# INVESTIGATIONS INTO THE REPRODUCTIVE PERFORMANCE AND LARVAL REARING OF THE BROWN SHRIMP, *Farfantepenaeus aztecus*, USING CLOSED RECIRCULATING SYSTEMS

A Dissertation

by

RYAN LEIGHTON GANDY

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

December 2004

Major Subject: Wildlife and Fisheries Sciences

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## ABSTRACT

Investigations into the Reproductive Performance and Larval Rearing of the Brown Shrimp, *Farfantepenaeus aztecus*, Using Closed Recirculating Systems.

(December 2004)

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The effects of unilateral eyestalk ablation, diets and sex ratios were evaluated on two wild populations of *Farfantepenaeus aztecus* in a closed recirculating maturation system. Ovarian development and spawning frequencies of ablated females in both studies were higher than the non-ablated females. Replacement of bloodworms in maturation diet with enriched adult *Artemia* sp. had no negative effect on the number of eggs spawned and resulted in increased hatch and survival rates from Nauplius I to Zoea I. Life span of ablated females fed enriched *Artemia* sp. was longer than ablated females fed bloodworms. Replacement of the expensive bloodworm diet component with adult enriched *Artemia* sp. is possible without negative impact on female reproductive performance. Reducing male to female ratio from 2:1 to 1:1 resulted in a 1.25% decrease in spawning activities of ablated females. The life cycle of pond-raised F1 generation *F. aztecus* also was completed in the closed recirculating system using unilateral eyestalk ablation as previously described. This study found diets that

contained an enriched adult *Artemia* sp. component performed superior (i.e. hatch rate, nauplii and zoea production) to a diet containing bloodworms.

Six consecutive larval rearing trials evaluated changes in select water quality indicators and their effect on growth, survival, and stress tolerance of *F. aztecus* postlarvae cultured in artificial seawater under closed recirculating and flow-through conditions. The closed recirculating larval rearing system successfully produced five-day-old postlarvae (PL) from Zoea I ($Z_1$) with similar dry weights, lengths and stress resistance to PL produced under standard water exchange practices. The trickling biofilters were found to be a limiting component of this system. A submerged coral biofilter was added to the system and effectively processed culture water for re-use. Addition of the submerged biofilter resulted in improved survival rates in Trials 4, 5 and 6.

These studies demonstrate maturation and larval rearing of *F. aztecus* is feasible in closed recirculating systems. Implementation of these systems in hatcheries bolsters biosecurity while reducing the environmental impact of hatchery effluent. Recirculating and re-use systems are therefore essential in the further development of sustainable hatchery programs for endemic species.

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# CHAPTER I

# INTRODUCTION

## Background

Over the past 50 years decreasing wild shrimp catch due to over exploitation of the fishery has caused increased governmental regulation. The reduction in wild catch coupled with increased demand for shrimp, has resulted in rapid growth of the shrimp farming industry. Development of alternative sources of edible shrimp, through aquaculture, to fill the increasing demand has led to the establishment of commercial shrimp farm operations. Commercial shrimp farms have significantly increased the volume of shrimp supplied to the world market. Three decades of research and the development of advanced farming techniques have allowed the production of food-grade shrimp to become economically profitable and for rapid development of shrimp farming worldwide. Over the past three years a trend of overproduction and competition from foreign sources has been driving wholesale market prices to historically low levels. These market trends are beginning to threaten the economic viability of United States shrimp farms. Domestic farmers are looking toward niche markets to insulate themselves from foreign competition. One example of this market strategy is the live bait shrimp market. The bait shrimp market is currently being evaluated because it requires a live native species as its main product, which is impractical to secure from foreign sources. The relatively insulated nature of the bait market, combined with reduced supplies of wild bait shrimp catches sets the stage for supplementation with a

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This dissertation follows the style and format of Aquaculture.

farm-raised product.

***History of United States Commercial Shrimp Industry: Commercial Development and Subsequent Government Regulation***

Commercial trawling of shrimp evolved from a small part-time industry after World War I to become the most valuable ocean fishery in the United States (Benefield and Moffett, 1990). During this time shrimp trawling began to change from cast net and beach seine methods to an efficiently mechanized trawler-based industry. The 1922 annual report of the Texas Game, Fish and Oyster Commission reported that shrimp could be caught with newly developed gear behind motorized trawls to a depth of 12.2 m. This mechanical advancement dramatically increased shrimp harvests in bay systems (Dokken et al., 1998). As the fishery became industrialized in the early 1900's the technological advancements to increase harvest capacities followed suit. Starting with the adoption of otter trawls in the early 1900's, the industry rapidly expanded. The otter trawl allowed for the use of smaller crews while increasing their catch per unit effort (Benefield and Moffett, 1990).

Historically, supplies of shrimp for the eastern Atlantic and Gulf of Mexico coasts has relied on the harvest of wild *Farfantepenaeus aztecus* (Atlantic brown shrimp*), F. duorarum* (Atlantic pink shrimp) and *Litopenaeus setiferus* (Atlantic white shrimp). Before the 1940's, the shrimp trawling industry was largely supported by catches of white shrimp. About 1947, the catch rapidly declined and shrimpers were forced to venture deeper into Gulf of Mexico waters where they found large quantities of brown shrimp (Benefield and Moffett, 1990). Since 1977, 74% of the shrimp landed in

Texas are brown shrimp (Benefield and Moffett, 1990). This "boom" of the commercial shrimp trawling industry and its implication of a high potential for over fishing in the early to mid 1900's was eventually followed by the development of federal fisheries programs.

Fishing effort and the result of exploitation can be seen in the catch history and government efforts to manage the United States trawl shrimp fishery via regulation. There have been 400% and 95% increases in Gulf and bay shrimp catches respectively, from 1961 to 1999 (TPWD, 2000a). This growth has represented an increase in bay harvests (135%) with a concomitant decrease in the Gulf of Mexico trawling (18%) since 1972 (TPWD, 1998). Commercial bay shrimp landings have increased significantly compared with the Gulf shrimp landings thus documenting an over fishing problem (TPWD, 1998). The over harvest of this natural resource forced the National Marine Fisheries Service (NMFS) to take action and impose additional regulations in order to maintain a sustainable yield of wild-caught shrimp (Warren, 1980; McKee, 1986; SFSC, 1992; Nance, 1993). In order to protect domestic shrimp stocks, the United States Congress enacted the Magnuson Fishery Conservation and Management Act in 1976. This act extended the United States jurisdiction from the "coastal borders of states" out to 200 nautical miles (Benefield and Moffett, 1990). It also laid the foundation for optimization of yield by initiating a nation-wide effort to manage the shrimp fishery. Fisheries biologists at the Texas Parks and Wildlife Department began to collect shrimp fishery statistics such as recruitment, population density, survival and essential habitat types to better manage this resource. These indices of population

dynamics are still employed by the NMFS in determining the annual closure of Gulf shrimping grounds to bolster recruitment. Despite these efforts, the trawler shrimp fishery continued to decline as state laws were slow to develop and were not contiguous or synchronous with adjoining state waters throughout the Gulf of Mexico. Further declines in the fishery prompted the establishment of a Gulf of Mexico Management Plan in 1981 by the South Atlantic Management Council to regulate the shrimp fishery along the eastern coast of the United States. Throughout the 1980's, catches continued to decline despite the concerted efforts of both national and state regulatory agencies. The Gulf of Mexico shrimp fishery for *F. aztecus, F. duorarum*, and *L. setiferus* was eventually declared over-exploited by the NMFS in 1990 (SFSC, 1992). The "catch" fishery has been overcapitalized since 1992 with more CPUE (catch per unit effort) expended than is economically profitable (SFSC, 1992). In an effort to reduce the negative effects of shrimping on the fishery, intensive scrutiny of gear modifications and the negative effects of trawling in ecologically sensitive areas have become issues discussed as part of the overall fisheries management strategy. The principal objective of state fisheries management has been to reduce trawling efforts in bays and estuaries which serve as essential nurseries for juvenile shrimp and are the source of recruitment stocks for the fishery.

***The Bait Shrimp Fishery***

Included in the commercial Gulf of Mexico shrimp fishery is a small subset of bay system trawlers which target smaller (e.g. 5 g) shrimp for the live bait industry.

These fishermen were long overlooked by regulators due to their typically limited economic impact, low biomass of shrimp harvested, and relatively small ecological impact on the overall fishery. However, bait shrimp trawlers have since been considered to have a significant ecological impact on the fishery due to the large numbers of small shrimp removed from these nurseries and recruitment grounds. In 1993, a proclamation issued by the Texas Parks and Wildlife Department recognized the continuing increase in the harvest of small (bait-sized) shrimp (<67 count) to be an ecologically unsustainable trend that would eventually result in the collapse of shrimp stocks (TPWD, 1993).

Negative impacts from over fishing resulted in a series of regulations in Texas to protect shrimp in bay nursery systems. In 1995, the State adopted House Bill HB750 that imposed new regulations on bait shrimpers. This bill created stricter eligibility standards (e.g., limiting the number of future bay bait shrimping licenses) with the goal of ultimately reducing the bait fishing fleet. This Bill was designed to provide long-term conservation of shrimp stocks and to create economic stability in the bay bait shrimp fishery (TPWD, 1995). The state stopped issuing new commercial bay and bait shrimp licenses in 1996 and has since implemented a statewide license "buy-back" program whereby the State purchases licenses back from commercial shrimpers (TPWD, 2000a). These "buy-back" efforts were increased when three decades of harvest data from TPWD revealed a rapid increase in bay bait shrimping effort and a 50% decline in the catch rate over this period (TPWD, 2000b). This information caused TPWD to change regulations to protect the smallest juvenile shrimp being harvested as live bait (TPWD,

2000c). To date, TPWD has purchased 554 bay and bait shrimping licenses from willing sellers (TPWD, 2001). From the beginning of this "limited entry" period, current license holders have been allowed to maintain their licenses and "grandfathered" until such time as they retire the license. If a license is "retired" under this buy-back program, it can not be replaced until the fishery shows signs that it could sustain increased fishing effort (TPWD, 2001). The buy-back program was combined with recently increased restrictions on bay shrimping to include: reducing fishing area by 18%, increased net mesh size, and decreased season length. These regulations supposedly allow more adult shrimp to migrate into spawning grounds to bolster recruitment. However, these regulations have severely decreased the availability of wild-caught bait shrimp in retail markets. This consistent lack of supply is compounded by its seasonal availability. In 1986, a decline in the harvest of wild bait shrimp was observed in the retail bait shrimp markets (McKee, 1986). McKee et al. (1989) observed that the retail demand for live bait shrimp in Texas waters exceeded the volume supplied by the bay bait shrimp fleet. Annual reductions in the supply of wild-caught live bait shrimp represent a clear opportunity to develop an alternative supply for this market.

### *Development of United States Shrimp Research*

Research to characterize the wild shrimp fishery eventually created an interest in culturing shrimp for retail markets. In 1929, federal response to increasing commercial pressure on the wild shrimp fishery established a research laboratory in Galveston, Texas. In 1950 a cooperative program involving Texas A&M University and the NMFS

intensified investigations into fisheries management issues (Temple, 1973). The NMFS Laboratory in Galveston was charged with investigating population dynamics, life histories, physiology and behavior of commercially important marine shrimp in the Gulf of Mexico. In the late 1960's this laboratory began to research the life cycles and basic physiology of local shrimp species which became the foundation of early culture technology for marine penaeid shrimp.

After observing shrimp farming developments in Asia, Allen (1963), reported a need to investigate the potential of developing a shrimp farming industry in the continental United States. The successful rearing of *Marsupenaeus japonicus* postlarvae (PL) had been reported in Japan by several researchers (Hudinaga, 1942; Hudinaga and Miyamura, 1962; Fujinaga, 1963; Hudinaga and Kittaka, 1967). The Galveston Laboratory, assisted by Hudinaga, attained successful early larval rearing of native Gulf of Mexico species (Cook, 1965; Cook and Murphy, 1966). These studies documented the production of *F. aztecus* postlarvae (PL) in small 250-ml beakers from eggs spawned by gravid females collected from the Gulf of Mexico. This ability to rear penaeid larvae to PL led to characterization of the larval stages of commercially important Gulf of Mexico penaeid species and resulted in the development of a generic key to the nauplii, protozoea, mysis and postlarval stages of native species (Cook, 1966). In addition, a basic protocol was established for small-scale PL production of six penaeid species from wild gravid females using diatoms and newly hatched *Artemia* sp. nauplii (Cook, 1969a).

In order to supply PL for further physiological studies, the initial larval rearing method was further refined for *F. aztecus* and *L. setiferus* (Cook and Murphy, 1969).

These studies, which were conducted in 15-L conical-bottomed tanks, documented the beneficial effect of ethylenediaminetetracetic acid (EDTA) on larval survival. In addition, studies were carried out to evaluate the effect of temperature and salinity on PL production.

The first attempt to raise shrimp in outdoor ponds was reported by Cook (1969b). In this study early PL of the *F. aztecus* produced in the Galveston Laboratory were stocked into two, 0.05-hectare ponds at a density of 20 PL/m$^2$ in Grand Terre, Louisiana. One pond was fertilized with rice husks to increase micro-organisms and pond substrate and the other pond was untreated. Both ponds were supplemented with a commercially available “rabbit chow” toward the end of the six-month study. This study concluded that the shrimp stocked in both ponds exhibited rapid growth initially with reduced growth as biomass density increased. The addition of “rabbit chow,” at 5% of the estimated biomass (dry weight feed/wet weight biomass) per day increased the growth rate in both ponds. However, Cook (1969b) listed two potential major limiting factors to large-scale production. These were: difficulty in obtaining large numbers of eggs from wild gravid females and inadequate production methodologies for producing large numbers of PL for stocking ponds. The developing interest in commercial shrimp farming of the late 1960's and subsequent need for large scale PL production techniques in the 1970's resulted in greater research interest in maturing native species in captivity (Cook, 1970).

Research efforts at the NMFS Galveston Laboratory intensified into the development of shrimp hatchery techniques to assist in the commercialization of shrimp

hatcheries (Mock, 1971). Until this time the most common method of larvae culture was the "Japanese Method." This method called for placing gravid females in previously fertilized large tanks (60 $m^3$ to 200 $m^3$). Wild gravid females are allowed to spawn then removed from the tank. Once nauplii are detected in the water column, the natural bloom of diatoms in the tank water is enhanced through various techniques (e.g., use of organic and inorganic fertilizers) (Mock and Neal, 1974). This enhanced natural algal bloom is established as a feed source for the shrimp larvae. Once the natural bloom of phytoplankton and zooplankton in the system is depleted, *Artemia* sp. cysts are added to the larval rearing tank to provide a food source as they hatch (Mock and Neal, 1974). Research efforts at the Galveston Hatchery became focused on the development of larval rearing techniques to increase production efficiency, reduce labor costs and lower initial capital investment of hatcheries (Brown, 1972; Mock, 1974). This new approach to larval rearing became known as the "Galveston Method" (Mock et al., 1980). This method created better control over the larval rearing process through using separate spawning, algae culture and *Artemia* sp. hatching tanks. The separation of spawning tanks from larval rearing tanks allowed for the hatchery manager to maximize larval rearing productivity by transferring only viable nauplii of known quality and quantity to larval rearing tanks. The use of certain species of unicellular algae cultured separately from larval tanks as well as hatching *Artemia* sp. in separate tanks provided greater control over feed densities and feed types. These changes combined with improvements in tank design and basic larval husbandry techniques increased control over PL production and was responsible for the "Galveston Method" becoming the hatchery

production method of choice in the western hemisphere. Nevertheless, it is important to note that both methods still relied heavily on collecting gravid females from the wild.

While the Galveston Method was being refined, efforts to develop more consistent supplies of fertilized eggs for larval rearing research were increased. Criteria for the various stages of ovarian developmental of wild *F. aztecus* were established to improve spawning success (Brusher et al., 1972; Brown and Patlin, 1974). Attempts were also made to raise fully-matured females in outdoor ponds from PL produced from wild spawners (Grajcer and Neal, 1972). This study met with little success as limited maturation and spawning was achieved. Without reliable sources of PL for stocking ponds, researchers continued to evaluate new methods for collecting wild PL using fine mesh trawls behind motorized tows in nearshore waters (Fontaine et al., 1972). Other studies investigated the use of *in vitro* fertilization of *F. aztecus* eggs through strip spawning (Clark et al., 1973). While success at fertilization was limited, these studies were essential in describing the sperm and eggs of this species.

The first steps toward true controlled reproduction under laboratory conditions in the United States took place at the University of Miami in 1970 when the technique of unilateral eyestalk ablation, the removal of one eyestalk, was applied to induce ovarian development in *F. duorarum* under laboratory conditions (Caillouet, 1972). It was concluded that this technique could help develop a genetic improvement program for this species while reducing dependence on wild populations.

During this period research into penaeid shrimp maturation under controlled conditions continued to develop in Galveston. Brown et al. (1979) reported successful

induced maturation and spawning of *L. setiferus* under controlled laboratory conditions. These "breakthroughs" with captive maturation techniques generated intense interest. Many researchers indicated that this "breakthrough" firmly placed commercialization of shrimp farming "on the cusp of development" (Lawrence et al., 1979). Consequently, Brown et al. (1980), using *L. stylirostris* (Pacific blue shrimp) collected from Pacific Mexican waters, were able to mature, mate and spawn this species under laboratory conditions. Following this success, more research was focused on different aspects of induced maturation and larval rearing. These studies targeted tank design, culture method, animal husbandry techniques, feed types, feed efficiency and cost effectiveness of hatcheries (Renaud and Caillouet, 1987). Through this research commercial hatcheries were able to rapidly develop and supply the growing demand for PL. *L. stylirostris* was selected as the target species for further research into induced maturation, spawning, and larval rearing due to its high growth rate and the ease of closing the life cycle in captivity (Brown et al., 1980). In 1984, Brown et al. (1984) reported successful production of an F2 generation of this species in captivity. These studies were the first step toward domestication of exotic species at the Galveston Laboratory on the Gulf of Mexico.

Domestic research on penaeid shrimp native to the Gulf of Mexico was at its peak from the late 1960's through the early 1980's. During this period research and preliminary grow-out attempts using these species were aided by the ease of acquiring wild gravid females from coastal waters. Production trials from 1967 through 1982 were aimed at determining the commercial potential of native species in the continental

United States and focused on their performance in outdoor ponds. Many of the early grow-out trials yielded similar findings to that of Lester (1983), where native species demonstrated rapid growth to 6 g, but limited growth to edible size.

Development of shrimp farming in the continental United States was negatively affected due to the difficulty of obtaining permits for discharge, the culture of non-indigenous species, the lack of a consistent national aquaculture policy, conflicts with environmental and recreational groups and climatological problems (McVey, 1980). On the other hand, the rapid development of shrimp farming in the year-round growing climate of Latin America in the early 1980's, especially in areas where *L. vannamei* and *L. stylirostris* were indigenous, shifted research and commercial interest towards the domestication of these two species. These research efforts were focused on induced maturation and larviculture technologies to provide dependable supplies of seed stock supplies. Demand for these technologies diverted research efforts in the United States and other countries to species used by commercial farms in Latin America (McVey, 1980).

In the 1980's, approximately 90% of the domestic United States shrimp research was focused in five laboratories, NMFS Galveston, Texas A&M University (Angleton, Texas), Texas Agricultural Experiment Station (Corpus Christi, Texas), Texas Agricultural Experiment Station (Port Aransas, Texas) and the Oceanic Institute (Makapuu Point, Hawaii) (McVey, 1980). Cooperative research efforts of these laboratories comprised the beginning of the United States Marine Shrimp Farming Program (USMSFP). Their subsequent identification of *L. vannamei* as the keystone

species for commercialization and domestication shifted domestic research from native species to this highly successful non-indigenous species. *L. vannamei* was of great commercial interest because it demonstrated superior growth rates under similar conditions used in previous studies for growing native species (McKee, 1986). Offspring raised in a selective breeding program by USMSFP from specific-pathogen-free (SPF) captive populations demonstrated fast growth and disease resistance which resulted in widespread use of this species in commercial operations all over the world (Pruder and Tchobanoglous, 1990; Wyban, 1992). This shift in research efforts allowed further development of maturation and larval rearing protocols which were ultimately applied to other species of commercial interest in captive breeding centers throughout the world: *L. stylirostris* in New Caledonia (Goyard et al., 2002), *P. monodon* in Thailand, Indonesia and the United States (Treece and Fox, 1993; Menasveta et al., 1993; Biswas, 1994; Menasveta et al., 1994; Benzie, 1997) and *Marsupenaeus japonicus* in Australia (Preston et al., 1999; Preston et al., 2004). Key achievements in maturation, larval rearing, genetic selection and SPF development have been summarized by many authors. The most complete training manuals and research summaries can be found in Wyban and Sweeney (1991), Treece and Fox (1993), Bray and Lawrence (1992), McVey (1993) and Browdy (1998).

