+} has been shown to depress K^+ concentrations of the carapace in juvenile *L. vannamei*, indicating a possible interaction between K^+ and Mg^{2+} (Davis et al., 1992). Furthermore, low levels of aqueous Mg^{2+} have been correlated to reduced survival and growth of post-larval and juvenile shrimp (Saoud et al., 2003; Davis et al., 2005). Few studies have examined the role of dietary Mg^{2+} for *L. vannamei* reared in low salinity environments (Gong et al., 2004, Cheng et al., 2005). Cheng et al. (2005) reported no differences in hepatopancreas Mg^{2+}-ATPase and Na^+/K^+ ATPase activities in diets containing various levels of Mg^{2+} for *L. vannamei* reared in low salinity waters. These authors also reported a dietary Mg^{2+} requirement for optimal growth of 2.60-3.46 g Mg^{2+} kg^{-1} for *L. vannamei* reared in low salinity waters. In the present work, supplementation of Mg^{2+} did not improve growth and survival of juvenile *L. vannamei* reared in waters deficient in Mg^{2+}. Differences in results among published reports are probably due to differences in ion profile of

culture waters used. Levels of Mg^{2+} vary considerably depending on the source of the low salinity water (Saoud et al., 2003). Thus, the reported requirement of Mg^{2+} for *L. vannamei* in low salinity waters (Cheng et al., 2005) might be beneficial only under the experimental conditions in which the experiment was carried out. Furthermore, supplementation of Mg^{2+} might affect K^{+} metabolism (Davis and Lawrence, 1997) masking any beneficial attributes of Mg^{2+} supplementation. The use of a chelated Mg^{2+} source might improve delivery of this divalent cation to the animal and thus should be examined in the future.

Dietary supplementation of NaCl has the potential to provide benefits for euryhaline species. In two separate studies with juvenile red drum (*Sciaenops ocellatus*) reared in freshwater, growth and feed efficiency were improved when fish were fed a diet supplemented with NaCl (Gatlin et al., 1992; Holsapple, 1992). Gatlin et al. (1992) observed that, in juvenile red drum, ion losses at low salinity can significantly impair growth, however, this limitation was overcome by dietary supplementation of NaCl. Shrimp in the present experiment that were offered a feed supplemented with NaCl appeared to survive better than in treatments without NaCl supplementation although differences were not significant. Sodium and chloride ions are the major osmolytes in the hemolymph of crustaceans, including shrimp (Castille and Lawrence, 1981; Mantel and Farmer, 1983; Pequeux, 1995). Dietary supplementation of these essential osmolytes potentially counteracts losses to the medium that cannot be counteracted by gill uptake in low salinity environments. The importance of NaCl regulation was demonstrated by McFarland and Lee (1963) who found that, in low salinity environments, *L. setiferus* maintains relatively high levels of serum sodium and chloride. However, there is a point beyond which organisms cannot

osmoregulate efficiently and thus become stressed. A dietary supplementation of salt was shown to remedy such stress in some cases. Gatlin et al. (1992) concluded that extra salt supplemented to diets of juvenile red drum compensated for salt deficiency observed in fish reared in dilute media. However, the benefits of NaCl supplementation are not universal for euryhaline species, as several studies have also documented the lack of beneficial effects on growth, feed efficiency and feed intake such as in Atlantic salmon (*Salmo salar*) and rainbow trout *(Oncorhynchus mykiss*) (Shaw et al., 1975; MacLeod, 1978). Dietary supplementation of NaCl in feed for shrimp reared in low salinity environments appears promising and merits further examination.

Hemolymph osmolality was not affected by dietary treatment. This is logical, because osmolality is usually a function of the salinity of the medium (Iwata and Shigueno, 1980; Gong et al., 2004) and not its ionic profile. It is reported that *L. vannamei* tend to be very euryhaline and have been shown to live in salinities ranging from 1 ppt to more than 40 ppt (Bray et al., 1994), which indicates that they are excellent osmoregulators as long as ion ratios in the water are adequate. Such is not the case in inland low salinity well waters, and that explains increased mortality and decreased growth even when measures of hemolymph osmolality were within adequate ranges. Gong et al. (2004) noted that shrimp reared with feed containing dietary sources of KCl, magnesium oxide, NaCl, phospholipids, and cholesterol had a more robust osmoregulatory capacity and a more stable osmolality compared to shrimp fed a diet containing no supplements. Their results may have been due to feed supplements other than the minerals, but they did not report effects of individual supplements. Levels of Cl^-, K^+, and Na^+ obtained in the present study were similar to those observed in other penaeids reared in low salinity water (McFarland and Lee,

1963; Dall and Smith, 1981). Supplementation of these minerals to the diet did not appear to affect levels in the hemolymph. This can be attributed to several facts such as leaching of minerals before consumption, the nibbling feeding typical of shrimp, and insufficient osmoregulatory capacity. Further studies where mineral levels in the water are modified concurrently with dietary supplements have to be performed before definitive conclusions can be made.

Levels of K^+, Na^+, Mg^{2+}, and Ca^{2+} in the hepatopancreas were not affected by dietary supplementation of K^+ , Mg^{2+}, or NaCl in either growth trials (Table 6). When K^+ levels in the hepatopancreas were compared among all shrimp offered diet 1 and diet 2, they were found to be higher in the second trial (3.749 - 4.127 mg g^{-1}) as compared to the first trial (2.628 - 3.453 mg g^{-1}). The observed results are probably due to the use of a coating agent for Mg^{2+} and NaCl diets, and the use of a chelated K^+ in the diets supplemented with K^+ in Trial 2. Additionally, it is noteworthy that although Ca^{2+} was not supplemented in excess of the dietary requirement in the present study, hepatopancreas levels of Ca^{2+} were higher in Trial 2 where a coating agent was utilized than in Trial 1 where no coating agent was added to the minerals (Table 6). Further growth trials utilizing diets containing a mineral coating agent in conjunction with diets receiving no coating agent might prove informative in terms of their application to feeds utilized for the culture of *L. vannamei* in low salinity environments.

In studies examining the effectiveness of dietary mineral supplementation, the amount of minerals that remains available to the organism after the feed was submerged in water was seldom examined. In the first growth trial there were no visible effects of the various minerals on growth. Thus, the question of availability of the minerals arose. In our experiment, approximately 45%

(0.5% supplement) and 43% (1.0% supplement)of the K^+ in the chelated K^+ amino acid complex leached from the feed after 30 minutes submersion, suggesting that a substantial amount of K^+ still remained in the feed and was available to the shrimp. Approximately 34.5%, 41%, and 44.5% of the Na^+ leached out of the 1% NaCl diet with the CA, 2% NaCl diet with CA, and the 2% NaCl diet without the CA, respectively. When comparing the 2% NaCl diets with and without the coating agent, the diet with the coating agent lost a slightly smaller amount of Na^+. Very little Mg^{2+} leached out of either the CA coated or non-CA coated feed. Based on these results, the CA was not effective in laboratory-prepared feed and the use of a chelated form of Mg^{2+} is worth examining.

Conclusions

Dietary supplementation of minerals essential for osmoregulatory processes appears to be a promising practice for enhancing growth and survival of *L. vannamei* cultured in low salinity waters. These results suggest that there are beneficial effects on growth of *L. vannamei* reared in 4.0 ppt inland low salinity well-water when the feed is supplemented with 1%K^+. Although there were no significant differences observed with Mg^{2+} and NaCl supplementation, there was an observable trend of increasing growth that should be investigated more closely. However, these results do not justify a recommendation that minerals be added to shrimp feed or that mineral supplements in feed can replace mineral supplements to culture medium. The degree to which specific osmolytes are regulated over a range of low salinity waters with non-oceanic ion ratio profiles needs to be studied. A balance between feed supplements and partial remediation of water

composition coupled with low discharge at harvest will be the optimal method to farm shrimp in inland low salinity well-water farms in west Alabama.

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CHAPTER 4

EFFECTS OF VARYING LEVELS OF AQUEOUS POTASSIUM AND MAGNESIUM ON SURVIVAL, GROWTH, AND RESPIRATION OF *Litopenaeus vannamei* REARED IN LOW SALINITY WATERS

Abstract

Inland shrimp culture is being practiced in several regions of the United States. In Alabama, the culture of shrimp (*Litopenaeus vannamei*) in inland low salinity well water (approximately 4.0 ppt) faces several challenges. The ionic composition of these waters is deficient in several key minerals, including potassium (K^+) and magnesium (Mg^{2+}). The objective of the present study was to evaluate the effects of several aqueous K^+ and Mg^{2+} concentrations on survival, growth, and respiration in juvenile *L. vannamei* . Two experiments (14-day trial with postlarvae, and a 7-week trial with juvenile shrimp) were conducted to evaluate effects of K^+ supplementation to culture water. Four different levels of K^+ (5, 10, 20, and 40 mg L^{-1}) were utilized and a treatment of 4 ppt reconstituted seawater was used as a reference for comparison to ideal ionic ratios. Additionally, a 6-week growth trial was performed to evaluate the effects of five concentrations of Mg^{2+} (10, 20, 40, 80, 160 mg L^{-1}). Following completion of growth trials,

measurements of basal respirometry rates were conducted to assess stress. Results from the 7-week K^+ growth trial indicated significant differences ($P < 0.05$) in survival and growth among treatments. Individual weight, specific growth rate, and percent weight gain appeared to increase with increasing K^+ concentration (decreasing Na:K ratios). Results from the Mg^{2+} experiment reveal a significant difference in survival between the lowest Mg^{2+} treatment (60%) and all other experimental treatments (90-97%). However, no differences in growth were observed. Shrimp respiration in the lowest Mg^{2+} treatment (10 mg L^{-1}) was significantly higher than in the 80 mg L^{-1} treatment. These results suggest a potentially higher energetic cost associated with depressed aqueous Mg^{2+} concentrations that are common in low salinity environments.

