Evaluating various management strategies of biofloc systems and understanding the physiological basis behind the thermal tolerance of Pacific white shrimp, *Litopenaeus vannamei* in low salinity waters

By

Aya Saied Hussain Mohamed ElSeadawy

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Approved by

D. Allen Davis, Chair, Alumni Professor, School of Fisheries, Aquaculture, and Aquatic Sciences
James A. Stoeckel, Co-chair, Associate Professor, School of Fisheries, Aquaculture, and Aquatic Sciences
Luke A. Roy, Associate Extension Professor, School of Fisheries, Aquaculture, and Aquatic Sciences
Claude E. Boyd, Professor Emeritus, School of Fisheries, Aquaculture, and Aquatic Sciences
Nahla M. Shoukry, Professor of Immunology and Parasitology, Zoology Department, Faculty of Science, Suez University.

Abstract

Biofloc technology (BFT), in its various types, has been known to be a realistic solution for efficiently managing water quality with low or no water exchange, enhancing shrimp growth performance and establishing an efficient and healthy shrimp culture with a better food conversion ratio in the shrimp aquaculture business. A series of laboratory-based trials were conducted at E.W. Shell Fisheries Center at Auburn University in Auburn, Alabama to evaluate the effects of applying different management strategies using prebiotics, probiotics, biofloc and synbiotic type systems in different production phases of *Litopenaeus vannamei*. Twenty-four 150 L polyethylene indoor tanks were used as static biofloc individual tanks for a nursery phase trial conducted for 28-days except for four tanks that were used as a clear water recirculating system (RAS; a reference). To evaluate the effects of using commercial probiotic products as a feed supplement and as water additive on the water quality, growth, and survival on nursery culture performance of Pacific white shrimp PLs. At the conclusion of the nursery trial, no significant differences existed in weight gain of shrimp post-larvae between treatments, however final biomass (g), and survival (%) of PLs were significantly higher between probiotic and clear water treatments. An 8-week indoor grow-out experiment was conducted to study the effect of culturing the Pacific white shrimp in "biofloc" and a "synbiotic" type system on the growth and immune responses of shrimp. The experimental system consisted of 24 static indoor circular polypropylene tanks (800-L water volume). At the conclusion of the grow-out study, it was detected that all treatments produced good survival, rapid growth, low FCR and physiological parameters indicating all are viable options. A higher level of total haemocyte count (THC) was noted in shrimp reared in the biofloc and synbiotic treatments as compared to the control, however, there was no significant differences between treatments. Additionally, a grow-out study was conducted to evaluate the performance of the Pacific white shrimp fed with four different protein-based extruded diets [plant-based (AP), 8% poultry by-product meal (PM8), 8% fishmeal (FM8) and 12% fishmeal (FM12)] while cultured in clear water and biofloc type systems. Results from the clear water experiment showed that shrimp fed with PM diet had the lowest final individual weight, biomass (g), and weight gain (g), and the highest

feed conversion ratio (FCR). Results from the biofloc experiment showed that shrimp fed with AP diet had the lowest biomass (g), weight gain (g), and thermal growth coefficient and the highest FCR. No significant differences in survival rate were observed between the four diets in both experiments. The low inclusion of fishmeal, as well as the use of alternative protein sources in these diets, did not adversely affect final weight, weight gain, and percent weight gain of Pacific white shrimp. Consequently, the choice of how to manage the bacterial community should be based on available resources.

Another major challenge facing the shrimp industry in inland, low-salinity ponds is a phenomenon called late-term mortality which is thought to be driven by thermal stress at the end of the growing season when water temperatures can reach or exceed 36 °C in shrimp production ponds. To investigate the physiological mechanisms behind upper lethal limits in shrimp, we evaluated linkages between empirically measured thermal limits and absolute aerobic scope (AAS), or ability to provide energy above that needed for basic maintenance. At each temperature, intermittent respirometry was used to estimate resting metabolic rate and lethal thermal tolerance by evaluating critical thermal maximum (CT_{max}) was directly measured. Additionally, the electron transport system assay was used to estimate maximum metabolic rate at temperatures from 9–45 °C. Small shrimp had a higher CT_{max} than large shrimp, with upper lethal limits of 40.6 and 39.0 °C, respectively. In this study, we tested whether thermal tolerance decreases with increasing shrimp age/size and whether AAS is a useful concept for understanding the physiological basis of thermal tolerance in shrimp. Two size classes of shrimp (small: 2.07 ± 0.86 and large: 24.64 ± 2.55 g) were exposed to increasing temperature at a rate of 1 °C/h from 28–42 °C. At the conclusion of the study, AAS reached its minimum (AAS_{min}) at temperatures within 2 °C of CT_{max} for both size-classes. Reductions in AAS appear to be one of the underlying physiological drivers of thermal tolerance in *L. vannamei* and an indicator of increasing thermal stress. Changes in the temperature at which AAS reaches its minimum may be a useful predictor of shifts in thermal tolerance among shrimp size-classes.

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CHAPTER 1

GENERAL INTRODUCTION

Penaeid shrimp are a highly valued seafood commodity in the global market (Tan et al., 2005). The primary cultured shrimp species worldwide is the Pacific white shrimp, *Litopenaeus* (*Penaeus*) *vannamei*, which is considered a high-value commodity with around 5.8 million metric tons produced in 2020 (FAO, 2022). The application of intensive cultivation to increase shrimp production has the potential to result in deterioration of water quality and increased stress which may influence disease susceptibility (Munaeni et al., 2014). There is great interest by the commercial aquaculture industry in closed aquaculture systems, mostly because of the biosecurity, environmental, and marketing advantages over traditional recirculating systems (Emerenciano et al., 2013). There are different biological control strategies through closed systems to improve growth and disease resistance for cultured organisms. Some of the commonly used biological control strategies in Penaeid shrimp culture are microalgal products (Ju et al., 2009), biofloc (Crab et al., 2012; Ray et al., 2010b), prebiotics (Zhang et al., 2012), probiotics (Ninawe and Selvin, 2009), and synbiotics (Hussain et al., 2021; Munaeni et al., 2014).

Biofloc technology (BFT) in its various forms, has been gaining importance and has been found to be a more efficient use of nutrient input through a closed culture system with limited water exchange. By manipulating the carbon/nitrogen (C/N) ratio in the culture water through the addition of an external carbon source (e.g., molasses, wheat, rice bran, etc.), the microbial biomass is enhanced (Avnimelech, 1999). These aggregated bacteria, algae, and protozoa are held together in a matrix along with particulate organic matter. Growing shrimp using BFT was proposed as a tool to reduce water exchange and minimize the introduction of viral pathogens through incoming water. Hence, observations on the effects of BFT on reducing viral disease outbreaks have been reported (Avnimelech, 2015).

Additionally, there are several other microbial control strategies that are used in shrimp aquaculture. Gibson et al. (2004) defined prebiotics as “non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/ or activating the metabolism of one or a limited number of health promoting bacteria in the intestinal tract, and thus improve

host health". Several definitions are found in the literature for the term "probiotics". The most widely quoted definition was made by Fuller (1989). He defined a probiotic as "a live microbial feed supplement which beneficially affects the host animal by improving its intestinal balance". Probiotics are diverse and are usually derived from the intestines of host animals (Chu et al., 2011; Sun et al., 2012), cultured in diverse environments (Yanbo and Zirong, 2006), and have been developed into commercial products which are also introduced and used (Fernandez et al., 2011).

In addition, one of the biological control strategies to improve growth and disease resistance in aquaculture organisms is synbiotic application (Huynh et al., 2018). The synbiotic concept is a variant of biofloc production which incorporates a nutritional supplement which is a combination of probiotics and prebiotics, thus it is usually done by fermenting a carbon source (prebiotic), such as rice bran or molasses, with probiotics as well as other supplements (such as enzymes) and applying it to the culture system (Munaeni et al., 2014). Dietary supplements such as probiotics, prebiotics, and synbiotics provide nonspecific disease protection and also act as growth promoting factors (Das et al., 2017).

Another major challenge faced by shrimp farmers is a phenomenon called late-term mortality. Due to environmental concerns and the higher cost of coastal real estate, aquaculture production of *L. vannamei* in inland, low-salinity ponds is becoming more common in many regions throughout the world, including the southeastern U.S. (Roy et al., 2010). In west Alabama, shrimp represent an important, high-value alternative to catfish production (Sun, 2012). Recent reports of late-term mortality by commercial producers are thought to be driven by thermal stress at the end of the growing season when water temperatures can reach or even exceed 36 ºC in shrimp production ponds.

One physiological approach to understanding thermal tolerance that is receiving increasing attention is the concept of aerobic (or metabolic) scope. Aerobic scope (AS) is defined as the difference between maximum metabolic rate (MMR: the maximum metabolic rate an organism is capable of) and resting metabolic rate (RMR: the minimum metabolic rate required for basic maintenance and survival; Verberk et al., 2016). Resting metabolic rate (RMR) is defined as the minimal maintenance of an unstressed, post-absorptive and non-breeding ectotherm acclimated to experimental conditions, below which physiological function is impaired (Chabot et al., 2016). RMR therefore represents the basic cost of living and is of major functional importance. For

example, life-history theory posits that acquired energy is allocated between functions such as growth, reproduction and self-maintenance (e.g. RMR; Roff, 1993). At the other end of the metabolic scale, MMR provides the upper boundary for aerobic energy metabolism (Norin and Clark, 2016). MMR is conventionally measured at the point of exhaustion (Rosewarne et al., 2016). In theory, the greater the metabolic scope, the greater the physiological capacity to generate energy in excess of that needed for basic maintenance. The temperatures at which aerobic scope is maximized represent the optimal temperature for that organism. The temperature at which aerobic scope declines to zero (AS_0) represents the upper thermal limit, as the organism is no longer physiologically capable of meeting its basic metabolic costs. However, while aerobic scope is increasingly being measured and reported in the literature, this hypothesis has rarely been empirically verified (Clark et al., 2013).

Acute thermal tolerance can be empirically measured as critical thermal maxima (CT_{max}), where the animal is exposed to temperature increases at a constant rate until a critical endpoint is reached (González et al., 2010; Kumlu et al., 2010). This approach has been used as a relevant ecological index for many taxa, including Pacific white shrimp (Re et al., 2012). Although CT_{max} may occur at different temperatures in different species, the behavioral responses of different species to temperature are mostly the same across diverse taxa. Common responses include loss of equilibrium (LOE), sudden onset of muscular spasms, and finally "heat rigor", "coma", or "death" (Lutterschmidt and Hutchison 1997a, 1997b).

Although extremely useful for quantifying thermal tolerance limits, indices such as CT_{max} do not provide information on the underlying physiological mechanisms that drive and control thermal tolerance. Understanding the physiological mechanisms behind thermal tolerance would allow for quantitative comparisons of specific physiological characteristics of various shrimp stocks and/or size classes concerning suitability for low-salinity culture, high temperature, and pond production. This information may help predict effects of multiple stressors on shrimp health at high temperatures and be a practical aid in developing management recommendations for the shrimp industry.

Therefore, the overall objectives of the study are:

1. To evaluate the effects of applying different management strategies through the use of prebiotics, probiotics, biofloc and synbiotic type systems on water quality, growth, and survival of Pacific white shrimp *Litopenaeus vannamei* cultured in nursery and grow out phases of production.
2. To improve our understanding of the physiological basis for upper thermal tolerance in shrimp, by using aerobic scope, calculated by a combination of respirometry and enzymatic assays, as a useful parameter for understanding the upper thermal limits of Pacific white shrimp in low-salinity inland culture.

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CHAPTER 2

EFFECT OF MANAGEMENT STRATEGY USING DIFFERENT COMMERCIAL PROBIOTICS ON NURSERY CULTURE PERFORMANCE OF PACIFIC WHITE SHRIMP, *Litopenaeus vannamei*

Abstract

Improvement of the rearing conditions of Pacific white shrimp, *Litopenaeus vannamei* during the early life stages would benefit shrimp performance as well as aquaculture profitability. This study aimed to evaluate the effects of using a combination of commercial probiotic products as a feed supplement and as a water additive on the water quality, growth, and survival on nursery culture performance of *Litopenaeus vannamei*. A four-week nursery trial was conducted using bacterial based water treatment. Two commercial probiotics were used as water additives singularly and in combination with a probiotic supplemented- feed, resulting in four treatments using static biofloc type systems. The fifth treatment was used as a reference by connecting to a recirculating aquaculture system (RAS). Results showed that the shrimp post larvae cultured in the four probiotic treatments had significantly higher final biomass (g) and survival (%) as well as significantly lower FCR than treatment 5 (reference; $p < 0.05$). There were no significant differences between dissolved oxygen (mg/L), temperature (ºC), pH, and total ammonia-nitrogen (mg/L) of the five treatments. However, a peak of total ammonia-nitrogen (1.11 ± 0.08 mg/L) and nitrite (0.74 ± 0.06 mg/L) was observed in the four probiotic treatments around day 20 of the experiment, that rapidly decreased by day 22. This is assumed to be due to the continuous addition of the fermented probiotic additive to the water as well as the restricted feed during these peaks. Overall, results of this study indicated that biofloc type systems have a number of advantages over clear water systems and that the use of commercial probiotics helps to develop and maintain biofloc based systems for enhancing shrimp performance.

Key words: Probiotics, probiotic-supplemented diet, nursery phase, biofloc, Pacific white shrimp

1. Introduction

The nursery phase of shrimp between hatchery and grow out can optimize space utilization, enhance biosecurity, and produce hardier juveniles to stock in production systems (Samocha, 2010). There are number of other potential benefits associated with the use of a nursery phase, including better control of stock inventory, greater uniformity of market-size shrimp PLs at harvest, and less predation. To optimize the use of space and energy, shrimp density in nurseries should be maximized; however, high density can impact shrimp growth, survival and water quality (Moss and Moss, 2004). Systems should also have environmentally friendly management practices that contribute to greater biosecurity (Boyd and Clay, 2002).

Recently, the interest in the use of probiotics in aquaculture to prevent diseases and increase resistance to pathogens has increased (Akhter et al., 2015). Gatesoupe (1999) defined probiotics as "microbial cells that are administered in such a way as to be kept alive, with the aim of improving health". The use of probiotics may benefit marine shrimp by improving the balance of intestinal microbiota (Vieira et al., 2016), survival (Pham et al., 2014), resistance to infection by pathogens (Aguilera-Rivera et al., 2014), immune stimulation (Ferreira et al., 2015), diet digestibility, growth enhancement as well as improving water quality (Liu et al., 2009; Zhang et al., 2011; Kongnum and Hongpattarakere, 2012; Zokaeifar et al., 2012).

Probiotics are diverse and are usually derived from the intestines of host animals (Chu et al., 2011; Sun et al., 2012), cultured in diverse environments (Yanbo and Zirong, 2006), and have been developed into commercial products (Fernandez et al., 2011). Commercial probiotics for aquaculture are usually divided into at least two dominant types; water-based probiotic and directly fed probiotic. Some commercial probiotics require the farmers to ferment the product before use to activate the bacteria and increase colony forming units.

Water based probiotic additives, as the name suggests, are administered directly to pond water. They work in several ways including; competitive exclusion and inhibition of pathogenic bacteria, as well as improvement of water quality. The strains of bacteria used for water probiotics include *Bacillus acidophilus*, *B. subtilis*, *B. licheniformis*, *Nitrobacter spp*., and *Aerobacter spp*. (Zhou et al., 2010). Moreover, to reduce the occurrence of shrimp diseases and control water quality, commercial farms started to culture shrimp in biofloc technology systems (BFT; Huerta-Rábago et al., 2019). Biofloc is a term used to designate the formation of aggregates of particles

in a colloidal dispersion. These aggregated bacteria, algae, and protozoa are held together in a matrix along with particulate organic matter (Hussain et al., 2021). In some cases, to promote and maintain the biofloc, shrimp farms use commercial probiotics (Huerta-Rábago et al., 2019) to initiate the system as well as recurring treatments. The gastrointestinal microbiota of fish and shellfish are peculiarly dependent on the external environment, due to the water flow passing through the digestive tract. Most bacterial cells are transient in the gut, with continuous intrusion of microbes coming from water and food (Gatesoupe, 1999).

Supplementing feed with probiotics is also an application in aquaculture; the aim of this method is to introduce live cells of probiotics to the host animal gut in order to potentially establish a more balanced gastrointestinal microbial flora and to improve digestive function or immune system responses (Tuan et al., 2013). Some probiotics that have been supplemented in animal feed include bacterial species, such as *Lactobacillus spp., Enterococcus faecium, Bifidobacterium thermophilum, Streptomyces spp., Micrococcus spp., Pseudomonas fluorescens*, as well as yeast, such as *Saccharomyces cerevisiae*, and herbs and extracted substrates, such as azadirachtin (Tuan et al., 2013).

Given the popularity of these products, there is a need for evaluation under controlled replicated conditions. Hence, the objective of this study was to evaluate the effects of using commercial probiotic products as a feed supplement and water additive on the water quality, growth, and survival on nursery culture performance of *Litopenaeus vannamei*.

2. Methods

2.1 Nursery trial

A four-week nursery trial was conducted at the E.W. Shell Fisheries Center at Auburn University in Auburn, Alabama in compliance with the Auburn University animal care policy. Pacific white shrimp PLs were obtained from American Penaeid (St. James City, FL, USA). The experimental system consisted of twenty-four 150 L polyethylene tanks. The system was used as static individual tanks except for four tanks that were used as a clear water recirculating system (RAS), which was used as a reference. Each tank was equipped with a heater to control temperature in addition to two air stones to maintain the dissolved oxygen level near saturation and suspend solids. No water exchange was done during the experiment. Approximately equal amounts of

dechlorinated freshwater were added to each tank to compensate for evaporation loss. Each tank was stocked with 300 PLs (mean initial weight 0.014 ± 0.003 g; 2 PL/ L). Daily feed rations were calculated based on a percent body weight of animals (10% at start and gradually reduced to 8%); PL were expected to double in size every 3 days, and size of feed provided was gradually increased accordingly over time. At the termination of the experiment, shrimp were counted, and the biomass weight was recorded to determine mean final weight, survival (%), and feed conversion ratio (FCR) of shrimp.

2.2 Experimental treatments

Two probiotic water additives were used during this nursery trial including Biowish® aqua-builder (contains a combination of *Bacillus subtilis, B. amyloliquefaciens, B. licheniformis, B. pumilis, Lactobacillus plantarum, Pediococcus acidilatici*, and *Pediococcus pentosaceus*, Cincinnati, OH, USA) and an Alltech® product that contains *Lactobacillus spp.* and *Saccharomyces spp.* (Nicholasville, KY, USA). Additionally, one probiotic was used as a feed supplement which contains *Bacillus subtilis* (1×10^7 UFC/g; Biowish® feed-builder, Cincinnati, OH, USA). The probiotic supplemented to the feed was mixed with commercial shrimp feed (Zeigler Bros. Inc., Gardners, PA, USA; protein ≥ 50 %, fat ≥ 15%, fiber ≤ 1%) at a ratio of 0.5 g probiotic/ kg of feed. As per the guidelines from Biowish® company, the probiotic supplemented-feed needed to be moistened with disinfected seawater to activate the probiotic prior offering that mixture to shrimp. The two-commercial water additive probiotics were used alone and with a combination of the probiotic supplemented- feed, resulting in four treatments. The fifth treatment was a clear water traditional recirculating aquaculture system (RAS) that was used as a reference. The treatments that did not receive the probiotic supplemented- feed, were fed with the same commercial shrimp feed (Zeigler Bros. Inc., Gardners, PA, USA; protein ≥ 50 %, fat ≥ 15%, fiber ≤ 1%). Description of the experimental treatments is presented in Table 1.

Biowish® and Alltech® water additives were prepared as per the directions of the products. Prior to application, a 3 L sample was prepared by mixing 3 g of the test product in disinfected sea water with 3 g of sodium bicarbonate and 3 g of molasses (Evolved Habitats, Plano synergy, LA, USA). These solutions were aerated at ~27 °C for more than 18 hours and a 300 mL sample of the fermented solution was added to each assigned treatment tank. A water additive was applied every other day in the first week and every third day thereafter, until the end of the experiment.

2.3 Water analysis

Water was prepared for all tanks by mixing manufactured sea salt (Crystal Sea® Marinemix, Baltimore, MD, USA) with dechlorinated freshwater and maintained at around 8 g/L during the trial. Water quality in the clear water treatment was maintained by recirculation through an Aquadyne bead filter (0.2 m^2 media, 0.6 m × 1.1 m) and vertical fluidized bed biological filter (600-L volume with 200-L of Kaldnes media) using a 0.25-hp. centrifugal pump. Mean water flow for these aquaria were ~4 L/min with an average turnover of ~30 minutes/tank. Dissolved oxygen was maintained near saturation using air stones in each culture tank and a sump tank using a common airline connected to a regenerative blower. During the feeding period, dissolved oxygen (DO), temperature and salinity were monitored twice daily (0830 and 1630) using an YSI 55 multi-parameter instrument (YSI, Yellow Springs, OH). Total ammonia-N (TAN) and nitrite-N were measured twice per week from each tank using YSI 9500 photometer (YSI, Yellow Springs, OH) using the water samples (supernatant) taken from 10 cm below in each tank after letting it settle. Total Ammonia- Nitrogen and Nitrite peaked up once during the trial, hence, during that time a restricted feeding regime was temporarily offered to shrimp. pH of the water in each of the experimental tanks was measured twice weekly during the experimental period using a pHTestr30 (Oakton Instrument, Vernon Hills, IL, USA).