### *Developing Commercial Interest in Bait Shrimp Production*

Increasing demand, inconsistent wild supply and relatively high sales price, since 1980, continue to attract investors interested in producing farm-raised live bait shrimp.

Initial investigations (McKee, 1986; McKee et al., 1989) into the commercial potential of farm-raised live bait shrimp concluded that it was not financially feasible due to low yields and high operating costs. Thus, with no alternative supply, the trend of over fishing and the lack of a consistent supply have resulted in a constant increase in wholesale prices.

To increase yields, researchers began to apply intensive production techniques developed for non-indigenous species, to species native to the coastal waters of the southeastern United States. Production trials with native species were undertaken in mid-1990. A production study by Burkott (1994) yielded *L. setiferus* stocked at a density of 700 PL/$m^2$ to bait size (6.2 g) in 94 d with a 73.6% survival and a yield of 3.1 kg/$m^2$ (Burkott, 1994; Samocha et al., 1998a). In addition, these authors concluded that a live bait shrimp operation would be profitable under a two crop per year production strategy. In 1996, investigators at the Texas Agricultural Experiment Station, Shrimp Mariculture Research Facility (TAES-SMRF) began a series of studies to determine the potential for developing farm-raised bait shrimp in Texas. This research focused on the production of *L. setiferus* and *F. aztecus* using intensive nursery and pond management practices (Samocha, 1997; Samocha et al., 1998b; Blacher et al., 1999). Gandy (1997) and Gandy et al. (2001) investigated the retail market structure of the live bait shrimp industry along the Texas coast and found the market was severely under supplied during an eight-month period when demand was at its peak.

The lack of a domesticated disease-free source of seed stock forced researchers to collect gravid females from the wild for production studies. This represents a severe limitation to the development of a sustainable and bio-secure farm-raised bait shrimp production industry in the United States. The risk of introducing, propagating and disseminating viral pathogens from wild populations is high. This risk was realized when wild shrimp populations from Texas coastal waters were found to be infected with White Spot Syndrome Virus (WSSV) (TPWD, 1998). Discovery of viral pathogens in wild native populations makes the practice of collecting wild gravid females for the production of PL a non-sustainable endeavor. In fact, this risk was demonstrated in 1996 when a wild broodstock population of *L. setiferus* was brought into TAES-SMRF, Corpus Christi. Heavy mortality due to infection by a white-spot-like virus required elimination of this population and a complete facility sterilization. The acquisition of captive broodstock populations of native species free of known viral pathogens is a prerequisite for the sustainable and bio-secure development of this industry. The discovery of the WSSV in *L. setiferus* populations in Texas coupled with male reproductive degeneration disease in induced maturation studies (Leung-Trujillo and Lawrence, 1987; Talbot et al., 1989; Alfaro et al., 1993) forced a shift in research efforts toward the commercially important Atlantic brown shrimp, *F. aztecus.*

The primary objective of the current research was to close the life cycle of *F. aztecus* in captivity. Achieving this goal would be the first step in the domestication process of this species which would secure a consistent supply of viral-pathogen-free PL for producers. In addition to development of viral-pathogen-free stocks, the technology

used to raise these shrimp must also focus on development of more sustainable practices to reduce potential negative impacts from effluent water on receiving streams. In the early stages of commercial shrimp farming in Texas, few regulations were present regarding effluent discharge from these facilities. Public opinion, politics and some science have led to increased restrictions. In order to comply with regulatory requirements, shrimp farmers have been working with regulatory agencies and researchers to minimize waste releases. Given the strict nature of regulatory compliance, further development of this industry must accommodate these criteria. For these reasons, a secondary research effort of the current study was developed to focus on the use of closed recirculating systems for induced maturation and larval rearing. Use of such systems could increase bio-security in the production of native species under controlled conditions. The absence of detailed maturation and larval rearing information regarding *F. aztecus* using modern hatchery management techniques under closed recirculating conditions has hampered research and development efforts. This lack of detailed information suggested the need for a study with an emphasis on providing guidelines for PL production methodology (e.g., maturation and larval rearing).

# CHAPTER II

# EVALUATION OF THE EFFECTS OF UNILATERAL EYESTALK ABLATION, DIET AND SEX RATIOS ON THE REPRODUCTIVE PERFORMANCE OF WILD-CAUGHT *Farfantepenaeus aztecus* USING A CLOSED RECIRCULATING SYSTEM

## Introduction

Development of sustainable commercial bait shrimp aquaculture requires securing a supply of viral-pathogen-free seed stock through domestication and the application of modern bio-secure induced maturation techniques to captive broodstock populations. Seed stock used in the grow-out of *F. aztecus* has historically come from wild-caught spawners (McKee, 1986). The use of seed stock originating from wild spawners represents a risk to the bio-security and sustainability of future commercial ventures. Therefore, development of captive breeding populations of *F. aztecus* and methods for commercial-scale induced maturation is essential to the further development of this industry.

Efforts to induce maturation in captivity through unilateral eyestalk ablation, of the three commercially important species native to the Gulf of Mexico have been successful in the production of fertile eggs by the closed-thelycum shrimp, *F. duorarum* (Caillouet, 1972) and *F. aztecus* (AQUACOP, 1975) as well as the open-thelycum shrimp, *Litopenaeus setiferus* (Brown et al., 1979). Studies with *F. aztecus* were conducted in outdoor, shaded tanks (12 $m^2$) with no environmental control and at

stocking densities of 3.3-8.3 shrimp/$m^2$ using a 1:1 male to female ratio. Broodstock were exposed to a natural photoperiod, ambient temperature (25-30 C), salinity (35 ppt) and pH (8.15-8.35) with high rate of water exchange (200-300%/day). In these studies, salinity and temperature tended to fluctuate on a daily basis due the high rates of daily water exchange (200-300%) with ambient seawater. The authors mentioned daily temperature fluctuations of 2-4 C and short-term salinity variation due to heavy rain events. Although three generations of *F. aztecus* were produced in these outdoor maturation systems the reproductive performance of this shrimp was poor compared to other species (*L. vannamei, L. stylirostris, Fenneropenaeus merguiensis* and *Marsupeneaus japonicus*). The average number of eggs spawned per female by unilaterally-ablated females (15-32 g) in these studies was only 45,000 eggs, with hatching rates termed "moderate." Ablation was the only method found to be effective in inducing ovary development and spawning in this species (AQUACOP, 1977). These studies documented mating, molting activities, spawning frequencies and egg characteristics for this species. In addition, the authors evaluated the effect of prepared maturation feeds containing different levels of protein on the reproductive performance of this species.

In the decades following the aforementioned AQUACOP studies, research efforts shifted away from species native to the Gulf of Mexico. *L. vannamei* was eventually chosen as the principal species for development of commercial shrimp farming in the western hemisphere. Commercial culture of this species benefited from two decades of intensive research into refinement of maturation techniques. The use of closed

recirculating systems for establishment of bio-secure controlled environments has allowed for consistent production of viable eggs from ablated *L. vannamei* females (Ogle, 1992a). Intensive research efforts to increase the reproductive performance of captive populations have also led to the development of a basal diet consisting of frozen bloodworms (*Glycera dibranchiata*) and squid (*Loligo opalescens*). This diet provided adequate nutrition leading to production of high-quality eggs (Middleditch et al., 1979; Wyban et al., 1987). In contrast, other studies have reported no adverse effect on reproduction when bloodworms were replaced by adult *Artemia* sp. (Browdy, 1985; Naessens et al., 1997). Replacement of high-cost bloodworms with nutritionally-enriched adult *Artemia* sp. could represent a significant economic savings for producers. The development and refinement of maturation techniques for open- and closed-thelycum species (Primavera, 1985; Wyban and Sweeney, 1991; Bray and Lawrence, 1992 and Browdy, 1992) ultimately provided a basis for investigation of the reproductive performance of *F. aztecus*.

## Materials and Methods

### *Maturation System*

The maturation system used in this study was adapted from that designed by Ogle (1992b) and used by the Gulf Coast Research Laboratory (Ocean Springs, Mississippi). Each of the three maturation tanks (9.6 $m^3$; 4.0 m dia. 0.8 m deep) was lined with a 1 mm PVC membrane and equipped with a center drain (8 cm dia.). Water which entered the center drain flowed by gravity into a shared common filter system

housed in an adjacent room (Figure 2.1).

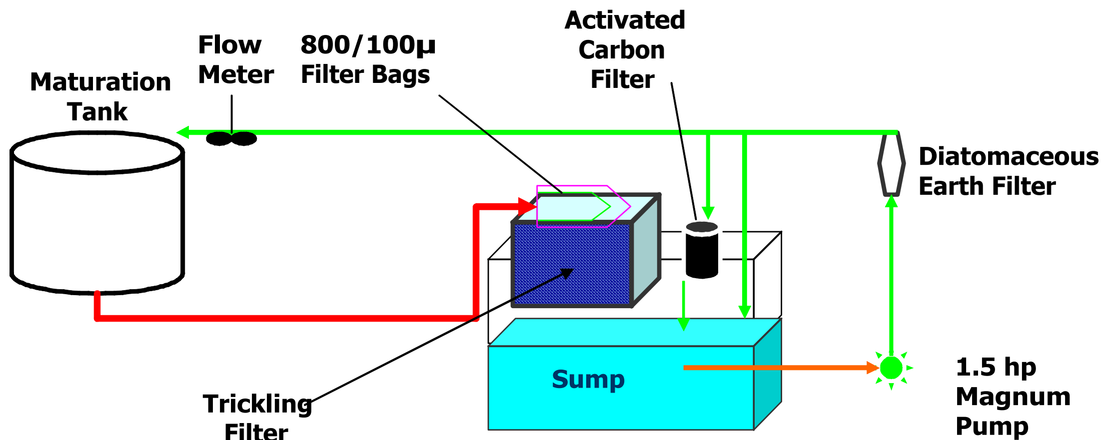



**Figure 2.1** Schematic diagram of the closed recirculating maturation system

Effluent from each maturation tank emptied into two filter bags; an inner 800 $\mu m$ bag for capture of large particles (e.g. leftover feed, feces and molts) and an outer 100 $\mu m$ which collected small particles and any eggs spawned within the maturation tanks. These filter bags were located on a perforated high-density polyethylene (HDPE) plate used to deliver a homogenized water flow over the subsequent trickling biofilter. Below this plate was a flat sheet of vinyl acetate filter material (Ogle, 1992b) for further particle filtration. The trickling biofilter (0.475 $m^3$) was constructed of extruded cage netting filled with 2.5-cm (1") Bio Pac media (Aquaculture Supply; Dade City, Florida) positioned over a 4-$m^3$ sump. A 1.5-hp centrifugal pump attached to the sump was used to discharge biologically-treated water into a 5-$\mu m$ Triton Nautilus FNS diatomaceous

earth (DE) filter (Purex; Sanford, North Carolina). About 5% of the water leaving the DE filter was diverted into a 7-L activated carbon-filter (Ocean Clear J320 Chemical Filter; Aquatic Ecosystems; Apopka, Florida) which discharged directly to the sump. The remainder of the processed water, from the common sump, returned to the maturation tanks. A hydraulic flow meter (FL7403; Omega Engineering; Stamford, Connecticut) was installed on the inflow line of each tank and used to maintain a flow rate of 32.2 liter/minute. Excess water in the filtration system was diverted back into the sump through a relief valve. The DE filter and in-line activated carbon filter components were added to the basic system design of Ogle (1992b) to improve water clarity and reduce daily system's maintenance.

Temperature in the system was maintained between 26.5 C and 27.0 C using two wall-mounted air conditioning units (total 18,000 btu) and a 12,000 watt submersible heater (Process Technology; Mentor, Ohio) placed in the sump. Lighting to maintain a photoperiod of 12 L:12 D over the maturation tanks was controlled using programable timers (Intermatic Corporation; Spring Grove, Illinois). Each tank had its own light bank which consisted of 25-, 50- and 75-watt incandescent bulbs and two 40-watt "cool white" fluorescent bulbs suspended 1.75 m from the water surface. Sunrise simulation was accomplished by activation of each bulb on a 15 minute transition from lowest wattage to highest. Once florescent lights were activated, the incandescent light sources were turned off completing the sunrise simulation process. Sunset was accomplished in reverse order of sunrise. The two fluorescent 40-watt "cool white" bulbs per tank yielded a maximum incident light intensity of 0.5 $\mu E/m^2/sec$. Biofilter media was

inoculated with Fritz-Zyme (Aquacenter; Leland, Mississippi). The pH of this system was maintained at 7.75 ± 0.5 during both studies by addition of 500 g of sodium carbonate (light soda ash) every five days.

***Spawning Room***

In a separate adjoining room, 12 individual 200-L (76 cm dia., 44 cm water depth) spawning tanks made of extrusion-welded black HDPE were used for spawning of individual females. Each spawning tank was equipped with one air stone (4 x 1.3 cm; Aquatic Ecosystems; Apopka, Florida), stand pipe (2.54 cm dia.) and an HDPE lid. Water in spawning tanks was maintained at 29 ± 1 C with a 300-watt submersible quartz heater (VT 301; Aquatic Ecosystems, Apopka, Florida). Source water was filtered to <5 $\mu m$ using a DE filter, foam fractionated and treated with 10 ppm ethylenedinitrotetracetic acid (EDTA, for chelation of heavy metals ions in the water). After each spawning event tanks were cleaned with a scrub brush and a solution of 10% sodium hypochlorite diluted to 10-ppm with tap water. Tanks were allowed to dry, in an inverted position to allow for complete drying, without rinsing the bleach.

***Water Quality***

Temperature, salinity, pH and dissolved oxygen (DO) levels were monitored daily. Temperature and DO were monitored using a YSI model 55 DO meter (Yellow Springs, Ohio), salinity was monitored by refractometer (model SR6; Aquatic Ecosystems; Apopka, Florida) and pH with a Cole Palmer Model 310 pH meter (Vernon,

Illinois). Nitrogen species ($NH_4$-N, $NO_2$-N and $NO_3$-N) were assayed weekly using HACH methods 10031, 8153 and 8039 (HACH, 1997) and a Perken-Elmer Lambda EZ201 spectrophotometer (Norwalk, Connecticut). Water in the experimental maturation system was maintained within the following parameters during the studies: temperature (26.5 $\pm$ 1.1 C), dissolved oxygen concentration (5.76 $\pm$ 0.51 mg/L), salinity (35 $\pm$ 1 ppt) and pH (7.75 $\pm$ 0.5) during both studies. System water during study 1 had mean $NH_4$-N, $NO_2$-N and accumulated $NO_3$-N concentrations of 0.50 $\pm$ 0.25 mg/L, 1.65 $\pm$ 0.95 mg/L and 48.5 mg/L, respectively. Study 2 had mean $NH_4$-N, $NO_2$-N and accumulated $NO_3$-N concentrations of 0.17 $\pm$ 0.10 mg/L, 1.05$\pm$ 0.75 mg/L and 52.6 mg/L, respectively.

***Daily System Maintenance***

Excess feed, molts and dead shrimp were removed daily before the first feeding of the day (0800 h) at which time the volume of excess feed, number and sex of molts, and tag number of dead shrimp was recorded. Other daily activities included monitoring maturation tank flow rates, feed rates (see subsequent section on Maturation studies), cleaning filter socks and checking for the presence of missed spawns in the 100 $\mu m$ filter bags. Dead females were replaced twice weekly with either ablated or non-ablated females. Female shrimp used for replacement were weighed and tagged upon introduction to the corresponding treatment tank. Activated charcoal and DE filters were recharged and changed, respectively, on a weekly basis.

***Experimental Populations of Shrimp***

Two experiments were conducted to evaluate the effects of unilateral eyestalk ablation, diet and sex ratio on the reproductive performance of wild (F) populations of *F. aztecus* in the aforementioned experimental system. Wild populations of shrimp were collected on two separate collection cruises at a depth of 50 m in the Gulf of Mexico near Freeport, Texas. Each population of broodstock was quarantined at the Texas Agricultural experiment Station-Shrimp Mariculture Research Facility for 30 days during which they were screened for known infectious viruses, including Infectious Hypodermal and Hematopoietic Necrosis Virus (IHHNV), Taura Syndrome Virus (TSV) and White Spot Syndrome Virus (WSSV). Upon notice of no viral presence, in the samples tested by the Texas Veterinary Medical Diagnostic Laboratory (College Station, Texas), populations were subdivided into three treatment groups.

The effect of sex ratio (2:1 and 1:1male to female sex ratio) was evaluated in the first and second experiments, respectively. The first population of 180 shrimp was sub-divided into three subgroups of 60 shrimp each and stocked into separate maturation tanks. Each subgroup consisted of 40 males (20 ± 3.6 g) and 20 females (36 ± 3.3 g) to achieve the desired sex ratio. The second population of 180 shrimp was also evenly distributed into three maturation tanks with 30 males (25 ± 4.6 g) and 30 females (41.6 ± 7.9 g) in each tank.

***Maturation Studies***

The two experiments, one for 142 days and the other for 132 days, were

conducted to evaluate the reproductive performance of the two broodstock populations under controlled conditions (Table 2.1). In both studies, females in two tanks were unilaterally ablated using a hot scalpel for cauterization. No eyestalk ablation was performed on the females in the third tank. In each experiment, shrimp in the non-ablated tank (Treatment 1) received a diet of frozen bloodworms (8% of the total wet shrimp biomass per day) and frozen squid (12% of the total wet shrimp biomass per day). The same diet was fed to shrimp in a second tank (Treatment 2) in which females were unilaterally ablated. Unilaterally ablated females in the third tank (Treatment 3) received a similar ration of squid; however, the bloodworm component was replaced with frozen adult enriched *Artemia* sp. To monitor the effect of diet and eyestalk ablation on reproductive performance, each female shrimp was tagged with a numbered latex ring mounted on one eyestalk (Browdy, 1985).

**Table 2.1** Maturation treatments

| Treatment | Ablation | Bloodworms[1] | Squid[2] | Enriched adult *Artemia* sp.[3] |
|---|---|---|---|---|
| 1 | No | Yes | Yes | No |
| 2 | Yes | Yes | Yes | No |
| 3 | Yes | No | Yes | Yes |

[1] Fed at 8% of the total wet shrimp biomass per day
[2] Fed at 12% of the total wet shrimp biomass per day
[3] Fed at 8% of the total wet shrimp biomass per day

***Data Collection***

Shrimp were placed in the closed-recirculating induced maturation system 2 weeks prior to initiation of each experiment. After acclimation all shrimp in their respective treatment tanks were weighed. Since larger females are known to produce larger spawn sizes (Primavera, 1985), the size consistency within a population at stocking was determined. Recording the weight at stocking allows for subsequent determination of average weight consistency among treatments to reduce the effect of weight differences on the overall reproductive performance of a treatment group. Following weight determination, identification tags were mounted on one eyestalk of the females in all treatments and females were ablated according to the experimental design. Observations of ovarian development commenced 24 h post-ablation. Following ablation, female shrimp in maturation tanks were surveyed nightly by flashlight at 1600 h for stage of ovarian development. Females with well developed ovaries (Stage 3-4) were removed from maturation tanks and placed individually in a spawning tank. The following morning (0800) previously-mated females were removed from spawning tanks and their ovaries were visually checked by shining light on the ventral side of the abdomen. Spawning as extent of residual egg mass (e.g. full, partial, none) was recorded and females returned to their respective treatment tanks.

Spawned eggs were homogenized within the spawning tank water column using a random manual stirring motion. Four, 261 mL samples were removed from each tank under continued mixing. Eggs were concentrated using a strainer made of 5.1-cm clear acrylic pipe with a 100-$\mu m$ sieve. Concentrated egg samples were then counted using

the following procedure: eggs were collected from the 100-$\mu m$ sieve using a Pasteur pipette (LPP10; Aquatic Ecosystems; Apopka, Florida) and placed as individual droplets on a glass petri dish with a 1-cm grid pattern on the bottom. The total number of eggs in the petri dish was then counted using a dissecting scope. This count was extrapolated to represent the total number of eggs spawned.