Introduction

The inland culture of shrimp, particularly the Pacific white shrimp, *Litopenaeus vannamei*, is becoming more widespread in the Western hemisphere. Depending on their source, inland waters available for shrimp culture are usually of different salinities and possess different ionic compositions (Boyd and Thunjai, 2003). The ability of *L. vannamei* to tolerate a wide range of salinities (0.5 - 40 ppt) has made it a popular species for low salinity culture (McGraw et al., 2002; Samocha et al., 1998; Samocha et al., 2002). Despite the relative success of some farmers in culturing *L. vannamei* in inland low salinity waters, problems still arise from deficiencies in the ionic profiles of pond waters (Saoud et al., 2003; Atwood et al., 2004). The lack of a necessary mix of essential ions, including potassium (K^+) and magnesium (Mg^{2+}), has been demonstrated to limit growth and survival of shrimp (Saoud et al., 2003; Davis et al., 2005).

Alabama has several saltwater aquifers (Feth, 1970) that are being utilized as sources of low salinity water for aquaculture (Saoud et al., 2003). Farmers in west Alabama have been successful in raising *L. vannamei* in inland low salinity waters by raising the K^+ and Mg^{2+} levels of their pond waters to correct ionic ratio imbalances (McNevin et al., 2004). McNevin et al. (2004) observed increased shrimp production in Alabama low salinity waters (2-4 ppt) by raising the levels of K^+ (6.2 mg L^{-1}) and Mg^{2+} (4.6 mg L^{-1}) to 40 mg L^{-1} and 20 mg L^{-1} using muriate of potash and potassium-magnesium sulfate, respectively. Furthermore, various studies have demonstrated a benefit to having appropriate levels or ratios of K^+ and Mg^{2+} as well as

other minerals during post larval acclimation to low salinity waters (McGraw et al, 2002; McGraw and Scarpa, 2003; Saoud et al., 2003; Davis et al., 2005).

Both K^+ and Mg^{2+} are ions essential for normal growth, survival, and osmoregulatory function of crustaceans (Mantel and Farmer, 1983; Pequeux, 1995). Potassium is the primary intracellular cation and is also important in the activation of the Na^+-K^+-ATPase (Mantel and Farmer, 1983), which is a key component of extracellular volume regulation. The lack of adequate levels of aqueous K^+ could thus be potentially detrimental in terms of the ability to effectively osmoregulate, because enzyme activity can be directly related to K^+ concentration (Bursey and Lane, 1971). In Penaeid shrimp, hemolymph K^+ is regulated within a narrow range despite decreases in external salinity of the medium (Dall and Smith, 1981). Closely linked to the function of the Na^+-K^+-ATPase is adequate availability of Mg^{2+}, which serves as a cofactor (Mantel and Farmer, 1983; Furriel et al., 2000). The lack of adequate Mg^{2+} or K^+ can affect Na^+-K^+-ATPase activity in crustaceans (Mantel and Farmer, 1983; Pequeux, 1995; Furriel et al., 2000). Magnesium also plays a role in the normal metabolism of lipids, proteins, and carbohydrates serving as a cofactor in a large number of enzymatic and metabolic reactions (Davis and Lawrence, 1997)

It is also well known that oxygen consumption can be affected by variations in environmental factors such as salinity,diet, activity level, temperature, and body weight (Mantel and Farmer, 1983; Brett, 1987). The impact of salinity on penaeid shrimp physiology has been examined by various authors such as Villareal et al. (1994) and Spanopoulos-Hernández et al.

(2005). Less studied, however, is the impact of various ionic profiles of iso-saline inland low salinity well-water on shrimp respiration. Consequently, the objective of the present study was to evaluate survival, growth, and respiration of *L. vannamei* maintained in artificial low salinity waters with different concentrations of K^+ and Mg^{2+}.

Materials & Methods

Culture Conditions

The following study was conducted at the North Auburn Fisheries Research Station in Auburn, Alabama. Post-larval *L. vannamei* were obtained from Harlingen shrimp farm (Bayview, TX, USA). Post larvae (PL) were acclimated to low salinity water (4.0 ppt) over a period of 8 hours and maintained in a 220-L, polyethylene nursery tank connected to a biological filter. During the first week, PL were maintained on a combination of artemia nauplii (100 nauplii per shrimp) and a commercial feed, PL Redi-Reserve (Ziegler Bros. Gardner, Pennsylvania, USA) at 25-50% body weight. Thereafter, shrimp were offered a commercial feed (Rangen 35% protein, Buhl, Idaho) and reared in the nursery system until they were of appropriate size for commencement of growth trials.

The experiment was conducted in twenty, 150-L polyethylene tanks. Each experiment consisted of five treatments with four replicate tanks per treatment. Individual tanks were equipped with an airlift biofilter, submerged air diffuser, and submersible heater to maintain an adequate temperature (27 ± 0.5 °C). Shrimp were offered a commercial feed four times daily

using an automatic feeder. Light control was set at 16 hours day and 8 hours night. Dissolved oxygen (DO), pH, salinity, and temperature were measured daily whereas ammonia nitrogen and nitrite nitrogen were measured twice weekly according to Solorzano (1969) and Parsons et al. (1985), respectively (Table 1).

Artificial low salinity water

Artificial low salinity water was prepared two weeks prior to the commencement of each experiment. Experimental waters for the 14 day PL trial and the K^+ growth trial were prepared using well water from the North Auburn Fisheries Station. Treatment waters were made up by filling the tanks with 0.5 ppt reconstituted seawater (Crystal Sea Salt, Baltimore, Maryland, USA) and supplementing with 40 mg L^{-1} calcium from $CaCl_2.2H_20$, and 40 mg L^{-1} magnesium from $MgCl_2.6H_20$. Thereafter, KCl was added to four replicate tanks per treatment at levels of 5, 10, 20, and 40 mg L^{-1} K^+. Finally, salinity in all treatments was raised to 4.0 ppt using rock salt (NaCl). A 4 ppt reconstituted seawater treatment was also added as a reference. The reconstituted seawater reference had K^+ levels (and thus Na:K ratios) similar to that of the treatment with the highest K^+ supplement (40 mg L^{-1}).

Artificial low salinity waters for the Mg^{2+} growth trial were prepared similarly to the waters with potassium. Reconstituted seawater (0.25 ppt) was supplemented with 40 mg L^{-1} calcium from $CaCl_2.2H_20$ and 40 mg L^{-1} potassium from KCl. After that, magnesium ($MgCl_2.6H_20$) was added to provide concentrations of 10, 20, 40, 80, and 160

Table 1. Mean water quality parameters for growth trials with juvenile *L. vannamei* reared in low salinity waters. Values represent the mean ± standard deviation.

Parameter	14 day K^+ trial	K^+ growth trial	Mg^{2+} growth trial
Dissolved O_2 (mg L^{-1})	7.98 ± 0.04	7.24 ± 0.05	7.3 ± 0.13
Temperature (C)	25.2 ± 0.07	27.3 ± 0.1	27.0 ± 0.1
Salinity (ppt)	4.2 ± 0.02	4.1 ± 0.05	4.1 ± 0.06
pH	8.1 ± 0.0	8.0 ± 0.02	8.1 ± 0.03
TAN* (mg L^{-1})	0.08 ± 0.01	0.09 ± 0.04	0.03 ± 0.01
NO_2 (mg L^{-1})	0.54 ± 0.1	0.06 ± 0.04	0.04 ± 0.01

* Total ammonia nitrogen

mg L^{-1} per tank to four replicate tanks per treatment, respectively. The waters were then raised to 4.0 ppt using rock salt (NaCl). Experimental waters were analyzed for major ions using ICAP (Inductively coupled argon plasma spectrophotometry) (Table 2) and flame photometry (Cole Parmer digital flame photometer, Model 2655-00, Vernon Hills, Illinois) (Clesceri et al., 1998).

Effect of K^+ on postlarvae

Twenty PL_{39} (Mean initial weight, 30 mg) were stocked into each of the 20 experimental tanks with four replicate tanks per treatment (5, 10, 20, and 40 mg L^{-1} K^+). Shrimp were fed ad libitum 4 times daily for 14 days. At the end of the 14 day trial, shrimp were harvested and survival and growth were assessed.

Effect of K^+ on juveniles

The same artificial low salinity waters used in the first experiment were utilized in the present experiment. Fifteen juvenile shrimp (mean individual weight: 280 mg) were stocked per replicate tank. Shrimp in each tank were counted weekly and ration was calculated assuming a 1.75 feed conversion ratio and a doubling in size until individual shrimp weighed one gram. Thereafter, a growth rate of one gram per week was assumed. At the end of a 49-day growth period, shrimp were harvested, counted and group weighed. Hemolymph was collected using a 1cc syringe, and all samples were pooled in a 1.5-ml Eppendorf microfuge tube. Hemolymph

Table 2. Ionic composition (mg L^{-1}) of artificial low salinity waters (4.0 ppt) used to culture *L. vannamei* at North Auburn Research Unit compared to seawater.

K^+ Trial	5	10	20	40	Reference	Dilute SW 4.0 ppt
K	9.1	16.8	22.4	39	40.1	47.3
Mg	156.2	170.3	155	169.9	154.1	132.3
Ca	80.1	86.1	81.6	86.2	82.5	42.4
Na	1085	1137	1068	1114	1214	1316.3
Na:K ratio	119:1	68:1	48:1	29:1	30:1	28:1
Mg:Ca ratio	1.95:1	1.98:1	1.90:1	1.97:1	1.87:1	3.1:1

Mg^{2+} Trial	10	20	40	80	160	Dilute SW 4.0 ppt
K	37.4	36.3	35.9	38.9	33.2	47.3
Mg	16.8	26.5	46.3	92.4	162.6	132.3
Ca	69.6	71.1	69.5	76.3	66.6	42.4
Na	1397	1331	1308	1334	1069	1316.3
Na:K ratio	37:1	37:1	36:1	34:1	32:1	28:1
Mg:Ca ratio	0.24:1	0.37:1	0.67:1	1.21:1	2.44:1	3.1:1

osmolality, chloride, Na^+, and K^+ levels were measured and the remainder of each sample stored at -70 °C pending further analysis.