2.4 Quantification of suspended solids

At the end of the nursery trial, water in each tank was well-mixed to attain a 200 mL sample for the determination of total suspend solids (TSS) in the medium. Each water sample was vacuum filtered through a pre-weighted glass microfiber filter (1.2 mm pore size, Whatman GF/C, St. Louis, Missouri, USA), dried for approximately 24 hours at 60 ºC and the weight was recorded (Da Silva et al., 2013). TSS in each tank was quantified as the fraction retained in the filter paper (weight difference) and expressed as grams per liter for each treatment (Table 3).

2.5 Statistical analysis

Data were analyzed using SAS (V9.4, SAS Institute, Cary, NC, USA). The performance of shrimp and water quality parameters tested during the study were analyzed using one-way analysis of variance (ANOVA). The level of significance (alpha) used was 0.05, while Tukey's multiple comparison test was used to evaluate significant differences between treatment means.

3. Results

3.1 Growth parameters

The performance of the shrimp post larvae at the end of the 28-day nursery culture is shown in Table 2. It was observed that shrimp cultured in the four probiotic treatments had significantly higher final biomass (g), survival (%) and significantly low FCR than treatment CW (reference; $p < 0.05$). There was no significant difference in the final mean weight (g), weight gain (g), and weight gain (%) of shrimp cultured among the five treatments.

3.2 Water quality parameters

Water quality data recorded during the four-week nursery trial is presented in Table 3. There were no significant differences between dissolved oxygen (mg/L), temperature (ºC), pH, and total ammonia-nitrogen (mg/L) of the five treatments. Additionally, it was observed that the probiotic treatments had significantly higher nitrite (mg/L) compared to the clear water treatment (reference). Moreover, a peak of total ammonia-nitrogen (1.11 ± 0.08 mg/L) and nitrite (0.74 ± 0.06 mg/L) was observed in the four probiotic treatments around day 20 of the experiment (Figure 1).

3.3 Quantification of suspended solids

The amount of total suspended solids collected at the end of the experiment is shown in Table 3. Average suspended solids (± SD) in the probiotic treatments were 0.19 ± 0.003 mL/L. Although, there was no significant difference between the four probiotic treatments, it was observed that the control had significantly lower suspended solids ($p < 0.001$).

4. Discussion

Pacific white shrimp are one of the most commercially important species grown all over the world. The improvement of rearing conditions during early life stages would benefit shrimp performance as well as aquaculture profitability (Huerta-Rábago et al., 2019). In the current study, it was observed that the shrimp cultured in the four biofloc type systems had significantly higher final biomass (g) and significantly lower FCR than the reference treatment (control). Even though the final shrimp weight (g), weight gain (g) and weight gain (%) were not significantly different between the five treatments, it was noted that the survival in the clear water treatment (32 %) was

extremely low compared to the probiotic treatments (~ 90%). The reason for such very low survival in the CW treatment was not clear. However, similar results reported by Schveitzer et al., (2013), who obtained a mean survival of 93.9% versus 42.5% with or without the addition of substrates, respectively, in an *L. vannamei* BFT culture for 34 days. Reflecting on these results, the final biomass in the biofloc treatments with artificial substrates was higher than that in the control. The use of substrates in that study was to increase the surface area to allow the promotion of a biofilm community on artificial substrates in addition to the biofloc in the water. The conclusion of that study strengthening the role of the use of biofloc systems (with or without substrate) in decreasing the stress on shrimp caused by the intensification of the culture.

A study by Hussain et al., (2021) reported that shrimp cultured in a biofloc treatment had higher growth with no significant differences in survival when culturing the Pacific white shrimp in biofloc and synbiotic treatments compared to a control. The control in that study consisted of static tanks with no added carbon source. Although, these authors did not add a carbon source to the control tanks, they transitioned to biofloc systems albeit with low amount of floc (7 mL/L) as compared to the biofloc treatment (26 mL/L) due to the occurrence of nutrients from the feed and feces with no solids removal. In that study, the presence of various bacterial communities produced good survival, rapid growth, low FCR and enhanced physiological parameters of shrimp in all treatments. Clear water systems "as typically described" have an external biofilter to provide surface area and an aerobic environment for nitrifying bacteria, and they have one or more solids filters to remove most or nearly all solids from the water with no accessibility to the natural productivity (Ray et al., 2017). While the clearwater treatment in the current study acted as a better control to evaluate shrimp performance without the influence of environmental factors, it imposed a greater survival challenge because of the stocking density of shrimp and the lack of naturally occurring food organisms. Esparza-Leal et al., (2015) stated that the increase in stocking density in the traditional nursery systems (RAS) may affect the growth and survival of shrimp. In contrast to our results Ray et al., (2017) observed that individual shrimp weights, total biomass, and FCR of shrimp were all significantly better in a clear water (CW) treatment compared to a biofloc treatment. The authors stated that the exact reasons for differences in shrimp production were not clear; however, the dissimilarities in water quality may have played a role as the biofloc treatment had significantly higher nitrite.

Previous research has demonstrated the beneficial effects probiotics can provide, such as enhanced feed utilization of cultured aquatic animals through the supplementation of digestive enzymes, improved feed efficiency and higher growth, the prevention of intestinal disorders, enhanced survival rate and the pre-digestion of anti-nutritional factors present in mixed feed (Ziaei-Nejad et al., 2006; Widanarni et al., 2010; Vieira et al., 2016; Arias-Moscoso et al., 2018; Kewcharoen and Srisapoome, 2019; Ali et al., 2021). Nevertheless, the use of the two-commercial probiotic as water additive and/or supplementing the feed with a probiotic during the current study did not show any differences in growth performance, survival, or feed conversion between treatments. Although effects of commercial probiotic on aquaculture have been investigated by many researchers, some of this research has not shown any positive effects on growth parameters or survival rate or any promising result on culture conditions. For instance, Shariff et al. (2001) found that treatment of *P. monodon* with a commercial *Bacillus* probiotics did not significantly increase survival. It's difficult to directly assess different studies using probiotic, because the efficacy of a probiotic application depends on many factors (Gomez-Gil et al., 2000), such as species composition, application level, frequency of application and environmental conditions.

Water quality parameters in this study including dissolved oxygen, temperature and pH were not significantly different between treatments ($p < 0.05$). Moreover, Total Ammonia-Nitrogen and Nitrite showed a peak around day 20 of the experiment (1.11 ± 0.08 and 0.74 ± 0.06 mg/L, respectively). Interestingly, these peaks rapidly decreased by day 22 of the experiment. This is likely due to the combined effect of the continuous addition of the fermented probiotic additive to the water as well as restricted feeding during the period of these peaks. Currently, there are some techniques implemented in the culture system to avoid problems with high ammonia and nitrite concentrations. These practices include biofloc maturation prior to the start of the culture with organic fertilizers and nitrogen salts (Lara et al., 2016); reuse of older biofloc water as inoculum (Krummenauer et al., 2014); control of total suspended solids by removing organic matter, feces and uneaten feed (Gaona et al., 2011); and production control measures when culture is already underway such as reducing or completely ceasing feeding during nitrite peaks to not overload the system with more nitrogen inputs (de Lara et al., 2021). Moreover, Wang et al. (2005) investigated the effect of commercial probiotics on water quality in *L. vannamei* ponds and the results showed that probiotics could significantly reduce concentrations of nitrogen and phosphorus in pond water compared with the control.

The amount of total suspended solids is an effective parameter for evaluating the efficiency of the bacterial community to process waste products. In this study, there was no significant difference in total suspended solids (~ 0.2 g/ L) between probiotic treatments. Yet, they were significantly higher than the clear water treatment (0.04 g/ L) for which solids were removed by physical filtration. It was noted that the amount of total suspended solids in the probiotic treatments of the current study did not exceed the maximum recommended level (0.5 g/ L) for shrimp culture in a bacterial based system specified by Samocha et al., (2007). These results were in agreement with the findings of Gaona et al., (2011) who reported a total suspended solid of 0.4 g/ L by the fourth week of their experiment. In that particular trial, they had to apply clarifiers to reduce the amount of suspended solids when the concentration reached 0.5 g/ L among their experimental period. These authors stated that maintaining the concentration of suspended solids in a biofloc system at suitable levels contributed to the improvement of water quality and the growth performance of shrimp. According to Ebeling et al., (2006), one of the biggest problems affecting water quality in heterotrophic closed systems is the rapid eutrophication of tanks and ponds. This problem is the result of increased concentrations of nutrients and organic matter during culture. This will lead to elevation in the production of bacterial biomass, which will consequently increase the suspended solids in the culture medium.

5. Conclusion

The information concerning probiotic utilization in shrimp aquaculture is growing; nevertheless, it is still difficult to define optimal supplementation practices. Overall, results of this work and previous studies indicated that the use of commercial probiotic to develop and maintain biofloc based systems are a viable tool. The use of two-commercial water additive probiotics singularly or in combination with the probiotic supplemented-feed, did not show any differences in growth performance parameters among treatments. It should be noted that these results were based on laboratory conditions which may differ from commercial conditions. Although shrimp survival in the clear water treatment was significantly lower than in biofloc treatments, that's not necessarily always the case as reported in other studies. The Total Ammonia- Nitrogen and Nitrite in the four probiotic treatments showed a peak of total ammonia- and nitrite around day 20, which rapidly decreased by day 22 of the experiment. The reduction of ammonia and nitrite is attributed to the increase in the production of bacterial biomass resulting from the continuous addition of the

fermented probiotic additive to the water. Since these organisms exhibit a high capacity to absorb inorganic nitrogen and are subsequently able to control the level of ammonia in the culture systems. Therefore, our observations reinforce the necessity to test in the field several dosages of commercial probiotic additives, within the range of the recommended levels by manufacturers, to achieve functional dosages in practical settings, given the unique microbiota in the environment in different areas that culture shrimp.

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Table 1: Different management strategies evaluated during the study.

Treatment (abbreviation)	Water additives	Feed additive	# of reps
AP*	Alltech® fermented solution	-	5
AP+BF	Alltech® fermented solution	Biowish® probiotic-supplemented feed	5
BP*	Biowish® AquaBuilder	-	5
BP + BF	Biowish® AquaBuilder	Biowish® probiotic-supplemented feed	5
CW*	-	-	4

AP: Alltech® probiotic, BF: Biowish® probiotic-supplemented feed, BP: Biowish® probiotic, CW: clear water

*Treatments received commercial feed (Zeigler Bros. Inc., Gardners, PA, USA; protein ≥ 50 %, fat ≥ 15%, fiber ≤ 1%).

Table 2: Response of Pacific white shrimp postlarvae (0.014 ± 0.003 g) nursed in different culture mediums (8 g/L salinity) for 28 days. Values represent the mean of five/four replicates ± standard deviation.

Treatment	Final Biomass (g)	Final weight (g)	Weight gain (g)	Weight gain (%)	FCR	Survival (%)
Treatment AP	78.3 ± 3.5[a]	0.31 ± 0.05	0.30 ± 0.05	2095 ± 346	1.2 ± 0.1[b]	85 ± 11[a]
Treatment AP + BF	80.1 ± 1.2[a]	0.28 ± 0.01	0.27 ± 0.01	1905 ± 80	1.2 ± 0.0[b]	94 ± 3[a]
Treatment BP	74.2 ± 8.4[a]	0.27 ± 0.04	0.26 ± 0.03	1826 ± 241	1.3 ± 0.2[b]	91 ± 7[a]
Treatment BP + BF	75.3 ± 3.1[a]	0.28 ± 0.02	0.26 ± 0.02	1870 ± 147	1.2 ± 0.1[b]	90 ± 4[a]
Treatment CW	28.6 ± 4.1[b]	0.31 ± 0.09	0.30 ± 0.08	2096 ± 591	3.3 ± 0.5[a]	32 ± 10[b]
PSE	4.03	0.01	0.01	65.53	0.17	4.89
p-value	0.00	0.57	0.55	0.58	0.00	0.00

AP: Alltech® probiotic, BF: Biowish® probiotic-supplemented feed, BP: Biowish® probiotic, CW: clear water
PSE= Pooled standard error
Feed conversion ratio= feed offered/ (final weight-initial weight)
Weight gain%= ((final weight-initial weight)/initial weight) × 100

Table 3: Dissolved oxygen (DO), temperature, salinity, pH, total ammonia-nitrogen (TAN), nitrite-nitrogen, and total suspended solids (TSS) of waters (8 g/L salinity) used in a 28-day nursery trial with Pacific white shrimp post-larvae. Values represent the mean of five/ four replicates ± standard deviation (SD).

Treatment*	Dissolved oxygen (mg/L)	Temperature (ºC)	Salinity (g/L)	pH	TAN (mg/L)	Nitrite (mg/L)	TSS (g/L)
Treatment AP	6.51 ± 0.63	27.83 ± 0.88	8.05 ± 0.30 [a]	7.63 ± 0.30	0.38 ± 0.35	0.38 ± 0.19 [a]	0.20 ± 0.02 [a]
Treatment AP + BF	6.55 ± 0.60	27.73 ± 0.94	8.11 ± 0.34 [a]	7.59 ±0.34	0.37 ± 0.33	0.37 ± 0.16 [a]	0.19 ± 0.02 [a]
Treatment BP	6.48 ± 0.58	27.98 ± 0.81	8.07 ± 0.32 [a]	7.64 ±0.29	0.39 ± 0.35	0.41 ± 0.23 [a]	0.19 ± 0.02 [a]
Treatment BP + BF	6.50 ± 0.60	27.76 ± 0.80	8.05 ± 0.30 [a]	7.63 ±0.31	0.42 ± 0.40	0.45 ± 0.17 [a]	0.19 ± 0.02 [a]
Treatment CW	6.73 ± 0.43	27.98 ± 0.60	7.78 ± 0.19 [b]	7.62 ± 0.25	0.37 ± 0.50	0.09 ± 0.12 [b]	0.04 ± 0.00 [b]
PSE	0.03	0.05	0.04	0.01	0.01	0.06	0.01
p-value	0.126	0.285	< 0.001	0.955	0.516	< 0.001	< 0.001

AP: Alltech® probiotic, BF: Biowish® probiotic-supplemented feed, BP: Biowish® probiotic, CW: clear water
PSE= Pooled standard error

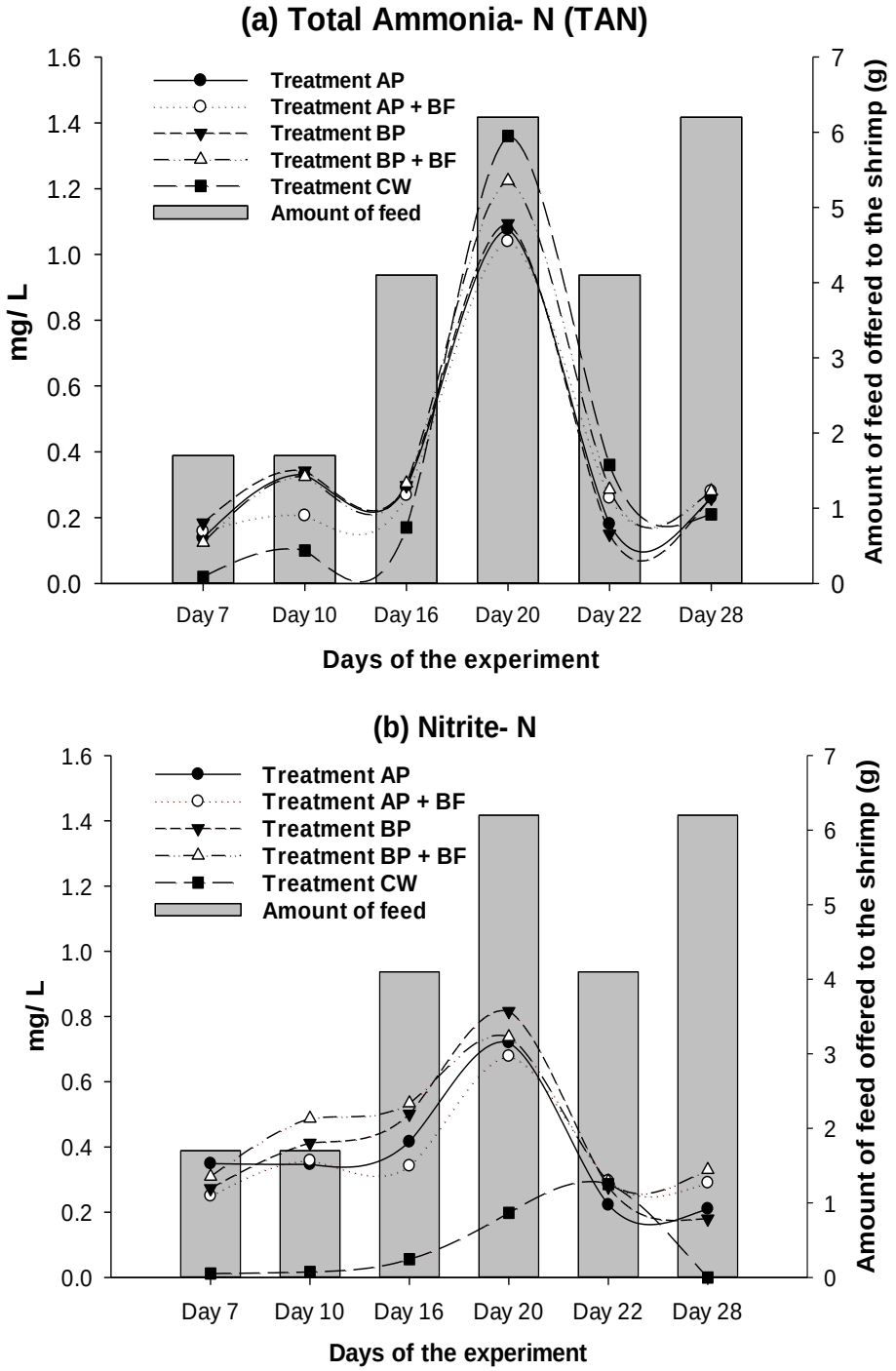

Figure 1: Fluctuation in total ammonia-nitrogen (a) and nitrite-nitrogen (b) over the rearing period of Pacific white shrimp post-larvae in each treatment (probiotic treatments n = 5, reference treatment n = 4). AP: Alltech® probiotic, BF: Biowish® probiotic-supplemented feed, BP: Biowish® probiotic, CW: clear water.

CHAPTER 3

EFFECTS OF CULTURING THE PACIFIC WHITE SHRIMP *Litopenaeus vannamei* IN "BIOFLOC" VS "SYNBIOTIC" SYSTEMS ON THE GROWTH AND IMMUNE SYSTEM

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Abstract

An 8-week indoor experiment was conducted to study the effect of culturing the Pacific white shrimp in "biofloc" and "synbiotic" type system on the growth and immune responses of the shrimp. The experimental system consisted of 24 static indoor circular polypropylene tanks (800L water volume). Each of the twenty-four tanks were stocked at a density of 125 shrimp/m^2 and eight treatments with 3 replicates were assigned using a 4 x 2 factorial design. Four primary treatments included biofloc (T1), synbiotics with β-xylanase enzyme (T2), synbiotics without enzyme (T3), and a control (T4). These were further assigned to have or not have a small, fluidized bed biofilter. The mean initial weight of the shrimp stocked in the treatments with biofilter was 0.39 ± 0.02 g, while the mean initial weight in the treatments without biofilter was 0.84 ± 0.01 g. The growth performance of the whole data set was analyzed by two-way ANCOVA, using the four treatments (T) as the first main factor, the biofilter (B) as the second main factor, and the initial weight as covariable. The results from two-way ANCOVA identified significant differences between treatments in final weights, weight gain, TGC, and FCR with shrimp reared with biofloc management performing the best. In contrast, there was no significant differences in survival (%) of the shrimp cultured between the four treatments. The levels of metabolic enzyme like Alkaline phosphatase (ALP) and Alanine aminotransferase (ALT) were significantly lower in the biofloc and synbiotic with enzyme, indicating better physiological performance in terms of improving of digestive enzymes giving a better growth index. Gamma-Glutamyl Transferase (GGT) was not significantly different. In addition, the cholesterol (CHOL) level of the hemolymph was significantly higher in shrimp produced from the biofloc and synbiotic treatments. A higher level of total haemocyte count (THC) was noted in the shrimp reared in the biofloc and synbiotic treatments as compared with control, however, there was no significant differences between

treatments. All treatments produced good survival, rapid growth, low FCR and physiological parameters indicating all are viable options. Consequently, the choice of how to manage the bacterial community should be based on the available resources.

Keywords: Biofloc, Synbiotic, probiotic, prebiotic, *Litopenaeus vannamei*, immune system.

1. Introduction

Penaeid shrimps are highly valued seafood commodity in the global markets (Tan et al., 2005). The primary cultured shrimp species worldwide is the Pacific white shrimp, *Litopenaeus vannamei*, which is considered a high-value commodity with around 4.2 million metric tons produced in 2016 (FAO, 2018). The application of intensive cultivation to increase shrimp production has also resulted in deterioration of water quality which may influences disease susceptibility (Munaeni et al., 2014).

There is great interest in closed aquaculture systems, mostly because of the biosecurity, environmental, and marketing advantages over conventional systems (Emerenciano et al., 2013). There are different biological control strategies through closed systems to improve growth and disease resistance for the cultured organisms. Some of the commonly used biological control strategies in penaeid shrimp culture are microalgal products (Ju et al., 2009), biofloc (Crab et al., 2012; Ray et al., 2010b), prebiotics (Zhang et al., 2012), probiotics (Ninawe and Selvin, 2009), and synbiotics (Munaeni et al., 2014).