Two sets of three 50-mL samples were removed by a Hansen Stempel Pipette (1805-C42; Wildco; Saginaw, Michigan) from the homogenized spawning tank and placed individually in 100-mL petri dishes for incubation. Three petri dishes were used for (24 h incubation) nauplii counts and the remaining three petri dishes were reserved for $Zoea_1$ ($Z_1$) counts performed after 48 h. All petri dish samples were placed in an incubator maintained at 29 C for the required period. To minimize potential errors, all petri dishes were labeled with a tag color and number corresponding to the treatment and individual female identification tag. Stage of ovarian developmental at spawning, degree of spawn (e.g., partial or complete), total eggs spawned, hatch rate and survival from Nauplius to $Z_1$ were obtained from these observations.

### *Statistical Analysis*

Data were evaluated using the Statistical Package for the Social Sciences (SPSS version 11.0.1 2003, Chicago, Illinois). All data was evaluated to determine if requirements for normality and equality of error variances were met prior to analysis. In the event of inequality or presence of non-normal data, appropriate transformations were made to satisfy these requirements. Data represented as percentages (e.g., survival to $Z_1$,

hatch rate and percent spawn per night) were arcsine transformed for analysis. Untransformed data was used in the reported descriptive statistics. Analysis of Variance was used to evaluate treatment differences using a significance level of $\alpha = 0.05$. When a significant treatment effect was observed, the Tukey's Highly Significant Difference (HSD) was used to compare treatment means. The use of two different wild populations of broodstock collected at different dates in both studies and their differences in size prevented any statistical comparisons between the two experimental groups.

## Results

### *Experiment I*

Experiment one was terminated after 142 d. Non-ablated females lived longer, spawned less frequently, attained a more advanced ovary developmental stage at spawning, and spawned more completely than ablated females regardless of diet ($p<0.05$)(Table 2.2). Ablated females in both treatments had similar life spans, spawning frequency, ovary developmental stage at spawning, and completeness of spawn. Average egg production per female was similar for all three treatments. Hatch

**Table 2.2** Maturation statistics experiment 1 population 2♂:1♀ ratio

| | Treatment 1 | Treatment 2 | Treatment 3 |
|---|---|---|---|
| Ablation | No | Yes | Yes |
| Diet | Squid + BW [3] | Squid + BW [3] | Squid + FEAA [4] |
| Experimental Period (Days) | 142 | 142 | 142 |
| Female Weight (g) [1] | 35.1 ± 7.0 [a] | 39.0 ± 7.0 [b] | 36.4 ± 5.2 [ab] |
| Female Life Span (Days) [1] | 85 ± 45 [a] | 45 ± 39 [b] | 53 ± 49 [b] |
| Female Population Spawning/Night (%) [1] | 2.61 ± 2.5 [a] | 8.53 ± 5.3 [b] | 8.88 ± 5.7 [b] |
| Ovary Stage at Spawning (0-4) [1] | 3.7 ± 0.56 [a] | 3.37 ± 0.55 [b] | 3.40 ± 0.55 [ab] |
| Spawning activity [1,5] | 2.85 ± 0.46 [a] | 2.56 ± 0.64 [b] | 2.56 ± 0.05 [b] |
| Egg Production/Female/Spawn (x $10^3$) [1] | 136 ± 69 [a] | 124 ± 53 [a] | 115 ± 56 [a] |
| Total Egg Production (x $10^6$) [1] | 3.7 | 18.0 | 18.0 |
| Hatch Rate (%) [1] | 57.2 ± 39.1 [ab] | 44.1 ± 35.7 [a] | 58.2 ± 33.3 [b] |
| Nauplii Count/Female/Spawn (x $10^3$) [1,2] | 93 ± 58 [a] | 77 ± 50 [a] | 81 ± 46 [a] |
| Survival(N1 to Z1)/Female/Spawn (%) [1, 2] | 43.3 ± 37.7 [a] | 46.9 ± 37.4 [a] | 55.0 ± 32.7 [a] |
| Zoea 1 Count/Female/Spawn (x $10^3$) [2] | 74 ± 57 [a] | 67 ± 49 [a] | 71 ± 44 [a] |

[1]Values are means ± s.d. and means not sharing a common superscript letter within a row are significantly different ($P< 0.05$)
[2] excluding spawns with zero hatched eggs
[3] BW = bloodworms
[4]FEAA = frozen enriched adult *Artemia* sp.
[5]0 = None; 1 = Partial; 2 = Full

rate for eggs from ablated females fed bloodworms was significantly lower than ablated females fed enriched *Artemia* sp. which performed similar to non-ablated females. The three treatments showed similar results with respect to hatched nauplii per female, survival to $Z_1$ and $Z_1$ production per female.

***Experiment II***

Experiment two was terminated after 132 d. Reproductive performance of non-ablated females fed bloodworms and ablated females fed enriched *Artemia* sp. was similar with regard to life span, ovary developmental stage at spawning, completeness of spawn and hatch rate ($p>0.05$)(Table 2.3). Reproductive performance of ablated females fed bloodworms was inferior to non-ablated females fed bloodworms and the ablated females fed enriched *Artemia* sp. for the aforementioned reproductive indicators. Ablated females spawned more frequently than the non-ablated shrimp. Average egg production per female over the period for all three treatments was similar. No differences in treatments were found for: nauplii count per female for hatched eggs, survival rate to $Z_1$ sub-stage and Zoea 1 production per female.

**Table 2.3** Maturation statistics experiment 2 population 1♂:1♀ratio

| | Treatment 1 | Treatment 2 | Treatment 3 |
|---|---|---|---|
| Ablation | No | Yes | Yes |
| Diet | Squid + BW [3] | Squid + BW [3] | Squid + FEAA [4] |
| Experimental Period (Days) | 132 | 132 | 132 |
| Female Weight (g) [1] | 34.7 ± 4.4 [a] | 45.6 ± 11.4 [b] | 45.7 ± 10.3 [b] |
| Female Life Span (Days) [1] | 116 ± 23 [a] | 82 ± 39 [b] | 122 ± 19 [a] |
| Female Population Spawning/Night (%)[1] | 2.67 ± 0.63 [a] | 7.44 ± 1.21 [b] | 7.50 ± 1.92 [b] |
| Ovary Stage at Spawning (0-4) [1] | 3.84 ± 0.36 [a] | 3.59± 0.49 [b] | 3.75 ± 0.43 [a] |
| Spawning activity[1,5] | 1.96 ± 0.19 [a] | 2.15 ± 0.42 [b] | 1.95 ± 0.23 [a] |
| Egg Production/Female/Spawn (x $10^3$) [1] | 143 ± 48 [a] | 144 ± 48 [a] | 151 ± 59 [a] |
| Total Egg Production (x $10^6$) [1] | 4.0 | 22.0 | 23.0 |
| Hatch Rate (%)[1] | 62.9 ± 26.3 [a] | 52.6 ± 29.5 [b] | 55.7 ± 31.7 [ab] |
| Nauplii Count/Female/Spawn (x $10^3$) [1,2] | 94 ± 45 [a] | 76 ± 53 [a] | 83 ± 59 [a] |
| Survival(N1 to Z1)/Female/Spawn (%)[1, 2] | 51.6 ± 35.5 [a] | 37.2 ± 32.7 [a] | 44.8 ± 31.6 [a] |
| Zoea 1 Count/Female/Spawn (x $10^3$) [2] | 89 ± 61 [a] | 63 ± 46 [a] | 73 ± 55 [a] |

[1]Values are means ± s.d. and means not sharing a common superscript letter within a row are significantly different ($P$< 0.05)
[2] excluding spawns with zero hatched eggs
[3] BW = bloodworms
[4]FEAA = frozen enriched adult *Artemia* sp.
[5]0 = None; 1 = Partial; 2 = Full

**Discussion**

Results from the two studies corroborate findings of AQUACOP (1975) in which eyestalk ablation was demonstrated to induce ovary development and spawning in *F. aztecus* females. Nevertheless, the fact that un-ablated females were able to mature and spawn viable eggs in the present studies and not in the AQUACOP (1975) study could have been due to improved maturation conditions in the present studies. Ablated females in both experiment 1 and experiment 2 matured and spawned more frequently

than non-ablated females. Percentage spawning per night in experiment 2, in which a male to female ratio of 1:1 was evaluated, was 1.25% less than experiment 1 (male to female ratio of 2:1). This apparent reduction in percent spawn per night was possibly due to increased female density and suggests a trend similar to that observed by Alva and Primavera (1980) for *P. monodon*. These authors found percentage spawn per night, total egg production and hatch rate in *P. monodon* decreased due to increased male to female ratio beyond 1:2. Results from the present studies suggest a 1:1 male to female ratio may be limiting prior to occurrence of major negative reproductive effects. Further studies would be needed to test this hypothesis.

Under the conditions present in experiment 1, non-ablated females attained longer life spans than ablated females regardless of diet. Ablated females in this experiment fed enriched *Artemia* sp. had a typically 10 day longer life span than those fed bloodworms; albeit, this difference was not statistically significant. In experiment 2 there was no significant difference in life span between non-ablated females fed bloodworms and ablated females fed enriched *Artemia* sp. (116 and 122 days, respectively). These findings suggest the enriched *Artemia* sp. diet has the potential to fortify females against the stress of frequent handling and the energetic demand associated with ablation.

The number of eggs spawned by female shrimp in either experiment was similar regardless of treatment. These findings suggest eyestalk ablation had no negative effect on mean egg production per female in either experiment. In addition, the use of enriched *Artemia* sp. had no adverse effect on number of eggs spawned indicating its potential as

a dietary replacement for bloodworms. In both studies no difference in hatch rate were found between non-ablated females fed bloodworms and those from ablated females fed enriched *Artemia* sp. These findings are similar to those of Naessens et al. (1997) in which substitution of bloodworms in the diet with frozen enriched adult *Artemia* sp. significantly increased reproductive performance of *L. vannamei* broodstock. The number of eggs spawned by ablated and non-ablated females in both studies was greater than those reported by the AQUACOP (1977) for unilaterally ablated *F. aztecus* females (45,000 eggs per spawn). The hatch rate was 3.1% higher in experiment 1 and 12.7% higher in experiment 2 for ablated females fed *Artemia* sp. vs. ablated females fed bloodworms; albeit, not statistically different. The increased hatch rate of eggs from broodstock fed enriched adult *Artemia* sp. is consistent with findings of Naessens et al. (1997) for *L. vannamei* broodstock.

Survival from $N_1$ to $Z_1$ in experiment 1 showed a similar trend to that of observations regarding hatch rate. Survival to $Z_1$ in experiment 1 was significantly higher (7.9%) for nauplii from ablated females fed enriched *Artemia* sp. vs. those from females fed a bloodworm diet. Survival to $Z_1$ in experiment 2 was 7.6% higher for the nauplii from ablated females fed the enriched *Artemia* sp. diet than those from ablated females fed the bloodworm diet; however, it was not statistically significant. The trend of higher hatch rates and survival rates to $Z_1$ for these two populations of larvae from ablated females fed a diet containing frozen enriched adult *Artemia* sp. indicates high-cost frozen bloodworms can be replaced by lower cost enriched *Artemia* sp. without losses in reproductive performance.

# CHAPTER III

# COMPLETING THE LIFE CYCLE OF POND-RAISED *Farfantepenaeus aztecus* USING UNILATERAL EYESTALK ABLATION AND MATURATION DIETS IN A CLOSED RECIRCULATING SYSTEM

## Introduction

Dependence on wild caught broodstock represents a major limitation in the development of sustainable commercial bait shrimp aquaculture on the Gulf of Mexico and southeast Atlantic coasts. Securing consistent supplies of viral-pathogen-free broodstock is essential to further sustainable development of this industry in a sustainable manner. To date, domestic studies on the production potential of *F. aztecus* as a bait shrimp have relied on wild gravid broodstock to supply PL (McKee, 1986). Initial investigations by AQUACOP (1977) into the reproductive potential of various species of Penaeid shrimp were successful in producing three generations of *F. aztecus* in an outdoor system. However, the reproductive performance of these shrimp was poor compared to other species (*Litopenaeus vannamei, L. stylirostris, Fenneropenaeus merguiensis* and *Marsupeneaus japonicus*) also investigated. These findings shifted research efforts away from *F. aztecus* as a potential candidate for commercialization.

The developing need for supplementing the wild live bait shrimp industry with a farm-raised product has subsequently renewed interest in commercial production of *F. aztecus*. Previous studies (see Chapter II) showed that the reproductive performance of unilaterally eyestalk-ablated wild *F. aztecus* females matured in an indoor closed

recirculating tank system was superior to that found by AQUACOP (1977). These findings suggest sufficient and predictable numbers of larvae could be produced using this method. Without a source of domesticated viral-pathogen-free broodstock, further sustainable development of the farm-raised live bait shrimp industry is questionable.

Successful development of multiple generations of commercially important penaeid shrimp from pond-reared broodstock has been reported for several species including: *L. vannamei* (AQUACOP, 1979; Ogle, 1992a; Ibarra et al., 1997; Palacios et al., 1999), *L. stylirostris* (Brown et al., 1984, Ottogalli et al., 1988; Alfaro, 1993; Mendoza, 1997), *Penaeus semiselactus* (Browdy et al., 1986), *P. monodon* (Beard and Wickins, 1980; Menasveta et al., 1993; Makinouchi and Hirata, 1995; Pratoomchat et al., 1993) and *F. paulensis* (Cavalli et al., 1997; Peixoto et al., 2003). Closing the life cycle of commercially important species has improved the sustainability of the world shrimp farming industry. Furthermore, genetic selection of cultured stocks has been applied to increased control through domesticating shrimp lines which are specific-viral-pathogen-free (SPF), disease-resistant and have improved growth rates (Browdy, 1998). Similarly, the selection of *F. aztecus* broodstock has the potential to assist in the sustainable development of the farm-raised live bait shrimp industry. Production of pond-raised broodstock will eventually lead to development of reliable supplies of SPF PL as well as genetic selection program to meet the needs of the industry. Before these goals can be realized, the reproductive performance of pond-raised *F. aztecus* population should be determined. The objectives of the present study were to evaluate the reproductive performance of pond-raised *F. aztecus* in a closed recirculating tank system

and the effect of partial replacement of frozen squid with a commercially available dry maturation diet on reproductive performance.

## Materials and Methods

### *Maturation System*

The maturation system used in this study was similar to the one described previously (see Chapter II), with one exception, the drain for each maturation tank was modified by diverting un-filtered water from the center drains directly into the sump tank (Figure 3.1). Re-diverting this water into the sump greatly reduced fouling of biofilter media and allowed for the elimination of the self-cross-linking vinyl-acetate-polymer which served to trap particles previously missed by the filter bags in the previous design. A second flow meter was installed downstream of the diatomaceous earth (DE) filter. It was used to supply a constant flow rate of 68 lpm (18 gpm) of filtered water to the biofilter media via rotating spray bars (RB16, Aquatic Ecosystems, Apopka, Florida) positioned over biofilter media. This modification additionally resulted in improved culture water contact with biofilter media and allowed for the elimination of the high-density polyethylene (HDPE) plate. A foam fractionator, designed and constructed 'in house,' was added in a side loop to the sump (Figure 3.1). The use of the foam fractionator reduced the amount of light soda ash (sodium carbonate) added to the system for pH maintenance from 500 g every 4 d to 500 g every 4 wk.

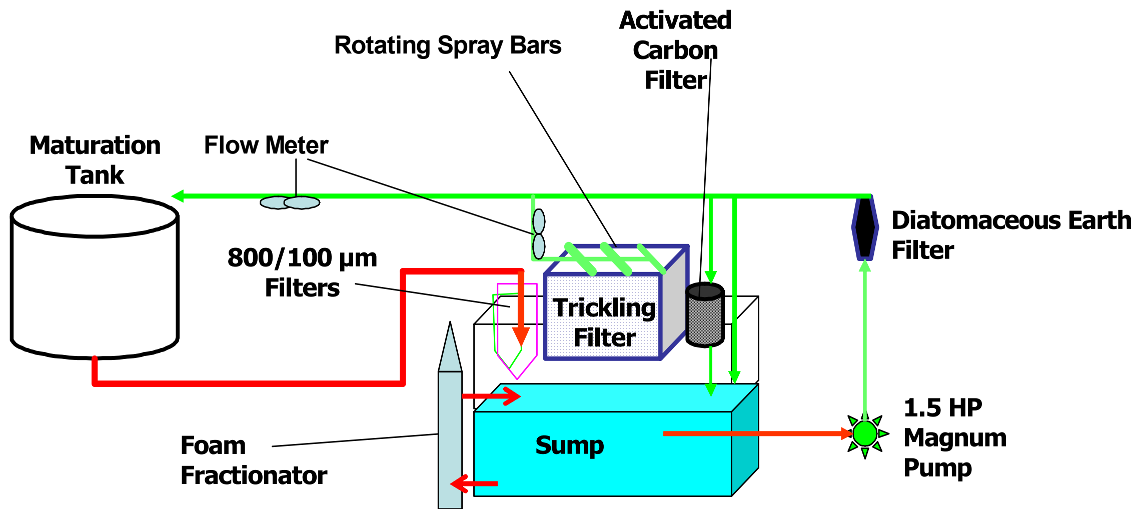



**Figure 3.1** Schematic drawing of the closed recirculating maturation tank system

***Water Quality***

Water in the maturation tank system was maintained at a temperature of 26.5 ± 1.1 C, dissolved oxygen (DO) concentration at 5.89 ± 0.68 mg/L, salinity at 35 ± 1 ppt and pH at 7.79 ± 0.3 during this study. Average levels of $NH_4$-N were 0.27 ± 0.15-mg/L, while $NO_2$-N and $NO_3$-N were 0.65 ± 0.45-mg/L and 30.5-mg/L, respectively. Temperature, salinity, pH and DO levels were monitored daily. All water quality factors were measured using the same instruments and procedures described in Chapter II. Daily system maintenance followed the protocol established in the previous studies.

***Population Used for Study***

Offspring of wild sourced broodstock used in Studies 1 and 2 (Chapter II) were raised for 9 months in ponds and then transferred for 2 months into greenhouse-enclosed raceways. The parent population (F generation) and the offspring (F1 generation) were routinely screened for known infectious viruses using pleopod tissue samples and a two-step polymerase chain reaction (PCR) detection technique. Specifically, this on-going screening process tested for presence of Infectious Hypodermal and Hematopoietic Necrosis Virus (IHHNV), Taura Syndrome Virus (TSV) and White Spot Syndrome Virus (WSSV). The parent population (F) and their offspring (F1) broodstock were determined as virus-free by the Texas Veterinary Medical Diagnostic Laboratory (TVMDL), College Station, TX. for these known pathogens. The F1 broodstock used in this study were re-tested for these known viral-pathogens prior to initiation of the study. In addition, health status of these pond-raised F1 broodstock was surveyed by histological procedures. Although analysis by PCR testing was negative for viral infections, histologic evaluation of the population found a slight presence of ectopic spheroids in the lymphoid organ (TVMDL Accession #: C032660303). These findings indicate an immune response to a foreign antigen; however, no specific agent was identified. Culture conditions could not be ruled out as the causative stressor and the maturation study continued as planned. Concurrently, TVMDL started to look into the cause of the immune response of this population. Further histological work with moribund shrimp during this study revealed large numbers of ectopic spheroids in the lymphoid organ of these shrimp (TVMDL Accession #: C032590290). These findings

suggested that a previously unknown viral pathogen could have been responsible for the abnormal histology and general poor health of this population. Based on the poor reproductive performance of this population and recommendations from TVMDL, the study was terminated 39 days after its initiation. At that point all remaining shrimp were destroyed, followed by a complete disinfection of the closed recirculating system.