Effect of Mg^{2+} on juveniles

Fifteen juvenile shrimp (mean individual weight 1.2 g) were stocked into 4 replicate tanks per treatment (10, 20, 40, 80, and 160 mg L^{-1} Mg^{2+}). Shrimp were counted weekly and were fed as described in the K^+ growth trial. At the end of a 42-day growth period, shrimp were harvested, counted and group weighed. Hemolymph was extracted as previously described and extracts from each tank were pooled into a 1.5-ml Eppendorf microfuge tube. Hemolymph osmolality, chloride, Na^+, and K^+ levels were determined and the remainder of each sample stored at -70 °C pending further analysis.

Hemolymph osmotic and ionic concentrations

In order to determine hemolymph osmotic and ionic concentrations, stored samples were thawed on ice and then sonicated (25 W, 30 sec, Heat Systems Microson ultrasonic cell disrupt or, Farmingdale, New York) to disrupt the clot (Henry et al., 2003). The samples were then centrifuged (10 000 x g for 60 sec, Fisher 235B microfuge) to separate the clot from serum. Total osmolality was measured using a Wescor 5100C vapor pressure osmometer. Hemolymph chloride ion concentration was determined by Ag titration (LabconCo chloridometer, Petaluma, California). Finally, hemolymph Na^+ and K^+ concentrations were

measured by flame photometry (Cole Parmer digital flame photometer, Model 2655-00, Vernon Hills, Illinois).

Hepatopancreas mineralization

Whole shrimp were thawed and the hepatopancreas of five intermolt shrimp from each tank removed. Hepatopancreas samples were pooled per tank, then oven dried and wet ashed according to established methods (Association of Official Analytical Chemists, 1984). Both K^+ and Mg^{2+} levels were measured using atomic absorption spectrophotometry.

Respirometry

A Strathkelvin respirometer (Model 928) was utilized to determine oxygen uptake by shrimp. Following the completion of growth trials, respiration was measured daily in 6-8 shrimp of similar size using flow through respirometry. Oxygen consumption of shrimp from each treatment was performed in water identical in ionic makeup to the one in which the shrimp were reared. Four experimental chambers were constructed using 2" diameter transparent plexiglass pipe cut into 6" sections, capped with quick-disconnect PVC caps and fitted with a small mesh false bottom. The two ends of the chamber connected to tygon tubing for a water inlet and outlet. The volume of each chamber was 200 mL. Each chamber was set up on a magnetic stir plate, and a micro stir-bar was placed underneath the false bottom in order to assure adequate mixing. An oxygen probe was fitted through the top of each chamber. Oxygen saturated water

was gravity fed into each chamber from a 40 L tank of previously prepared 4.0 ppt low salinity water. In experiments where aqueous concentrations of K^+ and Mg^{2+} were evaluated, a separate water source containing the appropriate concentration of the ions examined were utilized for each treatment. Temperature was maintained at 27.8 °C throughout the experiment using a heater submerged in the supply tank. Water exiting the chamber was controlled by a flow-restrictor attached to the respirometry chamber and flow rate was set at 5 ml min^{-1}. As such, water was exchanged within the chamber every 40 minutes.

Oxygen sensors were calibrated using an oxygen saturated sample of the experimental water. Following calibration, intermolt shrimp were blotted dry, weighed, and one shrimp stocked per respirometry chamber. Four replicate respirometry estimations were performed simultaneously. An oxygen probe was placed in each of the chambers, flow rate was measured and shrimp were allowed to acclimate 5-7 hours or until respiration rate remained stable for at least one hour. Oxygen concentration following the acclimation period was measured in mg L^{-1}. Respiration rate (R) in mg O_2 g $shrimp^{-1}$ hr^{-1} was calculated using the following equation:

$$R = Q \times ([O_2 \text{ initial}] - [O_2 \text{ final}])/ W$$

In this equation Q is flow rate (L hr^{-1}) and W is the weight of the shrimp in grams. The oxygen electrode was placed inside the actual chamber where the shrimp was resting because of constraints pertaining to the availability of supplies for the chamber. The placement of the electrode inside the chamber created a scenario in which fresh oxygenated saturated water was mixed with water inside the chamber. However, as the flow rate was low (complete exchange

in 40 minutes) and the chamber was adequately stirred to maintain fairly stable oxygen concentration within the chambers. As such, the respiration values obtained in this experiment could potentially be underestimates of true basal metabolic respiration rates, but do allow for a comparison of respiration rates of shrimp reared in waters containing different ionic profiles.

Statistical Analysis

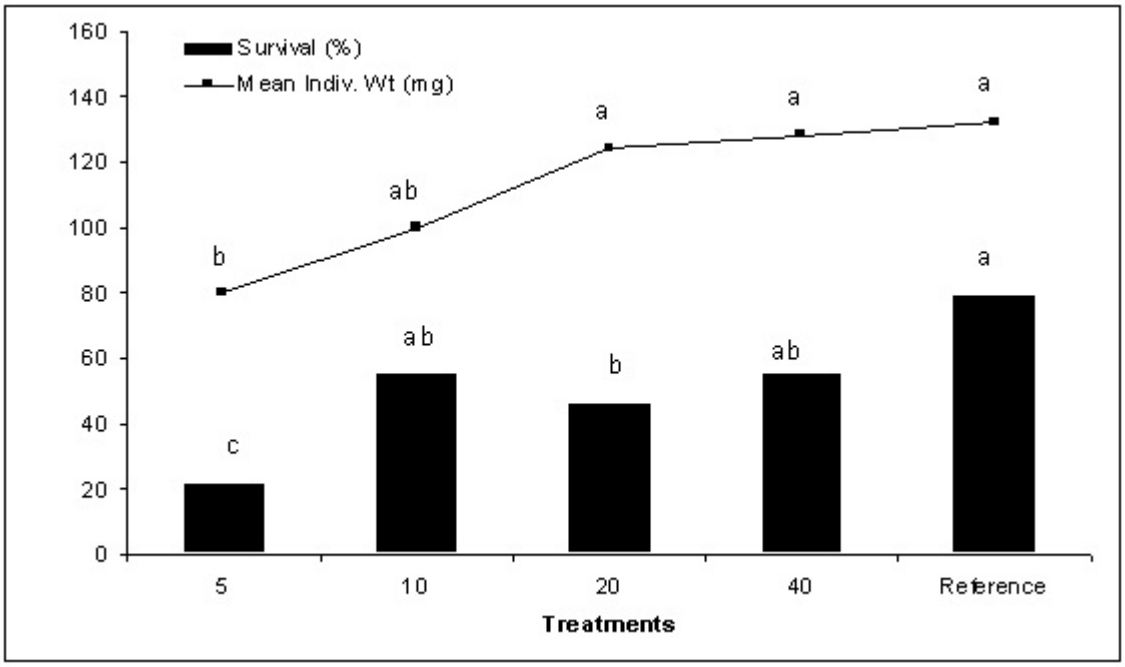
Statistical analyses were performed using SAS (version 8.2, SAS Institute, Cary, North Carolina). Data from both experiments were analyzed using one-way analysis of variance to determine if significant differences ($P \leq 0.05$) existed among treatment means. Student-Newman-Keuls multiple comparison test (Steel and Torrie, 1980) was utilized to determine differences among treatment means.

Results

Effect of K^+ on postlarvae

Results from the preliminary K^+ trial revealed significant differences in survival and mean individual weights after 14 days of culture (Figure 1). Survivals were low across all treatments with the exception of the reference (78%) which contained ionic profiles most similar to full strength seawater. The lowest K^+ treatment yielded the lowest survival (21.25%) and was significantly different from all other treatments ($P < 0.05$). Survivals in the 10, 20, and 40 mg L^{-1} K^+ treatments ranged from 46.3-55.0%. The 10 mg

Figure 1: Survival (%) and mean final weight (mg) of postlarval *L. vannamei* stocked in the 14-day preliminary growth trial with K^+.



L^{-1} treatment (46.3%) was significantly different from the control. Individual final weight was highest in the reference (0.132 g) and was significantly greater than weights in either the 5 mg L^{-1} (0.80 g) or 10 mg L^{-1} (0.99 g) treatments. Individual weights of the 20 mg L^{-1} and 40 mg L^{-1} K^+ treatments were 0.124 g and 0.128 g, respectively, and were also significantly different from the lowest treatment. Water quality parameters during the experiment remained within acceptable limits for the species.

Effect of K^+ on juveniles

Significant differences in final weight, survival, and percentage weight gain were observed in the 49-day growth trial in waters with various levels of K^+ (Table 3). Shrimp reared in the 4 ppt reference yielded the highest final weight (4.90 g) and weight gain (1854.6%), significantly different from all other treatments. The final weights and percentage weight gain of shrimp reared in the 20 and 40 ppm K^+ amended waters were significantly different from the 5 mg L^{-1} K^+ amended treatment. In general, as K^+ concentration increased, so did mean individual weight and percent weight gain. Survival in the 10, 20, 40 mg L^{-1} and reference ranged between 93.3-96.7%, and was significantly higher than survival in the 5 mg L^{-1} K^+ treatment (23.3%).

Table 3: Final weight (g), Survival (%), Weight Gain (%), hemolymph osmolality (mmol kg^{-1}), chlorides (mEq L^{-1}), K^+ (mEq L^{-1}), and Na^+ (mEq L^{-1}) for L. vannamei reared in low salinity water containing varying levels of K^+ and Mg^{2+}. Values represent the mean of 4 replicates. Letters that are different are significantly different ($P < 0.05$).