Biofloc technology (BFT) in its various forms, has been gaining importance and is found to be a more efficient use of nutrient input through a closed culture system with limited water exchange. By manipulating the carbon/ nitrogen (C/N) ratio in the culture water through the addition of an external carbon source (e.g., molasses, wheat, rice bran, etc.), the microbial biomass is enhanced (Avnimelech, 1999). These aggregated bacteria, algae, and protozoa are held together in a matrix along with particulate organic matter. Growing shrimp using BFT was proposed as a tool to reduce water exchange and minimize the introduction of viral pathogens through incoming water. Hence, observations on the effects of BFT on reducing viral disease outbreaks have been reported (Avnimelech, 2015).

Additionally, there are several other microbial control strategies that are used in shrimp aquaculture. Gibson et al., (2004) defined prebiotics as “non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/ or activating the metabolism of one or a limited number of health promoting bacteria in the intestinal tract, and thus improve host health”. Several definitions are found in the literature for the term “probiotics”. The most widely quoted definition was made by Fuller (1989). He defined a probiotic as “a live microbial feed supplement which beneficially affects the host animal by improving its intestinal balance”.

Prebiotics have several advantages, but the main advantage of prebiotics over probiotics is that they are natural feed ingredients. Their incorporation in the diet does not require particular precaution and their authorization as feed additives may be more easily obtained, in spite of some concerns about their safety and efficacy (Yousefian and Amiri, 2009). There are several foods, mainly carbohydrates, used as prebiotic currently, but for a food to be classified as a probiotic, it must have certain characteristics. Gibson et al., (2004) noted that most fulfill the following criteria: resistance to gastric acidity, resistance to hydrolysis by digestive enzymes and gastrointestinal absorption, fermentation by intestinal microflora and selective stimulation of the growth and/ or the activity of intestinal bacteria associated with health.

In addition, one of the biological control strategies to improve growth and disease resistance in aquaculture organisms is synbiotic application (Huynh et al., 2018). The synbiotic concept is a variant of biofloc production which incorporates a nutritional supplement which is a combination of probiotics and prebiotics, thus it is usually done by fermenting a carbon source (prebiotic), such as rice bran, with probiotics as well as other supplements (such as enzymes) and applying it to the culture system (Munaeni et al., 2014). Dietary supplements such as probiotics, prebiotics and synbiotics provide nonspecific disease protection and also act as growth promoting factors (Das et al., 2017).

Exogenous enzymes, including protease, lipase, carbohydrate enzymes and phytase, are widely used as additives in fish feed worldwide (Zheng et al., 2020). The chemical effects of these enzymes are well understood; however, their benefits to the fish or shrimp remain controversial. It has been reported that supplementation exogenous enzymes (pentosanase, cellulose and xylanase) into diets leads to an increase in growth of Japanese sea bass (*Lateolabrax japonicus*), where more than 40% of the protein extracted from plant feed (soybean, rapeseed, peanut meal and flour), indicating that the carbohydrate enzyme is an effective factor in reducing the anti-nutritional factor and improving the performance of the cultured animals (Zheng et al., 2020). Although synbiotic concepts appeared early, the first introduction of a synbiotic was reported in *L. vannamei* in 2009 (Li et al., 2009), yet very limited studies have been conducted on the use of synbiotics with carbohydrate enzyme supplement in aquaculture systems.

Thus, this study was conducted to test the effects of culturing the Pacific white shrimp in different management styles of bacterial community derived systems including biofloc type

environments and synbiotic type systems on the growth performance, immune system of the shrimp, and water quality of the cultured water. Also, a second objective was to evaluate the effect of culturing the shrimp in a synbiotic system with and without using beta-xylanase enzyme supplement, a carbohydrate enzyme supplement.

2. Methods

2.1 Experimental setup

An eight week grow-out experiment was conducted at E.W. Shell Fisheries Center, Auburn University, AL, USA. The experimental system consisted of 24 static indoor circular polypropylene tanks (800L) with a flat bottom surface of 0.8 m. The twenty-four tanks were filled with dechlorinated water, and manufactured sea salt (Crystal Sea® Marinemix, Baltimore, MD, USA) was then dissolved into the water to raise the salinity to 4 ppt and the water was recirculated for 2 days to allow consistent mixing. Following the mixing period, the circulation was closed, and the tanks were used as a static system. Vertical fluidized bed mini-biological filter containers (4.5 L volume) filled with about 2 L of K1 Kaldnes biofilter media (AnoxKaldnes^{TM} Company, Lund, Sweden) were installed in half of the system (12 tanks) to act as a reservoir for bacteria and help in stabilize the water quality, especially in the beginning of the experiment. The other twelve tanks were used without the mini-biofilters. No water was exchanged throughout the experiment; however, dechlorinated water was added to compensate for the losses through evaporation to maintain a consistent volume.

Post-larvae Pacific white shrimp (*L. vannamei*) were obtained from Shrimp Improvement Systems (Islamorada, FL, USA) and nursed in an indoor recirculating nursery system using commercial feeds (Zeigler® Bros. Inc. Gardners, PA, USA; protein ≥ 50 %, fat ≥ 15 %, fiber ≤ 1 %) using an automatic feeder for ~2 week and then switched to 1.5-mm crumbled commercial shrimp feed (Zeigler Bros. Inc., Gardners, PA, USA; protein ≥ 40 %, fat ≥ 9 %, fiber ≤ 3%) for ~1 week. Each of the twenty-four tanks were stocked with 125 shrimp/ m^2. The mean initial weight of the shrimp stocked in the treatments with a biofilter was 0.39 ± 0.02 g, while the mean initial weight in the treatments without a biofilter was 0.84 ± 0.01 g. Four treatments of different bacterial community management were evaluated: biofloc (T1), synbiotics with enzyme (T2), synbiotics without an enzyme (T3), and a control (T4). There was no addition of a carbon source in the control treatment. Each treatment had six replicates; three without the biofilter and the other three with the

biofilter. To promote the development of the bacterial communities in all treatments, ten liters of water from a system with tilapia culture were added to each tank as an inoculum a week prior to starting the experiment. For T1, C: N ratio of the daily organic matter addition to each of the treatment tanks was approximately 15: 1 using rice bran as a carbon source (Avnimelech and Kochba, 2009).

Prime® smart pellets (Keeton Industries, Inc., Colorado, USA) were used in the synbiotic treatments as a probiotic. The pellets are composed of *Bacillus amyloliquefaciens*, *Bacillus licheniformis*, *Bacillus pumilus*, and *Bacillus subtilis* at a concentration of 3×10^9 CFU/gram. The synbiotic was initiated by sterilizing one liter of seawater from each treatment tank (T2 and T3) with a few drops of hydrogen peroxide. As to T2, one pellet of the probiotic (~3 g), 100 g rice bran (carbon source) and 0.5 ml β-xylanase enzyme (Carbohydrate enzyme) were added to the sterilized seawater sample and the components were mixed (David Kawahigashi, Vannamei 101, Talang, Phuket, Thailand, personal communication). T3 consisted of the same components except for the β-xylanase enzyme, which was not included. An air stone was installed in each of the synbiotic mix. The mixed sample was left to ferment overnight at approximately 30 ºC. After the fermentation process, each sample was filtered, and 20 ml of the filtered solution was added to each of the treatments' tanks. This fermentation process was repeated every 2-3 days and added to the tanks of T2 and T3.

During the first week, shrimp were fed a 2-mm commercial diet (40% crude protein and 9% crude lipid) produced by Zeigler®, Inc. (Gardners, PA, USA). Starting from the second week to the end of the experiment, shrimp were fed 2.4-mm Protein soy optimized feed (35 % crude protein and 8 % crude lipid) produced by Ziegler®, Inc. (Reis et al., 2020). The daily feed was calculated according to the formula of Garzade Yta et al., (2004).

$$Feed(daily) = (Number\ of\ shrimp \times Expected\ weekly\ growth\ rate \times Expected\ FCR)/7 \quad (1)$$

The feeding rates were calculated, assuming feed conversion ratio (FCR) was 1.4 and 1 g of weight gain per week. Shrimp in all treatments received equal amounts of feed throughout the experiment. The feed was offered four times (~ 0830, 1100, 1400 and 1700) per day manually.

2.2 Determination of water quality parameters

During the experimental period, dissolved oxygen (DO), temperature and salinity were measured twice daily using a YSI 650 multi-parameter instrument (YSI, Yellow Springs, OH, USA) for each individual tank. pH was tested twice weekly using a waterproof pH Testr 30 (Oakton instrument, Vernon Hills, IL, USA). Sodium bicarbonate ($NaHCO_3$) was added as needed to each of the treatment tanks to maintain the pH above 7.6 throughout the experiment. For the determination of total ammonia nitrogen (TAN) and nitrite-nitrogen, water samples were collected twice per week about 10 cm below water surface in each tank and samples were allowed to settle, hence the supernatant water was used for analysis according to the methods of Solórzano, (1969) and Spotte, (1970), respectively. Floc volume in the different treatments' tanks were determined by sampling 1 L water into a series of Imhoff cones every 10 days. The volume of the floc plug accumulated on the bottom of the cone for ~20 min was read and noted as ml L^{-1} (Avnimelech and Kochba, 2009).

2.3 Growth performance

Final weight and survival were determined at the end of the eight-week experiment after the tank had been drained. The growth performance was assessed in terms of percentage weight gain, feed conversion ratio (FCR), Thermal Growth Coefficient (TGC) and survival (Kumar et al., 2018) using the following formulae:

$$Weight\ gain\ (\%) = 100 \times (final\ weight - initial\ weight)/initial\ weight$$

$$Feed\ conversion\ ratio\ (FCR) = Feed\ given\ (as\ is)/\ weight\ gain\ (wetweight)$$

$$TGC = \left(\frac{FBW^{\frac{1}{3}} - IBW^{\frac{1}{3}}}{\Sigma(Temp \times days)}\right) \times 100$$

The percentage survival rate was estimated at the end of the experiment as:

$$Survival\ rate\ (\%) = 100 \times \frac{Total\ number\ of\ shrimp\ harvested}{Total\ number\ of\ shrimp\ stocked}$$

2.4 Proximate composition of shrimp body

On the day of harvest, twenty shrimp from each tank were randomly sampled for proximate composition analysis of the whole body. Proximate composition analysis of crude protein, crude lipid, and ash contents of the shrimp samples were performed by the standard methods of AOAC (1995). Moisture of the shrimp sample was determined by oven drying at 90 °C for 24 h.

2.5 Hemolymph Enzymes and Total Hemocyte count

Samples of hemolymph were obtained from shrimp collected at the end of the trial. Hemolymph was withdrawn from pericardial cavity of the shrimp using a 25-gauge needle and 1-cc syringe inserted beneath the carapace at the cephalothorax-abdominal junction (Roy et al., 2009b). The 1 ml syringe was preloaded with 0.3 ml of an anti-coagulant and used to collect approximately 0.3 ml of hemolymph. To maintain semi-quantitative data, the weight of anticoagulant and hemolymph were determined gravimetrically. The anticoagulant solution (Liu et al., 2004) contains 30 mM Sodium Citrate Tribasic Dihydrate (Sigma S4641); 0.34 M Sodium Chloride (NaCl); 10 mM EDTA – Ethylene Diamine Tetra acetic Acid (Sigma, E9884); in de-ionized (DI) water. Hemolymph samples were withdrawn from all shrimp in the experiment (one composite sample obtained per tank), centrifuged for 20 min at 8000 RPM and stored at -80 ºC. Concentrations of the following haemolymph constituents were measured: Alkaline phosphatase (ALP), Alanine Aminotransferase (ALT), Gamma-Glutamyl Transferase (GGT), and cholesterol (CHOL) using Abaxis, VetScan® VS2 analyzer (Union City, CA). For each sample, 100 µL of haemolymph was used to determine the previous parameters using a Comprehensive Diagnostic Rotor.

The measurement of total haemocyte count (THC) was performed for two shrimp from each replicate tank according to Liu and Chen, (2004). Briefly, 0.2 mL of haemolymph sample was taken with a 1 mL syringe containing 0.2 mL of precooled anticoagulant solution (30 mM trisodium citrate, 0.34 M sodium chloride, 10 mM EDTA, 0.12 M glucose, pH 7.55). The number of haemocytes was determined in the Haemolymph samples using a haemocytometer under a light microscope.

2.6 Statistical analysis

The results were expressed as the mean ± standard error (SE). The statistical analyses were performed using R software for windows (version 4.1.1; R Core team, 2021). Data were analyzed by One-way ANOVA for each of the shrimp growth performance data of the three replicates: with and without the biofilters for the four treatments. The whole set of data composed of the six replicates per treatment was analyzed by a two-way ANCOVA, using the four treatments (T) as the first main factor, the biofilter (B) as the second main factor, and the initial weight as a covariate. Water quality, proximate composition, and immune parameters of the shrimp was analyzed using Two-way ANOVA, using the treatments (T) as one independent variable and biofilter (B) as the

second independent variable. Significant differences were considered at $P<0.05$. When significant differences were found, Tukey's test was used to identify differences among experimental treatments.

3. Results

3.1 Water quality parameters

The results of water temperature, salinity, DO, and pH are presented in Table 1. A greater amount of $NaHCO_3$ was required to maintain pH with the increase in C/N ratios in T1. There was no significant difference in the previously mentioned water quality parameters between treatments. As it would be excepted that Total Ammonia-Nitrogen and Nitrite concentrations behaved differently due to the presence or absence of the fluidized biofilters, so they were plotted in separate figures for each of the replicates with and without the biofilter. Figure 1 shows the Total Ammonia-Nitrogen and Nitrite in the four treatments with presence/absence of biofilters. Total Ammonia- Nitrogen and Nitrite ranged from 0.03 to 0.6 mg/L and 0.02 to 0.4 mg/L, respectively. Total Ammonia-Nitrogen and Nitrite in the four treatments without the biofilters is shown in Figure 2. In contrast, a peak in Total Ammonia- Nitrogen from the beginning of the experiment reached 2.5 mg/L followed by a peak in Nitrite that reached 2 mg/L. The mean TAN and Nitrite is shown in Table 1, with a significant difference between the treatments with and without biofilter.

The average volume of floc that settled in the Imhoff cones from the three replicate tanks as a function of culture time is shown in Figure 3 (a: without biofilters and b: with biofilters). The initial development of the floc was slow in all treatments then it grew rapidly after 10 days of culture in the replicates without the biofilters (Figure 3; a). Floc grown in the replicates without the biofilters was significantly higher after 10 days than the ones grown with the presence of the biofilters ($P < 0.05$). The floc development in the control treatment was not significantly different with or without the biofilters ($P = 0.63$).

3.2 Growth performance

Growth performance of the Pacific white shrimp throughout the 8-week experimental period with and without the presence of the biofilters for each of the four treatments are presented in Table 2. One-way ANOVA for the growth performance of the shrimp in the replicates with biofilters showed that final weights of the shrimp in the biofloc treatment (T1) were significantly

higher ($P < 0.05$) from the control (T4). The same trend was observed in the shrimp weight gain, weekly gain, and TGC. No Significant difference was observed in biomass, percentage weight gain, survival and FCR between treatments.

Results of one-way ANOVA for the growth performance of the shrimp in the replicates without the biofilter showed that biomass weight of the shrimp cultured in the biofloc (T1) and synbiotics with enzyme (T2) treatments were significantly higher from the synbiotics without enzyme (T3) and the control (T4). In contrast, FCR for the biofloc (T1) and synbiotic with enzyme (T2) were significantly lower from the other treatments. There was no significant difference in weight gain, survival and TGC between treatments.

Two-way ANCOVA showed that most of the growth parameter values were significantly higher in the biofloc treatment (T1) followed by synbiotic with enzyme (T2), synbiotic without enzyme (T3) and the control (T4). Additionally, a significantly lower FCR in the biofloc treatment (T1) was observed. In contrast, there was no significant differences between survival of the shrimp cultured in the four treatments.

3.3 Proximate composition of shrimp body

Proximate composition of the shrimp whole body is shown in Table 3. No significant differences ($P > 0.05$) were found in the protein, fat content and ash between the treatments, the presence or absence of the biofilters, nor interaction between them.

3.4 Hemolymph Enzymes and Total Hemocyte count

ALP, ALT and Cholesterol were significantly different between treatments (Table 4). The biofloc and synbiotic with enzymes treatments had significantly lower values of ALT and ALP than the control. However, there were no significant difference in GGT between treatments. Culturing the Pacific white shrimp in biofloc type environment did numerically increase the Total Haemocyte Count (THC), yet there was no significant difference between treatments (Table 4). The replicates without biofilters showed higher Total Haemocyte Counts in each treatment than the ones with biofilters, although there were no significant differences between the replicates with and without biofilters.

4. Discussion

The use of probiotics and prebiotics has been gaining attention during recent years as an alternative viable therapy in fish or shrimp culture. They are appearing to be a promising biological control strategy and becoming an integral part of the aquaculture practices for improving growth and disease resistance, and improving the water quality for the cultured organisms (Cerezuela et al., 2011).

In the present study, water quality parameters including dissolved oxygen (DO), temperature and pH were maintained in the favorable conditions for culturing the Pacific white shrimp (Fast and Lester, 1992). As would be expected, a greater amount of sodium bicarbonate ($NaHCO_3$) was required to maintain pH with the increase in C/N ratios in T1 (the biofloc treatment) due to the greater development of the microbial communities through the carbon source addition, which will consequently decrease the pH. It is important to take into account that all the cultured tanks started with clear water, which was inoculated from another system, hence, the bacterial communities in both synbiotic and biofloc systems took about 10 days or more during the experimental period to fully develop. Using the mini-biofilters in three replicates of each treatment promoted the uptake of ammonia nitrogen, which stabilized the water quality particularly in the early startup phase. It was clear that the other three replicates without the biofilters experienced high ammonia (above 2 mg/L) followed by a peak of nitrite (about 1.5 mg/L; Figure 1). It is also interesting to note that the level of floc as measured by settled solids was lower in the systems with the mini-fluidized beds which appeared to fractionate the biofloc. The biofilters served as a bacterial reserve, which seems to be helpful during the startup process or alternatively higher levels of an inoculate may be required to jump start systems with biofilters.

Although synbiotic concepts appeared early, the first introduction of a synbiotic was reported by Li et al., (2009). These authors added the synbiotic to the diet of Pacific white shrimp *L. vannamei*, which was composed of probiotic bacterium *Bacillus OJ* and Isomaltooligosaccharides (IMO-900P) that were made from high quality corn starch (as prebiotic). Cerezuela et al., (2011) stated that the synbiotic positively affects the host by improving the survival and inserting live microbial dietary supplements in the digestive tract by selectively stimulating the growth and/or by triggering the metabolism of one or a limited number of health-promoting bacteria, hence promoting host “welfare”. On the other hand, in a biofloc system; increasing the input C/N ratio

by adding carbohydrates can be a practical way to promote ammonia nitrogen uptake by heterotrophic bacteria in BFT systems as well as enhancing the growth of the cultured species (Avnimelech, 2015, 1999; Crab et al., 2007).

In the present study, the two- way ANCOVA results showed that there was significantly higher final weight of the shrimp, weight gain (g), final biomass (g) and TGC in the biofloc treatment (T1) followed by the synbiotics with enzyme (T2), and the synbiotics without enzymes (T4), than the control (T4). Similarly, FCR was significantly lower in shrimp reared in T1 and T2 as compared to T3 and T4. This indicates that the diverse microbial community of bioflocs not only provides supplemental nutrition, but also acts as nutrient recyclers. These findings are in agreement with the data obtained by many researchers (Dimitroglou et al., 2011; Merrifield et al., 2010; Nayak, 2010; Zhang et al., 2012) who found that the use of prebiotics (carbon source) and probiotics improved the feed conversion, growth rates, weight gain, immune system and disease resistance of shrimp. Although there was a lower amount of floc volume in the replicates with the biofilters compared to the replicates without biofilters (Fig. 3), there were no significant differences for the biofilter and a significant difference for the initial weight as a covariable in the different growth performance parameters. This may be because the effect of initial weight and biofilter are correlated and they are overshadowing each other.

Shrimp in replicates without biofilters of each treatment were more stressed than the replicates with biofilter by experiencing higher Total Ammonia-Nitrogen and Nitrite (Fig. 1 and 2), which could be a reason for the lower survival. These findings are supported by Liang et al., (2016) who stated that ammonia, as a major toxicant in aquatic systems, could cause the growth inhibition and immune depression of shrimp and increase their susceptibility to WSSV. Additionally, Gross et al., (2004) suggested that long-term exposure to nitrite concentrations of more than 1 mg/ L $N0_2$-N might have negative effects on *L. vannamei* growth and survival when grown in low salinity water.

Pacific white shrimp are well known for their ability to thrive in low salinity seawater, which makes them an especially good species for inland aquaculture (Pan et al., 2007). The reduction of salinity in the water used within recirculating aquaculture systems (RAS) or biofloc systems can lead to the production of shrimp at lower costs because of the need for less salt and less management of wastewater. However, the reduction of salt can lead to problems for intensely

grown shrimp, including a decrease in the resiliency of the shrimp to water quality concerns such as high ammonia and nitrite levels (Chen and Lin, 1992; Lin and Chen, 2003).