***Maturation Studies***

Table 3.1 summarizes the experimental design for this study. A sex ratio of 1:1 male (18.5 ± 2.1 g) to female (26.1 ± 2.9 g) was used in this study. Previous studies (Chapter II) showed significantly better reproductive performance of ablated versus non-ablated females. For this reason, all females used in this study were ablated. Shrimp in each tank received a different maturation diet as summarized in Table 3.1. Treatment 1 served as the control diet and consisted of frozen adult enriched *Artemia* sp. (8% wet body weight as wet weight feed per day (bwd)) and frozen squid (12% bwd). Treatment 2 consisted of enriched *Artemia* sp. (8% bwd) and dry maturation diet (Maturation Diet, Rangen Inc., Buhl, ID.) which was offered at 6% bwd and the squid component (6% bwd). Treatment 3 consisted of bloodworms, dry maturation diet and squid at 8, 6, and 6% bwd, respectively. To facilitate data collection, each female shrimp was tagged with a colored numbered latex ring mounted on one eyestalk (Browdy, 1985). All shrimp appeared healthy when placed into the closed recirculating system. Ablation was performed three days after this transfer.

**Table 3.1** Maturation diet treatments

| Treatment | Squid | Dry maturation diet | Bloodworms | Enriched adult *Artemia* sp. |
|---|---|---|---|---|
| 1 | Yes[2] | No | No | Yes[1] |
| 2 | Yes[3] | Yes[3] | No | Yes[1] |
| 3 | Yes[3] | Yes[3] | Yes[1] | No |

[1] Fed at 8% of the total wet shrimp biomass per day
[2] Fed at 12% of the total wet shrimp biomass per day
[3] Fed at 6% of the total wet shrimp biomass per day

***Data Collection***

After a 3-day acclimation period, all shrimp were weighed and females tagged using the same method described in Chapter II. Because larger females are known to spawn larger numbers of eggs (Primavera, 1985), female wet weight consistency within a population was monitored to ensure all tanks were stocked with similar-sized shrimp. Methodology regarding monitoring of ovarian development, selection of gravid females, spawning characteristics (full, partial), spawning tanks, and sampling, counting, and incubation procedures followed the same methods outlined in Chapter II.

***Statistical Analysis***

Data were evaluated using the Statistical Package for the Social Sciences (SPSS version 11.0.1 2003, Chicago, Illinois). All data were evaluated to determine if requirements for normality and equality of error variances were met prior to analysis. In the event of inequality or presence of non-normal data, appropriate transformations were

made to satisfy these requirements. Data represented as percentages (e.g., zoea survival, hatch rate and percent spawn per night) were arcsine transformed for analysis. Untransformed values were used in the reported descriptive statistics. Analysis of Variance (ANOVA) was used to evaluate treatment differences using a significance level of $\alpha = 0.05$. When a significant treatment effect was observed, Tukey's Highly Significant Difference (HSD) test was employed to compare treatment means. No statistical comparison of the reproductive performance of the F broodstock from Chapter II and this F1 broodstock were made due to large differences in shrimp mean weight of these two populations.

**Results**

The study was terminated after 39 days. Females fed all three diet treatments achieved similar life spans, level of ovarian development at spawning, total number of eggs spawned, and survival of hatched nauplii to $Z_1$ sub-stage ($p<0.05$). Females in Treatment 2 spawned more frequently than shrimp fed the other two diets ($p<0.05$). No statistically significant differences were found in hatch rate, nauplii and zoea count between Treatment 1 and 2. The reproductive performance of females fed Treatment 3 was significantly lower than those fed the other two treatments ($p<0.05$, Table 3.2).

**Table 3.2** Reproductive performance of pond-raised broodstock by diet treatment

| | Treatment 1 | Treatment 2 | Treatment 3 |
|---|---|---|---|
| Ablation | Yes | Yes | Yes |
| Diet | Squid + FEAA [1] | Squid+ Dry[2]+FEAA [1] | Squid + Dry [2]+ BW [3] |
| Study Period (days) | 39 | 39 | 39 |
| Female Weight (g) | 27.7 ± 0.62 [a] | 26.9 ± 0.62 [a] | 26.8 ± 0.54 [a] |
| Female Life Span (days) | 37.6 ± 1.28 [a] | 39.7 ± 0.82 [a] | 38.9 ± 1.30 [a] |
| Female Population Spawning/Night (%) | 2.7 ± 0.01 [a] | 4.2 ± 0.01 [b] | 2.4 ± 0.06 [a] |
| Ovary Stage at Spawning (0-4) | 2.87± 0.07 [a] | 2.82 ± 0.06 [a] | 2.64 ± 0.06 [a] |
| Spawning activity[4] | 1.84 ± 0.07 [a] | 1.90 ± 0.05 [a] | 1.96 ± 0.04 [a] |
| Egg Production/Female/Spawn (x $10^3$) | 59 ± 3.7 [a] | 58 ± 4.0 [a] | 48 ± 3.8 [a] |
| Total Egg Production (x $10^6$) | 1.84 | 1.57 | 1.36 |
| Hatch Rate (%) | 12.8 ± 5.7 [a] | 11.5 ± 4.7 [a] | 3.2 ± 1.2 [b] |
| Nauplii Count/Female/Spawn (x $10^3$)* | 47.0 ± 6.0 [a] | 31.0 ± 7.0 [a] | 7.0 ± 0.8 [b] |
| Survival (N1 to Z1)/Female/Spawn (%)* | 77.0 ± 7.3 [a] | 82.2 ± 5.1 [a] | 69.7 ± 7.0 [a] |
| Zoea 1 Count/Female/Spawn (x $10^3$)* | 34.8 ± 3.5 [a] | 26.0 ± 5.0 [a] | 4.50 ± 0.60 [b] |

Values are means ± s.d. and means not sharing a common superscript letter within a row are significantly different ($P < 0.05$)
* excluding spawns with 0 hatch rate
[1] FEAA = Frozen enriched adult *Artemia* sp.
[2] Dry = Rangen Maturation Diet
[3] BW = Frozen Bloodworms
[4] 0 = None; 1 = Partial; 2 = Full

**Discussion**

This study was successful in completing the life cycle of pond-raised *F. aztecus* using unilateral eyestalk ablation in a closed recirculating tank system. Results of this study and the conclusions of Chapter II corroborate findings of AQUACOP (1975) in

which eyestalk ablation induced ovary development and spawning in *F. aztecus* females. Additionally, the current research demonstrated that feeding shrimp with adult enriched *Artemia* sp. and equal amounts of squid and dry maturation diet significantly improved spawning frequency. Differences in all other reproductive performance criteria between females fed this diet and those fed the *Artemia* sp. and full ration of squid were not significant. The reproductive performance of female shrimp fed treatments containing enriched *Artemia* sp. was significantly better than that of females fed bloodworms and squid. These findings are similar to the findings in Chapter II of this research and to the findings of Naessens et al. (1997) that found maturation diets for *L. vannamei* broodstock which included a frozen enriched adult *Artemia* sp. significantly out performed diets which did not contain the frozen enriched adult *Artemia* sp.

Though the aforementioned findings are significant, further comparison of these data to other published literature is not relevant due to the premature termination of the study on day 39. Declining broodstock health in both the closed recirculating tank system and in raceways in which the remainder of the population was being held forced this premature termination. It is postulated that this decline in health was probably responsible for a gradual reduction in mating upon molting of females. During the first 20 days, all molts removed from the maturation tanks contained remains of spermatophores. After day 20, no spermatophores were found in the molts of females. Over the 39-day period, despite all females molting, no spermatophores were found in the newly molted females. The lack of mating severely interfered with spawning of fertilized eggs and subsequent hatch rate data reflect this. Therefore, comparison of

hatch rate, nauplii count, survival to $Z_1$ and $Z_1$ count to other published literature is not feasible due to interference from the lack of mating.

Routine health surveys of this population by histology identified a significant immune reaction within this population. An extremely high prevalence of ectopic spheroids in the lymphoid organ and heart suggested the presence of an infective agent. The infectivity of this unknown pathogen was demonstrated by TVMDL in bioassays using frozen tissue from infected shrimp fed to *L. vannamei* and *F. duorarum*. These bioassays produced significant immune reaction in both *L. vannamei* and *F. duorarum*; however, no mortality was noted in either species over the bioassay period. In these bioassays an identical immune response was clearly reproduced (Dr. Ken Hasson, personal communication). Although, this pathogen has still not been identified, ongoing studies at TVMDL have produced evidence suggesting this is a previously unknown viral-pathogen. All broodstock were subsequently destroyed and the facility sterilized to reduce potential spread of this potentially infectious undescribed viral disease.

Future researchers who are required to acquire endemic species of shrimp as eventual broodstock for the production of PL should be aware of the potential threat from this and other viral-pathogens which show few clinical signs of infection (i.e. those which can only be detected through histology). The potential for collecting endemic populations of shrimp with unidentified viral-pathogens is high. Continued extensive monitoring of broodstock using standardized examination methodology (e.g. Manual of Diagnostic Tests for Aquatic Animals) is essential. If viral-pathogen-free broodstock of

endemic species are to be developed, the identification of presently unknown viral pathogens and establishment of methods for their detection needs to be developed.

Sustainability of the bait shrimp industry will only be feasible if a concerted effort is made to identify and exclude viral-pathogens known to affect Penaeid shrimp endemic to the Gulf of Mexico and Southeastern Atlantic coasts of the United States. Only after sufficient efforts have been made to identify and detect highly lethal endemic viral-pathogens should domestication efforts continue along the lines of the SPF program developed by the USMSFP. The first step in this domestication process is the isolation of a SPF stock in a bio-secure nucleus breeding center. The 'SPF stream' is contained in a 'high-security' isolation nucleus breeding center which is responsible for isolation and development of pathogen-free lines of shrimp (Lotz, 1997). Isolation of wild populations for the nucleus breeding center begin with a wild population which is quarantined for 2-5 months during which they are screened using PCR techniques for known viral pathogens. Those which pass primary quarantine are then considered candidates for SPF stocks. These candidates are moved to a secondary quarantine facility where they are kept for 5-12 months during which time further PCR screening is performed as this population is matured and reproduced. Only offspring of the broodstock in the secondary quarantine facility, that are free of specified viral-pathogens, are then moved to the nucleus breeding center. During any stage in the process if a broodstock population is found to be infected with a viral-pathogen that population is immediately destroyed and the facility disinfected (Pruder, 2004). Future domestication efforts for bait shrimp farms must follow the precedent set forth by the

USMSFP with its emphasis on bio-security and SPF stocks. Only through the establishment of an SPF program for native species will sustainable farm-raised bait shrimp aquaculture develop in the southeastern United States.

# CHAPTER IV

# EVALUATION OF MULTIPLE WATER RE-USE IN A CLOSED RECIRCULATING SYSTEM FOR PRODUCTION OF *Farfantepenaeus aztecus* POSTLARVAE

## Introduction

Developing interest in raising indigenous species for the live bait shrimp industry necessitates the production of large numbers of PL. The larval rearing procedure known as the "Galveston Method" was developed for indigenous species *F. aztecus* and *L. setiferus* by Cook and Murphy in 1969 at the National Marine Fisheries Laboratory in Galveston, Texas. This method, which streamlined PL production, achieved high efficiency (70% to 90% survival rate) that made it the method of choice in the western hemisphere (McVey, 1993). High stocking densities (100 to 150 larvae/L) associated with this method force heavy water exchange (50% to 80% or higher/day) to reduce metabolite buildup from late zoea stages until harvest (McVey, 1993).

Increasing restrictions on effluent release from aquaculture facilities in the coastal zone of the United States has caused the development of management techniques and technologies to reduce discharge to receiving streams. Trials in Chapter II and III documented the ability of *F. aztecus* broodstock to mature and produce viable offspring in a closed recirculating maturation system. The next phase of study sought to integrate recirculating systems into the larval rearing process to maximize water re-use and reduce effluent discharge.

Studies on the use of closed recirculating systems for larval rearing of the marine shrimp *Penaeus monodon* have been described by Menasveta et al. (1989) and Millamena et al. (1991). In these studies, the water used for the production of the PL was discarded at the end of each production cycle. These studies have not focused on the re-use of the culture water for multiple consecutive PL productions in a closed recirculating system.

In contrast, production of freshwater prawn *Macrobrachium rosenbergii* PL under recirculating brackish water conditions (12 to 15 $^{0}/_{00}$ salinity) has been thoroughly investigated. Valenti and Daniels (2000) documented the development and use of two types of recirculating systems for commercial PL production of this species. These authors classified the recirculating systems as either static-closed or dynamic-closed. The basic configuration of the dynamic-closed system for PL production of *M. rosenbergii* maintains a continuous water flow through the culture tanks and the water treatment systems. In these systems, culture water flows from the larval rearing tank into a mechanical filter where solids are removed. Filtered water proceeds to a biological filter where ammonium and nitrite are converted to nitrate. The authors also indicate that some hatcheries use disinfection with ozone or ultra violet light to sterilize the culture water prior to reintroduction to the larval rearing tank. On the other hand, management of water in a static-closed system is best described as a "batch" type treatment. In these systems, waste water is discharged from the larval rearing tanks into treatment tanks daily during a water exchange. Waste-laden water is processed in the treatment tank first by particle filtration to 1 $\mu$m followed by disinfection with 2.5 mg/L

active chlorine for 40 minutes. Culture water is subsequently de-chlorinated with sodium thiosulfate before it is pumped into the biofiltration tank where it is processed for three to four days before its re-use. The comparison of the two systems by these authors revealed the dynamic-closed system was superior. The dynamic-closed system was found to have low operating cost, reduced water loss that minimized environmental impact while maximizing water quality and productivity.

In the current study a hybrid of the dynamic-closed and static-closed systems was developed to study the potential effects of multiple water re-use on the production of marine shrimp *F. aztecus* PL in a closed recirculating system. This system was divided into two treatment areas. The first was a dynamic-closed, *in-situ*, treatment which was used during the larval culture period. At the end of each production cycle (about 12 days) water from the recirculating system was pooled into a common reservoir. The reservoir was also fitted with a dynamic-closed system for culture water treatment between production cycles. The hybrid system was used to test the effects of multiple water re-use on water quality, growth, health and development of *F. aztecus* to a postlarval stage. The collected data was further used to determine the feasibility and limiting factors involved in production of marine shrimp PL under recirculating and multiple re-use conditions.

## Materials and Methods

### *Shrimp Larval Population*

Larvae for the trials were produced by a captive broodstock of *F. aztecus* which was maintained in a closed recirculating tank system. Larvae from several spawns were acquired at the Nauplius five sub-stage ($N_5$ ) and pooled into a single 200-L larval rearing tank (0.91 m (h) x 0.48 m (w) x 0.61 m (d)) (Rowland Fiberglass, Ingleside, Texas) equipped with three air stones (AS1, Aquatic Ecosystems, Apopka, Florida), stand pipe (3.81 cm dia.), High Density Polyethylene (HDPE) lid and 300-watt submersible quartz heater (VT 301; Aquatic Ecosystems, Apopka, Florida). Larvae were provided with 1.5 x $10^5$ cell/ml of *Chaetoceros muelleri* during their metamorphosis to $Z_1$ sub-stage. Once 90% of the population metamorphosed to the $Z_1$ sub-stage, larvae were acclimated to artificial seawater over a 4-h period. Acclimation was accomplished by removing and replacing 25% of the tank volume over the first hour, 50% over the second hour, 75% over the third hour, and 100% over the fourth hour. Following the acclimation, larvae were concentrated into 20 L of artificial seawater. The 20 L of water was homogenized by random manual stirring and ten, 50-ml samples were collected by a Hansen Stempel Pipette (1805-C42, Wildco, Saginaw, Michigan). Postlarvae in each sample were counted in the laboratory to determine the density of PL in the tank. All ten samples were counted only if the coefficient of variation of the first five samples was above 5%. Once the shrimp density in the larval concentration tank was established, 20,000 larvae were stocked into each of the ten

larval rearing tanks to provide a density of 100 $Z_1$/L. Each trial was terminated 5 days after 75% of the PL had reached $PL_1$.

### *Artificial Seawater*

To minimize variations due to seasonal changes in natural seawater composition, all larval rearing trials were conducted in artificial seawater. Culture medium was prepared from artificial sea salt (Instant Ocean, Aquarium Systems, Mentor, Ohio) and municipal freshwater treated by reverse osmosis (RO)(US 1800; Alamo Water Refiners Inc; San Antonio, Texas). Artificial seasalt was dissolved in RO water and aged for 24 h under aeration to ensure all mineral components were dissolved and the pH had stabilized. This artificial seawater was then treated with 10 ppm EDTA at the start of each trial to chelate heavy metals in the water prior to use. Control larval tanks, recirculating larval treatment tanks and their associated algae cultures all received the same batch of artificial seawater at the start of the study. Once the study was underway, the recirculating treatment and its associated algae culture re-used all culture water for subsequent larval production cycles. During the six trials only RO water was added to offset evaporative losses and no new artificial seawater was added to the recirculating system. At the end of each larval production cycle, water from the recirculating treatment tanks and its associated algae culture was collected in a common reservoir and processed by mechanical and chemical filtration. This processed water was used on the following recirculating larval production trial. In contrast, the control treatment received

new artificial seawater daily during water exchanges. New artificial seawater was also used to maintain the algal culture associated with these control tanks.

***Larval Rearing Systems***

Larval rearing tanks (LRT) consisted of ten, 200-L U-shaped tanks [0.91 m (h) x 0.48 m (w) x 0.61 m (d)] (Rowland Fiberglass, Ingleside, Texas) arranged as two sets of five replicates. Each LRT tank was equipped with three air stones (AS 1, Aquatic Ecosystems, Apopka, Florida), a stand pipe (3.81 cm dia.) and a HDPE lid (Figure 4.1). Water temperature was maintained with a 300-watt submersible quartz heater (VT 301; Aquatic Ecosystems, Apopka, Florida).

**Figure 4.1** 200-L larval rearing tank with aeration, water inlet and stand pipe

The first five replicates served as the control (Figure 4.2) and were managed under a standard larval rearing and water exchange practice (Table 4.1). Suitable water quality in the control tanks was maintained by daily flushing of the tanks with freshly made artificial seawater.

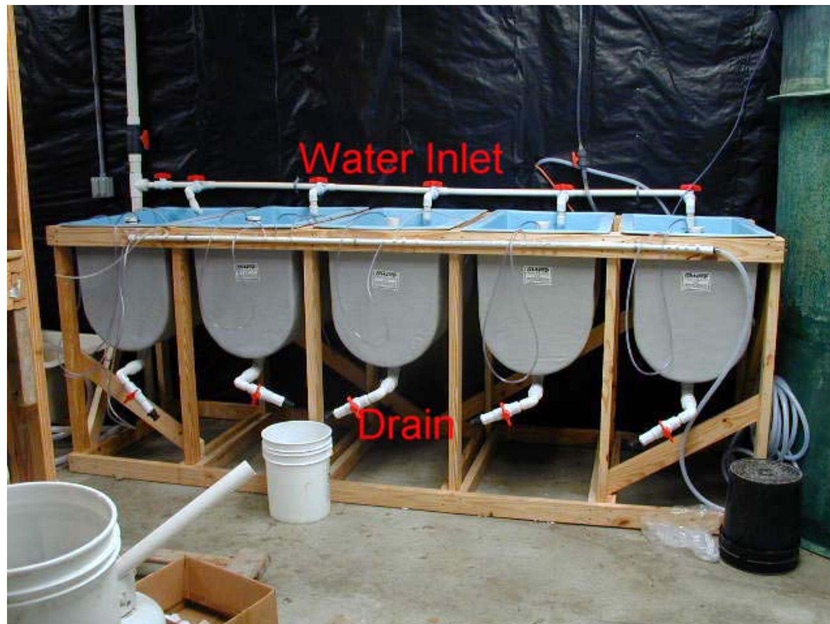



**Figure 4.2** Control tanks with water inlet, air distribution manifold and drain

**Table 4.1** Control tanks larval rearing management schedule

| Developmental Stage | Daily Water Exchange | Screen Size $\mu m$ | *Chaetoceros muelleri* x $10^5$ cell/ml | *Artemia* sp. nauplii/ml |
|---|---|---|---|---|
| $Z_1$ | 25% | 290 | 150 | 0 |
| $Z_2$ | 25% | 290 | 150 | 0 |
| $Z_3$ | 50% | 350 | 200 | 0.25 |
| $M_1$ | 75% | 350 | 200 | 1 |
| $M_2$ | 75% | 400 | 200 | 3 |
| $M_3$ | 100% | 400 | 200 | 4 |
| $PL_1$ | 100% | 500 | 100 | 6 |
| $PL_2$ | 100% | 500 | 50 | 8 |
| $PL_3$ | 100% | 500 | 0 | 8 |
| $PL_4$ | 100% | 500 | 0 | 10 |
| $PL_5$ | 100% | 500 | 0 | 12 |

A second set of five replicate tanks used a recirculating system consisting of particle filtration and biofiltration to control water quality during each trial (Figure 4.3). Recirculating treatment tanks were maintained under the same feeding regime as the control (Tables 4.1 and 4.2). However, there was no water exchange for these tanks; water quality in each tank was maintained by particle and biologic filtration (Table 4.2).