K^+ Trial	Indiv. Wt. (g)	Weight Gain (%)	Survival (%)	Osmolality (mmol Kg^{-1})	Cl (mEq L^{-1})	K^+ (mEq L^{-1})	Na^+ (mEq L^{-1})
5	2.40 ^{c}	852.8 ^{c}	23.3 ^{b}	629.8 ^{a}	261.4 ^{a}	7.4 ^{a}	283.8 ^{a}
10	2.79 ^{bc}	1064.8 ^{bc}	95.0 ^{a}	647.0 ^{a}	260.8 ^{a}	7.7 ^{a}	282.2 ^{a}
20	3.18 ^{b}	1145.5 ^{b}	96.7 ^{a}	629.3 ^{a}	267.5 ^{a}	7.3 ^{a}	289.2 ^{a}
40	3.30 ^{b}	1208.9 ^{b}	93.3 ^{a}	609.8 ^{a}	269.6 ^{a}	8.0 ^{a}	260.5 ^{a}
Reference	4.90 ^{a}	1854.6 ^{a}	93.3 ^{a}	631.3 ^{a}	264.8 ^{a}	7.6 ^{a}	275.7 ^{a}
PSE [1]	0.211	63.4	4.1	12.2	9.32	0.45	11.21
P-value	< 0.0001	< 0.0001	< 0.0001	0.37	0.96	0.39	0.77
Mg^{2+} Trial							
10	3.25 ^{a}	144.0 ^{a}	60.2 ^{b}	616.6 ^{a}	254.6 ^{a}	[2]	269.7 ^{a}
20	3.41 ^{a}	151.5 ^{a}	90.0 ^{a}	655.3 ^{a}	249.0 ^{a}	7.7 ^{a}	272.2 ^{a}
40	3.33 ^{a}	156.2 ^{a}	96.5 ^{a}	673.1 ^{a}	246.7 ^{a}	8.1 ^{a}	268.9 ^{a}
80	3.37 ^{a}	156.9 ^{a}	93.2 ^{a}	634.8 ^{a}	246.4 ^{a}	7.6 ^{a}	249.9 ^{a}
160	3.67 ^{a}	181.4 ^{a}	95.0 ^{a}	658.6 ^{a}	233.5 ^{a}	7.7 ^{a}	263.5 ^{a}
PSE [1]	0.167	11.87	4.1	22.1	7.31	0.42	14.23
P-value	0.49	0.28	0	0.44	0.6	0.84	0.8

[1] Pooled Standard Error

[2] Not enough hemolymph to run samples

Effect of Mg^{2+} on juveniles

There were no significant differences in final weights or weight gain (%) of shrimp reared in low salinity waters containing various levels of Mg^{2+} (Table 3). Final weights ranged from 3.25-3.67 g, while weight gain ranged from 144.0-181.4% across treatments. Nevertheless, the trend for the treatment with the highest concentration of Mg^{2+} (160 mg L^{-1}) to yield the largest weight gain, while the treatment with the lowest concentration of Mg^{2+} (10 mg L^{-1}) to yield the lowest weight gain was worth noting. Survival in the lowest Mg^{2+} treatment (10 mg L^{-1}) was 60.2%, significantly lower than all other treatments which ranged from 90.0 - 96.5%.

Hemolymph osmotic and ionic concentrations

There were no significant differences in hemolymph osmolality, Cl^-, Na^+, or K^+ in either the K^+ or Mg^{2+} supplementation experiments (Table 3). In the K^+ trial, mean hemolymph osmolalities were 629 mmol kg^{-1}, while hemolymph chloride was 264.8 mEq L^{-1}. Water osmolality in this experiment was 149.0 mmol kg^{-1} . In the Mg^{2+} growth trial, mean hemolymph osmolality was 647.7 mmol kg^{-1}, whereas mean hemolymph chloride was 246 mEq L^{-1}. Water osmolality was 130.0 mmol kg^{-1} , slightly lower than levels in the K^+ trial. In the K^+ trial, mean hemolymph K^+ and Na^+ was 7.6 mEq L^{-1} and 278.3 mEq L^{-1}, respectively. In the Mg^{2+} trial, mean hemolymph K^+ and Na^+ were also similar to those in the K^+ trial, 7.8 mEq L^{-1} and 264.8 mEq L^{-1}, respectively.

Hepatopancreas mineralization

In the K^+ trial, shrimp in the treatment with the lowest K^+ concentration (5 mg L^{-1}) had a significantly higher storage of Na^+ (1.78 mg g^{-1}) compared to those in the 4 ppt reference treatment made of reconstituted seawater (1.08 mg g^{-1}). Levels of Na^+ in shrimp hepatopancreas at 10, 20, and 40 mg L^{-1} K^+ treatments were not significantly different from the reference (Table 4). In the K^+ trial, there were no significant differences observed among treatments in storage of K^+, Mg^{2+}, or Ca^{2+} . However, in the Mg^{2+} trial there were significant differences in the storage of Mg^{2+}. The 160 mg L^{-1} treatment had significantly higher hepatopancreatic storage of Mg^{2+} (0.85 mg g^{-1}) when compared to the 10 (0.62 mg g^{-1}) , 20 (0.61 mg g^{-1}), and 40 (0.67 mg g^{-1}) mg L^{-1} Mg^{2+} treatments. The control treatment was not significantly different from the 80 mg L^{-1} (0.74 mg g^{-1}) treatment. In the Mg^{2+} trial there were no significant differences in hepatopancreas storage of Na^+, K^+, or Ca^{2+} .

Respirometry

Respirometry trials conducted with shrimp reared in low salinity waters containing various levels of K^+ ions revealed no significant differences in respiration rates (Table 5). Respiration rates ranged between 0.336 and 0.415 mg O_2 g shrimp^{-1} hr^{-1} for all treatments. However, significant differences in respiration ($P < 0.05$) were observed among respirometry trials containing various levels of Mg^{2+} ions (Table 5). Respiration rates ranged between 0.299 and 0.494 mg O_2 g shrimp^{-1} hr^{-1}. The 10 mg L^{-1} Mg^{2+} treatment yielded the highest respiration

Table 4: Selected mineral content (mg g^{-1}) of the hepatopancreas for *Litopenaeus vannamei* in growth trials. Values represent the mean of 4 replicates.

K^+ Trial	Minerals			
	Na^+	K^+	Mg^{2+}	Ca^{2+}
5	1.78 [a]	3.74 [a]	1.09 [a]	3.74 [a]
10	1.42 [ab]	4.10 [a]	1.02 [a]	4.55 [a]
20	1.33 [ab]	3.29 [a]	0.93 [a]	5.23 [a]
40	1.40 [ab]	4.06 [a]	1.03 [a]	4.32 [a]
Reference	1.08 [b]	4.11 [a]	0.98 [a]	4.09 [a]
PSE *	0.14	0.47	0.1	0.62
P-value	0.049	0.7	0.84	0.54

Mg^{2+} Trial	Minerals			
	Na^+	K^+	Mg^{2+}	Ca^{2+}
10	13.44 [a]	4.96 [a]	0.62 [b]	3.09 [a]
20	11.48 [a]	4.08 [a]	0.61 [b]	3.09 [a]
40	13.28 [a]	4.75 [a]	0.67 [b]	3.26 [a]
80	11.19 [a]	4.26 [a]	0.74 [ab]	3.18 [a]
160	11.43 [a]	4.28 [a]	0.85 [a]	3.10 [a]
PSE *	1.03	0.14	0.048	0.046
P-value	0.38	0.46	0.019	0.97

* Pooled Standard Error

Table 5: Respiration rate (mg O_2 g^{-1} shrimp hr^{-1}) of *L. vannamei* cultured in 4.0 ppt low salinity waters with different levels of K^+ and Mg^{2+}.

K^+ Trial Treatment	n	Weight (g)	Respiration Rate (mg O_2 / g shrimp / hr)
5	7	3.91	0.375[a]
10	7	4.11	0.415[a]
20	6	5.45	0.356[a]
40	6	5.46	0.336[a]
Reference	6	4.91	0.358[a]

Mg^{2+} Trial Treatment	n	Weight (g)	Respiration Rate (mg O_2 / g shrimp / hr)
10	8	3.96	0.494[a]
20	6	4.39	0.434[ab]
40	6	4.42	0.344[ab]
80	6	5.23	0.299[b]
160	6	4.17	0.317[ab]

rate (0.494 mg O_2 g shrimp^{-1} hr^{-1}) and was significantly different from the 80 mg L $^{-1}$ treatment (0.299 mg O_2 g shrimp^{-1} hr^{-1}). The 160 mg L $^{-1}$ Mg^{2+} treatment produced the second lowest respiration rate (0.317 mg O_2 g shrimp^{-1} hr^{-1}), however, this was not significantly different from the 10 mg L $^{-1}$ Mg^{2+} treatment.

Discussion

Various strategies have been proposed to improve the growth and survival of shrimp reared in inland low salinity well-waters. These strategies were devised for specific species reared in inland low salinity waters. However, inland low salinity waters differ from each other, and variations in ionic profiles occur even in waters derived from the same saline aquifer (Saoud et al., 2003). Thus, it is common for saline well waters to have ionic profiles unsatisfactory for shrimp culture. Mineral supplements, in the form of fertilizers rich in K^+ and Mg^{2+}, have been suggested as remediation methods whereby the osmoregulatory capacity and thus growth and survival of shrimp cultured in low salinity waters might be ameliorated (Saoud et al., 2003; Gong et al., 2004; McNevin et al., 2004). Results of the present study demonstrate an effect of K^+ and Mg^{2+} on shrimp physiology.

K^+ Trials

Results from the 14-day trial with post-larvae indicate a response to K^+ concentration in the rearing medium. The fact that better growth was observed in the reference treatment was

probably because ion ratios were similar to those of natural oceanic water. In experimental treatments, we observed an increase in PL survival and mean individual weight concurrent with an increase in the amount of K^+ (resulting in a decrease in Na:K ratio). Similar results were reported by McGraw et al. (2002) and McGraw and Scarpa (2004), who found that PL age, salinity endpoint, and rate of salinity reduction influence PL survival following acclimation to low salinity rearing environments. In a study with PL_{18} and PL_{28} ,(McGraw and Scarpa, 2003) determined that a minimum concentration of 1 mg L^{-1} K^+ was necessary for adequate survival following a 48 hour acclimation period in freshwater (0.7 ppt). Saoud et al. (2003) and Davis et al. (2005) also observed a positive correlation between PL survival following acclimation to low salinity water and levels of K^+. However, the low survival observed in the preliminary trial suggests that the effects of low salinity acclimation can extend beyond the 24-48 hour period assayed by Saoud et al. (2003) suggesting that although short term bioassays are a good way to quickly screen waters, longer term studies should also be conducted.