With respect to immune parameters, ALP is one of the enzymes responsible for biochemical reactions in the metabolism of amino acids with other metabolic intermediates (Abdollahi-Arpanahi et al., 2018). ALT in liver, kidney, heart, and muscle tissue catalyzes the transamination reaction. The enzyme of ALP in the cells ducts of the liver can be found on the mucosal epithelium of the small intestine, kidneys, bone and liver tissues. It plays an important role in lipid transposition in the small intestines and calcification of bones in vertebrates. Hepatopancreatic disorders cause an elevation in these enzymes. For, example a rise in ALT is associated with reduced insulin response, reduced glucose tolerance, and increased free fatty acids and triglycerides (Abdollahi-Arpanahi et al., 2018).

In studies by Anand et al., (2015) and Anand et al., (2017), the application of biofloc and periphyton reduced levels of AST and ALT, and enhanced growth and immune responses in *P. monodon* compared to a control group. Therefore, measuring of these enzymes is an indicator to assess the hepatopancreas functions (Van de Braak et al., 2002). In this study, a lower level of ALP and ALT enzymes was measured in the shrimp raised in biofloc and synbiotic with enzymes than the control, which indicates a better function of hepatopancreas in these groups compared to other treatments. This could be in part due to a better physiological performance in terms of improving of digestive enzymes giving a better growth index as we observed better growth in the biofloc and synbiotic with enzyme treatments than the control.

In the present study, the physiological condition of shrimp after the trial (as indicated by the hemolymph parameters), showed differences between treatments in cholesterol, suggesting that the culturing of the shrimp in biofloc and synbiotic systems may enhance the physiological status of shrimp, hence it's a good health indicator for the shrimp (Hamidoghli et al., 2018). These results are in agreement with the study by Martinez-Porchas et al., (2020) who reported that there was no change in the hemolymph physiological parameters except for a small increase in cholesterol when using the biofloc as complementary food for the pacific white shrimp.

The culture of the white shrimp in the biofloc and the synbiotics did numerically increased the number of the total hemocyte count (THC), yet there were no significant differences between all treatments neither with or without the biofilters. Xu and Pan, (2013) reported that the total

haemocyte count and phagocytic activity of the haemocyte of the shrimp from biofloc groups were significantly higher than those of the shrimp in the non-biofloc control group indicating a good health index.

Furthermore, the authors also noted that there were no significant differences in the protein, fat content, and ash in the proximate composition of the whole body of the shrimp. These results are in accordance with the results reported by Hussain et al., (2015) who stated that the quality of flesh was not affected by culturing the green tiger shrimp *Penaeus semisulcatus* in a biofloc system and a control treatment.

5. Conclusion

Comparing the efficacy of various bacterial based culture techniques have has numerous confounding factors as responses can be system and management specific. Each culture technique has advantages and disadvantages to each style of system. Clearly the use of a biofilter as a bacterial reserve did reduce TAN and Nitrite level during the initial startup but otherwise was probably not required. The biofloc treatment did produce significantly better results than the control but was not different from the other treatments. Each of the tested management variables requires a different amount of work to prepare the inoculants. All treatments produced good survival, rapid growth, low FCR and enhanced physiological parameters. Hence, all are viable options, albeit requiring different inputs.

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Table 1: Water quality from individual culture systems in which each tank was stocked juvenile shrimp at a stocking density of 125 shrimp per m^2. Four treatments (T) with three replicates were run with (1) and without (0) mini fluidized bed biological filters (B). Values represent means (± SD) between the three replicates of each treatment.

Treatment	Biofilter	DO[a] (mg/L)	Temperature (ºC)	Salinity (ppt)	pH	TAN (mg/L)	Nitrite (mg/L)	Floc volume (mL/L)
T1-Biofloc	1	6.83 ± 0.13	28.09 ± 0.18	4.59 ± 0.33	7.57 ± 0.21	0.18 ± 0.15	0.23 ± 0.10	12 ± 10
T2-Synbiotic with Enzyme	1	6.86 ± 0.13	27.90 ± 0.18	4.55 ± 0.30	7.60 ± 0.20	0.16 ± 0.12	0.25 ± 0.09	12 ± 10
T3-Synbiotic without Enzyme	1	6.80 ± 0.12	28.45 ± 0.06	4.59 ± 0.30	7.68 ± 0.20	0.21 ± 0.17	0.24 ± 0.11	10 ± 9
T4-Control	1	6.83 ± 0.11	28.14 ± 0.14	4.56 ± 0.31	7.61 ± 0.20	0.20 ± 0.16	0.23 ± 0.10	6 ± 5
T1-Biofloc	0	6.88 ± 0.19	28.73 ± 0.30	4.38 ± 0.00	7.63 ± 0.21	0.59 ± 0.66	0.75 ± 0.62	26 ± 20
T2-Synbiotic with Enzyme	0	6.90 ± 0.16	28.12 ± 0.33	4.42 ± 0.04	7.54 ± 0.21	0.72 ± 0.82	0.63 ± 0.52	24 ± 20
T3-Synbiotic without Enzyme	0	6.86 ± 0.13	28.93 ± 0.31	4.44 ± 0.03	7.60 ± 0.21	0.62 ± 0.76	0.73 ± 0.59	24 ± 19
T4-Control	0	6.98 ± 0.20	28.40 ± 0.37	4.21 ± 0.00	7.32 ± 0.22	0.62 ± 0.69	0.67 ± 0.61	7 ± 6
Two-way ANOVA P-value								
Treatment (T)		0.740	0.748	0.634	0.945	0.773	0.632	0.051
Biofilter (B)		0.258	0.394	0.543	0.797	<0.001	<0.001	0.030
Interaction T × B		0.548	0.558	0.262	0.598	0.730	0.677	0.257
PSE		0.02	0.14	0.05	0.03	0.10	0.08	2.13

* DO: dissolved oxygen, TAN: Total Ammonia- Nitrogen, SD: standard deviation, PSE: pooled standard errors

Table 2: Growth performance of the Pacific white shrimp cultured over an 8-week culture period in biofloc and synbiotic type systems. Each tank was stocked with juvenile shrimp at a stocking density of 125 shrimp per m^2. Four treatments (T) with three replicates were run with (1) and without (0) mini fluidized bed biological filters (B).

Treatment	Biofilter	Initial wt (g)	Final wt (g)	Survival (%)	Weight gain (g)	WG (%)	Biomass (g)	Weekly gain (g)	TGC	FCR
T1-Biofloc	1	0.39	9.36[a]	90.7	8.97[a]	2306.9	847.8	1.12 [a]	0.089 [a]	1.09
T2-Synbiotic with Enzyme	1	0.41	8.50[ab]	94.7	8.09[ab]	1996.7	804.2	1.01[ab]	0.084 [b]	1.16
T3-Synbiotic without Enzyme	1	0.39	8.43[ab]	95.7	8.04[ab]	2056.9	806.4	1.00 [ab]	0.084 [b]	1.15
T4-Control	1	0.38	8.10[b]	95.7	7.72[b]	2022.7	774.0	0.96 [b]	0.083 [b]	1.20
One-way ANOVA P-value		0.349	0.027	0.310	0.028	0.092	0.174	0.035	0.043	0.196
T1-Biofloc	0	0.83	11.70	89.0	10.87	1304.9	1041.2[a]	1.36	0.086	1.21 [b]
T2-Synbiotic with Enzyme	0	0.84	11.03	91.0	10.19	1213.8	1000.6[a]	1.27	0.083	1.27 [b]
T3-Synbiotic without Enzyme	0	0.84	9.94	86.5	9.11	1086.6	858.4[b]	1.14	0.078	1.50 [a]
T4-Control	0	0.84	11.42	79.7	10.58	1255.9	903.5[b]	1.32	0.085	1.42 [a]
One-way ANOVA P-value		0.890	0.230	0.235	0.225	0.171	0.002	0.239	0.235	0.002
Two-way ANCOVA P-value										
Treatment (T)		0.045	0.045	0.358	0.044	0.037	<0.001	0.045	0.017	0.005
Biofilter (B)		0.623	0.623	0.247	0.615	0.916	0.206	0.652	0.689	0.764
Initial weight		<0.001	<0.001	0.003	<0.001	<0.001	<0.001	<0.001	0.528	<0.001
Interaction (T × B)		0.249	0.249	0.197	0.250	0.232	0.021	0.288	0.636	0.015
PSE		0.05	4.76	1.40	0.27	97.40	20.50	0.03	0.001	0.03

*TGC: Thermal Growth Coefficient, FCR: Feed Conversion Ratio, PSE: pooled standard errors

Table 3: Proximate composition (% wet weight basis) of the whole body of *Litopenaeus vannamei* cultured over an 8-week culture period in biofloc and synbiotic type systems. Each tank was stocked at a stocking density of 125 shrimp per m^2. Four treatments (T) with three replicates were run with (1) and without (0) mini fluidized bed biological filters (B).

	Biofilter	Moisture	Crude protein	Crude fat	Ash
T1-Biofloc	1	4.10	70.90	7.53	11.00
T2-Synbiotic with Enzyme	1	4.05	71.25	8.22	10.80
T3-Synbiotic without Enzyme	1	4.79	70.53	7.64	11.17
T4-Control	1	3.78	71.43	7.88	10.80
T1-Biofloc	0	4.10	70.90	7.53	11.00
T2-Synbiotic with Enzyme	0	4.05	71.25	8.22	10.80
T3-Synbiotic without Enzyme	0	4.79	70.53	7.64	11.17
T4-Control	0	3.78	71.43	7.88	10.80
Two-way ANOVA P-value					
Treatment (T)		0.746	0.072	0.838	0.197
Biofilter (B)		0.906	0.142	0.859	0.352
Interaction T × B		0.892	0.207	0.695	0.395
PSE		0.23	3.11	0.35	0.48

*PSE: pooled standard errors

Table 4: Hemolymph enzyme constituents and Total Hemocyte Count (THC) of *Litopenaeus vannamei* in the four treatments with and without biofilters at the end of experiment. Four treatments (T) with three replicates were run with (1) and without (0) mini fluidized bed biological filters (B).

	Biofilter	ALP (U/L)	ALT (U/L)	GGT (U/L)	CHOL (mg/L)	THC ($\times 10^7$)
T1-Biofloc	1	127.13	194.63	4.37	19.27	1.38
T2-Synbiotic with Enzyme	1	187.23	187.20	5.47	14.27	1.39
T3-Synbiotic without Enzyme	1	148.63	307.00	4.73	10.67	1.33
T4-Control	1	430.97	428.20	4.17	10.10	1.00
T1-Biofloc	0	102.93	265.20	6.73	19.07	2.28
T2-Synbiotic with Enzyme	0	136.80	325.73	6.37	16.37	2.07
T3-Synbiotic without Enzyme	0	138.45	367.40	7.70	9.90	1.65
T4-Control	0	191.17	410.90	7.20	12.90	1.45
Two-way ANOVA P-value						
Treatment (T)		0.008	0.034	0.451	0.015	0.363
Biofilter (B)		0.047	0.185	0.112	0.597	0.050
Interaction T × B		0.143	0.671	0.620	0.879	0.957
PSE		26.15	26.11	1.51	1.06	1.64×10^6

*ALP: Alkaline phosphatase, ALT: Alanine Aminotransferase, GGT: Gamma-Glutamyl Transferase, and CHOL: cholesterol, U/L: units per liter, PSE: pooled standard errors

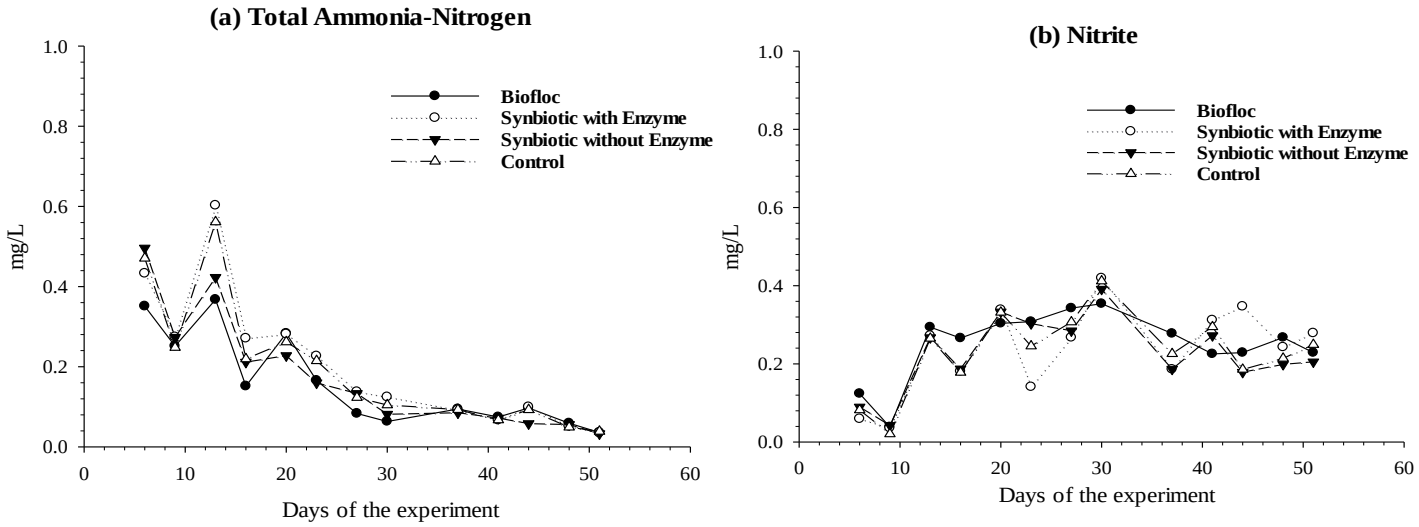

Figure 1: (a) Total Ammonia-Nitrogen and (b) Nitrite results for the four treatments throughout the experimental period. Values represent the means of the three replicates with biofilters for each of the four treatments.

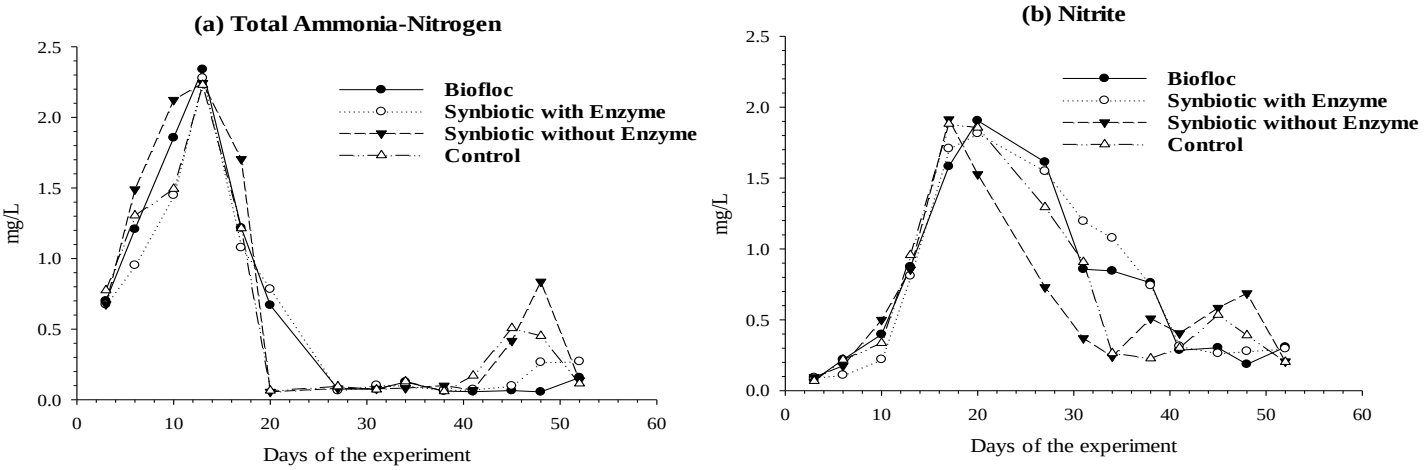

Figure 2: (a) Total Ammonia-Nitrogen and (b) Nitrite results for the four treatments throughout the experimental period. Values represent the means of three replicates without biofilters for each of the four treatments.

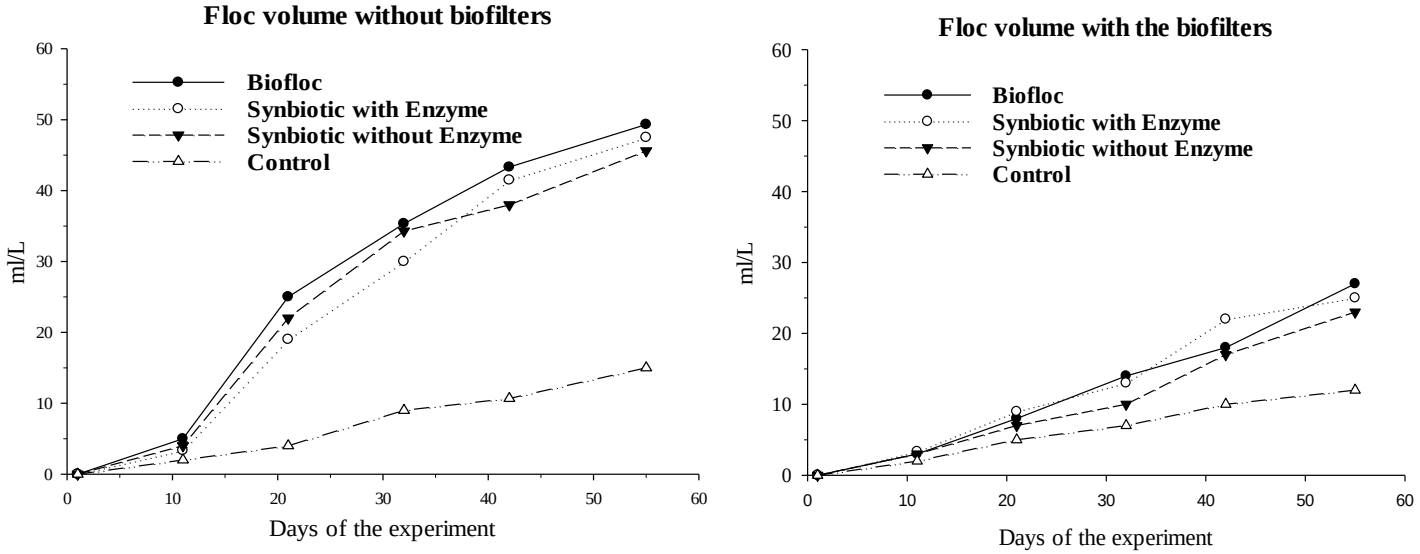

Figure 3: Dynamic changes of floc volume (settled solids in 1 L Imhoff cone) in different treatments throughout the experimental period. Values are means of the three replicates; (a) without biofilters and (b) with biofilters.

CHAPTER 4

EVALUATION OF THE GROWTH PERFORMANCE OF THE PACIFIC WHITE SHRIMP *Litopenaeus vannamei* FED WITH FOUR DIFFERENT PROTEIN-BASED DIETS IN CLEAR WATER AND BIOFLOC SYSTEM

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Abstract

Shrimp research has been focused on the development of feeds with minimal levels of fish meal, as well as using alternative, lower cost protein sources. The study objective was to evaluate the performance of the Pacific white shrimp *Litopenaeus vannamei* fed with four different protein-based extruded diets [plant-based (AP), 8% poultry by-product meal (PM8), 8% fishmeal (FM8) and 12% fishmeal (FM12)] while cultured in clear water and biofloc type systems. Additionally, the pellet durability index (PDI) and hardness of these diets was determined. Results from the clear water experiment showed that the shrimp fed with PM diet had the lowest final individual weight, biomass (g), and weight gain (g), and the highest feed conversion ratio (FCR). It was observed that the shrimp fed with AP, FM8 and FM12 diets had significantly higher weight gain than shrimp offered the PM8 diet. Results from the biofloc experiment showed that shrimp fed with AP diet had the lowest biomass (g), weight gain (g), and thermal growth coefficient and the highest FCR. No significant differences in survival rate were observed between the four diets in both experiments. The extruded diets showed high PDI when measured using the tumbling box and the Holmen tester. Additionally, the pellet hardness showed no significant differences among the four diets. The low inclusion of fishmeal, as well as the use of alternative protein sources in these diets, did not adversely affect the final weight, weight gain, and percent weight gain of *L. vannamei.*

Keywords: extruded feed, protein source, plant based-diet, pellet durability index, poultry by-product, penaeid shrimp

1. Introduction

Pacific white shrimp (*Litopenaeus vannamei*) farming is one of the major aquaculture activities in many countries contributing 53% of the global shrimp production in 2016 (FAO, 2018). In semi-intensive and intensive shrimp farming, artificial feed is the main nutrient source, which must be both nutritionally and economically adequate for the culture system. Feed represents up to 60% of total production costs (Rego et al., 2017), therefore, the quality of the feed is important in shrimp aquaculture. Protein is the most critical ingredient in shrimp diets from standpoint of cost and growth response (Sánchez-Muros et al., 2020).

Due to desirable characteristics such as high quality protein, amino acid profile, palatability, lipid content, high digestibility, and nutrient content, fishmeal has been the most important protein source in shrimp feeds (Tacon & Metian, 2008). The amount of fishmeal in commercial shrimp feeds usually varied between 25 to 50%, depending on the species and culture phase (Lim & Dominy, 1990; Richard et al., 2011). Although the fishmeal supply is limited in the word market (Hardy, 2010), its increasing demand has resulted in high prices for the commodity. The reduction of fishmeal in feed formulations is considered one of the most important requirements for continued growth of the aquaculture industry (Koshio et al., 2013).