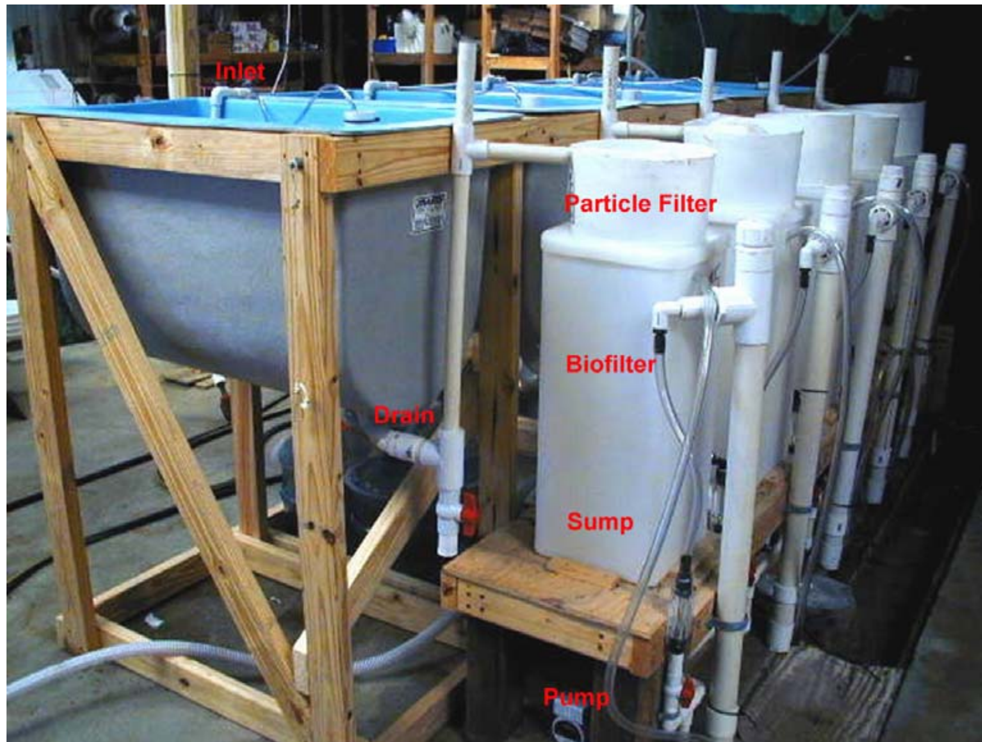



**Figure 4.3** General view of the recirculating tank system using a modified dynamic-closed system

**Table 4.2** Recirculating larval rearing feed and water turnover rate associated with larval developmental stage

| Developmental Stage | Flow (Turnover/24 h) | Screen Size $\mu m$ | *Chaetoceros muelleri* x $10^5$ cell/ml | *Artemia* sp. nauplii/ml |
|---|---|---|---|---|
| $Z_1$ | 3.6 | 290 | 150 | 0 |
| $Z_2$ | 3.6 | 290 | 150 | 0 |
| $Z_3$ | 3.6 | 350 | 200 | 0.25 |
| $M_1$ | 3.6 | 350 | 200 | 1 |
| $M_2$ | 3.6 | 400 | 200 | 3 |
| $M_3$ | 4.6 | 400 | 200 | 4 |
| $PL_1$ | 4.6 | 500 | 100 | 6 |
| $PL_2$ | 5.1 | 500 | 50 | 8 |
| $PL_3$ | 5.1 | 500 | 0 | 8 |
| $PL_4$ | 5.1 | 500 | 0 | 10 |
| $PL_5$ | 5.1 | 500 | 0 | 12 |

Water from each recirculating LRT flowed by gravity through a center filter pipe, positioned at one end of the tank, into a filter bag of 100 $\mu$m which collected feces and uneaten *Artemia* sp. nauplii. Algal cells (< 5 $\mu$m) were not collected by the particle filter and were allowed to circulate back to the LRT. Water from the filter bag spread evenly over the trickling biofilter using a perforated HDPE plate placed over a flat filter sheet of vinyl acetate filter material (Figure 4.4).

**Figure 4.4** Top view of filtration, HDPE plate, trickling biofilter and sump

The trickling biofilter (6.0 x $10^3$ $cm^3$) was made of extruded cage netting filled with a 2.5 cm (1") Bio Pac media (BB-1, Aquaculture Supply, Dade City, Florida) positioned over 50.0 x $10^3$ $cm^3$ sump. A 45 lpm centrifugal pump (P95V, Aquatic Ecosystems, Apopka, Florida) was used to pump the water from the sump into two flow meters (FL7503, Omega Engineering, Stamford, Connecticut). One flow meter diverted a portion of the water back over the biofilter at a rate of 15.0 lpm while the remainder was returned to the LRT at 0.5 lpm resulting in 3.6 system turnovers per day. From the $M_3$ sub-stage to one day-old-PL ($PL_1$) the flow rate was increased to 3.33 lpm for one hour in the morning, before feeding, to allow removal of excess feed and fecal material

from the previous day. The one hour flow rate increase resulted in one complete turnover, thus adding one extra turnover for the 24 h period. An additional turnover per day was provided from $PL_2$ to $PL_5$ by increasing the flow rate to 5.0 lpm for one hour in the morning to flush old fecal strands and excess feed from the LRT into the filter.

At the end of each of the six larval rearing trials, water from the five recirculating treatment tanks was collected in a main reservoir and processed using particle filtration to 1 $\mu$m (VF125, Aquatic Ecosystems, Apopka, Florida), UV disinfection (15,000 $\mu$ws/cm$^2$ at a flow rate of 25 gpm) (UV32, Aquatic Ecosystems, Apopka, Florida) and carbon adsorption (Ocean Clear J320 Chemical Filter, Aquatic Ecosystems, Apopka, Florida). Further water purification was accomplished by foam fractionation with a unit produced "in house" for this project. These water processing units prepared the culture water for re-use in the next larval rearing trial (Figure 4.5). Once processed, the water from the reservoir was used for algae production and for re-filling all five recirculating tanks.

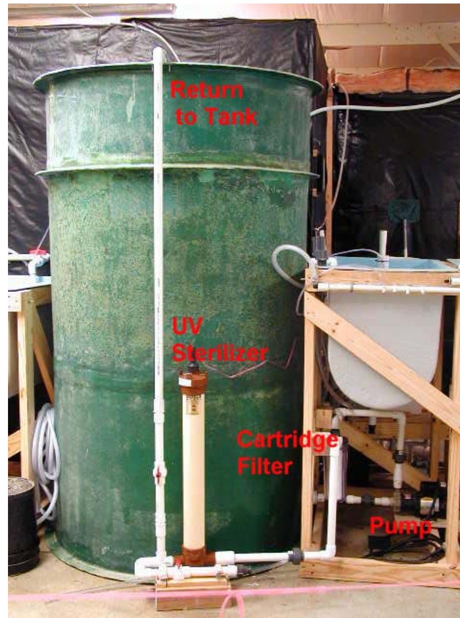



**Figure 4.5** Water treatment reservoir using a modified static-closed system, foam fractionator installed behind the reservoir and not pictured

***Biofilter Pre-Conditioning***

Biofilters used in the five recirculating treatment tanks were pre-conditioned prior to use in a manner similar to that used by Carmignani and Bennett (1977) and Daniels et al. (1992). Pre-conditioning of the biofilter was accomplished over a 7-d period in a separate tank. All 'Bio-Pack' media was removed from the recirculating treatment biofilters, washed with fresh water and submerged in a 10 mg/L active chlorine solution for 24 h. Following this disinfection the media was rinsed with fresh water to remove the chlorine and placed in a preconditioning tank of 1 $m^3$. The preconditioning tank was equipped with four air stones (AS 1, Aquatic Ecosystems, Apopka, Florida) and three 300-watt submersible quartz heaters (VT 301; Aquatic Ecosystems, Apopka, Florida) which maintained a DO of 5.25 ± 1.25 mg/L and a

temperature of 29.5 C ± 0.90 C. During the first 4 days of the biofilter conditioning period filter media was inoculated daily with 946 ml of Fritz Zyme #9 inoculum (Aquacenter, Leland, Mississippi). On day 1 ammonium chloride ($NH_4Cl$) and sodium nitrite ($NaNO_2$) were added to the tank to achieve levels of 25 mg/L and 10 mg/L for the $NH_4$-N and $NO_2$-N, respectively. Levels of ammonium, nitrite and nitrate in the preconditioning tank were monitored on day 2, 4 and 6 and any adjustments in concentration were made at that time. Monitoring these concentrations indicated the nitrogen processing ability of the biofilter. Nitrogen species concentrations ($NH_4$-N, $NO_2$-N and $NO_3$-N) were assayed using HACH methods 10031, 8153 and 8039 (HACH, Loveland, Colorado) for color development and the HACH DR 2000 spectrophotometer for the color analysis.

***Algae Culture***

Two sets of three 200-L transparent fiberglass tanks (TC185, Aquatic Ecosystems, Apopka, Florida) were used for algal culture. One set of three algal tanks was used for culturing the algae for the control treatments with freshly made artificial seawater. Algal production in the second set of three algal tanks was accomplished with water taken from the recirculating reservoir tank. Water in both systems was treated with an initial dosage of 10 mg/L active chlorine 24 h prior to use. This water was monitored twice over the 24-h period to be sure the active chlorine concentration was above 5 mg/L. If the concentration of active chlorine fell below 5 mg/L additional sodium hypochlorite (10% solution) was added to the system to raise the concentration

above this level. Prior to use, algae water was de-chlorinated with sodium thiosulfate. A 5.0 L culture of *Chaetoceros muelleri* was split between the two sets of algal culture tanks to ensure a similar initial algal concentration at inoculation (>2.5 x $10^5$ cell/ml). These algal cultures were provided with Guillards nutrient formulation (F/2) (McVey, 1993), enriched with sodium meta-silicate (Mass Pack with silicate, Florida Aqua Farms Inc., Dade City, Florida). Carbon dioxide ($CO_2$) was injected into algae cultures' air supply once daily during which the culture pH was continuously monitored. Once the algae culture pH reached 7.0, $CO_2$ injection ceased. After 72 h, one algal culture from each set of tanks was partially harvested and fed to the corresponding treatment. The algal culture tanks were topped off with either new artificial seawater (control) or the re-use artificial seawater (recirculating treatment).

***Water Quality Monitoring***

Performance of the recirculating systems and the potential for repeated use was evaluated by monitoring changes in levels of physio-chemical factors on day 0, 4, 8 and 12 for each larval-rearing cycle. Physio-chemical factors (Table 4.3) monitored on day 0 and 12 were total Kjeldahl nitrogen (TKN), ammonium-nitrogen ($NH_4$-N), nitrite-nitrogen ($NO_2$-N), nitrate-nitrogen ($NO_3$-N), reactive phosphorus (RP), alkalinity, chemical oxygen demand (COD), total suspended solids (TSS), volatile suspended solids (VSS) and cell density of *Vibrio* sp (Table 4.3).

**Table 4.3** Water quality indicators monitored and methods used for analysis

| Parameter | Method |
| --- | --- |
| Alkalinity | HACH[a] Method #8221 |
| Ammonium ($NH_4$-N) | Artiola[b]<br>Perken-Elmer Lambda EZ201 Spectrophotometer |
| Chemical oxygen demand (COD) | HACH[a] method #8000<br>Perken-Elmer Lambda EZ201 Spectrophotometer |
| Dissolved oxygen (DO) | YSI[c] model 55 |
| Total Kjeldahl nitrogen (TKN) | Artiola[b]<br>Perken-Elmer Lambda EZ201 Spectrophotometer |
| Nitrate ($NO_3$-N) | HACH[a] Method 8039<br>Perken-Elmer Lambda EZ201 Spectrophotometer |
| Nitrite ($NO_2$-N) | Standard Method[d] 4500-$NO_2^-$ B[a]<br>Perken-Elmer Lambda EZ201 Spectrophotometer |
| pH | Cole Palmer[e] Model 310 pH meter |
| Reactive phosphorus (RP) | Standard Method[d] 4500-P E[a]<br>Perken-Elmer Lambda EZ201 Spectrophotometer |
| Salinity | Aquatic Ecosystems[f] Refractometer Model SR6 |
| Temperature | YSI[c] model 55 |
| Total suspended solids (TSS) | Standard Method[d] 2540 D[a] |
| Volatile suspended solids (VSS) | Standard Method[d] 2540 E[a] |
| *Vibrio* sp density | Standard Method[d] 9215D<br>culture with TCBS agar |

[a] HACH Company, Loveland, Colorado.
[b] Aritola, 1989.
[c] YSI Inc., Yellow Springs, Ohio.
[d] APHA et al. 1995.
[e] Cole Palmer Inc., Vernon, Illinois.
[f] Aquatic Ecosystems, Apopka, Florida.

Excessive variability within and between culture cycles for the Colony Forming Unit (CFU) data of *Vibrio* sp. prevented further use of these data in this study. Therefore, no further reference to these data will be made. Physio-chemical factors monitored on day 4 and 8 were $NH_4$-N, $NO_2$-N and $NO_3$-N in both systems to characterize culture water prior to and post activation of the recirculation filter. Collection and analysis of culture water at these intervals provided a profile of system performance within and between trials for the duration of the study. Culture water was monitored daily for temperature, DO and pH during each culture cycle. These analyses provide information for evaluation of performance and the potential for long-term use of this type of system.

***Shrimp Performance Data***

Differences between the two systems were additionally evaluated based on larval performance (e.g., growth, survival and dry weight). Each trial was terminated 5 days after 75% of the shrimp in a tank had reached $PL_1$. Survival to harvest was determined by completely harvesting the LRT and placing all PL in 20 L of seawater. The 20 L of water was homogenized by random manual stirring and ten 50-ml samples were collected by a Hansen Stempel Pipette (1805-C42, Wildco, Saginaw, Michigan). PL in each sample was counted in the laboratory and the survival rate calculated and recorded for each LRT. Once survival was determined, 120 PL were removed from each tank. A sub-population of 100 PL was dried (60 C for 24 h) on labeled and pre-weighed microscope slides. Prior to drying the 100 PL were rinsed with DI water to remove

adhered salts then blotted dry before being placed on the microscope slide. This sample of 100 PL was taken as a representative sample of the LRT biomass and provided the overall average PL weight of the tank. However, the sample was not sub-divided into PL stages (e.g., $PL_4$ or $PL_5$) for weights of those individual stages; this weight is intended for use as a representative sample of the PL weight 5 days after reaching $PL_1$. The sample was dried to a constant weight over a 24-h period before slides were weighed. Postlarval dry weight at harvest was calculated by subtracting the initial pre-dried slide weight from the final dry weight of slide and subsequently divided by 100 (number of PL in the sample) to determine the 5 day post-$PL_1$ dry weight. Length measurement was performed on the remaining 20 PL by placing them on a microscope slide. The microscope slide was then placed on top of a metric ruler under a dissecting scope and PL were measured in millimeters from the distal end of the telson to the distal end of the first antennae.

A stress-test (Samocha et al., 1998) was slightly modified from its original format which called for a single group of 100 $PL_5$ to be transferred into 1 liter of seawater which was 19 $^0/_{00}$ lower than the culture water for a period of 2 h. The stress test used here was performed on a group of 50 PL from each of the ten LRT. The 50 PL from each tank were subdivided into five replicate groups each containing ten PL. Postlarvae were exposed to an instantaneous salinity decrease of 20 $^0/_{00}$ that lasted for 2 h. After the period had elapsed each group was checked for dead PL and survival was recorded.

### *Statistical Analysis*

Data was evaluated using the Statistical Package for the Social Sciences (SPSS version 11.0.1 2003, Chicago, Illinois). All data was evaluated to determine if requirements for normality and equality of error variances were met prior to analysis. In the event of inequality or presence of non-normal data, appropriate transformations were made to satisfy these requirements. Data represented as percentages (survival) were arcsine transformed for analysis. Untransformed data is used in the reported descriptive statistics. Analysis of Variance (ANOVA) was used to evaluate treatment differences using a significance level of $\alpha = 0.05$.

## Results

### *Water Quality*

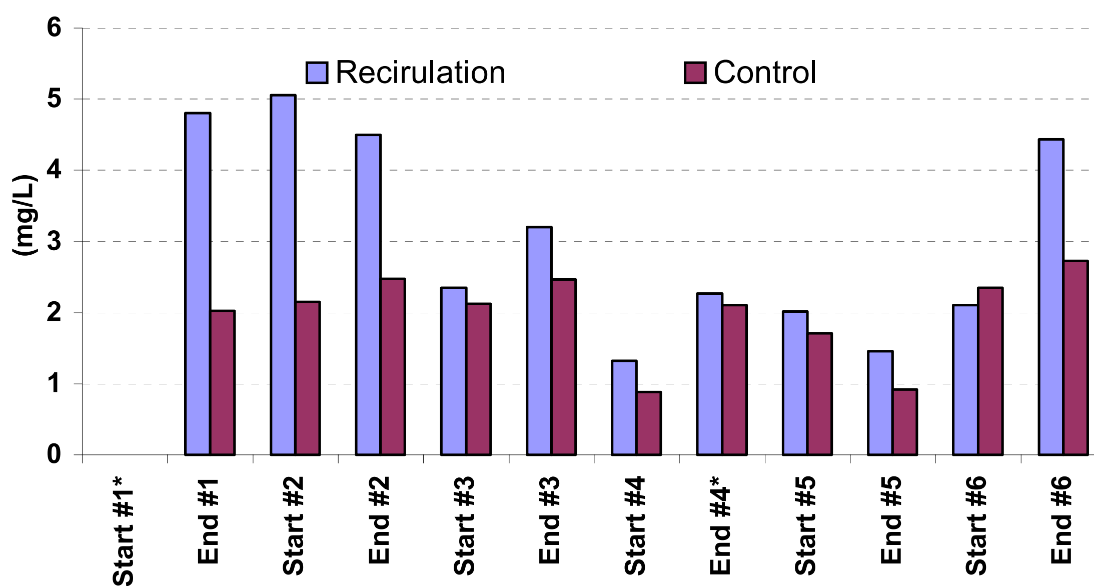
Basic water quality indicators of temperature, DO and pH were within acceptable ranges for larval culture for all six of the larval rearing trials (Table 4.4).

**Table 4.4** Basic water quality parameters during the six larval rearing runs

| | Recirculating Treatment | Control |
|---|---|---|
| Temperature (C) | 29.29 ± 1.10 | 29.07 ± 0.87 |
| Dissolved Oxygen (mg/L) | 5.82 ± 0.21 | 5.78 ± 0.25 |
| pH | 7.79 ± 0.26 | 7.81 ± 0.13 |

***Ammonium***

Changes in ammonium ($NH_4$-N) concentration in both systems were similar for the start of trial 1 and the end of trial 4 ($p > 0.05$). Ammonium concentrations in the recirculating larval rearing treatments were significantly higher than the control with the exception of the start of trial 6 which was significantly lower (Figure 4.6). During recirculating trials 1-6 the ammonium nitrogen concentrations significantly increased from the start to the end of each trial with the exception of the trial 2 and trial 5 culture periods.