In the present experiment, shrimp reared in 4 ppt reconstituted seawater had significantly higher weight gain when compared to all other treatments. Because the reference and the 40 mg L^{-1} K^+ treatment had nearly identical Na:K ratios (29:1 vs 30:1), other ions or ion ratios must have influenced growth and well being. Furthermore, results of the present work indicate that the closer the Na:K ratio is to 28:1 (which is the ratio found in full strength seawater) the better the growth of the animals. The poor survivals and slower growth in the treatment with the lowest concentration of K^+ had the highest Na:K ratio (119:1). In a series of farm trials conducted in west Alabama where shrimp were reared on two separate farms

containing different ionic profiles, better growth and survival were obtained in the farm with the Na:K ratio most similar to full strength seawater (Roy et al., 2006). Our results are similar to those obtained by Zhu et al. (2004) who reported that an Na:K ratio of 40-43:1 was adequate for *L. vannamei* reared at 30 ppt. In the study by Zhu et al. (2004), the lack of an adequate Na:K ratio resulted in low activity and death of juvenile shrimp at a salinity of 30 ppt. Fielder et al. (2001) also reported an influence of Na:K ratio on growth of Australian snapper, *Pagrus auratus*, cultured in saline groundwater deficient in K^+.

There were no differences in respiration rate among shrimp in the different treatments of the K^+ Trial. The respiration rates for the two lowest K^+ treatments were numerically higher (0.375 - 0.415 mg O_2 g shrimp^{-1} hr^{-1}) than the other three treatments (0.336 - 0.358 mg O_2 g shrimp^{-1} hr^{-1}), but were not significantly different. The fact that differences in survival and growth were not apparent in respirometry measurements suggests that respirometry studies are not always adequate to evaluate stress in penaeid shrimp. Saoud et al. (2004) suggest that scope for growth studies that evaluate total energy assimilation and expenditure are better indicators of shrimp well being. In the present study, K^+ levels might have affected feed uptake or assimilation thus affecting growth.

Mg^{2+} Trials

Results from the growth trial using various levels of magnesium indicate that shrimp growth was not influenced by depressed Mg^{2+} concentrations. There was a significant effect of

aqueous Mg^{2+} concentration on hepatopancreas storage of Mg^{2+}. Furthermore, survival in the treatment with the lowest Mg^{2+} concentration (10 mg L^{-1}) was significantly lower than survival in all other treatments, indicating a stressful environment due to depressed aqueous Mg^{2+} levels. Low aqueous Mg^{2+} concentrations were also associated with an increase in respiration rate in the shrimp (Table 5). The higher respiration rate of shrimp reared in low salinity water containing the lowest Mg^{2+} levels indicates that the shrimp were stressed and corroborates results of the survival experiment in which shrimp reared in high magnesium waters survived better than shrimp in the lowest magnesium waters.

The present study demonstrated an effect of potassium on survival and growth but not on respiration and osmoregulation. Such results suggest a possible effect of potassium on ingestion or assimilation or other functions that do not strongly affect metabolism. Conversely, magnesium levels affected survival and metabolism but not osmoregulation. Hepatopancreas levels of Mg^{2+} in various treatments suggest an active regulation of magnesium levels, which probably explain differences in respiratory rates. The green gland probably had to work much more in shrimp maintained in waters with 10 mg L^{-1} Mg^{2+} for hepatopancreatic levels of magnesium to be similar to those of shrimp at 80 mg L^{-1} Mg^{2+} (Mantel and Farmer, 1983).

Conclusions

Farmers growing shrimp in inland LSWW with depressed levels of K^+ and Mg^{2+} should continue to supplement K^+ and Mg^{2+} directly to ponds using fertilizers. Potassium and magnesium should be maintained at adequate concentrations to ensure optimum growth and survival. Ratios of Na:K should approximate the ratio of these two ions found in full strength seawater (28:1). Magnesium levels are also important for shrimp well-being, and can be maintained by regulation ratios of divalent cations in the water. Thus, we suggest that ratios of Mg:Ca should also approximate those found in natural seawater (3.1:1) to ensure adequate survival of *L. vannamei* reared under low salinity conditions. Before stocking post-larvae, farmers should check Na^+, K^+, and Mg^{2+} concentration to ensure that ratios and concentrations of these specific ions are not deficient. Further research on rapid assays of stressful environments are also needed. The present experiment demonstrates that respirometry studies are adequate in some cases but not always.

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CHAPTER 5

BRANCHIAL CARBONIC ANHYDRASE ACTIVITY AND NINHYDRIN POSITIVE SUBSTANCES IN THE PACIFIC WHITE SHRIMP, *Litopenaeus vannamei*, ACCLIMATED TO LOW AND HIGH SALINITIES

Abstract

The Pacific white shrimp, *Litopenaeus vannamei*, acclimated to 30 ppt salinity, was transferred to either low (15 and 5 ppt), or high (45 ppt) salinity for 7 days. Hemolymph osmolality, branchial carbonic anhydrase activity, and total ninhydrin-positive substances (TNPS) in abdominal muscle were then measured for each condition. Hemolymph osmotic concentration was regulated slightly below ambient water osmolality in shrimp acclimated to 30 ppt. At 15 and 5 ppt, shrimp were strong hyper-osmotic regulators, maintaining hemolymph osmolality between 200 and 400 mOsm above ambient. Shrimp acclimated to 30 ppt were slightly hypo-osmotic to the medium, and the degree of hypo-osmotic regulation increased dramatically at 45 ppt. Branchial carbonic anhydrase (CA) activity was low and uniform across all 8 gills in shrimp acclimated to 30 ppt, but CA activity increased in all gills after exposure to both low and high salinity. Anterior gills had the largest increases in CA activity, and levels of increase were approximately the same for low and high salinity exposure.

Branchial CA induction appears to be functionally important in both hyper- and hypo-osmotic regulation of hemolymph osmotic concentrations. Abdominal muscle TNPS made up between 19 and 38% of the total intracellular osmotic concentration in shrimp acclimated to 5, 15, and 30 ppt. TNPS levels did not change across this salinity range over which hemolymph osmotic concentrations were tightly regulated. At 45 ppt, hemolymph osmolality increased, and muscle TNPS also increased, presumably to counteract intracellular water loss and restore cell volume. *L. vannamei* appears to employ mechanisms of both extracellular osmoregulation and intracellular volume regulation as the basis of its euryhalinity.

Introduction

The Pacific white shrimp, *Litopenaeus vannamei*, which is found on the Pacific coast of the Americas from northern Mexico to Northern Peru, is a euryhaline species capable of tolerating a wide range of environmental salinity (0.5 to 40 parts per thousand) (Menz and Blank, 1980; Bray et al., 1994). *L. vannamei* is commonly found in offshore waters at depths of up to 72 m (Bailey-Brock and Moss, 1992), so adults typically experience stable salinities circa 35 ppt. The penaeid life cycle, however, involves a variety of stages that occur in a number of different habitats and salinities. While adults generally inhabit open ocean waters, larvae migrate to low salinity estuaries where they remain until the juvenile stage and then migrate back to oceanic waters. As such, adults are rarely exposed to low salinity environments or fluctuating salinities.

L. vannamei is also economically important, being the most widely cultured species of shrimp in the western hemisphere. Furthermore, since much of the culture occurs in low salinity environments (ponds and embankments), various researchers studied the osmotic tolerance of this species (e.g., Gong et al., 2004; Palacios et al. 2004a). In general, penaeid shrimp are osmotic and ionic conformers in the range of 24-26 ppt (700-780 mOsm kg H_2O) (Castille and Lawrence, 1981). Below that range they are hyper-osmotic regulators, maintaining hemolymph osmotic concentrations as much as 500 mOsm above that of the ambient medium; and above that range they are hypo-osmotic regulators, maintaining hemolymph osmotic concentrations as much as 300 mOsm below ambient (Bray et al., 1994; Castille and Lawrence, 1981; Gong et

al., 2004). Despite these studies, little is known about the actual physiological mechanisms of salinity adaptation in *L. vannamei*.

Euryhaline marine invertebrates respond to changes in salinity using intracellular volume regulation and hemolymph ionic/osmotic regulation. Volume regulation, in response to altered hemolymph osmotic concentration and subsequent cell swelling or shrinking, is typically accomplished by adjusting the size of the intracellular pool of organic osmolytes (e.g., free amino acids and/or quaternary ammonium compounds) (Pierce and Amende, 1981; Henry, 1995). Extracellular osmotic regulation, on the other hand, is a result of active ion uptake from dilute sea water, or ion excretion into concentrated seawater (Pequeux, 1995). These two mechanisms are linked: in organisms that are strong osmotic regulators, hemolymph osmotic concentrations change very little despite large changes in external salinity, and as a result, the need for intracellular volume adjustment is minimal. Most euryhaline shrimp are believed to fall into this category.

The mechanism of ion uptake in shrimp has not been systematically studied, but in other groups of decapod crustaceans (e.g., crabs, lobsters and crayfish) specific ion transport proteins and transport-related enzymes have been identified to play a role in the active uptake of the major ions, Na^+ and Cl^- (Mantel and Farmer, 1983). One of the central proteins in this mechanism is believed to be the transport-related enzyme carbonic anhydrase (CA). CA is known to be present in high levels of activity in the organ of ion transport, the gill, and its activity is sensitive to changes in environmental salinity (Henry, 1984; Henry, 1988a,b). For

brachyuran crabs, only the posterior 3 pairs of gills are involved in ion transport and have high, salinity-sensitive levels of CA activity. In fresh water crustaceans, such as crayfish, all gills have high CA activity (Wheatly and Henry, 1987). The distribution and salinity-sensitivity of branchial CA has not been examined in a hyper/hypo regulating species such as *L. vannamei*. One study reported no difference in CA activity in either anterior or posterior gills of *L. vannamei* in 35 vs. 10 ppt (Palacios et al., 2004a,b), but the period of low salinity exposures (3 hr and 24 hr) was much too short for low salinity mediated CA induction to occur (Henry and Watts, 2001; Henry et al,. 2002). Bouricha et al. (1991) reported no differences in CA activity between larval and postlarval stages of *Penaeus japonicus*.

The present study reports on CA activity in individual gills in shrimp fully acclimated to salinities in which they are isosmotic, hyper-regulating, and hypo-regulating. Furthermore, we present data on the relationship between the size of the intracellular pool of organic osmolytes, acclimation salinity, and hemolymph osmotic concentrations.