In order to relieve the pressure on fish meal in the steadily increasing aquaculture sector, alternative feed ingredients from plant, microbial and other animal sources have been a prioritized field of research for many years (Sudaryono et al., 1995; Samocha et al., 2004; Hardy, 2010; Sørensen, 2012; Sookying et al., 2013; Sánchez-Muros et al., 2020). Ingredients obtained from terrestrial animals such as poultry meal, poultry by-product meal, and meat and bone meal have been considered suitable protein sources for shrimp feeds (Amaya et al., 2007). Additionally, soybean products have also been used in shrimp feeds as alternatives for fishmeal due to their balanced nutritional composition, high digestibility, acceptable price and widely available supply (Amaya et al., 2007; Sookying et al., 2013; Galkanda-Arachchige & Davis, 2020). Even though soybean products have been used over the years in many experimental diets for shrimp or fish, yet the farmers still have concerns of using a plant-based diets to feed their animals. The efficiency of these alternative products in shrimp diets may also be affected by the culture method such as the biofloc technology system for which natural foods are present. Some management approaches in biofloc systems have focused on reducing protein content in feeds (Wasielesky et al., 2006; Xu et

al., 2012) as well as incorporating alternative ingredients (e.g. floc meal, plant-based; Ray et al., 2010; Bauer et al., 2012; Dantas et al., 2016).

Most studies evaluate the value of new ingredients in terms of nutritional quality with the main focus on digestibility, growth performance, health and feed intake. However, little attention has been paid on the effects of ingredients on physical quality of fish or shrimp diets. Ingredient composition affects directly not only the physical quality of steam pelleted feeds (Behnke, 1996), but also extruded feeds (Sørensen et al., 2009; Sørensen, 2012; Soares et al., 2021a). Extrusion processing has become the primary technique used for shrimp feed production, mainly because of the high physical and nutritional quality of the feed (Soares et al., 2021a).

It is well known that aquaculture feeds must resist to different mechanical damages related to transportation, handling and automatic feeders (Poveda & Ortega, 2021). Physical quality of feed varies with ingredient composition and processing condition and may interfere with feed intake, nutrient digestibility and therefore growth performance of the cultured organism (Sørensen, 2012). Tests to evaluate pellet durability are performed to simulate forces such as those applied when filling bins, transportation and feeding activities at the farm (Sørensen et al., 2009). Pellets with high durability form fewer small particles and fines during bagging and storage and finally, show low degradation in pneumatic feeding devices when fed to the shrimp (Sørensen, 2012). Several methods exist to determine the physical quality in terms of durability of the pellets including the tumbling box and Holmen durability tester. The tumbling box method is an accepted standard in the feed industry in North America (ASAE, 1997) and is used to simulate formation of fines during mechanical handling. Holmen durability tester has been developed to test effects of impact and shear forces during pneumatic conveying (Sørensen, 2012). Hardness or strength at rupture, defined as the maximum force needed to crush a pellet, is commonly determined using a texture analyzer. The pellet breaking resistance to external pressure can be related to the stress-force applied during storage (e.g. bins or silos) and crushing (e.g. screw conveyor or animal teeth) of the pellets (Sørensen, 2012).

Therefore, the objective of this study was to evaluate the growth performance of the Pacific white shrimp *Litopenaeus vannamei* fed with four different protein-based diets including soybean based, poultry-by product meal, 8% fishmeal and 12% fishmeal while cultured in clear water and

biofloc system. Additionally, the pellet durability index (PDI) and hardness of these extruded diets was determined.

2. Methods

2.1 Experimental setup

Four different protein-based practical diets manufactured by Ziegler®, Inc. (Gardners, PA, USA) were used in two 8-week growth trials. The four protein sources of the diets evaluated including: Plant-based (AP), 8% poultry by-product meal (PM8), 8% fishmeal (FM8) and 12% fishmeal (FM12) diets. The formulation and proximate composition of each of the diets are shown in Table 1. Additionally, Amino acid profile for the four diets is presented in Table 2. The trials were conducted indoors at E.W. Shell Fisheries Center, Auburn University, AL, USA. Post-larvae of the Pacific white shrimp were obtained from American Penaeid (St. James City, FL, USA) and nursed in an indoor biofloc nursery system and fed four times per day with commercial feeds (Zeigler® Bros. Inc. Gardners, PA, USA; protein ≥ 50 %, fat ≥ 15 %, fiber ≤ 1 %; ranging from 400 to 1200 microns) for ~3 week and, then 1.5-mm crumbled commercial shrimp feed (Zeigler Bros. Inc., Gardners, PA, USA; protein ≥ 40 %, fat ≥ 9 %, fiber ≤ 3%) for ~1-2 week until they reached an appropriate size for research.

The first experiment was conducted in a clear water recirculation system which consisted of 20 glass aquaria (50 x 50 x 50 cm) filled with 70 L of water with constant aeration. Water quality was maintained by recirculation through an Aquadyne bead filter (0.2 m^2 media, 0.6 m × 1.1 m) and vertical fluidized bed biological filter (600-L volume with 200-L of Kaldnes media) using a 0.25-hp. centrifugal pump. The second experimental system consisted of sixteen 400-L square polyethylene indoor tanks recirculating with a sump (~ 800L) as a common biofloc system. Dissolved oxygen was maintained near saturation using air stones (culture tanks and sump tank) connected to a regenerative blower. To promote the development of the natural productivity in the biofloc system, tilapia culture water was added as an inoculum a week prior to the start of each experiment. Molasses was used as a carbon source to maintain C: N ratio at 15:1 (Avnimelech, 1999). Although, molasses has been added daily in the beginning of the experiment, yet no more addition of molasses once the water quality was stabilized. There was no water exchange throughout the biofloc experiment and dechlorinated water was added to compensate evaporation losses. In the clear water system, each tank was stocked with 15 shrimp (0.10 ± 0.01g), however

100 shrimp (0.23 ± 0.01g) was stocked in each tank of the biofloc system. Five replicates were assigned for each of the four diets in the clear water system, while in the biofloc system there were four replicates.

During the first week in both trials, the four diets were grinded to about 2-mm pellets to be suitable for the shrimp feeding. Starting from the second week to the end of the experiment, shrimp were fed the 2.4-mm diets. The daily feed in both experiments was calculated according to the formula of Garza de Yta et al., (2004). The feeding rate was estimated, by assuming 1 g of weight gain per week, and feed conversion ratio (FCR) of 1.8 and 1.4, for the clear water and biofloc system, respectively. At the end of both growth trials, shrimp were counted, and the biomass weight was recorded. Performance of the cultured shrimp was calculated in terms of the mean final weight, weight gain (g), weight gain (%), feed conversion ratio (FCR), Thermal Growth Coefficient (TGC) and survival rate (%).

2.2 Water quality measurements

Since both systems were recirculating, hence, one reading of water quality was measured from the sump of each system. Dissolved oxygen (DO), temperature and salinity were measured twice daily using a YSI 650 multi-parameter instrument (YSI, Yellow Springs, OH, USA). pH was checked twice weekly using a waterproof pH Testr 30 (Oakton instrument, Vernon Hills, IL, USA). Sodium bicarbonate was added as needed to maintain the pH above 7.6 throughout both experiments. For the determination of total ammonia nitrogen (TAN) and nitrite, water samples were collected twice per week and analyzed using a YSI photometer 9500 kit (YSI, Yellow Springs, OH, USA). For the biofloc system, the sample was collected about 10 cm below water surface and the sample was allowed to settle, hence the supernatant water was used for analysis.

2.3 Physical properties of the diets

The tumbling box and Holmen tester were used to determine the pellet durability index (PDI). For the tumbling box method, a sample of 500 g of sifted pellets was placed in a box that revolves for a period of 10 min at a speed of 100 rpm. Eight replicate samples were performed for each of the four diets. After testing, the pellets were screened on a mechanical sieve shaker with a sieve size of about 0.8 times the pellet diameter. The tumbling box pellet durability index (PDI) was calculated as the mass of the pellets retained on the screen divided by the total mass of pellets.

While ten 100 g samples of sifted pellets from each diet were conveyed using the Holmen tester with high air velocity through a tube with right angled bends for 30–120 s. Fracture occurred when pellets strike the right-angle corners of the tester. The Holmen PDI was calculated using the same procedure as for the tumbling box.

The pellet hardness of all diets was measured using a texture analyzer (TA XT plus, Stable Microsystems Inc., Godalming, Surrey, UK) following the protocol described by Peixoto et al. (2021). The cutting force (N) was recorded by placing a single pellet perpendicularly to the blade and this procedure was repeated 15 times for each diet.

2.4 Statistical analysis

Growth performance parameters of the shrimp and water quality parameters for each system were analyzed using one-way ANOVA. Results for pellet durability index (PDI) and the hardness for each of the four experimental diets were analyzed using one-way ANOVA. The results were expressed as the mean ± standard deviation (SD). Significant differences were set as $P < 0.05$ among treatments followed by the Tukey's multiple comparison test to evaluate significant differences between treatment means. Statistical analyses were performed using R software for windows (version 4.1.1; R Core team, 2021).

3. Results

3.1 Growth performance

Response of the shrimp cultured for 8 weeks in both clear water and biofloc systems is presented in Table 3. Results from the clear water experiment showed no significant difference in weight gain (%) and survival rate (%) among treatments. However, it was observed that there was a significant difference between final weight (g), final biomass (g), weight gain (g), FCR and TGC. Although higher values for final biomass (g) and weight gain (g) were observed in the FM12 treatment, yet they did not differ significantly from FM8 and AP diets. Meanwhile, there was a significant difference between final biomass (g), weight gain (%), FCR and TGC of the shrimp in the biofloc system. It was observed that there was no significant difference in shrimp final weight (g), weight gain (g) and survival rate (%) between treatments. While higher values for final weight (g) and weight gain (g) were observed in the FM8 and FM12 treatment, they did not differ significantly from AP and PM diets.

3.2 Water quality parameters

Water quality parameters recorded during the clear water experiment including dissolved oxygen (DO), temperature, salinity, and pH, were 6.75 ± 1.30 mg/L, 28.52 ± 0.57 ºC, 7.53 ± 0.46 ppt and 7.51 ± 0.28, respectively. Additionally, Total Ammonia- Nitrogen and Nitrite were as follows; 0.39 ± 0.44 mg/L, 0.04 ± 0.03 mg/L, respectively. Moreover, average water quality parameters recorded during the biofloc water experiment including dissolved oxygen (DO), temperature, salinity, and pH, were 6.72 ± 0.50 mg/L, 27.48 ± 0.45 ºC, 6.79 ± 0.12 ppt and 7.72 ± 0.70, respectively. Total Ammonia- Nitrogen and Nitrite were as follows; 1.00 ± 1.00 mg/L, 0.32 ± 0.38 mg/L, respectively.

3.3 Physical properties of the diets

Pellet durability index and hardness of the four extruded diets is shown in Table 4. All four different protein-source diets showed high durability index (97.83 ± 0.66 %) using the tumbling box and Holmen tester. It was observed that there was no significant difference between the four diets durability index when measured with the tumbling box. Although, the four diets showed high PDI using the Holmen tester (97.33 ± 0.14 %), there was a significant difference between them. It was observed that the pellet hardness did not differ significantly among the four diets.

4. Discussion

Successful replacement of fishmeal (FM) by alternative ingredients varies widely in shrimp feeds. This can be attributed not only to the nutritional quality of these ingredients in comparison to FM, but also to their completeness of the required nutrient and feed formulations (Bulbul et al., 2013). There is a need to develop diets that can meet the protein requirement and cost for shrimp production while reducing nitrogen amount introduced into the culture medium (Correia et al., 2014). Results from this study provide important information regarding the ability of Pacific white shrimp to utilize alternative feed formulations with low levels of animal protein sources under two different culture conditions.

In the clear water experiment, it was observed that the shrimp fed with all plant, 8% and 12% fishmeal diets had significantly higher weight gain than the shrimp offered the 8% poultry by-product diet. Although, the shrimp fed with the poultry by-product diet had the lowest performance in the clear water system, the shrimp fed with the same diet in the biofloc system was not

significantly different from the fishmeal treatments. Davis & Arnold, (2000) found that *L. vannamei* did not demonstrate palatability problems when poultry by-product meal was used to replace fish meal in the diet. Amaya et al., (2007) used a green water outdoor tank system to evaluate the efficacy of using commercial extruded diets including different levels of fishmeal (0, 3, 6 and 9%) and plant based (Solvent extracted soybean meal) diets fed to Pacific white shrimp. These authors did not observe significant differences between any of their treatments. Roy et al., (2009) conducted a similar study with four portions at 8% of the diet (PM, pea meal, distillers' dried grains with solubles (DDGS) and FM) in shrimp feeds using indoor clear water and farm trial. They found that shrimp weight gain, survival and feed conversion ratio were similar among dietary treatments, suggesting that PM, pea meal and DDGS could be alternative protein sources to FM for *L. vannamei*. However, in that study, the authors observed different ranking of the shrimp growth response fed with the four diets in clear water and farm trial. The variation seen between system types and diet formulations could be simply due to intrinsic variability in shrimp growth or slight differences in nutrient availability from natural productivity when present.

It was also observed that the shrimp cultured in the biofloc system in all treatments had higher final weight (g) than the shrimp produced from the clear water system after the same culture period. Many studies reported that microorganisms forming the bioflocs serve as an additional food source of amino acids, proteins, fatty acids and lipids, and thus substantially reduces external feed supply to make it more economical (Hussain et al., 2015; Lara et al., 2017; Panigrahi et al., 2018; Hussain et al., 2021). Moreover, It has been suggested that using this culture method may contribute to better shrimp growth and feed conversion efficiency (Xu et al., 2012; Gao et al., 2020).

Results from the biofloc experiment showed that the shrimp fed with the plant-based diet (AP) had the lowest final biomass (g), weight gain (g), and TGC and the highest FCR. Yet, there was no significant difference of these parameters between the shrimp fed with the PM, 8% FM and 12% FM. This could be because of the relatively low survival in the plant-based treatment (86%) compared to the other ones. Even though some of the growth performance parameters of the shrimp fed with the AP diet was lower than the others in the biofloc system, this was not the case in the clear water system. In addition, the survival rate of the shrimp fed with AP diets was not significantly different from the other treatments. Soybean meal (SBM) in combination with other plant based protein sources is known to be one of the most successful replacers of FM, because of

its favorable protein content and amino acid profile, less expensive price than FM and high availability in the market (Yun et al., 2017). It was observed that the four diets used in this study had balanced amino acid profile (Sookying et al., 2013). Soybean meal has been used as the primary protein in replacement strategies for fishmeal in many laboratory-based diets for shrimp (Davis & Arnold, 2000; Samocha et al., 2004; Sørensen et al., 2009; Galkanda-Arachchige et al., 2021).

Results of the growth performance in both experiments showed reasonably good values under laboratory conditions. It was clear that the low inclusion of fishmeal (8 and 12 %) as well as the use of alternative protein sources in these practical diets did not adversely affect the final weight, weight gain, and percent weight gain of the shrimp. The shrimp survival in both experiments was greater than 85% in all treatments, with no significant differences between treatments. These results could be supported by the findings of Samocha et al., (2004) who indicated that shrimp offered soybean poultry by-product meal co-extruded laboratory based diets had a good growth and survival rate. Hence, these authors concluded that there were no indications of the feed with no fish meal being rejected or being less palatable. To improve the palatability of the soybean based diets, Soares et al., (2021b) suggested that low addition level (10 g/kg) of krill meal, krill oil or fish hydrolysate to a soy-based diet can improve food consumption and stimulate feeding activity of *L. vannamei*. Overall, there was no clear trend of one diet being superior in the current study in terms of shrimp growth and survival among both trials.

Soybean meal was used as a primary protein source in all of the tested diets. Soy proteins are known to improve the durability of feed (Cavalcanti & Behnke, 2005b), and in the food industry soy proteins are known for a wide range of functional properties that help to stabilize food systems and improve texture. The adhesiveness of soy protein is caused by intermolecular forces, basically its electrostatic and covalent disulfide bonding pattern (Kumar et al., 2002). The use of extruded diets in aquaculture has been extensively investigated for fish nutrition, but limited studies are available regarding shrimp farming (Soares et al., 2021a). In the current study, the four extruded diets showed a high pellet durability and hardness which indicates that these diets will show low degradation in pneumatic feeding devices when fed to the shrimp. These diets had basically the same size to allow a fair comparation of the extrusion effect using different protein sources to produce them. Despite the difference in protein composition, all diets presented high, but slightly

different durability index (PDI), indicating no further effect of the production method "extrusion". Therefore, this indicates that extrusion processing has no negative effect on the physical properties tested of the diets. These results were in accordance with Soares et al., (2021b), who observed a significantly higher PDI for extruded shrimp feed (97%) than pelleted shrimp feed (92%) used during their trial. Additionally, these authors reported that the extruded diet had significantly higher hardness than pelleted diet; and suggested that the diet hardness is closely related with the ingredients' composition, extrusion moisture, cut, and temperature adjustments.

Welker et al., (2018) stated that there are several advantages of using extrusion systems to produce aquafeeds, such as better digestibility, food conversion, deactivation of antinutritional factors, water stability, floatability control, and cost effectiveness. Furthermore, extruded aquaculture feeds have been reported to be more hydrostable and keep its shape longer due to the higher degree of starch gelatinization attained by the extrusion process (Misra et al., 2002; Welker et al., 2018). As acoustic-based feeding technology became more common in commercial settings, Peixoto et al., (2020) tested the acoustic and hardness of extruded vs. pelleted commercial diets to feed Pacific white shrimp and they found a higher acoustic intensity when extruded diet was offered dry probably due to its greater hardness in comparison to pelleted food. The authors suggested that textural properties of aquaculture foods should gain more attention with the increasing use of automatic acoustic feedback systems in shrimp farming.

5. Conclusion

The current study evaluated the use of alternative protein sources along with the low inclusion of fishmeal (8 and 12%) in the Pacific white shrimp diets. Shrimp cultured in both clear water and biofloc systems showed good growth performance and survival rates. No difference in survival rate was observed between the shrimp fed with the four diets. Results from the clear water experiment showed that the shrimp fed with PM diet had the lowest final individual weight, biomass (g), and weight gain (g), and the highest FCR. Results from the biofloc experiment showed that the shrimp fed with AP diet had the lowest biomass (g), weight gain (g), and TGC and the highest FCR. Difference in performance across systems with and without natural foods have been reported by other researchers indicating system interactions. The four extruded diets showed high durability index (PDI) when measured using the tumbling box and the Holmen tester. Therefore, these results indicated that the different ingredients tested did not affect the PDI or hardness of the

diets produced by extrusion. The use of extrusion systems to produce feeds enables production of pellets with high physical quality for a range of ingredients. Results of this study were conducted in laboratory-based conditions, and practical shrimp farming using alternative protein sources may differ in some properties. Hence, the authors encourage shrimp farmers to use alternative protein source diets in their farms in order to decrease the pressure on the fishmeal as well as increasing profit since plant-based products are typically less expensive.

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Table 1: Formulation (g/ kg) of each 2.4mm 35% crude protein extruded sinking feed with various protein sources used to assess shrimp growth in both clear water and biofloc recirculating systems. Proximate analysis performed by Midwest Laboratories (Omaha, NE, USA) with results expressed as g/100 g.

Ingredients	AP	PM8	FM8	FM12
Soybean meal	560.0	500.0	537.0	575.0
Wheat	191.0	231.0	219.0	216.0
Fish meal[a]	0.0	0.0	80.0	120.0
Poultry-by product meal	0.0	80.0	0.0	0.0
Corn Gluten	120.0	80.0	60.0	0.0
Dicalcium Phosphate	41.3	31.3	26.3	16.3
Fish Oil	60.0	50.0	45.0	50.0
Bentonite	15.0	15.0	15.0	15.0
Lecithin	10.0	10.0	10.0	10.0
Vitamin Premix [b]	1.2	1.2	1.2	1.2
Mineral Premix [b]	1.2	1.2	1.2	1.2
Stay C-35% active	0.2	0.2	0.2	0.2
Copper sulfate	0.1	0.1	0.1	0.1
Proximate Composition (g/100g as is) [c]				
Phosphorus	1.47	1.28	1.41	1.32
Crude Protein	37.5	38.1	37.7	37.9
Moisture	8.99	9.62	8.44	9.41
Crude Fat	6.90	7.54	7.68	7.02
Crude Fiber	8.8	9.2	9.9	12.0
Ash	8.57	8.89	8.95	8.99

[a] Menhaden fishmeal 62% protein: Special Select.

[b] Premixes are proprietary products therefore composition is not listed.

[c] Proximate analysis performed by Midwest Laboratories (Omaha, NE, USA) with results expressed as g/100g. AP: All plant, PM8: Poultry by-product meal 8%, FM: Fish meal 8% (FM8) and 12% (FM12).

Table 2: Amino acid content (as is) of each 2.4mm 35% crude protein extruded sinking feed with various protein sources used to assess shrimp growth in both clear water and biofloc recirculating systems. Proximate analysis performed by University of Missouri Laboratory (Columbia, MO, USA) with results expressed as g/100 g.