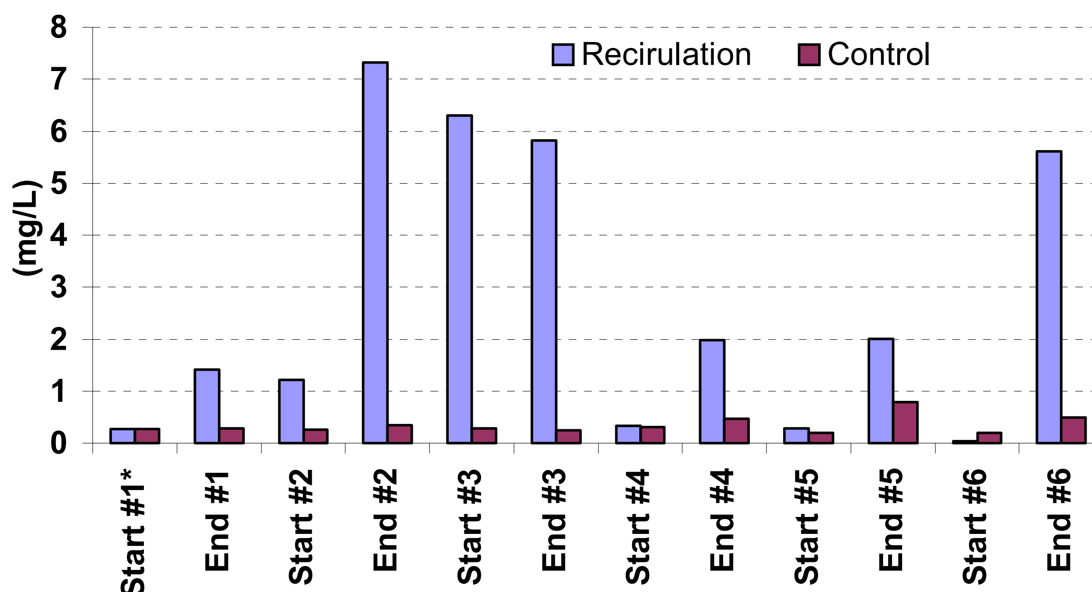



* Indicates no significant difference in values ($p > 0.05$)

**Figure 4.6** Ammonium ($NH_4$-N) concentrations of control and recirculating treatments during trials 1-6

*Nitrite*

Concentrations of nitrite ($NO_2$-N) in recirculating treatments were similar to that of the control at the start of trial 1 ($p > 0.05$). In the remainder of the recirculating trial periods, nitrite concentrations were significantly higher than the control (Figure 4.7). With the exception of recirculating trial 3, nitrite concentrations increased significantly from the start to the end of each trial in the recirculating system. Beginning with the start of recirculating trial 2 and lasting to the end of trial 3, average nitrite concentrations of 6.48 ± 0.04 mg/L were experienced. Addition of a biofilter to the water processing system on the reservoir, between the end of trial 3 and start of the trial 4, significantly reduced nitrite concentration in the recirculating treatment's culture water. Further results are presented in the re-use reservoir results section.

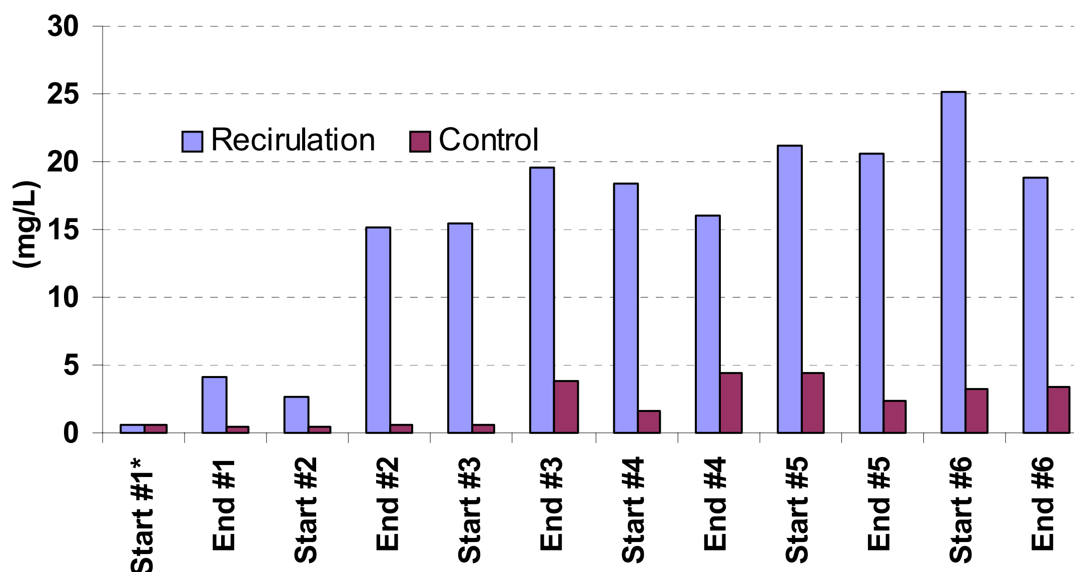



* Indicates no significant difference in values ($p > 0.05$)

**Figure 4.7** Nitrite ($NO_2$-N)

*Nitrate*

Nitrate ($NO_3$-N) concentration for the recirculating treatment was similar to the control concentration at the start of trial 1 ($p > 0.05$). Concentrations in the remainder of the recirculating trials were significantly higher than the controls (Figure 4.8). Nitrate concentrations increased significantly from the start to the end of the treatment trials 1, 2 and 3. In treatment trials 4, 5 and 6, nitrate concentrations decreased from the start to the end of each trial. Overall the nitrate concentration in the recirculating treatments increased from 0.81 ± 0.01 mg/L at the start of trial 1 to a high of 25.22 ± 0.39 mg/L at the start of trial 6. Over the same period the nitrate concentration in the control treatments ranged from a low of 0.81 ± 0.01 mg/L to a high of 4.39 ± 0.02 mg/L.

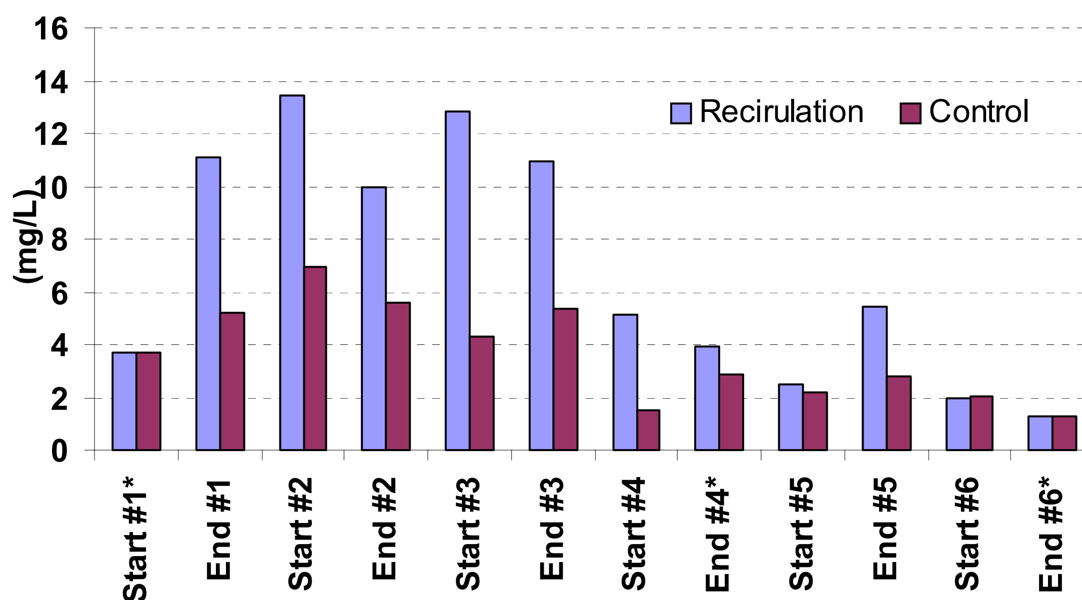



* Indicates no significant difference in values ($p > 0.05$)

**Figure 4.8** Nitrate

***Total Kjeldahl Nitrogen (TKN)***

Total Kjeldahl nitrogen concentration for the recirculating treatments were similar to the control concentrations at the start of trial 1, the end of trial 4 and the end of trial 6 ($p > 0.05$). The remainder of the recirculating larval rearing trial periods demonstrated significantly higher concentrations than the control (Figure 4.9). TKN concentrations increased significantly from the start to the end of the recirculating trial 1 while the remainder of the recirculating larval rearing trials demonstrated a significant reduction in the TKN level from the start to the end of each trial. The level of TKN increased from $3.72 \pm 0.10$ mg/L at the start of recirculating trial 1 to $13.5 \pm 0.10$ mg/L at the start of recirculating treatment trial 2 and decreased from this point successively until reaching a level of $1.28 \pm 0.05$ mg/L by the end of recirculating trial 6. Total Kjeldahl nitrogen concentrations in recirculating treatments at the end of trial 6 were not statistically different than the TKN concentration of the control group.



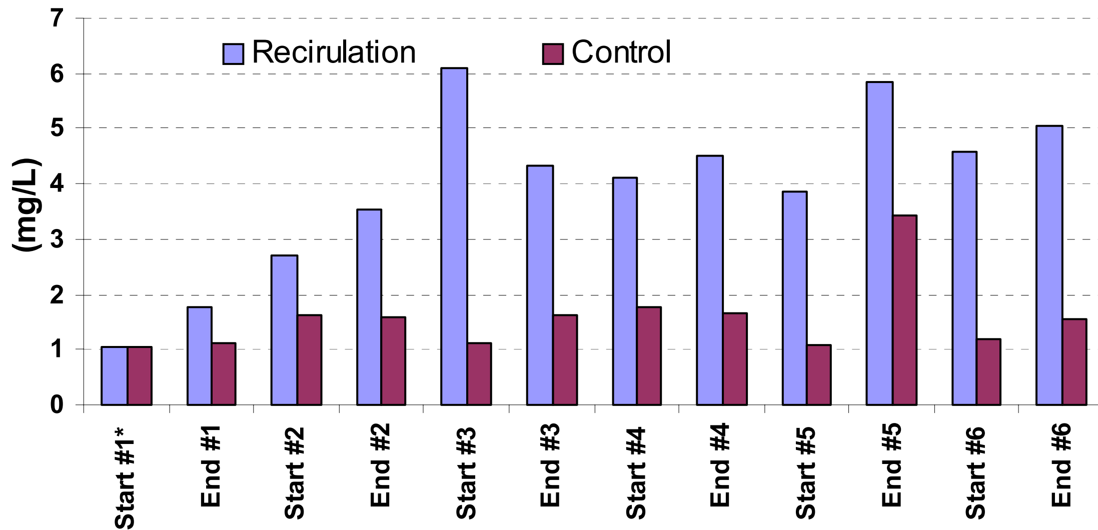

* Indicates no significant difference in values (p> 0.05)

**Figure 4.9** Total Kjeldahl nitrogen

***Reactive Phosphorous (RP)***

Reactive phosphorous concentrations of the recirculating treatment were similar to the control concentration only for the start of trial 1 ($p > 0.05$). The concentrations of the recirculating treatments were significantly higher than the control group with the exception of the end of the control treatment for trial 5 (Figure 4.10). Reactive phosphorous concentrations ranged between 1.04 ± 0.10 mg/L and 1.67 ± 0.35 mg/L for the control group over the six larval rearing trials. Reactive phosphorus levels in the recirculating treatments dramatically increased from 1.04 ± 0.10 mg/L at the start of the trial 1 to 6.08 ± 0.09 mg/L by the start of trial 3. Reactive phosphorus level decreased

significantly from the start to the end of recirculating trial 3 then returned to rise significantly from the start to the end of the trials 4, 5 and 6.



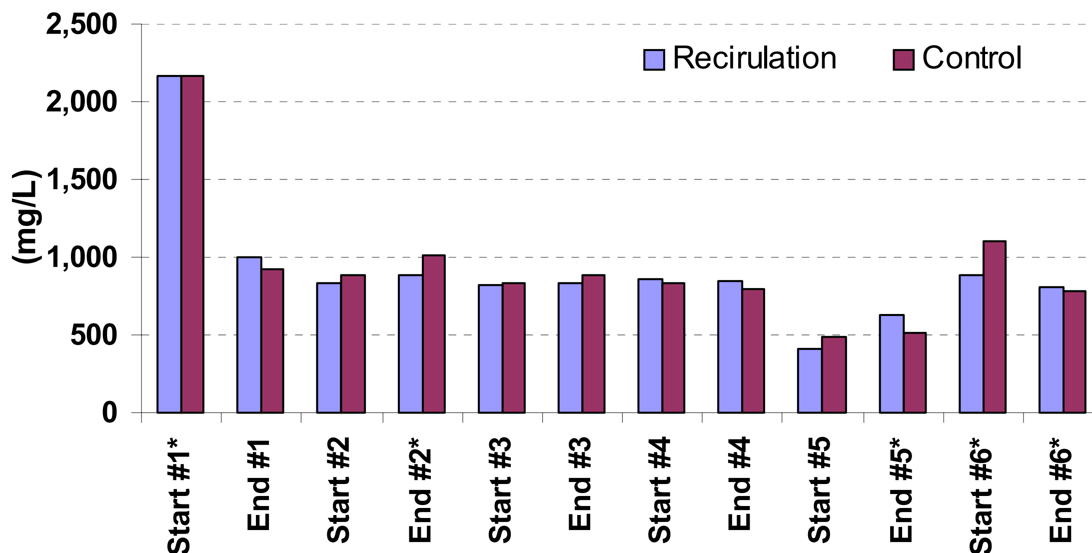

* Indicates no significant difference in values ($p > 0.05$)

**Figure 4.10** Reactive phosphorous

***Chemical Oxygen Demand (COD)***

Chemical oxygen demand for the recirculating treatments was similar to the control concentration at the start of trial 1, the end of trial 2, end of trial 5, start of trial 6 and the end of trial 6 ($p > 0.05$). The remainder of the recirculating treatment trials demonstrated COD levels were similar to the control however they were statistically different (Figure 4.11). Elevated COD (2,171 ± 21.7 mg/L) was found at the start of the treatment and control trial 1. The level of COD for the remainder of the recirculating

treatment trials 2-6 ranged from 406 ± 4.09 mg/L to 1,001 ± 25.4 mg/L and the remainder of the control trials 2-6 ranged from 485 ± 1.84 mg/L to 1,007 ± 206 mg/L.

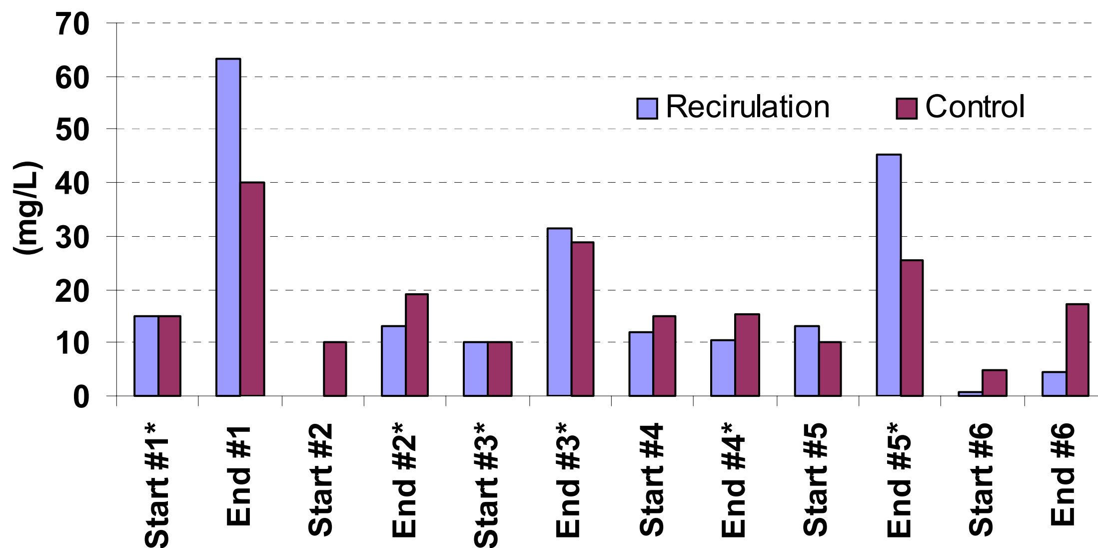



* Indicates no significant difference in values (p> 0.05)

**Figure 4.11** Chemical oxygen demand

***Total Suspended Solids (TSS)***

Total suspended solids concentration for recirculating treatments were similar to the control concentrations at the start of trial 1, end of trial 2, start of trial 3, end of trial 3, end of trial 4 and the end of trial 5 (p> 0.05)(Figure 4.12). With the exception trial 4, the TSS concentrations increased significantly from the start to the end of each trial (p<0.05).

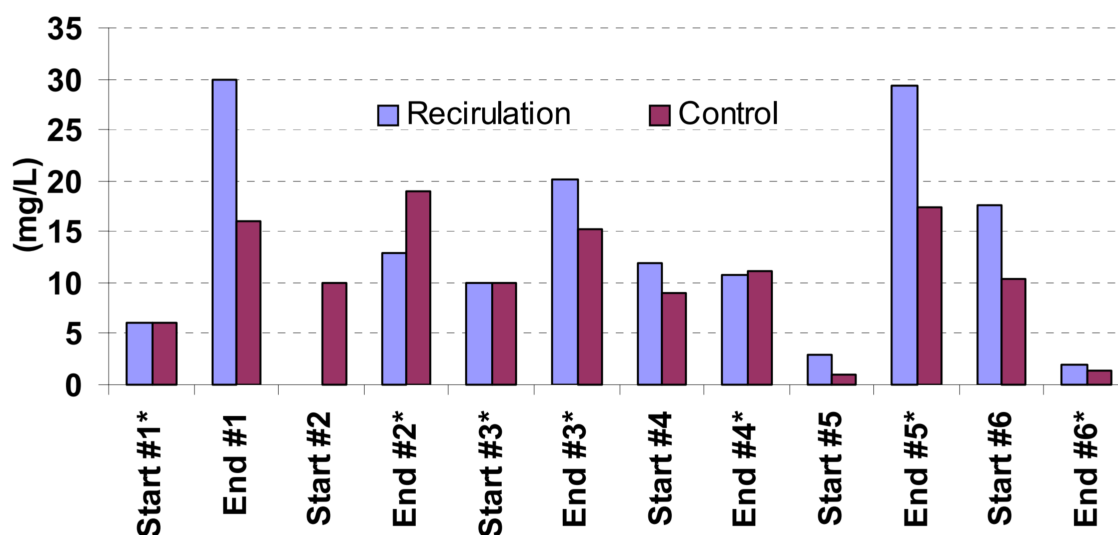



* Indicates no significant difference in values (p> 0.05)

**Figure 4.12** Total suspended solids

***Volatile Suspended Solids (VSS)***

Volatile suspended solids concentrations of the recirculating treatments were similar to the control concentrations at the start of trial 1, end of trial 2, start of trial 3, end of trial 3, end of trial 4, end of trial 5 and end of trial 6 (p> 0.05)(Figure 4.13). Volatile suspended solids concentrations from the start to the end of trial 4 in the recirculating and control treatments remained stable; however, these concentrations were statistically different. Volatile suspended solids concentrations significantly decreased from the start to the end of trial 6 within the recirculating treatment and control groups. The remainder of the VSS data for recirculating and control treatments showed an increase in VSS concentrations from the start to the end of each trial.

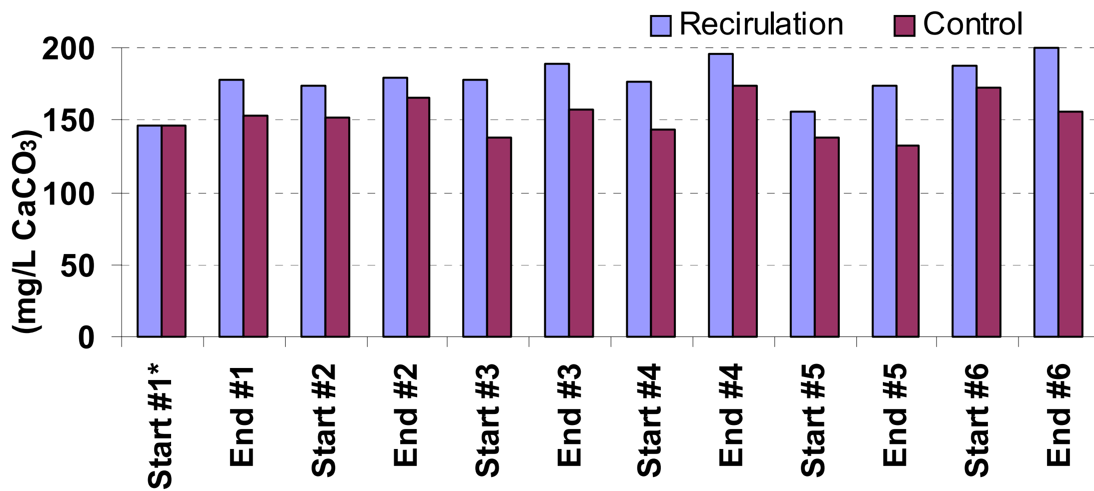



* Indicates no significant difference in values (p> 0.05)

**Figure 4.13** Volatile suspended solids

### *Alkalinity*

Alkalinity levels for the recirculating treatments were similar to the control concentrations at the start of trial 1 (p> 0.05), and the remainder of the trials demonstrated significantly higher alkalinity in the recirculating treatments than the control in each trial (Figure 4.14). Alkalinity in the control treatments ranged between 132 ± 2.83 mg/L and 174 ± 4.00 mg/L $CaCO_3$ for trials 1-6. Alkalinity in the recirculating treatments increased from a low of 146 ± 1.20 mg/L at the start of trial 1 to 199.6 ± 2.19 mg/L by the end of trial 6.




