NUCLEOTIDE NUTRITION OF HYBRID STRIPED BASS, *Morone saxatilis* × *Morone chrysops*: EFFECTS ON GROWTH PERFORMANCE, NUTRIENT DIGESTIBILITY, DIGESTIVE ENZYMES, AND IMMUNE SYSTEM MODULATION AFTER ACUTE AND CHRONIC STRESS

A Dissertation

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ABSTRACT

Several feeding trials were conducted with juvenile hybrid striped (HSB) to determine potential effects of supplementing purified dietary nucleotides. The basal diet in these trials was formulated to contain approximately 40% protein and 10% lipid. Moreover, the nucleotide supplements were added to the basal diet at the expense of cellulose. Adenosine monophosphate (AMP), uridine monophosphate (UMP), cytidine monophosphate (CMP), guanosine monophosphate (GMP), and inosine monophosphate (IMP) were individually supplemented at 0.5% of diet and evaluated in a 9-week trial. Results showed significant enhancement of weight gain and innate immunity of fish fed the AMP-supplemented diet compared to those fed the basal diet.

Furthermore, the effects of dietary AMP and IMP, each at 0.5% of diet, or combinations of both (equaling 1% of diet) were evaluated in a 9-week trial. No significant effects of AMP or IMP were observed on growth performance, whole-body composition or innate immunity; however, fish fed IMP had significant enhancement of lymphocyte proliferation compared to fish fed the basal diet. These dietary treatments were retained and evaluated for immune modulation prior to and after acute-stress challenge. Result indicated some of the dietary nucleotides provided significant enhancement of innate immunity at 0.5 h and 12 h post stress challenge, imposed by 1-minute air exposure, compared to those fed the basal diet. Supplementation of AMP at 0.5% of diet provided the greatest enhancement of innate immunity during post stress.

The minimum dietary AMP requirement was estimated based on weight gain responses in a two-slope broken-line model to be 0.5% of diet in a dose-response 8-week trial. Therefore, the AMP diet supplemented at 0.5% of dry weight was evaluated against the basal diet in four other trials. Dietary supplementation of AMP significantly improved apparent digestibility coefficients for organic matter and energy after a 4-week trial, trypsin enzymatic activity after a 8-week trial, innate immunity after a 6-week trial and acute stress challenge (air exposure), as reflected in anti-protease activity at 0.5 and 1 h after stress, and elevated plasma lysozyme and anti-protease activity after fish were subjected to chronic stress of high salinity for 4 weeks. Based on results from these various trials, this study demonstrated that an exogenous supply of AMP at 0.5% of diet was able to modulate immune responses under stressful conditions, and to a limited extent, improve growth performance, and nutrient digestibility of hybrid striped bass.

DEDICATION

This dissertation is dedicated to my mother, family members, and sweetheart. Their continuous love and support have kept me going.

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NOMENCLATURE

ANOVA	Analysis of Variance
AMP	Adenosine Monophosphate
IMP	Inosine Monophosphate
UMP	Uridine Monophosphate
GMP	Guanosine Monophosphate
CMP	Cytidine Monophosphate
FER	Feed Efficiency Ratio
PRE	Protein Retention Efficiency
LRE	Lipid Retention Efficiency
HSI	Hepatosomatic Index
IPF	Intraperitoneal Fat
K	Fulton's Condition Factor
ADC	Apparent Digestibility Coefficient
LPS	Lipopolysaccharide
C. Impex	Chem-Impex International
Sig.	Sigma
L	Linear
Q	Quadratic
NOS	No structure
SBL	Slope Broken Line