	AP	PM8	FM8	FM12
Alanine	1.94	1.94	1.84	1.76
Arginine	2.33	2.40	2.40	2.54
Aspartic Acid	3.68	3.61	3.73	3.91
Cysteine	0.60	0.59	0.54	0.53
Glutamic Acid	7.21	6.80	6.79	6.62
Glycine	1.49	1.86	1.67	1.79
Histidine	0.91	0.89	0.92	0.94
Hydroxylysine	0.08	0.09	0.08	0.09
Hydroxyproline	0.03	0.28	0.15	0.20
Isoleucine	1.75	1.71	1.72	1.75
Lanthionine	0.00	0.00	0.00	0.00
Leucine	3.51	3.20	3.11	2.85
Lysine	1.96	2.10	2.21	2.42
Methionine	0.61	0.62	0.62	0.64
Ornithine	0.02	0.03	0.03	0.03
Phenylalanine	2.05	1.94	1.92	1.89
Proline	2.45	2.63	2.38	2.27
Serine	1.62	1.59	1.56	1.55
Taurine	0.13	0.15	0.17	0.19
Threonine	1.40	1.41	1.40	1.45
Tryptophan	0.41	0.40	0.35	0.41
Tyrosine	1.48	1.41	1.35	1.35
Valine	1.82	1.82	1.84	1.85
Total	37.48	37.47	36.78	37.03

* AP: All plant, PM8: Poultry by-product meal 8%, FM: Fish meal 8% (FM8) and 12% (FM12).

Table 3: Response of Pacific white shrimp grow-out in the clear water and biofloc systems for 8 weeks. Values represent the mean of replicates ± standard deviation.

	Initial weight (g)	Final weight (g)	Final Biomass (g)	Weight gain (g)	Weight gain (%)	Survival (%)	FCR	TGC
Clear water								
AP	0.10 ± 0.00	3.62 ^{ab} ± 0.08	50.60 ^{ab} ± 2.16	3.51 ^{ab} ± 0.08	3412.07 ± 147.97	93.33 ± 4.71	2.02 ^{ab} ± 0.08	0.068 ^{b} ± 0.001
PM8	0.09 ± 0.01	3.51 ^{a} ± 0.41	45.32 ^{a} ± 4.23	3.41 ^{a} ± 0.41	3564.02 ± 533.17	86.67 ± 9.43	2.12 ^{a} ± 0.27	0.068 ^{b} ± 0.004
FM8	0.09 ± 0.01	3.59 ^{a} ± 0.12	51.69 ^{b} ± 2.60	3.49 ^{ab} ± 0.12	3621.11 ± 415.27	96.00 ± 3.65	2.02 ^{ab} ± 0.11	0.068 ^{b} ± 0.002
FM12	0.09 ± 0.01	4.03 ^{b} ± 0.18	53.87 ^{b} ± 3.00	3.93 ^{b} ± 0.17	3984.04 ± 254.34	89.33 ± 5.96	1.83 ^{b} ± 0.03	0.072 ^{a} ± 0.001
p-value	0.48	0.015	0.003	0.015	0.132	0.134	0.042	0.035
Biofloc system								
AP	0.24 ± 0.01	4.65 ± 0.25	400.93 ^{a} ± 35.13	4.41 ± 0.25	1838.84 ^{a} ± 61.39	86.25 ± 8.02	1.61 ^{a} ± 0.15	0.069 ^{a} ± 0.002
PM8	0.23 ± 0.00	5.00 ± 0.32	455.55 ^{b} ± 19.70	4.77 ± 0.32	2052.21 ^{ab} ± 163.69	91.50 ± 8.74	1.40 ^{ab} ± 0.07	0.072 ^{ab} ± 0.003
FM8	0.24 ± 0.01	5.25 ± 0.13	471.23 ^{b} ± 14.15	5.02 ± 0.14	2137.74 ^{b} ± 109.55	89.75 ± 4.11	1.35 ^{b} ± 0.04	0.074 ^{b} ± 0.001
FM12	0.23 ± 0.00	5.17 ± 0.41	460.08 ^{b} ± 24.48	4.94 ± 0.40	2122.05 ^{b} ± 128.06	89.25 ± 6.24	1.39 ^{ab} ± 0.08	0.074 ^{ab} ± 0.003
p-value	0.310	0.062	0.007	0.057	0.016	0.764	0.049	0.029

AP: All plant, PM8: Poultry by-product meal 8%, FM: Fish meal 8% (FM8) and 12% (FM12).
Feed conversion ratio= feed offered/ (final weight-initial weight)
Weight gain%= [(final weight-initial weight)/initial weight] × 100
TGC = (final weight 1/3-initial weight 1/3)/ Σ (temperature * days) *100

Table 4: Physical properties of the four practical extruded diets including pellet durability index (PDI; %) and hardness (N). Values of the Tumbling box represent the mean of eight replicates ± standard deviation. Values of the Holmen tester represent the mean of ten replicates ± standard deviation.

Treatment	Tumbling box method (Mean ± SD)	Holmen durability tester (Mean ± SD)	Hardness (Mean ± SD)
AP	99.05 ± 0.55	97.52 ± 0.15 [a]	9.58 ± 1.75
PM8	98.96 ± 0.64	97.22 ± 0.09 [b]	9.93 ± 2.73
FM8	97.65 ± 1.42	97.21 ± 0.16 [b]	9.40 ± 0.83
FM12	97.69 ± 2.08	97.37 ± 0.15 [ab]	8.75 ± 2.83
p-value	0.07	0.001	0.52

AP: All plant, PM8: Poultry by-product meal 8%, FM: Fish meal 8% (FM8) and 12% (FM12).

CHAPTER 5

RELATIONSHIP BETWEEN AEROBIC SCOPE AND UPPER THERMAL LIMITS OF PACIFIC WHITE SHRIMP (*Litopenaeus vannamei*) IN LOW-SALINITY CULTURE SYSTEMS

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Abstract

Aquaculture of the Pacific white shrimp *Litopenaeus vannamei* in low-salinity water is a viable industry and production strategy in the southeastern United States. A major challenge facing this industry is a phenomenon called late-term mortality which is thought to be driven by thermal stress at the end of the growing season when water temperatures can reach or exceed 36 °C in shrimp production ponds. To investigate the physiological mechanisms behind upper lethal limits in shrimp, we evaluated linkages between empirically measured thermal limits and absolute aerobic scope (AAS), or ability to provide energy above that needed for basic maintenance. In this study, we tested whether thermal tolerance decreases with increasing shrimp age/size and whether AAS is a useful concept for understanding the physiological basis of thermal tolerance in shrimp. We exposed two size classes (small: 2.07 ± 0.86 and large: 24.64 ± 2.55 g) of shrimp to increasing temperature at a rate of 1 °C/h from 28–42 °C. At each temperature, we used intermittent respirometry to estimate resting metabolic rate and we directly measured lethal thermal tolerance by evaluating critical thermal maximum (CT_{max}). Additionally, we used the electron transport system assay to estimate maximum metabolic rate (RMR) at temperatures from 9–45 °C. Small shrimp had a higher CT_{max} than large shrimp, with upper lethal limits of 40.6 and 39.0 °C, respectively. For both size-classes, AAS reached its minimum (AAS_{min}) at temperatures near the peak RMR (RMR_{peak}) and within 2 °C of CT_{max}. Large shrimp exhibited a lower temperature at AAS_{min} than that of the smaller shrimp. Reductions in AAS appear to be one of the underlying physiological drivers of thermal tolerance in *L. vannamei* and an indicator of increasing thermal stress. Changes in the temperature at which AAS reaches its minimum may be a useful predictor of shifts in thermal tolerance among shrimp size-classes.

Keywords: Aerobic scope, thermal tolerance, critical thermal maximum, electron transport system, shrimp

1. Introduction

Pacific white shrimp, *Litopenaeus vannamei*, are the most intensively cultivated shrimp in the world (Anderson et al., 2019). Due to environmental concerns and the higher cost of coastal real estate, production of *L. vannamei* in inland, low-salinity ponds is becoming common in many regions throughout the world, including the southeastern U.S. (Roy et al., 2010). Recent research has shown that high variation in shrimp production among ponds can be attributed in large part to differences in thermal regime (Abdelrahman, 2016; Abdelrahman et al., 2019). However, no work has yet been conducted on upper thermal tolerance in low-salinity ponds. Water temperatures as high as 36 °C have been documented in production ponds, which are approximately one meter in depth and can heat up considerably by late afternoon during the summer months. Increased summertime mortality is frequently reported by commercial shrimp farmers in Alabama when shrimp are approaching harvest size – a pattern labeled “late-term mortality” (Roy et al. 2018).

Acute thermal tolerance can be empirically measured as critical thermal maxima (CT_{max}), where the animal is exposed to temperature increases at a constant rate until a critical endpoint is reached (González et al., 2010; Kumlu et al., 2010 Re et al., 2012). Common responses include loss of equilibrium (LOE), sudden onset of muscular spasms, and finally "heat rigor", "coma", or "death" (Lutterschmidt and Hutchison 1997a, 1997b). Although useful, direct indices such as CT_{max} do not provide information on the underlying physiological mechanisms that drive and control thermal tolerance. Understanding the mechanisms behind thermal tolerance may allow for quantitative comparisons of specific physiological characteristics of various shrimp stocks and/or size classes concerning suitability for low-salinity, high temperature, pond production.

One approach to understanding the physiological underpinnings of thermal tolerance is the concept of aerobic (or metabolic) scope. Absolute aerobic scope (AAS) is defined as the difference between maximum aerobic metabolic rate (MMR) and standard aerobic metabolic rate (SMR: the minimum oxygen consumption rate required for basic maintenance and survival at a given temperature; Halsey et al., 2018; Verberk et al., 2016; Fig. 1A). In theory, the greater the aerobic scope, the greater the physiological capacity to generate energy above that needed for basic maintenance. Typically, although SMR and MMR initially increase with increasing temperature, MMR peaks sooner than SMR, resulting in a decrease in AAS with further increases in temperature (Fig. 1A, B). The temperature at which aerobic scope equals zero (MMR = SMR) represents the

critical temperature (T_{crit}), above which the organism is no longer physiologically capable of meeting its basic metabolic costs via aerobic respiration and increasingly relies on anaerobic respiration. The CT_{max}, indicated by LOE, occurs at a temperature beyond which the organism has an insufficient capacity for anaerobic respiration (Ern, 2019).

In fishes, MMR is typically measured as the maximal oxygen consumption rate in a respiration chamber after intense and exhaustive exercise via methods such as swimming against a current or manual chasing. We hereafter refer to this measurement as MMR_{resp}. Although considered generally accurate and repeatable, there are some concerns and challenges associated with these approaches to measure MMR_{resp} (Killen et al., 2017; Rosewarne et al., 2016). Also, approaches used for measuring MMR_{resp} in highly mobile taxa cannot be used for organisms such as bivalves that simply close in response to disturbance. In this study, we explore the use of an enzymatic approach for estimating MMR that could potentially be applied to a wide range of taxa, including shrimp.

The electron transport system (ETS) assay has been used in various studies investigating thermal tolerance of various invertebrate taxa and fish (Bielen et al., 2016; Simčič et al., 2017, 2014, 2010, 2005; Westhoff et al., 2021; Žagar et al., 2018; Horne et al., 2022). The ETS assay measures the electron transfer activity of dehydrogenases and cytochromes in the presence of saturating levels of substrates (Maldonado et al., 2012) and has been used as a proxy for maximum potentential cellular respiration (Bielen et al. 2016; Zagar et al. 2015, 2018). We hereafter refer to this measurement as MMR_{ets}. Because thermal tolerance of enzymes is frequently higher than that of whole organisms (Pörtner et al., 2017), and the ETS assay is not conducted on live organisms, MMR_{ets} has the advantage of being measurable across a temperature range that approaches and exceeds the lethal limits for whole organisms, whereas MMR_{resp} cannot be easily measured at temperatures that quickly become lethal to whole organisms (i.e. far right sides of Fig. 1 A, B). We expect that the greater upper range in temperatures available for direct testing will result in modifications to the expected patterns in respiration rates and aerobic scope (Fig. 1 C, D).

We use a combination of empirical, respirometry, and enzymatic assays to address the following questions : 1) Does CT_{max} differ between small and large size classes of shrimp?; 2) Do AAS_{min} and CT_{max} occur at similar temperatures in shrimp?; and 3) Is a higher AAS_{min} indicative of increased upper thermal tolerance?

2. Materials and Methods

2.1. Experimental shrimp and acclimation procedures

Experiments were conducted using a "small" and "large" size class of shrimp with a mean weight ± *SD* of 2.07 ± 0.86 g (n= 44) and 24.64 ± 2.55 g (n=34), respectively. The experiments were conducted at E.W. Shell Fisheries Center, Auburn University, Alabama, USA.

For small-size class experiments, 400 specific-pathogen-free (SPF) shrimp postlarvae (PLs) were obtained from Shrimp Improvement Systems (SIS; Islamorada, Florida, USA). This hatchery supplies many of the shrimp larvae utilized for aquaculture in west Alabama. Upon receipt in the lab, shrimp were acclimated to a laboratory holding temperature of 28 ℃ by changing the shipping water temperature at a rate <4 ℃/h (Davis et al., 2004), and salinity was slowly decreased from shipping salinity (15–32 ppt) to typical inland farm salinity (4–6 ppt). The PLs were nursed in an indoor recirculating nursery system using particle size #1 (400–600 µ), then size #2 (600–850 µ), then followed by size #3 (850–1,200 µ) commercial feeds (Zeigler® Bros. Inc. Gardners, Pennsylvania, USA; protein ≥ 50 %, fat ≥ 15 %, fiber ≤ 1 %) for ~3 weeks and then switched to 1.5-mm crumbled commercial shrimp feed (Zeigler® Bros. Inc.; protein ≥ 40 %, fat ≥ 9 %, fiber ≤ 3 %) for ~1 week. Prior to the experiment, PLs were distributed into four, 100-L tanks (100 PLs/ tank) to ensure proper acclimation and during this time were fed 1.5-mm crumbled commercial shrimp feed (Zeigler® Bros. Inc.).

For the large-size class experiments, 200 shrimp were transported from Greene Prairie Aquafarm, Boligee, Alabama, acclimated to the experimental conditions (using the same method as with the small shrimp), and distributed in seven, 100-L square tanks. Shrimp were fed 2.4-mm commercial feed (Zeigler® Bros. Inc.; 35% crude protein and 8% crude lipid).

For both experiments, artificial seawater (ASW) was prepared using water from a reverse osmosis (RO)/ deionization (DI) system (BAR-50-CB-W; AquaFX Barracuda; Winter Park, Florida) and mixed with sea salt (Crystal Sea® Marinemix, Baltimore, Maryland, USA) for a final salinity of 6 ppt. A biofilter was installed in each of the holding tanks to help maintain water quality. For both experiments, shrimp were cultured at a holding temperature of 28 ℃ for two weeks prior to the onset of the experiment to stabilize their physiology and allow metabolic compensation (Castille and Lawrence, 1981; Re et al., 2005). Daily feed was calculated based on the methodology of Garza de Yta et al. (2004), assuming a feed conversion ratio of 1.8 and a

weight gain of 1 g/week. The feed was manually offered 4 times/day at ~08:00, 11:00, 13:00, and 15:45.

2.2 Water quality

Dissolved oxygen (DO) and water temperature of holding tanks, prior to experiments, were measured twice daily using a YSI 650 multi-meter (YSI®, Yellow Springs Instrument; Yellow Springs, Ohio, USA), and salinity was measured twice daily using ExStik® EC400 Conductivity/TDS/Salinity Meter (Extech Instruments, Waltham, Massachusetts, USA). Water from the RO/DI system was added as needed to compensate for evaporative water loss. pH was measured twice weekly using a waterproof pHTestr30 (Oakton instrument, Vernon Hills, Illinois, USA). Sodium bicarbonate was added as needed to each of the holding tanks to maintain pH between 7.6 and 8.2 throughout the experiment. Total ammonia-nitrogen (TAN) and nitrite were analyzed twice/week using a YSI 9300 Spectrophotometer (YSI®). In holding tanks, when TAN increased above 1 mg/L, a partial water change was conducted, and feeding was reduced by half until the TAN declined below 0.5 mg/L.

2.3 Critical thermal maximum determination

Critical thermal maximum was determined for both size classes. A total of 20 shrimp (10 individuals/run) were tested for the small-size class and 14 shrimp (7 individuals/run) were tested for the large-size class. To initiate each run, shrimp were placed in individual 1-L plastic beakers containing ASW that were partially submerged in a water bath containing 6 ppt salinity water (Fig. 2A). Each beaker had a mesh screen on each side wall to allow for water circulation. To ensure proper mixing and aeration, air stones and small pumps were installed within the tank (Fig. 2A), and the whole system was covered with a mesh screen to prevent the shrimp from jumping out of cups. Shrimp were acclimated to the beakers for ~12 h prior to the start of each run. At the initiation of the corresponding respirometry run (see section 2.4), the CT_{max} water bath and associated beakers were heated at a rate of 1°C/h starting from 28 °C until all shrimp had reached CT_{max}. Every 30 minutes, shrimp were flipped on their sides. CT_{max} was defined as the temperature at which shrimp could not right themselves within 30 seconds (LOE; Lutterschmidt and Hutchison, 1997a; Halsey et al., 2018).

2.5 Resting metabolic rate measurement

Because SMR is difficult to measure accurately, resting metabolic rate (RMR: metabolic rate with minimal activity levels) is frequently used as a practical approximation of SMR. Also, because SMR requires multiple measurements of respiration at a constant temperature over an extended period of time, it is not a practical metric for acute thermal ramps. For this study, we measured RMR which caters for low levels of spontaneous activity but still represents the lower-range metabolism of an individual in a relatively quiescent state (Burton et al., 2011; Chabot et al., 2016).

Respirometry experiments were conducted in an 8-chamber fiber-optic respirometry system using AutoResp™ 2.3.0 software (Loligo® Systems, Viborg, Denmark). Chambers were made of acrylic and the chamber volumes were 200 mL for small- and 600 mL for large-size class. Each chamber was connected to two Eheim submersible pumps (300 L/h; EHEIM GmbH & Co., Deizisau, Germany). Where one pump circulated fresh, oxygenated water through the chamber during flush cycles, and the other pump re-circulated water through the chamber during the measurement (closed) cycles (Fig. 2B; Haney et al., 2020). A flow-through oxygen cell with an optical DO sensor was inserted in the recirculation tubing of each chamber (Fig. 2B). Respirometry chambers, associated pumps, and sensors were submerged in a ~300-L rectangular trough filled with 6 ppt water. Water was aerated within the trough to maintain near 100% DO saturation. Water temperature was controlled by two aquarium heaters (Finnex Deluxe Titanium Tube Heater; 800 W, JSK Merchandise Inc., Countryside, Illinois). To reduce ambient bacterial oxygen demand, chambers and tubing associated with the respirometry setup were soaked in a 3% bleach solution prior to each experiment and then rinsed with tap water.

Intermittent respirometry was used to estimate RMR of small and large shrimp. In the small shrimp experiments, six respiration chambers contained single animals and two served as controls to calculate the background respiration rate due to bacteria. A total of 12 animals were tested in two runs. In the large shrimp experiments, five large chambers were used, four containing single animals and one serving as a control. A total of eight animals were tested in two runs. Prior to each experiment, shrimp were weighed (gWW) and fasted for ~12 h to minimize effects of feeding and digestion on RMR. Fasting shrimp were acclimated to respiration chambers and pumping patterns overnight (~12 h). Chambers were of sufficient size to be within the recommended respirometer: organism volume ratio of 20 to 50 (Svendsen et al., 2016). During acclimation and subsequent

experimental periods, flush pumps were turned off and on using AutoResp™ version 2 automated intermittent respirometry software (Loligo® Systems) to create alternating "measuring", "flush", and "wait" periods. During each measuring cycle, the flush pump was turned off and internal water recirculated through each chamber via its closed pump. This was followed by a flush period, where water from the respirometry trough was continuously pumped through each chamber to restore DO to ~100% saturation and flush out any accumulated wastes. The flush pump was then shut off and a short wait period accounted for the lag in system response before starting the next measurement period.

To assess the effects of temperature on RMR, the temperature in the respirometry trough was increased at a rate of 1 °C/h from an initial temperature of 28 °C, with the first measurement of RMR occurring at 0900 for a given run. The duration of each measurement cycle was adjusted as temperature increased due to associated changes in respiration rate, to ensure that shrimp did not draw DO below 80% saturation. In general, 1–2 measurement cycles (≤ 30 m each) were completed every hour. Each respirometry run was terminated at the temperature at which all shrimp in the concurrent thermal tolerance experiment (see section 2.3) were observed to have reached CT_{max}. Respiration rate (mL O_2/gWW/h) was calculated for each shrimp during each measurement cycle by AutoResp™ software using the formula:

RMR (mg O_2/gWW/h) = $\frac{V([O_2]_{t0}-[O_2]_{t1})}{t \times BW}$ where

$[O_2]_{t0}$ = DO at time t0 (mg O_2/L)

$[O_2]_{t1}$ = DO at time t1 (mg O_2/L)

V = respirometer volume (L) – volume of shrimp (L)

t = time t1 (h) – time t0 (h)

BW = whole shrimp body weight (g)

To correct for any background oxygen demand from bacteria within the respirometry chambers, the RMR of the control chamber(s) were subtracted from the RMR of the shrimp chambers during each measurement period.

To allow for calculation of AAS, the RMR was then converted from mg O_2/gWW/h to mL O_2/gWW/h using the ideal gas law:

$PV = nRT$

Where P = pressure in atmospheres (atm), V = volume in liters, n = number of moles, R = universal gas constant (0.08206 L atm/K-mol), T = temperature in Kelvin.