* Indicates no significant difference in values ($p > 0.05$)

**Figure 4.14** Alkalinity

***Reservoir Water Quality Under Re-Use Conditions***

Reservoir water quality was monitored pre- and post-processing (end of trial and start of next trial) to determine the effects of processing methods. These data are divided into two sections: pre-biofilter addition (Table 4.5) and post-biofilter addition (Table 4.6). The first data set is a summary of the effects of reservoir treatment on water quality indicators for the interim between trials 1-2 and trials 2-3. Water processing used on these samples consisted of particle filtration to 1 $\mu$m, foam fractionation and UV sterilization (Table 4.5).

**Table 4.5** Changes in selected water quality indicators in the recirculating and re-use system over the first three trials (prior to biofilter addition to reservoir water processing after the end of trial 3)

| | Trial 1 Termination | Trial 2 Start | Trial 2 Termination | Trial 3 Start |
|---|---|---|---|---|
| $NH_4$-N* | 4.80 ± 0.64 [a] | 5.06 ± 0.01 [a] | 4.49 ± 1.60 [b] | 2.35 ± 0.01 [c] |
| $NO_2$-N* | 1.41 ± 0.31 [a] | 1.22 ± 0.01 [a] | 7.32 ± 2.00 [b] | 6.30 ± 0.10 [b] |
| $NO_3$-N* | 5.48 ± 1.07 [a] | 3.91 ± 0.00 [b] | 22.4 ± 3.60 [c] | 21.8 ± 0.00 [c] |
| TKN* | 11.1 ± 1.30 [a] | 13.5 ± 0.01 [b] | 9.93 ± 4.59 [c] | 12.8 ± 0.01 [c] |
| $PO_4$-P* | 1.77 ± 0.15 [a] | 2.70 ± 0.00 [b] | 3.53 ± 0.61 [c] | 6.08 ± 0.09 [d] |
| COD* | 1001 ± 25.4 [a] | 829 ± 0.10 [b] | 878 ± 31.5 [c] | 824 ± 1.00 [d] |
| TSS* | 63.0 ± 1.64 [a] | 0.00 ± 0.00 [b] | 13.0 ± 2.73 [c] | 10.0 ± 0.10 [c] |
| VSS* | 30.0 ± 0.00 [a] | 0.00 ± 0.00 [b] | 12.9 ± 2.60 [c] | 10.0 ± 0.00 [c] |
| Alkalinity* | 178 ± 3.90 [a] | 174 ± 0.01 [b] | 180 ± 12.7 [c] | 178 ± 0.10 [c] |

* Units mg/L ± s.d.
** Values with similar superscript letter are not statistically significantly different.

Ammonium concentrations increased during water processing in the reservoir tank between trial 1 and trial 2; however, the difference was not statistically significant ($p > 0.05$). Ammonium concentrations significantly decreased during the water processing periods between trials 2 and 3. Nitrite concentration increased from 1.41 mg/L at the end of the trial 1 to 6.30 mg/L at the start of trial 3. Nitrite concentrations were not affected by processing as no significant change in concentration was found before and after processing for the first and second processing periods between trial 1 and 2 and trials 2 and 3. Nitrate concentrations decreased significantly between trials 1 and 2 but not between trials 2 and 3. Nitrate level increased from 5.48 mg/L at the end

of trial 1 to 25.3 mg/L by the end of trial 3. Total Kjeldahl Nitrogen and RP levels increased significantly during both of these processing periods. In contrast, significant reductions during these processing periods were found for COD, TSS, VSS and alkalinity.

Table 4.6 shows the changes in selected water quality indicators after adding the biofilter for the processing periods between trials 3-4, 4-5 and 5-6.

**Table 4.6** Effect of recirculating and re-use of reservoir processing on select water quality parameters (after biofilter addition to reservoir water processing at the end of trial 3)

| | Trial 3 Termination | Trial 4 Start | Trial 4 Termination | Trial 5 Start | Trial 5 Termination | Trial 6 Start |
|---|---|---|---|---|---|---|
| $NH_4$-N* | 3.20 ± 0.50 $^{a}$ | 1.32 ± 0.01 $^{b}$ | 2.26 ± 0.14 $^{c}$ | 2.01 ± 0.00 $^{d}$ | 1.46 ± 0.15 $^{e}$ | 2.10 ± 0.01 $^{f}$ |
| $NO_2$-N* | 5.82 ± 0.05 $^{a}$ | 0.33 ± 0.00 $^{b}$ | 1.98 ± 0.48 $^{c}$ | 0.27 ± 0.10 $^{d}$ | 2.00 ± 0.39 $^{e}$ | 0.04 ± 0.00 $^{f}$ |
| $NO_3$-N* | 25.3 ± 1.86 $^{a}$ | 18.4 ± 1.10 $^{b}$ | 16.0 ± 1.86 $^{c}$ | 21.2 ± 0.11 $^{d}$ | 20.7 ± 5.26 $^{e}$ | 25.2 ± 0.00 $^{e}$ |
| TKN* | 10.9 ± 1.73 $^{a}$ | 5.15 ± 0.00 $^{b}$ | 3.94 ± 1.03 $^{c}$ | 2.49 ± 0.00 $^{d}$ | 5.41 ± 0.50 $^{e}$ | 1.96 ± 1.01 $^{e}$ |
| $PO_4$-P* | 4.32 ± 0.11 $^{a}$ | 4.11 ± 0.00 $^{b}$ | 4.52 ± 0.07 $^{c}$ | 3.85 ± 0.00 $^{d}$ | 5.86 ± 1.22 $^{e}$ | 4.60 ± 0.10 $^{f}$ |
| COD* | 832 ± 14.5 $^{a}$ | 864 ± 10.0 $^{b}$ | 846 ± 10.5 $^{c}$ | 406 ±0.05 $^{d}$ | 627 ± 38.8 $^{e}$ | 880 ± 0.17 $^{f}$ |
| TSS* | 31.6 ± 8.56 $^{a}$ | 12.0 ± 0.00 $^{b}$ | 10.4 ± 5.90 $^{c}$ | 13.0 ± 2.1 $^{c}$ | 45.3 ± 28.8 $^{d}$ | 0.90 ± 0.01 $^{e}$ |
| VSS* | 20.2 ± 1.64 $^{a}$ | 12.0 ± 0.00 $^{b}$ | 10.3 ± 2.28 $^{c}$ | 3.00 ± 0.01 $^{d}$ | 29.3 ± 7.60 $^{e}$ | 0.90 ± 0.10 $^{f}$ |
| Alk.* | 189 ± 2.19 $^{a}$ | 176 ± 0.00 $^{b}$ | 195 ± 2.60 $^{c}$ | 156 ± 13.0 $^{d}$ | 173 ± 4.15 $^{e}$ | 188 ± 0.00 $^{f}$ |

* Units mg/L ± s.d.
** Values with similar superscript letter are not statistically significantly different.

Ammonium concentrations were significantly reduced during the processing period between trials 3 and 4 and between trials 4 and 5 ($p < 0.05$). However,

ammonium concentration increased significantly in the final processing period between trials 5 and 6. Nitrite was significantly reduced in all three water periods. Nitrate was significantly reduced in the first processing period and was significantly increased during the last two processing periods. Reactive phosphorous and TKN levels were significantly reduced in all three processing periods. Chemical oxygen demand increased significantly because of the processing in all three processing periods. Total suspended solids and VSS decreased significantly during all three processing periods. Alkalinity of the processed water was significantly reduced during the first processing period and increased during the last processing period.

***Survival***

In five of the six trials, overall survival to $PL_5$ ranged from 28.8% to 69.0% for the control group and from 26.5% to 64.8% for the recirculating treatment group (Table 4.7). In the first and second trials, the survival to PL for the control group was 5.8% and 22.3% higher than the recirculating treatment ($p<0.05$). Trial 3 was unable to go to term due to elevated nitrite levels in the recirculating treatment and was terminated after 4 days at the $Z_3$ sub-stage due to the high mortality. In trials 4 and 5 the recirculating treatment attained similar survival rates as the control. In trial 6 the recirculating treatment achieved a significantly higher (9.1%) survival rate than the control.

**Table 4.7** Postlarval survival at harvest (5 days after reaching $PL_1$)

| Trial # | Survival (%) | |
|---|---|---|
| | **Recirculating** | **Control** |
| 1 | $44.6 \pm 0.01^{a}$ | $50.4 \pm 0.02^{b}$ |
| 2 | $46.7 \pm 0.09^{a}$ | $69.0 \pm 0.11^{b}$ |
| 3 * | $41.4 \pm 0.07^{a}$ | $90.3 \pm 0.04^{b}$ |
| 4 | $26.5 \pm 0.08^{a}$ | $28.8 \pm 0.08^{a}$ |
| 5 | $52.0 \pm 0.11^{a}$ | $61.7 \pm 0.02^{a}$ |
| 6 | $64.8 \pm 0.03^{a}$ | $55.7 \pm 0.07^{b}$ |

* Trial terminated at $Z_3$ due to mortality from high nitrite
Means ± s.d. not sharing a common superscript letter within a row are significantly different ($\alpha < 0.05$)

***PL Weight***

Overall the average dry weight of PL at harvest ranged from 0.017 mg to 0.031 mg in the control group and 0.014 mg to 0.030 mg in the recirculating treatment (Table 4.8). In trial 1 PL weights were similar between the recirculating treatment (0.03 mg) and the control (0.031 mg) ($p > 0.05$). These values are significantly higher than values found for trials 2, 3, 4, 5 and 6. In trial 2 the control produced significantly larger shrimp (0.017 mg) than the recirculating treatment (0.014 mg). The recirculating treatment in trials 4, 5 and 6 resulted in average PL dry weight values of 0.003 mg, 0.005 mg and 0.004 mg greater than the control.

**Table 4.8** Postlarval weight at harvest (5 days after reaching $PL_1$)

| Trial # | Average Dry Weight (mg) | |
|---|---|---|
| | **Recirculating** | **Control** |
| 1 | 0.030 ± 0.002 [a] | 0.031 ± 0.002 [a] |
| 2 | 0.014 ± 0.001[a] | 0.017 ± 0.000 [b] |
| 3 * | * | * |
| 4 | 0.020 ± 0.001 [a] | 0.017 ± 0.001 [b] |
| 5 | 0.023 ± 0.002 [a] | 0.018 ± 0.003 [b] |
| 6 | 0.027 ± 0.001 [a] | 0.023 ± 0.003 [b] |

* Trial terminated at $Z_3$ due to mortality from high nitrite in recirculating treatments
Means ± s.d. not sharing a common superscript letter within a row are significantly different ($\alpha < 0.05$)

***PL Length at Harvest***

Overall the length of PL at harvest ranged from 6.77 mm to 8.08 mm in the control group and 6.93 mm to 8.26 mm in the recirculating treatment (Table 4.9). Shrimp length for the recirculating treatment was similar to the control lengths for trials 1, 2, 4 and 5 ($p > 0.05$). Shrimp in the recirculating treatment were significantly longer than the control in trial 6.

**Table 4.9** Postlarval length at harvest (5 days after reaching $PL_1$)

| Trial # | PL Length (mm) | |
|---|---|---|
| | Recirculating | Control |
| 1 | 8.26 ± 0.22 [a] | 8.08 ± 0.40 [a] |
| 2 | 7.04 ± 0.08 [a] | 7.09 ± 0.13 [a] |
| 3 * | * | * |
| 4 | 6.93 ± 0.17 [a] | 6.77 ± 0.19 [a] |
| 5 | 7.16 ± 0.24 [a] | 6.79 ± 0.35 [a] |
| 6 | 7.91 ± 0.16 [a] | 7.34 ± 0.28 [b] |

* Trial terminated at $Z_3$ due to mortality from high nitrite in recirculating treatments
Means ± s.d. not sharing a common superscript letter within a row are significantly different ($\alpha < 0.05$)

***Postlarval Survival in Low Salinity Challenges***

Survival of PL, harvested 5 days post-$PL_1$, in low salinity challenges (15 $^0/_{00}$) ranged from 88.0 % to 98.7% in the control group and 87.9 % to 99.3% in the recirculating treatment (Table 4.10). Shrimp in the recirculating treatment for trials 1 and 2 performed significantly better in the salinity challenges than the control group. However, the low salinity stress tests for trials 4, 5 and 6 showed no statistically significant differences among treatments ($p > 0.05$).

**Table 4.10** Postlarval survival at harvest (5 days after reaching $PL_1$) in a 2 hour low salinity challenge

| Trial # | Survival (%) | |
|---|---|---|
| | Recirculating | Control |
| 1 | $95.8 \pm 0.02^{a}$ | $89.7 \pm 0.39^{b}$ |
| 2 | $99.9 \pm 0.00^{a}$ | $97.2 \pm 0.02^{b}$ |
| 3 * | * | * |
| 4 | $87.9 \pm 0.01^{a}$ | $88.0 \pm 0.03^{a}$ |
| 5 | $94.7 \pm 0.02^{a}$ | $95.8 \pm 0.02^{a}$ |
| 6 | $99.3 \pm 0.00^{a}$ | $98.7 \pm 0.01^{a}$ |

* Trial terminated at $Z_3$ due to mortality from high nitrite in recirculating treatments
Means ± s.d. not sharing a common superscript letter within a row are significantly different ($\alpha < 0.05$)

**Discussion**

The closed recirculating larval rearing system evaluated in this trial successfully produced PL of similar weight, length and stress resistance as PL produced under standard water exchange practices. *F. aztecus* PL were produced under recirculating and water re-use conditions from the $Z_3$ sub-stage in five of the six larval rearing cycles performed. Larval rearing trial 3 did not produce PL due to the accumulation of nitrite within the recirculating systems.

Increases in ammonium and nitrite concentrations indicate either an insufficient biomass of nitrifying bacteria in the biofilter or the conditions of the system are inadequate for bacterial growth (Valenti and Daniels, 2000). The data suggest that the size of the trickling biofilters used to process the larval rearing water during each of the

six recirculating larval rearing trials was not adequate for completely converting ammonia to nitrite and nitrite to nitrate. Poor performance of the trickling biofilters is evident in the significant increases in ammonia during trials 1, 3, 4 and 6 (Figure 4.6) and the increases in nitrite during all trials (Figure 4.7). The inability of the biofilter to reduce the nitrite levels during trial 3 resulted in exposure of larvae to lethal levels of nitrite (6.30 ± 0.10 mg/L $NO_2$-N). Chen and Nan (1991) found the susceptibility of *Metapenaeus ensis* larvae to nitrite decreases from early larval stages to postlarval stages, the reported 48 h $LC_{50}$ values were 20.67 and 27.10 mg/L nitrite-N for $M_3$ and $PL_1$, respectively. Wickins (1976) reported 48h LC $_{50}$ of 170 mg/L $NO_2$-N for 0.5-1.5 g juveniles of seven penaeid shrimp species. During this study *F. aztecus* larvae were exposed to lower levels of nitrite for longer periods of time than these 48 h tests and the susceptibility of larvae to these lower concentrations was evident during larval trial 3. Larval trial 3 was terminated at $Z_3$ sub-stage due to elevated nitrite and high mortality in all of the recirculating treatment replicates. This 72-h exposure of *F. aztecus* larvae ($Z_3$) to 6.30 ± 0.10 mg/L $NO_2$-N and its effects on survival are consistent with long-term (108 h) exposures of *M. ensis* $PL_1$ which had an $LC_{50}$ of 7.06 mg/L (Chen and Nan, 1991).

After the termination of trial 3 nitrite concentrations were effectively reduced by adding a submerged crushed coral media biofilter to the water processing system of the reservoir tank. The biofilter was selected based on findings of Daniels et al. (1992) which documented the efficiency of crushed coral biofilters in cycling nitrogenous waste of *M. rosenbergii* hatcheries. Yang et al. (1989) reported on the ability of submerged biofilters to maintain ammonia and nitrite concentrations below 0.05 mg/L in loliginid

squid culture. After implementation of the submerged biofilter for the start of each of the remaining three recirculating larval rearing trials, sub-lethal nitrite concentrations, which were only slightly higher than the control concentrations, were maintained. The reduction in culture water nitrite concentrations increased the survival rates of recirculating treatments. Increased recirculating treatment survival rates resulted in similar survivals as the control in trials 4 and 5. At the termination of trial 6 the survival rate for the recirculating treatments significantly exceeded that of the control group. The subsequent reduction of the nitrite by the submerged biofilter suggests the water quality of the culture water was sufficient for bacteria cycling, as was suggested in other trials (Valenti and Daniels, 2000). Therefore, the performance of the trickling biofilter used during the larval rearing trials is the weak link in this recirculating larval culture system.

Reduced filter efficiency from channeling is most likely responsible for the poor nitrogen cycling of those systems. Replacing the trickling biofilter with the submerged biofilter during the larval rearing cycle may eliminate the channeling problem and effectively reduce nitrite concentration. The ability of a submerged biofilter to handle nitrite concentrations in the reservoir suggests it has potential for use during the larval rearing cycle to properly process nitrite. However, the age of the biofilter's bio-film and the ability of the biofilm to adjust to increasing nitrite concentrations have not been ruled out. Bovendeur (1989) documented significant variation in the nitrification capacities of bio-films based on their degree of maturity. Thorn et al. (1999) documented a bio-film's loose attachment at the beginning of bio-film development and its irreversible attachment after 20 days. Given that biofilters were seeded with bacteria only 14 days

prior to the start of the each trial, there is potential that the bio-film was not properly attached to the media before it was moved to the larval rearing biofilters. The need for complete larval rearing system cleaning and sterilization at the end of each larval cycle for proper health maintenance forces biofilters to be started new with each larval rearing cycle. Therefore, cleaning of the larval rearing systems prevents the re-use of an established biofilter from a previous culture cycle. Seeding and maturing of bio-films on recirculating larval rearing trickling filter media under these health requirements needs further investigation to determine the cause of reduced nitrification during this research and to develop techniques for rapid start-up of a fully matured biofilter.

Alkalinity was found to have increased during each larval cycle (Figure 4.13) from a low of 146 ± 1.20 mg/L $CaCO_3$ at the start of trial 1 to 199.6 ± 2.19 mg/L by the end of trial 6. The increase in buffering capacity over time allowed for the pH to be maintained at 7.79 ± 0.26 over the six larval rearing trial periods without addition of buffer. Studies of *Macrobrachium rosenbergii* larvae cultured in closed recirculating brackish water hatcheries also found pH does not vary significantly over time due to the low biomass of these systems and buffering capacity of the culture water (AQUACOP, 1983; Valenti, 1993; Silva, 1995; Mallasen and Valenti, 1998; Valenti and Daniels, 2000). Maintenance of the pH in the current study eliminated the dependence on sodium bicarbonate ($NaHCO_3$) which is commonly used to maintain pH in the range of 7 to 8.5 in closed recirculating systems (Valenti and Daniels, 2000). Bower et al. (1981) found calcium carbonate based media such as limestone, crushed coral or crushed oyster shell were valuable prophylactics against reductions in pH below 7.5. Those authors findings

supported works by Hirayama (1970 and 1974) which found the pH decline reached an asymptote at 7.5 in the presence of calcium carbonate media of the biofilters (e.g. crushed oyster shell, limestone and dolomite). These authors found it necessary to add carbonate buffers to maintain pH above 7.5 in their systems regardless of the media used. Therefore, the addition of the submerged biofilter to the water processing system between trials may have added to the buffering capacity of the water and were responsible for a portion of the alkalinity increase during this study. Additionally, contributions to alkalinity may have arisen from off gassing of carbon dioxide ($CO_2$) produced in the culture system by trickling filters. It is understood that $CO_2$ can be stripped from water when it is aerated or passed over a packed column (Summerfelt et al., 2000). Summerfelt et al. (2000) further describes the chemical reaction of $CO_2$ stripping through a packed column which results in an increase in pH and reduces the total carbonate carbon in the water. Other contributions to alkalinity may have additionally arisen from alkaline bases released from the larvae and live feeds introduced to the system. Boyd (1990) describes the contribution to overall alkalinity from the reaction of ammonia ($NH_3$) released into an aquatic medium by cultured organisms. The current study demonstrates the complex nature of alkalinity in a recirculating seawater system. Further research into the implementation of recirculating systems during the larval rearing period should investigate the individual contributions of submerged crushed coral biofilter, trickling filter and metabolites from cultured organisms to alkalinity.