Materials & Methods

Experimental System

The following study was conducted at the North Auburn Fisheries Research Station in Auburn, Alabama. Juvenile shrimp were obtained from Harlingen shrimp farm (Bayview, TX, USA) and were held at 30 ppt for 4 weeks prior to commencement of experiments. Shrimp were maintained in a 220 l polyethylene nursery tank connected to a biological filter and were

offered a commercially prepared feed four times per day (Rangen 35% protein, Buhl, Idaho, USA). The experimental system consisted of a series of 150L tanks, each equipped with an airlift biofilter, air stone, and submersible heater to maintain constant temperature of 28.0 ˚C. Throughout the course of each experiment, shrimp were fed Rangen 35% protein feed four times per day using an automatic feeder. Light control was set at 16 hours day and 8 hours night. Dissolved oxygen (DO), pH, salinity, and temperature were measured daily, whereas ammonia (Solorzano 1969) and nitrite (Parsons et al. 1985) were measured twice weekly. In the CA experiment dissolved oxygen (5.96 ± 0.72 mg L^{-1}), temperature (26.6 ± 2.4 °C), pH (7.8 ± 0.2), total ammonia nitrogen (0.077 ± 0.10 mg L^{-1}), and nitrite nitrogen (0.24 ± 0.21 mg L^{-1}) remained within acceptable limits. Likewise, in the FAA experiment dissolved oxygen (6.03 ± 0.29 mg L^{-1}), temperature (27.6 ± 0.30 °C), pH (8.0 ± 0.2), total ammonia nitrogen (0.13 ± 0.06 mg L^{-1}), and nitrite nitrogen (0.16 ± 0.12 mg L^{-1}) also remained within acceptable limits.

Carbonic anhydrase

For the carbonic anhydrase experiment, four 150 L tanks were filled with reconstituted seawater of the following salinities: 5.0, 15.0, 30.0, 45.0 ppt. Shrimp were transferred directly from the 30.0 ppt nursery system into the target salinity water and allowed to acclimate for 7 days prior to CA activity evaluation. At the 7th day 6 postmolt and intermolt shrimp (mean weight: 9.6 ± 1.8 g) were selected from each salinity, and their gills were excised under a

dissecting microscope. In order to differentiate among gills, a classification system was devised (Table 1). The gill excision method utilized by Palacios et al. (2004a,b) grouped all gills from the 3rd maxilliped to the 2nd pereopod and designated them as "anterior", while all other gills were also pooled and designated "posterior." Our classification scheme delineates clear excision of individual gill-pairs and allows comparison of enzyme activity on an individual gill-pair basis. Individual gill pairs (left and right) from the same animal were identified, dissected out, and immediately homogenized in 2 ml of tris-phosphate buffer (225 mM mannitol, 75 mM sucrose, 10 mM Trizma base, adjusted to pH 7.40 with 10% H_2PO_4) using an Omni TH113 homogenizer (Waterbury, CT). The homogenate was centrifuged at 10,000 g for 20 min at 4°C (Sorvall RC5-B, Sorvall Instrumetns, Wilmington, DE), and the CA activity in the supernatant was assayed electrometrically using the delta pH method described by Henry (1991). Either 50 or 100 µl of supernatant (depending on gill size and acclimation salinity) was added to 6 ml homogenization buffer in a vigorously stirred, thermostated (4°C) reaction vessel. The reaction was initiated by adding 100 µl of $C0_2$-saturated water, and the subsequent change in pH was monitored via micro pH and reference electrodes (World Precision Instruments, Sarasota, FL) which were connected to a null point pH meter and linear chart recorder. Typically, a change in pH of around 0.2 unit was utilized to measure the initial velocity of the uncatalyzed hydration reaction. Protein concentrations were determined utilizing the Coomassie blue dye binding method (Bio Rad Laboratories, Hercules, CA) and CA activity was reported as µmol CO_2 mg protein^{-1} min^{-1} (Henry 2005).

Table 1: Gill number classification code and their corresponding location. The classification system begins with the anterior gills located at the anterior end of the gill chamber (closest to mouth) and runs backward to posterior gills.

Gill Number	Description
1 (G1)	anterior arthrobranch, corresponding to the third maxilliped
2 (G2)	posterior arthrobranch, corresponding to the soma of the first pair of pereopods
3 (G3)	pleurobranch, corresponding to soma of the second pair of pereopods
4-8 (G4-G8)	The remaining gills were excised in succession from the 3rd to the 5th pereopod.

Total ninhydrin positive substances

For the TNPS experiment 10 shrimp were transferred directly from the nursery system to salinities of 2.5, 5.0, 15.0, 30.0, 45.0 ppt and allowed to acclimate for 14 days. At the end of this period, shrimp were dried by blotting on filter paper, and hemolymph was withdrawn from the pericardial cavity using a 23 ga needle and 1 cc syringe inserted beneath the carapace at the cephalothorax-abdominal junction. Following hemolymph extraction, approximately 1 g of abdominal muscle was excised from each shrimp using a scalpel. Hemolymph samples were stored at -20 °C, while abdominal muscle samples were fixed in 5 ml of 80% ethanol and refrigerated. Total ninhydrin positive compounds were assayed colorimetrically utilizing the method of Lee and Takahashi (1966) using a norleucine standard. For TNPS assays with hemolymph, 100 µl of hemolymph was deproteinized in 400 µl of 80% ethanol and centrifuged at 10,000 g for 60 sec (Eppendorf USA/Brinkmann, Westbury, NY). A sub-sample of the supernatant (100 µl) was then used in the assay. Additionally, approximately 1 g of abdominal tissue was placed in 5 ml of 80% ethanol for 48 hours. The abdominal muscle extracts were then diluted 1:100 and TNPS concentration assayed, while TNPS in hemolymph samples were assayed directly with no dilution.

For analysis of individual amino acid composition, a sample (4 individual samples pooled into one composite sample) of both the muscle tissue extracted in 80% ethanol and hemolymph (non-extracted) from shrimp exposed to each salinity was sent off for analysis of physiological free amino acids by the Protein Chemistry, Laboratory, Texas A & M University,

Corpus Christi, TX. Free amino acids were extracted and quantified with reverse phase HPLC using a UV absorbance with a diode array detector.

Hemolymph osmotic and ionic measurements

In order to determine osmotic and ionic concentrations, hemolymph samples were thawed on ice and then sonicated (25 W, 30 sec, Microson, Heat Systems, Farmingdale, N.Y.) to disrupt the clot (Henry *et al.* 2003). The samples were then centrifuged at 10,000 g for 60 sec (Eppendorf USA/Brinkmann, Westbury, NY)) to separate the clot from serum. Total osmolality was then measured using 10 µl of serum by dew point depression (Wescor 5100C vapor pressure osmometer). Hemolymph chloride ion concentration was determined by Ag titration (Labcon Co. digital chloridometer, Petaluma, CA). Finally, hemolymph Na^+ and K^+ concentrations were measured by flame photometry (Cole Parmer digital flame photometer, Model 2655-00, Vernon Hills, Illinois).

Statistics

Statistical analyses were performed using SAS (version 8.2, SAS Institute, Cary, North Carolina). Data from both experiments was analyzed using one-way analysis of variance to determine if significant differences ($P \leq 0.05$) existed among treatment means. Student-Newman-Keuls multiple comparison test (Steel & Torrie, 1980) was utilized to determine

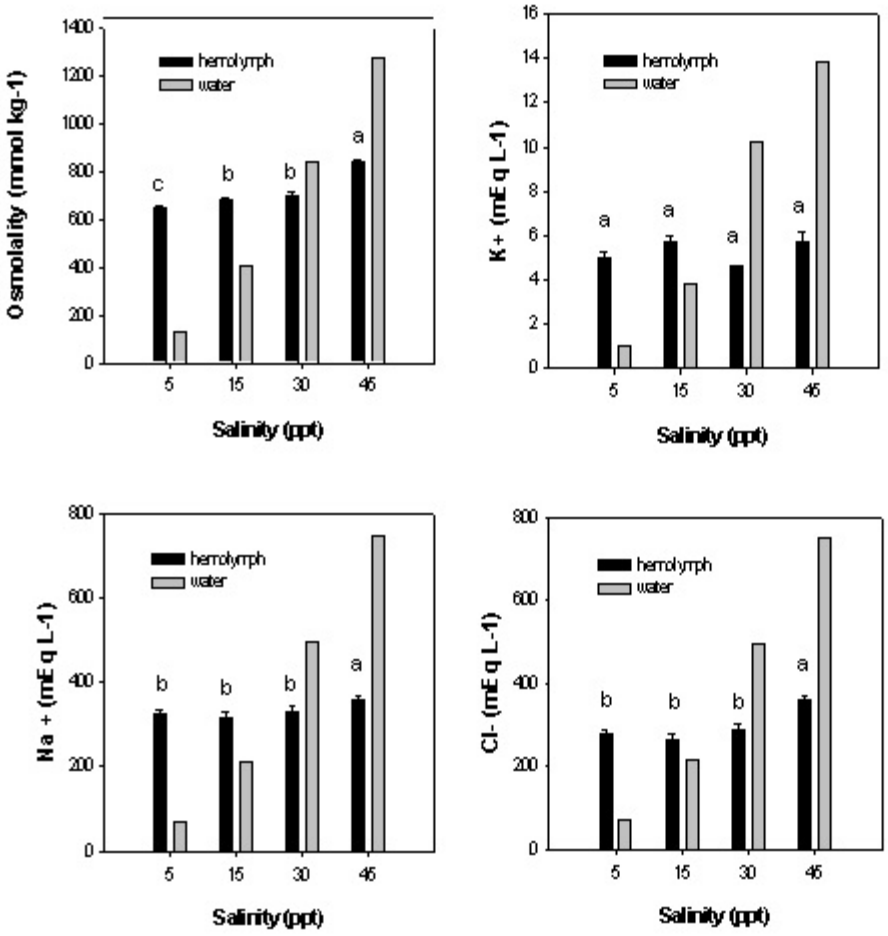
differences among treatment means in the TNPS experiment, while Dunnett's test was utilized to assess differences in carbonic anhydrase at the test salinities from activity at 30 ppt.