2.5 Maximum metabolic rate estimated via the ETS assay

Prior to each experiment, 12 shrimp acclimated at a holding temperature of 28 °C were randomly selected and frozen at –80 °C. Each shrimp was thawed and the whole body was homogenized on ice with an analytical grinding mill (Item No. 2900001, IKA Works Inc., Wilmington, North Carolina, USA). A subsample consisting of 25% of the total homogenized tissue mass of each shrimp was then diluted with reagent-grade deionized water (Cat. No. 9150-1, Ricca Chemical, Arlington, Texas) to obtain a final concentration of 1 mg tissue/mL. The diluted homogenate from each individual was distributed into 20, 2-mL micro-centrifuge tubes (MCTs) and stored at -80 °C. Homogenate samples were thawed while in an ice-water bath, and ETS activity was measured using a methodology originally developed by Packard (1971) and subsequently modified and used for aquatic invertebrates (Simčič et al., 2014; Westhoff et al., 2021). Electron transport system activity (mL O_2/gWW/h) was measured at each of 13 temperatures from 9 to 45 °C at 3 degrees intervals to generate a thermal performance curve (TPC) relating ETS activity to temperature. Because the enzymatic assay essentially measures electron transfer activity as opposed to organismal respiration rate, we refer to MMR_{ets} for the remainder of this paper to distinguish from the standard approach of measuring MMR using respirometry (i.e., MMR_{resp}).

2.6. Absolute aerobic scope calculations and statistical analyses

For each shrimp size class, a TPC was created by fitting a smoothing spline (SS) model to ETS activity data (y-axis) at tested incubation temperatures (x-axis). The selection of the smoothing parameter (λ) was based on the restricted maximum likelihood (REML) method to ensure the compromise between the smoothness of the function and the lack of fit (Berry and Helwig, 2021). The fitted SS models were used to predict MMR_{ets} at temperatures from 9–45 °C with an interval of 0.001 °C. For each shrimp size class, 95% confidence intervals (95% CI) of predicted MMR_{ets}

curves were created via bootstrapping (Efron and Tibshirani, 1993) implemented in the boot package (version 1.3-28; Canty and Ripley, 2021). Data were resampled with replacement 1,000 times, with the SS model re-fitted to these data each time, and the 95% CIs were determined from the 2.5^{th} and 97.5^{th} percentiles.

Similarly, SS models with the REML method were applied to RMR data to create TPC for each shrimp size class. The fitted SS models were used to predict RMR across the tested respirometry temperature ranges with an interval of 0.001 °C, and bootstrapping was used to create 95% CIs of predicted RMR curves.

For each shrimp size class, at any temperature, AAS range consisted of a central estimate (predicted mean MMR_{ets} – predicted mean RMR; Clark et al., 2013), an upper boundary (upper 95% CI of MMR_{ets} – lower 95% CI of RMR), and a lower boundary (lower 95% CI of MMR_{ets} – upper 95% CI of RMR).

Critical thermal maximum data were analyzed using survival analysis methods. Kaplan-Meier survival analyses were used to determine the median CT_{max} temperature for each shrimp size class, and log-rank tests were used to compare probabilities of CT_{max} among size class-molt status groups.

Statistical significance for survival analyses was set at P-value < .05, and data were presented as the median (95% CI). Data for MMR, RMR, and AAS_{min} were presented as mean ± *SE* (95% CI). For MMR_{ets} and RMR estimates we considered descriptors to differ significantly between size classes if their 95% CIs did not overlap. Survival analyses were performed in SAS® version 9.4 (SAS, 2013). All other statistical analyses were conducted using R software for windows (version 4.1.1; R Core team, 2021).

3. Results

3.1. Water quality parameters

The water quality measurements (mean ± *SD*) in the holding tanks for the small shrimp were 6.87 ± 0.23 mg/L for DO, 27.74 ± 0.08 °C for temperature, 6.38 ± 0.17 ppt for salinity, 7.82 ± 0.16 for pH, 0.65 ± 0.13 mg/L for TAN, and 0.56 ± 0.32 mg/L for nitrite. Water quality measurements in the large shrimp holding tanks were 7.25 ± 0.20 mg/L for DO, 27.79 ± 0.25 °C

for temperature, 6.09 ± 0.23 ppt for salinity, 7.70 ± 0.23 for pH, 0.69 ± 0.23 mg/L for TAN, and 0.46 ± 0.06 for nitrite.

3.2.Critical thermal maximum

During the CT_{max} experiments, we observed two of 18 shrimp molting in the small-size class and three of 11 shrimp molting in the large size class. When controlling for the effect of molt status, CT_{max} of small shrimp [median (95% CI); 40.60 °C (40.10–41.50) °C] was significantly higher than that of large shrimp [38.95 °C (38.20–39.80) °C; Log-rank test: $\chi^2_{(1)} = 17.08$, $P < .0001$; Fig. 3A]. When controlling for the effect of shrimp size, the CT_{max} of intermolt shrimp [40.30 °C (39.80–40.70) °C] was significantly higher than that of recently molted shrimp [39.00 °C (35.40–40.30) °C; Log-rank test: $\chi^2_{(1)} = 3.95$, $P = .0470$; Table 1; Fig. 3B).

3.3. Resting metabolic rate

The relationships between RMR and temperature for both shrimp size classes are summarized in Fig. 4. Estimated RMR peaked at a significantly higher rate for small shrimp than large shrimp [mean ± *SE* (95% CI); small: 0.65 ± 0.04 (0.57–0.74) mL O_2/gWW/h, large: 0.36 ± 0.01 (0.34–0.38) mL O_2/gWW/h; Table 1). However, there was no significant difference in the estimated temperature at RMR_{peak} between the two shrimp size classes [small: 39.02 ± 0.94 (37.18–40.86) °C, large: 37.70 ± 0.45 (36.81–38.60) °C; Table 1].

3.4.Maximum metabolic rate estimated via the ETS assay

The estimated MMR_{ets} peaked at a higher rate and a lower temperature for small shrimp compared to large shrimp (Fig. 5). Small shrimp exhibited a significantly higher MMR peak, referred to as ETS_{max}, [small: 2.43 ± 0.07 (2.30–2.56) mL O_2/gWW/h, large: 1.26 ± 0.03 (1.20–1.31) mL O_2/gWW/h; Table 1] than large shrimp. However, small shrimp exhibited a significantly lower temperature of ETS_{max} compared to large shrimp [small: 22.92 ± 0.51 (21.93–23.92) °C, large: 25.12 ± 0.29 (24.56–25.69) °C; Table 1].

3.5. Absolute aerobic scope

For both size classes of shrimp, RMR continued to increase with temperature until RMR_{peak} even as MMR_{ets} declined, resulting in decreasing ASS as temperatures increased above 28 °C. However, because RMR did not increase at a fast enough rate to meet or exceed the declining

MMR_{ets} values, aerobic scope never decreased to zero (Fig. 6A, B). For small shrimp, the central estimate for AAS_{min} was 0.16 mL O_2/gWW/h at 39.56 °C, with a lower boundary of 0.0003 mL O_2/gWW/h at 39.14 °C, and an upper boundary of 0.34 mL O_2/gWW/h at 39.49 °C (Fig. 6C). For the large shrimp, the central estimate for AAS_{min} was 0.28 mL O_2/gWW/h at 38.59 °C with a lower boundary of 0.21 mL O_2/gWW/h at 38.65 °C, and an upper boundary of 0.35 mL O_2/gWW/h at 38.53 °C (Fig. 6D). Small shrimp AAS was nearly twice that of large shrimp until temperatures rose to ~31°C, after which small shrimp AAS declined to that of large shrimp at ~36 °C, and subsequently declined to nearly half that of large shrimp when it reached its minimum value at ~39 °C (Fig. 7).

4. Discussion

The ability of juvenile *L. vannamei* to thrive in low-salinity water makes them an especially good species for inland aquaculture (Pan et al., 2007). In west Alabama, USA, shrimp represent a potential, high-value alternative to catfish production (Sun, 2012), but few shrimp farms are currently in production due to unpredictable yields. Production of shrimp in low-salinity ponds has been plagued by late-summer mortality events as shrimp approach a market size exceeding 25 g. These events are likely due in part to a decreasing ability to osmoregulate with increasing size (Lemaire et al., 2002; Vargas-Albores and Ochoa, 1992). The reduced CT_{max} of larger shrimp found in this study indicates that late-summer mortality is also due in part to decreasing tolerance of larger, older shrimp to acute thermal stress.

Temperature is one of the most important abiotic factors that affect survival and growth of shrimp and other aquatic organisms (Lutterschmidt and Hutchison, 1997a). The thermal preference of an individual is a species-specific response that can vary according to age, weight, food availability, season, water quality, or light intensity (Wedemeyer et al., 1999). Although it is relatively easy to empirically measure the upper thermal limits of organisms, it is more difficult to determine or explain the physiological drivers that determine the limits of thermal tolerance. It has long been hypothesized that cardiorespiratory system failure of the organism is the principal determinant of the upper critical temperature where animals lose equilibrium, as well as the realized environmental thermal niche that they occupy (Fry and Hart, 1948). According to the oxygen- and capacity-limited thermal tolerance (OCLTT) model, the upper thermal limit is reached when the cardiovascular system of an organism cannot supply enough oxygen to meet the oxygen

demand of the mitochondria and subsequently they depend on fermentation processes which are less efficient at producing adenosine triphosphate (Ern et al., 2015; Pörtner et al., 2017).

Aerobic scope, which can be described as the physiological capacity to generate energy above that needed for basic maintenance (Ern, 2019), is a potential metric to describe and measure the physiological underpinnings of thermal tolerance. Results of this study supported an OCLTT-based mechanism driving shrimp to reach CT_{max} when AAS had declined to a minimum value. For both size classes, the peak and subsequent decline of MMR_{ets} with increasing temperature occurred earlier than that of RMR. This resulted in a decreasing AAS for both size classes as temperatures warmed toward the peak RMR. Beyond this peak, RMR also began declining with further increases in temperature, resulting in a subsequent increase in AAS with further increases in temperature. Therefore, AAS_{min} occurred at temperature(s) near the onset of RMR_{peak}. If subsequent declines in RMR represented a compensatory response to the increasing temperature that helped to reduce thermal stress (i.e., Guppy and Withers, 1999) to shrimp, we would have expected CT_{max} to occur well beyond the RMR_{peak}. However, both size classes exhibited CT_{max} within 2 °C of RMR_{peak}, indicating that subsequent declines in metabolic rate were a result of respiratory failure rather than a physiological adjustment to reduce thermal stress.

Although it held true for the Pacific white shrimp in this study, the hypothesized pattern of a steady decline in AAS as organisms approach CT_{max} has been only partially supported by empirical studies of other taxa. Aerobic scope of rainbow trout has been shown to decrease as temperatures approached CT_{max} (Chen et al., 2015). However, for other fish (barramundi and halibut), MMR_{resp} continued to rise with increasing temperature, resulting in a maximum AAS value near the upper lethal limits of juvenile barramundi and halibut (Gräns et al., 2014; Norin et al., 2014). Crustacean patterns also appear to be somewhat inconsistent. In Tiger shrimp (Penaeus monodon) and giant river shrimp (Macrobrachium rosenbergii) MMR_{resp} leveled off or continued to rise with increasing temperature resulting in either a leveling off or only a slight decline in AAS near CT_{max} (Ern et al., 2015, 2020). In contrast, for noble crayfish (Astacus astacus), MMR_{resp} declined steadily with increasing temperatures resulting in a steady decline in AAS (Ern et al., 2015, 2014). It is unclear why the relationship between AAS and increasing temperature appears to vary widely among fish and crustacean taxa and whether these same differences would be observed if using MMR_{ets} rather than MMR_{resp} to calculate AAS across a wide array of taxa.

However, both size classes of Pacific white shrimp clearly showed the hypothesized decline in AAS with increasing temperature in this study, indicating that declining AAS is a physiological indicator of increasing thermal stress, and ultimately tolerance limits, for this species.

The temperature at which AAS reached its minimum value was a good general predictor of the temperature range where shrimp would reach their thermal maximum. It may also exhibit enough sensitivity to accurately reflect small (i.e. < 2 °C) differences in thermal tolerance between size classes. Large shrimp exhibited a median CT_{max} that was 1.65 °C lower than the small-size class and also exhibited a lower temperature at AAS_{min} than that of the smaller shrimp. However, although temperature at which AAS_{min} was observed showed promise as a predictor of CT_{max}, the difference in AAS_{min} (i.e. mL O_2/gWW/h) was not a good predictor of differences in thermal tolerance between the two size classes. As temperatures increased above 35 °C, the AAS of small shrimp declined to approximately half that of large shrimp – suggesting a reduced aerobic capacity and a greater degree of thermal stress. However, the CT_{max} of small shrimp was higherthan the large shrimp. Although both size classes exhibited a strong, continuous reduction in aerobic scope as temperatures approached CT_{max}, the size class with the lowest AAS_{min} was not the most thermally sensitive.

5. Conclusion

In conclusion, we found that upper thermal limits of *L. vannamei* in low-salinity aquaculture ponds are likely driven in part by OCLTT. Upper thermal limits were preceded by temperature-dependent declines in aerobic scope and occurred within a couple of degrees of RMR_{max}. Understanding the physiological underpinnings of thermal tolerance may ultimately be of use in evaluating thermal tolerance of genetic lines of *L. vannamei* for low-salinity aquaculture, with temperatures at AAS_{min} and RMR_{max} being particularly relevant endpoints. Additional studies are needed to determine whether this framework is useful for understanding the thermal tolerance of other high-valued aquaculture species experiencing unexplained mortalities, or even reductions in performance, during production cycles, particularly in situations where culture water temperature is high. The relationship between the endpoints of AAS_{min}, RMR_{max}, and CT_{max} may also be useful in understanding the physiological basis of performance (lethal limits, growth, and reproduction) in the presence of additional stressors such as suboptimal ionic balance or suboptimal water quality parameters that may also affect metabolic rates.

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List of acronyms and definitions

Acronym	Definition
AAS	Absolute aerobic scope (mL O_2/gWW/h)
AAS_{min}	Minimum observed AAS value (mL O_2/gWW/h)
CT_{max}	Critical thermal maximum; temperature at which an organism experiences loss of equilibrium (°C)
DO	Dissolved oxygen (mg/L)
ETS	Electron transport system
ETS_{max}	Maximum ETS or maximum MMR_{ets} activity (mL O_2/gWW/h)
LOE	Loss of equilibrium
MMR_{ets}	Maximum metabolic rate as estimated by the ETS assay
MMR_{resp}	Maximum metabolic rate as estimated by respirometry
OCLTT	Oxygen- and capacity-limited thermal tolerance
RMR	Resting metabolic rate
RMR_{peak}	Peak resting metabolic rate
SMR	Standard metabolic rate

Table 1. Mean, standard error (*SE*), and 95% confidence intervals (CI) for peak resting metabolic rate (RMR_{peak}), the temperature at which RMR_{peak} occurs, the highest estimate of maximum metabolic rate measure via the electron transport system assay (ETS_{max}) and the temperature at which ETS_{max} occurs for small and large shrimp. Central estimates, lower, and upper bounds for the minimum absolute aerobic scope (AAS_{min}) and the temperature at which AAS_{min} occurs, and the median and 95% CIs for the temperature at which shrimp reach critical thermal maximum (CT_{max}) for small and large shrimp size classes. Statistical differences between size classes for endpoints are denoted with an asterisk (*).

Endpoint	**Small**		**Large**		**Sig.**
	Mean (SE)	**95% CI**	**Mean (SE)**	**95% CI**	
RMR_{peak} (mL O_2/gWW/h)	0.65 (0.04)	0.57–0.74	0.36 (0.01)	0.34–0.38	*
Temp. at RMR_{peak} (ºC)	39.02 (0.94)	37.18–40.86	37.70 (0.46)	36.81–38.60	
ETS_{max} (mL O_2/gWW/h)	2.43 (0.07)	2.30–2.56	1.26 (0.03)	1.20–1.31	*
Temp. at ETS_{max} (ºC)	22.92 (0.51)	21.93–23.92	25.12 (0.29)	24.56–25.69	*
	Central estimate	**Lower and upper bounds**	**Central estimate**	**Lower and upper bounds**	
AAS_{min} (mL O_2/gWW/h)	0.16	0.0003–0.34	0.28	0.21–0.35	N.A.
Temp. at AAS_{min} (ºC)	39.56	39.14–39.49	38.59	38.65–38.53	N.A.
	Median	**95% CI**	**Median**	**95% CI**	
CT_{max} (ºC)	40.60	40.10–41.50	38.95	38.2-39.8	*

Figures

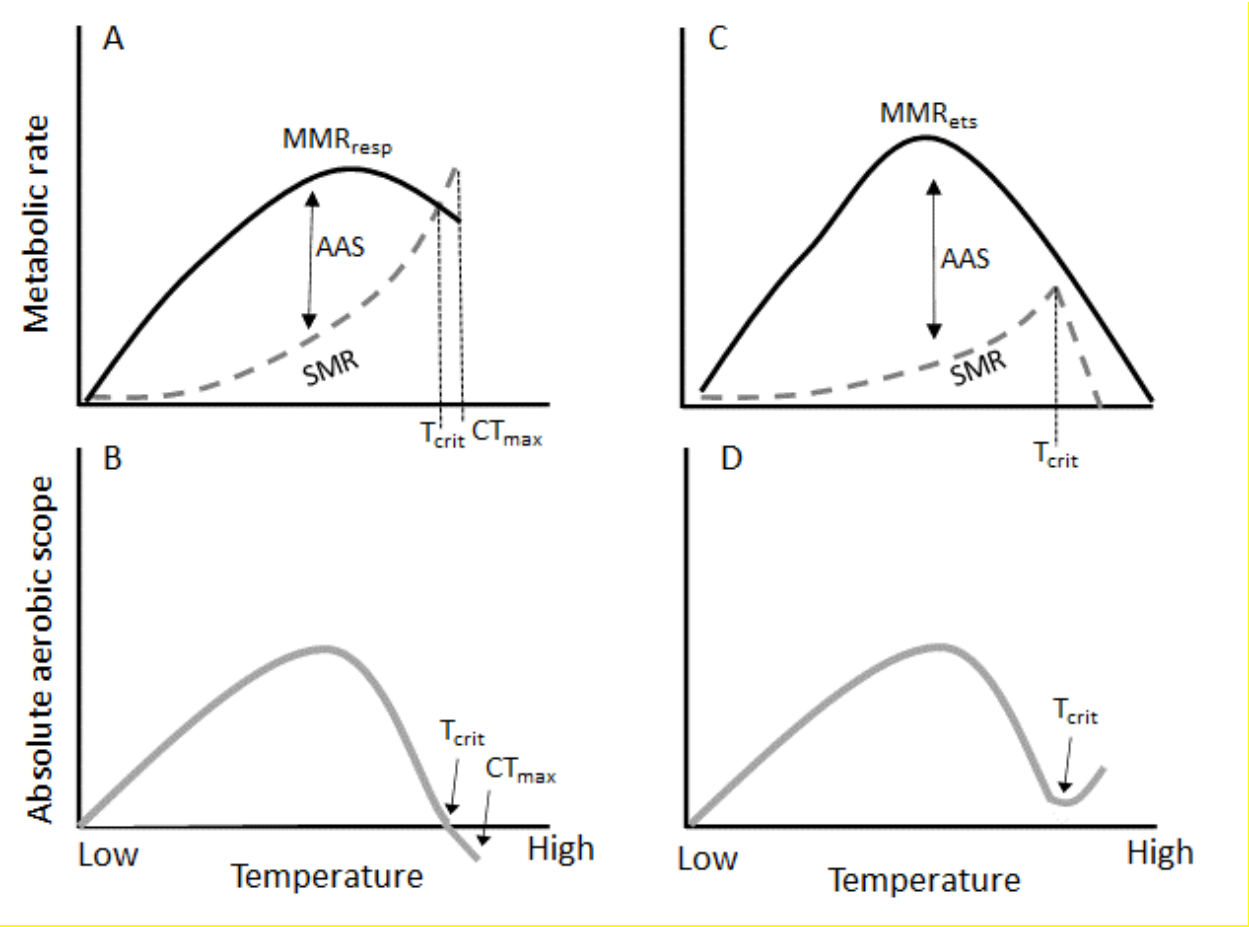

Figure 1. A) Theoretical relationships between MMR as measured by respirometry (MMR_{resp}), SMR, and temperature as modified from Verberk et al. (2016) and Ern (2019) under normoxic conditions. Absolute aerobic scope (AAS) is the difference between MMR_{resp} and SMR. The critical temperature (T_{crit}) indicates the temperature at which MMR_{resp} = SMR and CT_{max} indicates the temperature at which loss of equilibrium occurs due to insufficient oxygen supply and insufficient anaerobic capacity. B) The relationship between AAS and temperature when using MMR_{resp} to calculate AAS. C) Hypothesized relationship between MMR_{ets}, RMR, and temperature in this study when using the ETS assay to estimate MMR, when using RMR as a proxy for SMR, and when measuring RMR past the point of RMR_{peak}. D) The hypothesized relationship between AAS and temperature in this study where AAS does not necessarily decline to zero.