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CHAPTER I

INTRODUCTION AND RESEARCH GOAL

I.1 Introduction and justification

Nucleotide supplementation of diets for fish produced in aquaculture has received heightened worldwide attention over the last two decades. Aquaculture production has intensified in recent years resulting in increased demand for aquafeeds and many of the ingredients included in aquafeeds. In particular, marine ingredients such as fishmeal (FM) and fish oil have increased in price due to their rising demand but limited supplies. Many strategies have been implemented to reduce costs of aquafeeds (NRC, 2011). Replacement of FM with plant protein (PP) ingredients has been reported in many studies to reduce dependency on FM and also reduce the cost of aquafeeds (Bendiksen et al., 2011; Minjarez-Osorio et al., 2016). However, some PP ingredients have disadvantages including reduced palatability and the presence of anti-nutritional factors that may reduce the bioavailability of nutrients, thereby affecting the growth of the fish (Imorou et al., 2008). According to Gatlin et al. (2007), the use of PP feedstuffs to replace FM in the diet of carnivorous fish species may result in negative effects on the target organism. An alternative approach to combat poor performance of fish fed diets in which FM is replaced with certain PP ingredients is to supplement those diets with additives to provide nutrients or other constituents normally found in FM.

Feed additives in the strictest sense have been defined as non-nutritive ingredients that are included in the diet which may physically or chemically have

influences on the properties of the diet or affect target organism performance or quality of the resulting product (Barrows and Hardy, 2000). Numerous studies have demonstrated that various feed additives such as antioxidants, enzymes, organic acids, immunostimulants, prebiotics, probiotics, and nucleotides can improve the overall performance of various cultured fish and shrimp (Fuchs et al., 2015; Chakraborty and Hancz, 2011; NRC, 2011). It is well documented that PP ingredients such as soybean meal contain fewer nucleotides than FM (Mateo et al., 2004); therefore, supplementation of dietary nucleotides could be considered an important approach to compensate for the lack of such compounds in diets with PP ingredients replacing FM.

Nucleotides are monomers of nucleic acids. There are three parts in a single nucleotide that are covalently bonded together: a nitrogen base, a sugar base, and a phosphoric acid residue (Campbell and Farrell, 2011). The nucleobases consist of two types which are classified as either pyrimidines (cytosine, thymine, and uracil) or purines (adenine and guanine). Additionally, there are other bases that are found principally, but not exclusively in nucleotides such as hypoxanthine, N6-Dimethyladenine, 5-Methylcytosine, and 5, 6-Dihydrouracil (Campbell and Farrell, 2011). A nucleoside is different from a nucleotide because it does not have a phosphate group. It is formed from a base and a sugar through a glycosidic linkage from the N-l nitrogen of a pyrimidine or the N-9 of a purine and the C-l' carbon of the sugar (Rudolph, 1994). A nucleotide is formed when a phosphoric acid is esterified to one of the sugar hydroxyls of a nucleoside (Campbell and Farrell, 2011).

The potential benefits of nucleotide supplementation were reported over two decades ago; however, nucleotides are not considered essential given that *de novo* synthesis and salvage pathways exist in animals and plants (Cosgrove, 1998; Gil, 2002). However, during extraordinary stressful events, sufficient amounts of nucleotides may not be synthesized (Devresse, 2000). It is known in aquacultural production that the cultured organism may undergo stress associated with physiological responses including rapid growth, reproduction, resisting disease as well as recovering from injury. In addition, environmental changes, marginal water quality, and stress from confinement and handling are commonly encountered in aquaculture. These stressful conditions may require that the cultured organism be provided with additional nucleotides to be readily available for cell proliferation (Hoffmann, 2007). The essentiality of ribonucleotides is more apparent under these conditions. Also, *de novo* synthesis and salvage of nucleotides are metabolically costly processes; therefore, a supplemental source of exogenous nucleotides in the diet may enhance the functions of rapidly dividing tissues, principally when growth is rapid (Sanderson and He, 1994).

Exogenous nucleotides are commonly distributed in food especially those containing cellular elements and nucleoproteins such as animal tissues including organ meats and seafood (Kojima, 1994; Mateo et al., 2004). Dietary supplementation of nucleotides was not particularly relevant a few decades ago in aquaculture because most of the aquafeeds at that time had relatively high inclusion of FM which is a rich source of nucleotides (Mateo et al., 2004). Nonetheless, because FM is being increasingly replaced with alternative feedstuffs which have a lower concentration of nucleotides (Table 1);

therefore, supplemental sources of exogenous nucleotides in diets could be as important as supplementation of essential nutrients.