Results

Hemolymph Ion Regulation

Shrimp acclimated to 30 ppt salinity maintained their hemolymph slightly hypo-osmotic to that of the ambient seawater (Figure 1). At the lower salinities (15 and 5 ppt), shrimp were hyper-osmotic regulators with hemolymph osmolality being maintained at 274 mOsm above ambient at 15 ppt (682 vs 408 mOsm kg H_2O^{-1} for hemolymph and water, respectively, Figure 1) and 518 mOsm above ambient at 5 ppt (645 vs. 127 mOsm kg H_2O^{-1} for hemolymph and water, respectively, Figure 1). At 45 ppt, however, shrimp were hypo-osmotic regulators. Hemolymph osmolality was maintained 436 mOsm below that in the medium (838 vs. 1274 mOsm kg H_2O^{-1} for hemolymph and water, respectively, Figure 1). Similar patterns were observed for Na^+ and Cl^- (Figure 1). K^+ concentrations were held relatively constant at between 4 and 6 mM across the range of experimental salinities, and as such, this ion was strongly hyper-regulated at 5 ppt and strongly hypo-regulated at 30 and 45 ppt (Figure 1).

Figure 1. Hemolymph osmotic and ionic concentrations in Pacific white shrimp reared in 30 ppt salinity and acclimated to three different salinities for 7 days. Values are Mean ± SEM (N=3-6). Bars with the same letter are not significantly different ($P < 0.05$). Bars for the seawater values represent single measurements.



Carbonic anhydrase

For shrimp acclimated to 30 ppt salinity, CA activity was uniformly low (approximately 100 umol CO_2 mg protein^{-1} min^{-1}) across the 8 gills (Figure 2). CA activity increased in all gills in shrimp exposed to both low and high salinity. CA activity in anterior gills (G1-G6) were induced 2-4 fold, depending on salinity ($P < 0.05$, Dunnett's post-hoc comparison)(Figure 2). The magnitude of CA induction was approximately equal in 15 and 45 ppt. The changes in CA activity in posterior gills (G6-G8) were much less. The maximum CA induction was 2 fold, occurring at 15 and 45 ppt; but remarkably, CA activity in posterior gills was not different in shrimp acclimated to 30 ppt vs 5 ppt (Figure 2).

Total ninhydrin positive substances

In general, concentrations of TNPS in abdominal muscle were correlated with hemolymph osmotic concentration. While there were statistically significant differences in hemolymph osmotic concentrations for shrimp acclimated to 2.5 to 30 ppt, the changes were small and the values were relatively stable, varying no more than 120 mOsm kg H_2O^{-1} (Table 2). The concentrations of muscle TNPS, while also significantly different at some salinities, were also relatively stable across this range of salinity, varying between 90 and 170 mmol g dry weight^{-1} (Table 2). Assuming that wet muscle tissue has a water content of 70% total weight, TNPS made up between 18 and 38% of the total intracellular osmotic concentration of muscle, depending on acclimation salinity. Shrimp acclimated

Figure 2. Carbonic anhydrase activity in gills 1-8 of Pacific white shrimp acclimated to 30 ppt salinity and exposed to low (15 and 5 ppt) and high (45 ppt) salinities for 14 days. Values represent Mean ± SEM (n=5-6) Asterisks denote significant differences from 30 ppt ($P < 0.05$) .

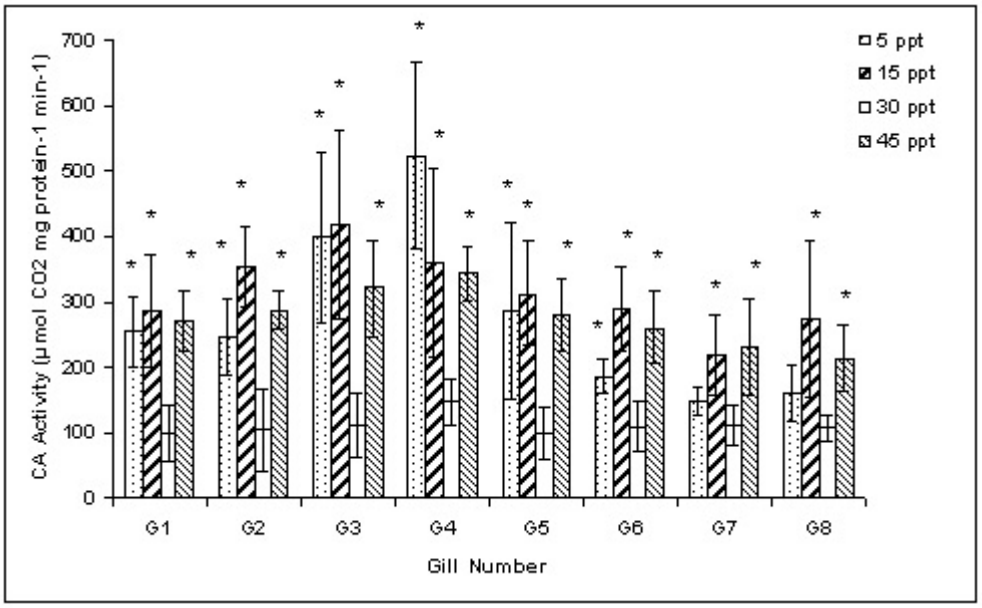


Table 2. Total ninhydrin positive compounds (TNPS) in the tail muscle and hemolymph of *L. vannamei* exposed to low and high salinity for 14 days. Values represent the mean ± standard deviation (n = 4-10). Values with different letters are significantly different from each other ($P < 0.05$).

Salinity	Tail muscle (mmol g^{-1} dry tissue)	Hemolymph (mmol L^{-1})	Water Osmolality (mmol kg^{-1})	Hemolymph Osmolality (mmol kg^{-1})
2.5	169.9 ± 41.1 [b]	0.291 ± 0.099 [a]	80	632.2 ± 17.8 [d]
5	132.9 ± 45.1 [bc]	0.133 ± 0.053 [b]	139	660.8 ± 12.9 [d]
15	91.0 ± 35.4 [c]	0.127 ± 0.068 [b]	408	702.0 ± 28.2 [c]
30	117.6 ± 21.6 [bc]	0.068 ± 0.036 [b]	889	759.9 ± 22.9 [b]
45	473.5 ± 69.8 [a]	0.149 ± 0.040 [b]	1355	854.14 ± 57.7 [a]
P-value	<0.0001	<0.0001	-	<0.001
PSE *	16.8	0.024	-	11.5

* Pooled standard error

- Only one water sample was taken

to 45 ppt were hypo-osmotic to the ambient seawater by 500 mOsm but still had elevated hemolymph osmolality compared to all other acclimation salinities (Table 2). Also, at 45 ppt, abdominal muscle has the highest concentration of TNPS, a 3-5 fold increase over values seen at the lower salinities (Table 2). TNPS made up 79% of the total intracellular osmotic concentration of abdominal muscle at 45 ppt. Hemolymph concentrations of TNPS were low and constant for all salinities except 2.5 ppt, at which there was a 2-fold increase (Table 2).

HPLC analysis of pooled abdominal muscle samples revealed that glycine (44.5-53.4%), alanine (8.8 - 12.0%), arginine (2.9 - 9.1%), and glutamine (3.2 - 6.2%) were the most abundant free amino acids regardless of salinity. The pooled hemolymph samples analyzed by HPLC revealed that proline (11.1 - 25.6%), taurine (11.0 - 20.0%), glutamine (12.1 - 16.2%), alanine (9.7 - 12.0%) and glycine (7.6 - 14.7%) were the predominant FAA.

Discussion

The present study confirms that *L. vannamei* is a strong hyper-osmotic regulator in low salinity and a hypo-osmotic regulator at high salinity. The isosmotic point for this species has been reported to be approximately 718 mOsm kg H_2O^{-1} (24 ppt)(Castille and Lawrence, 1981). Since the baseline salinity used in this study, 30 ppt, was slightly above that point, hemolymph osmolality was predictably slightly hypo-osmotic. That *L. vannamei* is a strong regulator is evidenced by the fact that at the two extremes of acclimation salinity, 5 and 45 ppt, hemolymph was regulated 500 mOsm above and below ambient values, respectively. This

magnitude of difference between the hemolymph and the ambient medium at 5 ppt is as large as that of some species of decapod crustaceans considered to be examples of the strongest osmotic regulators (e.g., the blue crab, *Callinectes sapidus*)(Henry, 2001; Henry and Watts, 2001). The same is true for hypo-osmotic regulation: *L. vannamei* can maintain its hemolymph osmotic concentration below that of a hypersaline medium to the same extent as a number of fiddler crab species (e.g., Rabalais and Cameron, 1985).

Hemolymph osmotic regulation was achieved by regulating the concentrations of the major ions, Na^+ and Cl^-. These ions make up over 90% of the total osmotic concentration of the hemolymph, and their concentrations changed little when the salinity was reduced from 30 to either 15 or 5 ppt. Shrimp acclimated to 45 ppt had a slight but significant increase in both hemolymph Na^+ and Cl^- levels; however, ionic concentrations in the hemolymph were still regulated significantly below those in the ambient medium. This is typical of the mechanism of hyper- and hypo- osmotic regulation both in other species of shrimp (McFarland and Lee, 1963), and in the more well-studied decapod crustaceans. A similar pattern was also seen for K^+, although this ion was more tightly regulated. Regulation of hemolymph K^+ within a relatively narrow range of concentrations appears to be a consistent trait in penaeid shrimp. Dall and Smith (1981) reported that *Penaeus plebejus*, *P. esculentus*, and *P. Merguiensis* regulated their K^+ levels within the range of 2 - 7 mEq L^{-1}.