Mechanical filtration with foam fractionation and cartridge filtration to 1 $\mu$m along with chemical adsorption via activated carbon in the reservoir water maintained TSS, VSS and COD at consistent levels throughout the six trial periods. During recirculating larval rearing cycles TSS and VSS load increased dramatically from the beginning to the end of each cycle (Figures 4.12 and 4.13). Total suspended solids and VSS loads were effectively reduced by the water processing system of the reservoir tank between the recirculating larval rearing trials. This reduction in both the TSS and VSS loads from the end of a trial to the start of the next trial (Figures 4.12 and 4.13) demonstrates the effectiveness of the foam fractionation and filtration to 1 *um*. The effect of particle filtration on catfish pond water was clearly illustrated by Ozbay and Boyd (2001) whereby TSS and VSS were reduced by 21% each with filtration to 41 $\mu$m which resulted in a general improvement in water quality.

Dwivedy (1973) found foam fractionation removed suspended and dissolved organic matter with the added effect of maintaining pH by removal of organic acids from marine oyster culture systems. The organic matter in the recirculating system, as indicated by the COD data, reveal that the recirculating system was able to maintain the organic matter at levels which were comparable to the control. Overall COD level increased from the beginning to the end of each recirculating trial and were reduced during water processing between the trials (Figure 4.11). However, each mechanical filtration component was not individually tested for its contribution to TSS, VSS and COD removal.

Weeks et al. (1992) documented the ability of foam fractionation to significantly reduce the concentrations of TSS, VSS and TKN in marine fish culture water. Total Kjeldahl Nitrogen increased from the start of trial 1 to the end of trial 2 and decreased thereafter. Therefore, foam fractionation during water processing is potentially responsible for the decrease in TKN over the six trials. Ammonium concentrations followed a similar trend, as is expected due to the TKN digestion, also accounting for the ammonium in solution in the water sample. However, an increase in ammonium concentration at the end of trial 6 was not observed in the TKN reading. The TKN reading at the end of trial 6 was significantly lower than the ammonium reading. Therefore, an analysis error may be involved in the lack of representation in the reading of TKN, though no error could be found when these data were reviewed. Overall the ammonium concentration of the culture water represented, on average, 40.5% of the TKN of the samples. The remaining 59.5% of ammonium in the TKN sample represented the nitrogen concentration of the organic matter within the culture water.

The addition of the submerged biofilter further complimented the water purification processing. Implementation of this type of water processing in future experiments or commercial applications must contain components consisting of cartridge filtration to 1 $\mu$m level, foam fractionation, carbon adsorption, submerged biofiltration and UV sterilization. This study demonstrated the effectiveness of the water processing components applied between trials to maintain an adequate culture environment. In the final three trials culture medium was significantly improved and allowed for survival in

the recirculating systems to first match (trial 5) then surpass (trial 6) the standard larval rearing batch culture system.

Continued biofiltration for continuous re-use will eventually lead to elevated levels of nitrate and the need for denitrification. One potential method for reducing the level of nitrate is in uptake by the unicellular algal culture. Unicellular algae are commonly provided with sodium nitrate ($NaNO_3$) in Guillards nutrient media f/2 (Guillard, 1983). Future research should focus on the effects of limiting the nitrate input in the nutrient media added to the algal system to enhance algal growth using available nitrate in the culture water. Thus, the unicellular algae could potentially be utilized to reduce culture water nitrate concentration. In addition, the culture of unicellular algae using artificial seawater could stand further scrutiny. Under the conditions of this study algal performance in artificial seawater was not specifically investigated. However, during the course of this study algae were produced in artificial seawater along side algae produced in natural seawater for other larval rearing trials. Algae cultured in the artificial and natural seawater media were maintained under the same management and used the same inoculum to start the culture. However, algae cultured in natural seawater attained average cell densities of 4.5 $x10^6$ cells/ml; whereas, algae cultured in artificial seawater only achieved average cell densities of 2.25 x $10^6$ cells/ml. Therefore, artificial seawater as the culture medium was the major difference. In order for the entire hatchery system to be fully functional and attain maximum production in all areas the algal culture in artificial seawater must be refined. Guillard (1983) indicated that algal

species react differently to the varieties of artificial sea salts available and enrichment of the artificial sea salt must be investigated to improve algal performance.

# CHAPTER V

# SUMMARY AND CONCLUSIONS

## Discussion

### *Domestication of* **Farfantepenaeus aztecus** *Broodstock*

The farm-raised bait shrimp industry in the southeastern United States is preparing to satisfy the chronic lack of live bait shrimp being supplied from a dwindling wild resource. In the interest of advancing commercial development, this dissertation took the first steps toward domestication of an endemic species by closing the life cycle of *Farfantepenaeus aztecus* in captivity. Founder populations of wild broodstock were acquired, quarantined and screened using a two step PCR procedure for known viral-pathogens (TSV, WSSV and IHHNV). Viral-disease testing determined the population free of these known viral-pathogens at which time they were moved from the quarantine to maturation. Periodic health monitoring of broodstock populations and their offspring, using the same procedure, ensured the populations remained free of these known viral-pathogens. However, a previously unknown potential viral-pathogen was identified in wild and F1 populations of *Farfantepenaeus duorarum* after shipments of wild broodstock were brought to the site and utilized in induced maturation studies outside the scope of the current research.

The origins and primary vector of this suspected viral-pathogen remain unknown and efforts are underway at the Texas Veterinary Diagnostic Lab under the direction of Dr. Kenneth Hasson to identify this pathogen. Bioassay with infected tissue produced

significant immune reaction in both *Litopenaeus vannamei* and *F. duorarum*; however, no mortality was noted in either species over the bioassay. A review of acquisition, culture and mortality records revealed broodstock mortality was absent in the *F. aztecus* population until the over wintering period from November 2002 to March 2003 when they were maintained in tanks adjacent to *F. duorarum* broodstock. Chronic low grade mortality was first observed in the *F. duorarum* population early on in this over wintering period. A similar low mortality rate was observed a few months later in the *F. aztecus* population. These records suggest that a potential viral-pathogen was introduced with the *F. duorarum* population and spread to the *F. aztecus* population during this over wintering period. However, there is insufficient evidence to conclusively determine the origin of this potential viral-pathogen. Further research and commercial production efforts using native species PL must be aware of the potential threat from this unknown potential viral-pathogen.

### ***The Future Development of Specific-Pathogen-Free (SPF)*** **F. aztecus** ***Broodstock***

This research effort demonstrates the elevated risk associated with collecting wild broodstock of endemic populations for domestication. These risks are not new to the shrimp farming industry, as severe economic losses and low production in the 1990's were encountered due to stocking wild and hatchery-reared PL infected with virulent viral-pathogens (Pruder, 2004). Virulent viral-pathogens were the principal threat to the sustainability of the shrimp farming industry during this period (Lotz, 1997). Pruder

(2004) describes the state of aquaculture as a 'maturing industry' where bio-security is a new concept that has not been refined to the level of other agricultural production such as in the poultry industry. The establishment of SPF-based shrimp aquaculture has become the cornerstone of bio-security management in commercial shrimp farming (Lotz, 1997).

Lotz et al. (1995) described an SPF-based industry as having three main components: 'SPF stream, high health stream and commodity production stream' which are based on the degree of control over the health of the population. The 'SPF stream' is contained in a 'high-security' isolation nucleus breeding center which is responsible for isolation and development of pathogen-free lines of shrimp (Lotz, 1997). Isolation of wild populations for the nucleus breeding center begins with a wild population which is quarantined for 2-5 months, during which they are screened using PCR techniques for known viral pathogens. Those which pass primary quarantine are then considered candidates for SPF stocks. These candidates are moved to a secondary quarantine facility where they are kept for 5-12 months during which time further PCR screening is performed as this population is matured and reproduced. Only offspring of the broodstock in the secondary quarantine facility, that are free of specified viral-pathogens, are then moved to the nucleus breeding center. During any stage in the process, if a broodstock population is found to be infected with a viral-pathogen, that population is immediately destroyed and the facility disinfected (Pruder, 2004). The next 'stream', as described by Lotz (1997), is the 'high health stream.' This is a medium-

security facility that receives broodstock from the ‘SPF stream’ and produces ‘high-health seed’ which flow to the third ‘stream’, the commodity production in ponds.

The effectiveness of the SPF program, which provided high-health shrimp to United States shrimp farms, was illustrated in the disease outbreaks of the 1990's. Shrimp farms in the U.S. which stocked high-health shrimp were seemingly unaffected by disease outbreak. Shrimp farms in South Carolina and Texas which were importing and stocking foreign produced seed of unknown health suffered severe losses due to those seed stocks being infected with viral pathogens (Pruder, 2004). Use of high-health stocks and bio-security management have been responsible for increased stability in commodity shrimp farms. This strategy is directly applicable to exclusion of the unknown potential viral-pathogen encountered during this research and would effectively assist in the development of sustainable bait shrimp culture in the U.S.

Sustainability of the bait shrimp industry will only be feasible if a concerted effort is made to identify and exclude viral-pathogens known to affect Penaeid shrimp endemic to the Gulf of Mexico and Southeastern Atlantic coasts of the United States. Only after sufficient efforts have been made to identify and detect highly lethal endemic viral-pathogens should domestication efforts continue along the lines of the SPF program developed by the USMSFP. The first step in this domestication process is the isolation of a viral-pathogen-free stock in a bio-secure nucleus breeding center. Future domestication efforts for bait shrimp farms must follow the precedent set forth by the USMSFP with its emphasis on bio-security and SPF stocks. Only through the

establishment of an SPF program for native species will sustainable farm-raised bait shrimp aquaculture develop in the southeastern United States.

***Recirculating Maturation Studies***

Once viral-pathogen-free broodstock of *F. aztecus* are developed, closed recirculating systems will be essential in maintaining them in a bio-secure manner. Establishment of SPF stocks in controlled and isolated environments is essential to assuring bio-security in commercial shrimp production (Lee et al., 2001). Studies undertaken for this dissertation evaluated maturation of *F. aztecus* under closed-recirculating conditions and demonstrated good reproductive performance under these conditions. The fact that un-ablated females successfully matured and spawned in this study clearly demonstrates the favorable breeding environment created by this system. The production of viable eggs by un-ablated females in the present studies and not in studies performed by AQUACOP in 1975, on the same species, suggest improved maturation conditions in the present studies. The results from this research suggest the highest reproductive performance was observed when females were ablated under a male to female ratio of 1:1 and density of 5.71 shrimp/$m^2$. Similar trends were observed by Alva and Primavera (1980) for *P. monodon* whereby nightly spawning activity, total egg production and hatch rate decreased due to increased male to female ratio beyond 1:2.

Reproductive performance of females was further enhanced by daily feeding of a diet containing frozen adult enriched *Artemia* sp. (8% body weight), dry maturation diet (Maturation Diet, Rangen Inc., Buhl, Idaho) (6% of the body weight) and the squid

component (6% body weight) per day. The enriched *Artemia* sp. portion of the diet was additionally suspected to have fortified females against the stress of frequent handling; the energetic demand associated with ablation and significantly increased female reproductive performance as seen in the life-span of females in this treatment. In a 132-d study, ablated females attained average life spans of 122 ± 19 days which were similar to the un-ablated control group. However, further research is needed to strengthen this observation. Additionally, increased hatch rates and survival rates to $Z_1$ sub-stage were found for ablated females provided a diet containing frozen enriched adult *Artemia* sp. These findings suggests that the high-cost frozen bloodworms component of a standard maturation diet can be completely replaced with a lower-cost frozen enriched adult *Artemia* sp. without losses in reproductive performance. These findings are similar to those of Naessens et al. (1997) in which substitution of bloodworms in the diet with frozen enriched adult *Artemia* sp. significantly increased reproductive performance of *L. vannamei* broodstock. Furthermore, in the present study when shrimp were fed adult enriched *Artemia* sp. and equal amounts of squid and dry maturation diet; spawning frequency significantly improved compared to treatments provided only squid and adult enriched *Artemia* sp. diet.

These maturation studies demonstrate the feasibility and long-term production potential of *F. aztecus* in a closed recirculating maturation system operated with limited water discharge. During the recirculating maturation studies, diatomaceous earth filter maintenance in the system resulted in an average weekly release of <0.05% of the total water volume. Reclaiming water discharged during filter maintenance with a settling

basin may further reduce the discharge from this type of system. Furthermore, the build up of nitrate as a by-product of biofiltration represents an eventual limiting factor of this system. Removal of nitrate from the system will have to be addressed to extend the period over which water is continuously re-used. Similar studies to the present research were performed by Lotz and Ogle (1994) on *L. vannamei* broodstock in recirculating maturation systems. These studies also did not include denitrification in the recirculating maturation system and resulted in findings suggesting nitrate concentrations up to 60 mg/L did not adversely affect reproductive performance of *L. vannamei* broodstock. Closed recirculating maturation systems will eventually need to utilize denitrification systems to be sustainable. Denitrification in recirculating maturation systems was successfully addressed in recirculating shrimp maturation system by Menasveta et al. (2001) whereby low nitrate concentrations (<25 mg/L) were maintained by a denitrification system.

***Sustainability of Re-Use Recirculating Larval Rearing Systems***

Larval rearing studies to investigate the feasibility of long-term repeated use of larval rearing culture water in closed recirculating systems was also investigated. These studies re-used culture water for six larval rearing and algae production cycles. During the first three larval production cycles, trickling filters were found to have less than adequate ability to convert nitrite to nitrate. Poor filter performance resulted in accumulation of toxic levels of nitrite by the beginning of the third larval rearing cycle. The elevated nitrite concentrations in recirculating treatments resulted in significantly

lower survival rates compared to the flow-through treatments during the first two larval rearing cycles. High mortality in recirculating treatments from elevated nitrite during the third larval rearing cycle resulted in early termination of trial 3. The inability of the individual trickling filter used in each individual larval rearing tank (LRT) during the culture cycle to handle nitrite production forced the addition of a submerged biofilter to further process the water collected at the end of each trial in order reduce nitrite concentrations to tolerable levels.

Processing of culture water between trials was initially designed only to reduce solids, organic load and for sterilization. Originally, no biofilter was included in the reservoir's water treatment system for processing water collected at the end of each trial. The biofilter mounted on each LRT was inadequate for maintaining ammonium and nitrite concentrations which rose with each of the first three larval rearing trials. Therefore, the biofilter was added to the reservoir's water processing system after larval rearing trial 3 in order to reduce this accumulation. As a result, nitrite concentration was significantly reduced to a similar concentration as the control at the start of trial 4. Additional evidence of the reservoir's submerged biofilter to reduce the nitrite accumulation is found in the subsequent increase in nitrate of the system. This reduction in nitrite level of the reservoir provided trials 4 and 5 with initial nitrite concentrations which were similar to their associated controls. The low nitrite concentrations at the start of trials 4 and 5 helped increase survival to PL stage in both recirculating trials to the same level found in the respective controls. It is interesting to note that the final

recirculating larval cycle attained survival to PL (64.8 ± 0.03%) which was significantly higher than the control system survival (55.7 ± 0.07%).

Nitrite concentrations during each larval trial continued to show a gradual increase over the culture period. Increasing nitrite concentration during larval rearing cycles clearly indicates the trickling biofilter used on each larval rearing tank was inadequate for nitrite cycling. Water channeling within the trickling filter media is suspected to have significantly reduced the filter's performance. This channeling problem could be eliminated by replacing the trickling biofilter with the submerged biofilter. Effective nitrite reduction by a submerged biofilter was demonstrated during this study when it was applied to the reservoir water processing system. In addition to the type of biofilter used, the bio-film associated with that biofilter must be fully mature to ensure adequate nitrogen cycling. The degree of bio-film maturity has been documented to significantly affect nitrification rates within a biofilter (Bovendeur, 1989). The rapid biofilter start-up over this period potentially created a situation whereby the bio-film was not properly attached to the media before it was moved to the larval rearing biofilters. Bio-films have been found to have a loose attachment during early their development and solid attachment after 20 days (Thorn et al., 1999). Proper health maintenance dictates complete cleaning and sterilization of the larval rearing system at the end of each larval cycle. Therefore, the biofilter must be started new with each larval rearing cycle. Cleaning of the larval rearing systems prevents the re-use of an established biofilter from a previous culture cycle. Seeding and maturing of bio-films on recirculating larval rearing trickling filter media under these health requirements

needs further investigation to determine the cause of reduced nitrification during this research and to develop techniques for rapid start-up of a fully matured biofilter.

The trickling filters used in this study are suspected to have had the added effect of off gassing carbon dioxide ($CO_2$) produced in the culture system. When culture water with elevated $CO_2$ concentrations is passed over a packed column a portion of the $CO_2$ is removed from the water (Summerfelt et al., 2000). Alkalinity was found to have increased during the six larval cycles which caused the pH to remain stable ($7.79 \pm 0.26$) over the study period without addition of a buffer. Stripping $CO_2$ with a packed column reduces the total carbonate carbon and increases the pH of the solution that was passed through the column (Summerfelt et al., 2000). Further research into recirculating systems for larval rearing should investigate a combination of a trickling filter for $CO_2$ off gassing to maintain higher pH and more effective nitrogen cycling by either developing better seeding techniques for trickling filter bio-film development or using a submerged crushed coral biofilter. Use of biofiltration for continuous re-use of culture water will eventually lead to elevated levels of nitrate and need for nitrate removal. Future research should focus on the effects of limiting the nitrate input in the algal production tanks, thus forcing unicellular algae to utilize the available nitrate in the culture water. Allowing the unicellular algae to use the available nitrate could reduce the nitrate concentration in the culture water and potentially reduce or eliminate the need for a denitrification unit.

Concentrations of TSS, VSS and COD were effectively maintained at consistent levels in the reservoir water throughout the six trial periods by mechanical filtration

(foam fractionation and cartridge filtration) and chemical adsorption (activated carbon). However, each of these components was not individually tested for their contribution to the removal of TSS, VSS and COD. Other researchers have documented significant improvements in water quality when these components have been implemented. Mechanical filtration effectively reduced TSS and VSS concentrations when culture water was filtered to 41 $\mu$m (Ozbay and Boyd, 2001). Foam fractionation effectively reduced concentrations of TSS, VSS and Total Kjeldahl Nitrogen (TKN) in marine fish culture water (Weeks et al., 1992). Additionally, foam fractionation in marine systems maintained pH by removing suspended and dissolved organic matter (Dwivedy, 1973). In future experiments or commercial applications the components of cartridge filtration (1 $\mu$m), foam fractionation, carbon adsorption, submerged biofiltration and UV sterilization should be considered as essential for effective water processing. Once applied in total, between the final three trials of this study, water quality significantly improved allowing increased survival of PL in the recirculating treatments. These systems performed similar for trial 5 then surpassed in trial 6 the standard larval rearing batch culture system. Additionally, the recirculating larval rearing systems produced PL with similar length, weight and stress tolerance as PL produced in the control systems.

Commercial applications of recirculating and re-use hatchery systems are inevitable. Data collected in this study showed that re-use maturation and larval rearing in hatcheries using standard water processing methods is feasible. Implementation of these re-use hatchery systems will eventually lead to significant reductions in hatchery effluents and subsequent increases in bio-security. Hatchery re-use water management

will bolster the sustainability of commercial hatcheries, especially in the increasingly restrictive regulatory environment of the continental United States.

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