Table 1. Nucleotide concentration in commonly used animal feed ingredients (as-fed basis)[1].

Ingredient	Nucleotide (mg/g)				
	5'CMP	5'AMP	5'GMP	5'UMP	5'IMP
Barley	0.002	0.001	0.001	0.000	0.001
Corn	0.003	0.002	0.003	0.000	0.001
Fish meal	0.026	0.011	0.002	0.001	0.035
Naked oats	0.003	0.003	0.003	0.001	0.001
Soybean meal, 44%	0.016	0.008	0.003	0.009	0.002
Soy protein concentrate	0.000	0.001	0.002	0.000	0.001
Whey, dried	0.270	0.019	0.000	0001	0.004

[1]Data adapted from Mateo and Stein (2004)

In comparison to animal tissues, single cell proteins have up to seven-fold the concentration of nucleic acids, and thus products such as yeast provide a good source of nucleotides (Ingledew, 1999). Commercial brewer's yeast (*Saccharomyces cerevisiae*) is inactive yeast remaining after the brewing process which has been used widely for commercial production of supplemental nucleotides (Ferreira et al., 2010).

One of the earliest works on dietary nucleotide supplementation in fish nutrition was reported by Ramadan et al. (1991 and 1994) who demonstrated that supplementation

of the commercial nucleotides Ascogen P® could improve growth performance and survival of hybrid tilapia. After a decade, Burrells et al. (2001a) reported the inclusion of exogenous nucleotides in salmonid diets increased disease resistance to challenges from viral and rickettsial diseases, as well as ectoparasitic infestation.

Supplementation of nucleotides in fish diets has received considerable amounts of attention in recent years, and the current knowledge of using such additives and their effects on fish have been widely reviewed (Li and Gatlin, 2006). Some of the recent studies have focused on commercial nucleotide products such as AccelerAid® and NuPro®. Berto et al. (2016) demonstrated that inclusion up to 8% of NuPro® in tilapia diets resulted in increased weight gain and feed efficiency. On the other hand, inclusion of 0.05-0.4% of AccelerAid® in tilapia diets did not have any significant effects on growth performance or immunological responses (Barros et al., 2015). A potential limitation of using these commercial products is that the concentrations and types of nucleotides present in such products are typically not disclosed due to trade secret. However, the efficacy of nucleotides is determined by the type and concentration of nucleotides incorporated in the diet (Hossain et al., 2016a; Song et al., 2012). Many studies have reported recommended nucleotide dosages between 0.2 and 0.5% of dry weight in the fish diet. Furthermore, more recent studies have indicated that, instead of using combinations of nucleotides, a single nucleotide supplementation may be more effective and sufficient to improve growth response, stress tolerance and immune responses (Hossain et al., 2016a; Song et al., 2012). To date, only one study (Li et al.,

2004) have evaluated the efficacy of using commercial dietary nucleotide in hybrid striped bass.

Hybrid striped bass (*Morone saxatili*s x *Morone chrysops*), a widely known and prized sports fish, is a commonly cultured carnivorous species (Garber and Sullivan, 2006). It has been established that hybrid striped bass can digest diets high in PP such as soybean meal without significant decreases in overall growth performance (Gallagher, 1994; Keembiyehetty and Gatlin, 1993; Savolainen and Gatlin, 2010). However, the susceptibility of diseases due to inclusion of high PP levels in the diet of hybrid striped bass is yet to be studied. According to Li et al. (2004), supplementing the nucleotide product Ascogen P® to hybrid striped bass diets resulted in a significant increase in innate immune responses and also survival after exposure to *Streptococcus iniae*.