The ability of euryhaline crustaceans to regulated hemolymph osmotic and ionic concentrations is believed to depend on the presence of a number of ion-transport proteins and

transport-related enzymes in the gills (Towle, 1984,1996; Henry, 2001). The two most important transport-related enzymes are the Na^+/K^+ ATPase and carbonic anhydrase (CA). When a euryhaline crustacean makes the transition from osmotic conformity to osmotic regulation, the expression and activity of these proteins are strongly up-regulated (Towle et al., 2001; Henry et al., 2002, 2003). Most previous studies have focused on the transition from conformity to hyper-regulation, for which CA is up-regulated by as much as 15-fold in decapod crustaceans, depending on species and salinity (Henry et al., 2003). This report is the first to show that branchial CA is up-regulated in response to both hyper- and hypo-osmotic regulation in a euryhaline marine organism. The degree of CA induction reported here is not as large as that observed in other decapod crustaceans, but the data here are most likely a conservative measure of the changes in CA activity. The baseline acclimation salinity used in this study was 30 ppt, a salinity that is slightly above the isosmotic point of the shrimp (Castille and Lawrence, 1981). CA induction in crustaceans has been shown to be highly sensitive to even small changes in salinity, doubling at the point of transition between conformity and regulation (Henry, 2005). CA activity in Pacific white shrimp acclimated to 30 ppt, therefore, was probably already above the baseline levels at that species' isosmotic point, and this most likely resulted in an underestimation of the degree of induction at either 5 or 45 ppt. Even so, it is interesting to note that the degree of CA induction observed here was equal for the transition to both hyper- and hypo-osmotic regulation, indicating that CA plays a role in both the active uptake and excretion of ions. In this case, the pattern of CA activity is similar to that of the Na^+/K^+ ATPase, whose activity is induced in the gills of fiddler crabs acclimated to hypersaline

conditions in which they are hypo-regulators (D'Orazio and Holliday, 1985). Preliminary results using fiddler crabs, indicate that CA activity is induced when the crabs are actively regulating their hemolymph either above or below the ambient seawater (L. Serrano and R. Henry, unpublished data).

Overall, *L. vannamei* displayed a pattern similar to that of freshwater crayfish (*Pacifastacus leniusculus*), in which CA induction occurred in both anterior and posterior gills in response to low salinity exposure (Wheatly and Henry, 1987). Branchial CA activity was uniformly low in all gills near the isosmotic point (750 mOsm), and activity was induced in gills 2-7 following a 3 week acclimation to low salinity (450 mOsm)(Wheatly and Henry, 1987). Although the distribution of CA activity across shrimp gills was not as homogeneous as in crayfish, differences between anterior and posterior gills were not as pronounced as in crabs (Henry, 1984, 2001). While CA activity in crabs in low salinity is greatest in posterior gills, in our study *L. vannamei* displayed slightly higher CA activity in the anterior gills. CA induction in anterior and posterior gills may be an adaptation of some species to low and high extremes of salinity, where ion uptake or excretion must occur at very high rates in order to ensure hemolymph osmotic regulation. This idea deserves more systematic investigation.

While the dynamics of CA activity has not been extensively examined in euryhaline shrimp, previous reports had indicated no differences in CA activity in gills of post-larval (PL_{20}) *L. vannamei* exposed to a 3 hr salinity challenge in which salinity was dropped from 35 ppt to 10 ppt (Palacios et al., 2004a). Palacios et al. (2004b) compared CA activity in shrimp

acclimated from 35 to 10 ppt over 3 and 24 hr periods in fed and starved post-larval shrimp and reported no differences in CA activity between anterior and posterior gills. However, in both of these studies insufficient time was allowed for salinity mediated induction of CA activity which generally takes several days to reach peak levels (Henry and Watts, 2001; Henry et al,, 2002). Bouaricha et al. (1991) examined CA activity in larval (nauplii, zooea, mysis) and postlarval (PL_1, PL_2, PL_4, PL_5 PL_{16}) *Penaeus japonicus* held in seawater and found no statistical differences in CA activity. In that study, however, CA activity was assayed on whole animal homogenates. CA induction occurs exclusively in the gills (e.g., Henry and Cameron, 1982), and thus, remaining tissue of adult or larval organisms would dilute changes in branchial CA activity to the point where they would not be measurable.

Despite the high degree of extracellular osmotic regulation, the hemolymph of *L. vannamei* does become more concentrated by about 150 mOsm when shrimp are acclimated to 45 ppt. At that point it appears the shrimp employ a mechanism common among invertebrates for adjusting intracellular volume: increasing the intracellular pool of organic osmolytes as measured by changes in muscle tissue TNPS. As extracellular fluid becomes more concentrated, the osmotic equilibrium with the intracellular fluid is disrupted, water is lost from the intracellular compartment, and cell shrinkage results (Pierce and Amende, 1981). Large changes in cell volume can disrupt normal cell function and lead to cell death (Deaton and Pierce, 1994). Many invertebrates adjust intracellular osmotic concentration (and therefore cell volume) by regulating the size of an intracellular pool of amino acids and quaternary ammonium compounds. This pool is increased in response to an increase in salinity in order to retain

osmotically obligated water and thus restore cell volume. In *L. vannamei*, the increase in the muscle TNPS pool coincided with the increase in hemolymph osmolality. The major components of the intracellular pool of organic osmolytes were glycine and alanine, and this is typical of crustacean tissue (Bishop et al., 1994) as well as muscle tissue in other invertebrate groups (Henry, 1995).

Conclusions

In summary, the Pacific white shrimp, *L. vannamei*, is able to withstand a wide range of salinity fluctuations by maintaining hemolymph osmotic and ionic concentrations within narrow levels. The enzyme carbonic anhydrase appears to be important in both hyper- and hypo-osmotic regulation, because it is induced in response to exposure to both low and high salinity. Furthermore, when hemolymph osmotic concentrations vary due to variations in environmental salinities, intracellular volume is adjusted by changing the size of the intracellular pool of organic osmolytes.

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SUMMARY & CONCLUSIONS

The culture of shrimp in inland low salinity well waters (LSWW) of Alabama faces a number of challenges before production can be maximized and a viable multimillion dollar industry can be established. These challenges pertain to economics, farmer education, marketing, and development of new technologies through sound applied science. The studies designed and carried out through this dissertation were aimed at furthering the knowledge of basic physiology and nutrition of *L. vannamei* in low salinity environments, while also improving upon existing technologies for the culture of this species in LSWW of west Alabama. These studies have improved our knowledge of the biology of *L. vannamei* as it relates to rearing in these unique low salinity environments.

The second chapter of this dissertation examines the possibility of supplementing cholesterol and lecithin to diets of *L. vannamei* reared in low salinity waters. Cholesterol is an essential sterol and phospholipids are important in normal gill membrane function. Several authors have suggested the use of dietary supplements of both cholesterol and lecithin as a means of improving growth, survival, and osmoregulatory capacity of shrimp reared in low salinity waters. Based on our study, there were no appparent benefits, in terms of survival and growth, incurred from dietary supplementation of either of these ingredients in excess of the

dietary requirement in the absence of an adequate ion profile for *L. vannamei* reared in LSWW. Thus, dietary supplementation of cholesterol and lecithin in excess of requirement is not warranted.

Dietary supplementation of potassium (K^+), magnesium (Mg^{2+}), and sodium chloride (NaCl) was examined in chapter 3 of this dissertation. All of these ions are essential in osmotic and ionic regulation in *Penaied* shrimp and are also important for maintenance and growth. Results from the first trial revealed no benefits of dietary supplementation of potassium chloride, magnesium chloride, or NaCl, although a nonsignificant trend was observed in the highest NaCl treatment. After examining the results, a second trial was designed to use ingredient alternatives that might reduce the amount of minerals leaching from the feed. A chelated potassium amino acid complex was utilized as the K^+ source, while a mineral coating agent was utilized for Mg^{2+} and NaCl treatments. In the second trial, improved growth, but not survival, was observed in the dietary treatment containing 1% K^+ utilizing the coating agent. No benefits were observed from the other diets, although once again a trend for improved growth was observed for the highest NaCl treatment. These results suggest that the use of a chelated K^+ source in the feed might prove beneficial for farmers growing shrimp in inland LSWW. The results obtained also warrant further examination, particularly the use of a chelated Mg^{2+} amino acid complex.

Aqueous supplementation of agricultural fertilizers containing sources of K^+ and Mg^{2+} (such as muriate of potash or K-Mag) have been utilized by shrimp farmers operating in LSWW to increase the levels of these ions in the water profile. Studies have demonstrated that

both K^+ and Mg^{2+} can be limiting factors in terms of growth and survival of both post-larval and juvenile shrimp. The concentration and also the ratio of these two ions in the water profile are important for normal growth and survival of a number of euryhaline fish and crustaceans. The 4th chapter of this dissertation examined the role of varying aqueous levels of K^+ and Mg^{2+} and the effects on survival, growth, and respiration in *L. vannamei*. In both experiments, the lowest Na:K ratio and Mg:Ca ratio resulted in reduced survival of *L. vannamei*. In the K^+ experiment, reduced growth was also observed at the lowest Na:K ratio. In addition, respirometry experiments with Mg^{2+} revealed an increased respiration rate at the lowest Mg^{2+} concentration and Mg:Ca ratio. Based on the results of our experiments, it appears that supplementation of K^+ and Mg^{2+} in the form of agricultural fertilizers is essential for farmers culturing *L. vannamei* in LSWW deficient in these two ions. In order to improve both survival and growth throughout the production cycle, supplementation of these ions is essential.

The 5th chapter of this dissertation examines the role of branchial carbonic anhydrase (CA) activity and free amino acids (FAA) in the muscle and hemolymph in *L. vannamei* exposed to low and high salinities. The patterns observed in branchial CA activity in *L. vannamei* were similar to those observed in euryhaline crayfish. Although not examined in this study, it is probable that the antennal gland has higher CA activity than the gills, as in crayfish. Free amino acids are important in isosmotic intracellular volume regulation. A decrease in salinity (2.5 ppt) caused an increase in TNPS levels in the hemolymph, while an increase in salinity (45 ppt) resulted in an increase in muscle TNPS levels. These patterns are consistent

with a number of other euryhaline crustaceans. The role of both CA activity and FAA needs to be further examined in *L. vannamei* reared in these unique LSWW environments.

The studies conducted herein have improved the knowledge base of farmers seeking to raise marine shrimp in inland LSWW. However, there is still a need for further examination of the problems associated with the culture of *L. vannamei* in LSWW of west Alabama. Improved production technologies and strategies are essential for the further growth of the inland shrimp industry in the state of Alabama.

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