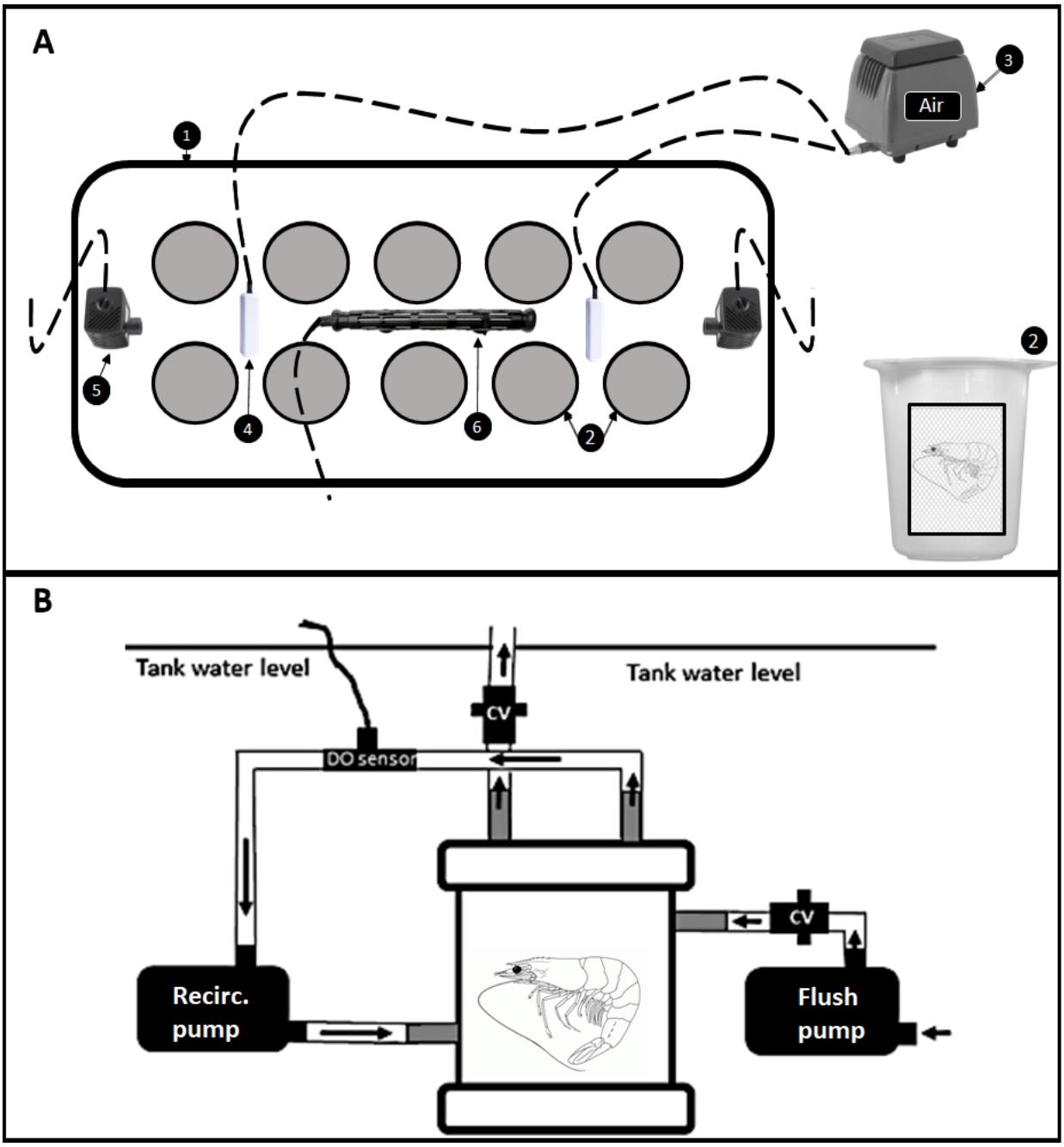

Figure 2. A) A general diagram of the tank for critical thermal maximum exposure (1) with 1-L mesh-sided beakers containing one experimental animal per beaker (2), an air pump (3) with airstones (4), pumps for water circulation (5) and an aquarium heater (6). B) A diagram of the intermittent respirometry system adapted from Haney et al., (2020) where shrimp were placed within an acrylic chamber and a flush pump pumped normoxic water through the chambers during flush cycles and the recirculating (recirc.) pump circulated water through the chamber during measure cycles. An optical dissolved oxygen (DO) sensor within the recirculation tubing measures oxygen and check valves (CV) to ensure water outside water and air do not enter the chamber when the flush pump is off. Arrows indicate the direction of water flow.

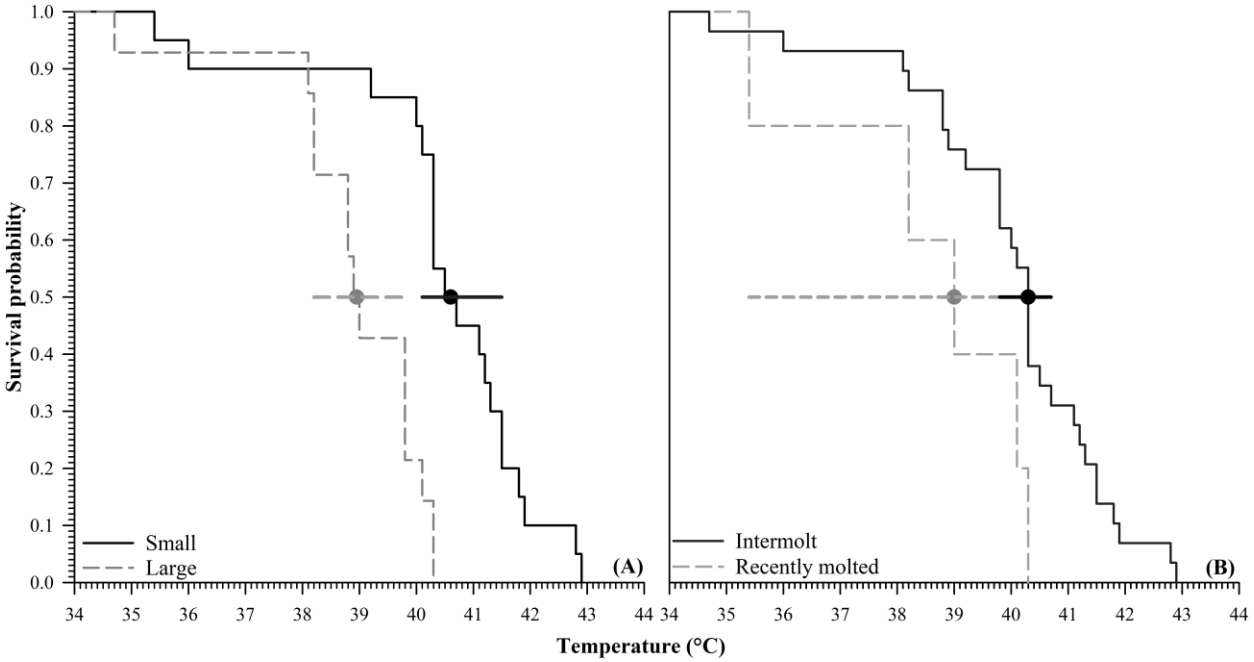

Figure 3. A) Comparison between the critical thermal maximum (CT_{max}) values for small and large shrimp size classes regardless of molt status, B) comparison between the CT_{max} values for premolt (recently molted) and intermolt regardless of shrimp size. Filled circles represent CT_{max} median estimates and horizontal error bars represent 95% CI.

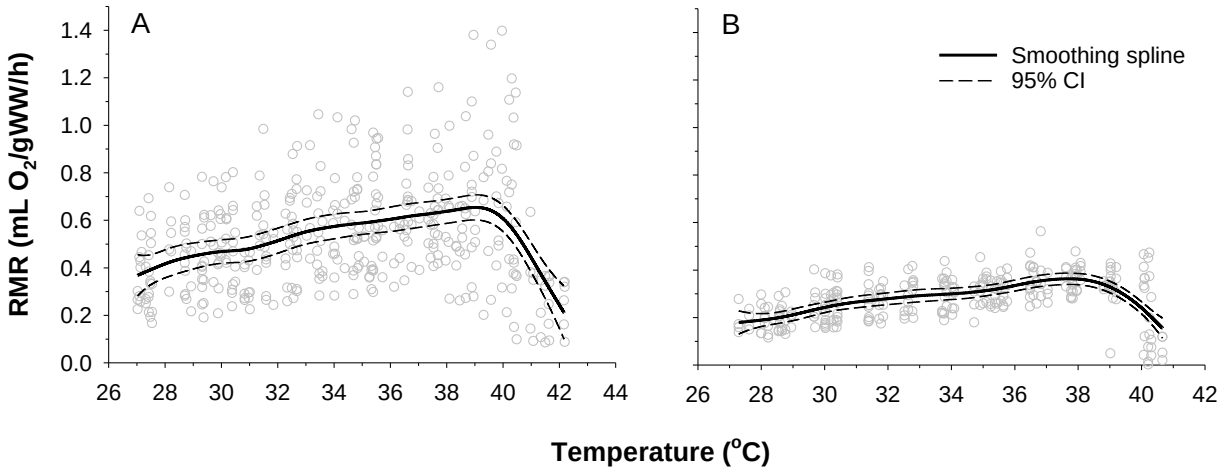

Figure 4. Relationship between individual resting metabolic rate (RMR) and temperature for small-size class shrimp (A) and large-size class shrimp (B) using a smoothing spline model and 95% confidence intervals (95% CI).

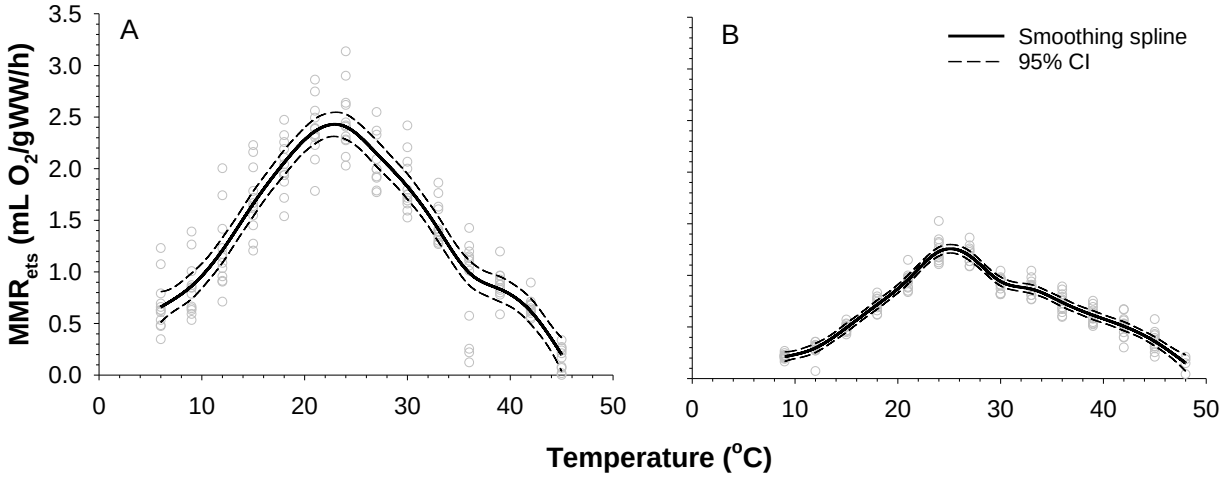

Figure 5. Relationship between MMR_{ets} and temperature for the small-size class shrimp (A) and large size class shrimp (B) using a smoothing spline model and 95% confidence intervals (95% CI).

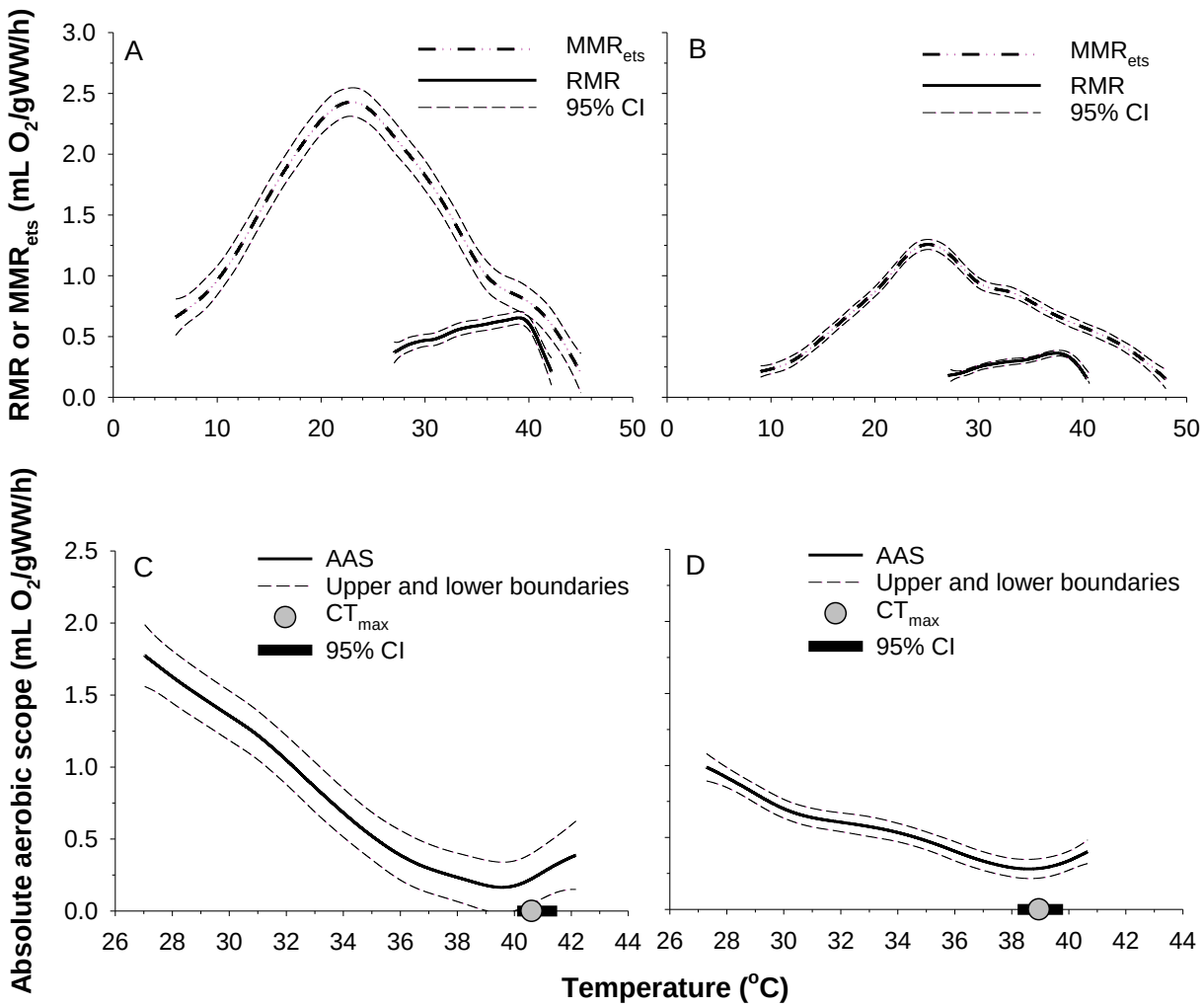

Figure 6. Relationships between ETS activity, resting metabolic rate (RMR), and temperature for (A) small and (B) large-size classes of shrimp. Relationships between absolute aerobic scope, CT_{max}, and temperature for (C) small- and (D) large-size classes of shrimp. Gray circles and solid lines on the X-axes indicate the median and 95% confidence intervals (CI) for CT_{max}.

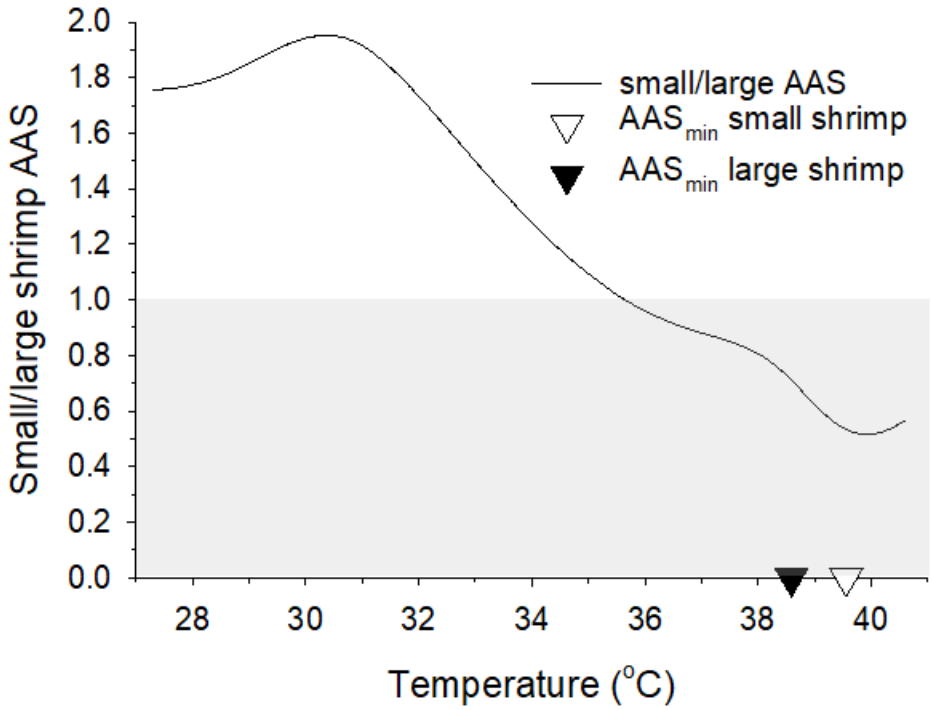

Figure 7. Changes in the ratio of small: large shrimp absolute aerobic scope (AAS) with increasing temperature. Black and white triangles on X-axis indicate temperatures where large and small shrimp exhibited AAS_{min}, respectively. Gray region of the graph indicates the region within which small shrimp AAS is equal to or lower than large shrimp AAS.

CHAPTER 6

SUMMARY AND CONCLUSION

A considerable interest in culturing Pacific white shrimp exists far from coastal areas either in inland ponds filled with low-salinity well water (2–5 g/L), or in indoor recirculating aquaculture systems (RAS). However, shrimp farming still has critical limiting factors regarding expansion, such as the use of large quantities of water and the potential deterioration of water quality. Moreover, the high price of shrimp feeds and cost of maintaining proper water quality represents a considerable financial burden making the profit margins much less robust. Biofloc technology (BFT) in its various types, has been known to be a realistic solution for efficiently managing water quality with low or no water exchange, enhancing shrimp growth performance and establishing an efficient and healthy shrimp culture with a better food conversion ratio in the shrimp aquaculture business. Hence, the promotion and optimization of these technologies could further advance farming techniques for shrimp.

The current line of research was designed to evaluate the effects of applying different management strategies for the improvement of BFT for the culture of shrimp. Towards this goal we evaluated the use of probiotics, biofloc and synbiotic type systems, alternative feeds, and evaluated the upper lethal limit of shrimp. Commercial probiotic products were used as a feed supplement and a water additive to evaluate shrimp nursery culture performance. All probiotic treatments had fast growth and high survival with no differences noted between the different treatments under nursery conditions. In a grow-out trial with juvenile shrimp, biofloc and synbiotic type systems were applied, and nonspecific immune response and growth were evaluated. All treatments had high growth performance with the biofloc treatment producing the highest biomass and lowest FCR. Total haemocyte count (THC) was highest in shrimp reared in biofloc and synbiotic treatments as compared with a reference “control”, however, there were no statistical differences between treatments. Demonstrating that these technologies may have benefits other than simply controlling water quality.

To evaluate shrimp growth fed with different feed type, a grow-out study was conducted using four different protein-based extruded diets (plant-based, 8% poultry by-product meal, 8% fishmeal and 12% fishmeal) while cultured in clear water and biofloc type systems. The low inclusion of fishmeal, as well as the use of alternative protein sources in these diets, did not adversely affect the final weight,

weight gain, and percent weight gain of Pacific white shrimp. It was noted that the biofloc system produced higher biomass than the clear water system and this could be attributed to the presence of natural productivity. Biofloc technology is known to make it possible to minimize water exchange and usage in aquaculture systems through maintaining adequate water quality within the culture unit, while producing low-cost protein-rich flocs (which can serve as feed for aquatic organisms). Overall, all nursery and grow-out trials produced good survival, rapid growth, low FCR and physiological parameters indicating that all are viable options. Therefore, the current study suggests that the use of low-level alternative protein diets can be used in favor of carbohydrate application to the water without compromising shrimp production which makes farming more economically viable.

Under commercial shrimp culture, farmers have been facing late-term shrimp mortality in inland, low-salinity ponds. To understand the physiological mechanisms behind upper lethal limits, a study of linkages between empirically measured thermal limits and absolute aerobic scope (AAS), or ability to provide energy above that needed for basic maintenance was investigated. At the conclusion of that study, small shrimp (2.07 ± 0.86g) had a higher critical thermal maximum (CT_{max}) than large shrimp (24.64 ± 2.55 g), with upper lethal limits of 40.6 and 39.0 °C, respectively. AAS reached its minimum (AAS_{min}) at temperatures within 2 °C of CT_{max} for both size-classes. Reductions in AAS appear to be one of the underlying physiological drivers of thermal tolerance in *L. vannamei* and an indicator of increasing thermal stress. Changes in the temperature at which AAS reaches its minimum may be a useful predictor of shifts in thermal tolerance among shrimp size-classes. This study suggests having an indoor system (biofloc system) aside from pond culture that could be used for PLs nursery phase and/ or additional grow-out production system using intensive or super-intensive shrimp culture. Additionally, it's critical to monitor water temperature throughout the cycle and be prepared to have an early harvest plan when shrimp are 18 g (more shrimp per pound but smaller size) to avoid late-term mortality. Lastly, different hatcheries have different genetic stocks, so it’s important to select family lines at these commercial hatcheries for characteristics such as growth.

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