The hybrid striped bass is produced in aquaculture for both stock enhancement and food production. Many aquaculture practices including seining, handling, crowding, grading, confinement, and transport have been established as stressors to fish (Wendelaar Bonga, 1997; Davis, 2004). Stress-induced by these practices has been long suspected of causing delayed mortalities and immunosuppression. Stress may be defined as a change in biological condition beyond the normal resting state that challenges homeostasis and, consequently, presents a threat to the fish's health (Barton and Iwama, 1991). The stress response is characterized by disruption of biochemistry and physiology, which may have effects on the non-specific immune response and can continue for hours or days. The primary immune response, also known as the adaptive response, enables fish to cope with stressful conditions when imposed on them by

maintaining homeostasis, which includes a rapid change in plasma catecholamines and corticosteroids (Mazeaud et al., 1977; Barton and Iwama, 1991; Barnett and Pankhurst, 1998). Conversely, when the response is forced beyond its normal limits, it leads to negative secondary effects such as increased blood glucose, lactic acid, free fatty acids as well as immunosuppression and osmotic disturbance (Mazeaud et al., 1977). Stress-related immunosuppression has been reported to be one of the leading causes of decreased disease resistance in aquaculture (Li and Gatlin, 2006). There are several studies reporting that supplementation of nucleotides in fish diets is able to mitigate stress by improving stress tolerance (Tahmasebi-Kohyani et al., 2011; Welker et al., 2011; Hossain et al., 2016a); however, the effects of nucleotide supplementation on stress responses of hybrid striped bass have not been reported. Also, there are a few recent studies reporting that nucleotide supplementation to fish diets improved digestive enzyme activity (Tahmasebi-Kohyani et al., 2011; Guo et al., 2019)

I.2 Goal and research objectives

The ultimate goal of this study was to identify the most effective type of nucleotide as well as establishing the optimal dose to incorporate in a practical hybrid striped bass diet. This assessment was based on the effects of dietary nucleotide treatments on growth performance, adaptive and innate immune responses, immune modulation during acute and chronic stress, as well as nutrient digestibility and digestive enzyme activity. Thus, the following objectives were investigated:

1) Evaluate the effects of singularly incorporating adenosine 5'-monophosphate (AMP), guanosine 5'-monophosphate (GMP), uridine 5'-monophosphate (UMP), inosine 5'-monophosphate (IMP), and cytidine 5'-monophosphate (CMP) at 0.5% by weight in plant protein (PP)-based diets for hybrid striped bass. The dosage was chosen at 0.5% based on existing literature in which the recommended range of dosage was 0.2%-0.5% for various species.

2) Evaluate the potential synergistic effects of AMP and IMP as feed additives in PP-based diets for hybrid striped bass (based on the objective 1 results, which showed that AMP and IMP nucleotides were the most beneficial).

3) Establish the minimum effective dosage of AMP (based on the objective 2 results, which showed that AMP was the most beneficial) in a PP-based diet for hybrid striped bass. Validate the effectiveness of using the optimal dose of AMP in PP-based diet against acute and chronic stress and compare to fish fed a basal diet without any supplementation.

CHAPTER II

EFFICACY OF PURIFIED NUCLEOTIDE SUPPLEMENTS ON THE GROWTH PERFORMANCE AND IMMUNITY OF HYBRID STRIPED BASS

II.1 Introduction

The hybrid striped bass is a carnivorous fish that is cultured in the United States for food production, sport fishing and stock enhancement programs (Smith et al., 1986; Schramm Jr. et al., 1991; Garber and Sullivan, 2006). Diet formulations for this fish have traditionally contained relatively high levels of fishmeal although various other protein feedstuffs including those of plant origin have been incorporated at relatively high levels (reference specifically on HSB nutrition; NRC, 2011). Fishmeal is one of the major feed ingredients in aquafeeds that is a dense source of protein and energy, which is preferentially utilized by carnivorous fish species. It also is an ingredient that has become increasingly more expensive as its demand has risen with the growth of commercial aquaculture. That has prompted various efforts to find less expensive and readily available alternative protein feedstuffs. For example, numerous alternative ingredients have been shown to partially replace fishmeal in diets for hybrid striped bass including various plant and animal feedstuffs such as soybean meal (Gallagher, 1994; Webster et al., 1997), meat and bone meal (Bharadwaj et al., 2002), poultry by-product meal (Rawles et al., 2006a), and microalgae concentrates (de Cruz et al., 2018; Perez-Velazquez et al., 2019). These feedstuff substitutions for fishmeal have become more feasible as the requirements of hybrid striped bass for specific nutrients such as amino

acids have become established over the years (NRC, 2011). However, fishmeal also is a rich source of other constituents such as nucleotides which have numerous physiological functions but whose concentration may be limited in other feedstuffs, especially those of plant origin (Li et al., 2015).

Nucleotides are monomers of nucleic acids that play numerous roles in important physiologic and biochemical processes including encoding genetic information and mediating energy metabolism and signal transduction (Carver and Walker, 1995). These biochemical are not considered essential nutrients given that *de novo* synthesis from amino acids and salvage pathways from the breakdown of nucleotides exist in animals and plants (Grimble and Westwood, 2001). However, during extraordinary stressful events such as rapid growth, reproduction, resisting disease as well as recovering from injury, sufficient amounts of nucleotides may not be synthesized (Devresse, 2000; Gil, 2002) in which dietary supplementation of nucleotides can positively influence various responses, thereby characterizing them as semi-essential nutrients.

Supplementation of nucleotides in fish diets has been reported to have various positive effects including boosting innate and adaptive immune responses of fish (Ramadan et al., 1994; Reda et al., 2018), enhancing the resistance to bacterial and viral infections (Burrells et al., 2001a), mediating stressors (Kenari et al., 2013), improving osmoregulation (Burrells et al., 2001b), up-regulating genes related to immune responses (Low et al., 2003; Guo et al., 2019) and improving growth responses of various fish species(Hossain et al., 2016a; Meng et al., 2017). Most studies concerning nucleotides during the late 90s and early 2000 focused on the efficacy of commercial nucleotide

products on fish health and immunity (Ramadan et al., 1994; Adamek et al., 1996; Burrells et al., 2001a; Burrells et al., 2001b; Low et al., 2003; Li et al., 2004; Li et al., 2005; Russo et al., 2006). However, many of the underlying mechanisms involving the functionality of nucleotides in fish were not well understood. In addition, a potential limitation of using these commercial products was the types and concentrations of nucleotides and other constituents present in such products were typically not disclosed due to trade secret. Therefore, more recent studies have focused on known single nucleotides or nucleotide mixtures to provide a better understanding on how these compounds influence numerous physiological responses of various fish species (Li et al., 2007; Song et al., 2012; Hossain et al., 2016a; Guo et al., 2017; Guo et al., 2019)

It is well known that aquaculture is moving towards more economically and environmentally sustainable methods of farming. Therefore, using nucleotides as feed additives due to their potential immunomodulatory effects (Bricknell and Dalmo, 2005; Ringø et al., 2012) could reduce or even prevent the inappropriate use of antibiotics that can cause pathogen resistance, antibiotics residue and eradication of normal or beneficial microbiota of fish (Alderman and Hastings, 1998; Dawood et al., 2018). To date, only one study has been conducted with hybrid striped bass using a commercial nucleotide product which was shown to positively influence immune responses and bacterial resistance of juvenile fish (Li et al., 2004). The effects of purified nucleotide on the growth and health of hybrid striped bass have not been explored to date. Thus, the objectives of this study were to evaluate the use of several single purified nucleotides on the growth performance, as well as innate and adaptive immunity of hybrid striped bass.

II.2 Materials and methods

II.2.1 Experimental design and diet formulations

The basal diet, which utilized menhaden fishmeal (25%) and soybean meal (75%) as protein sources, was formulated to contain 44% crude protein, 10% lipid and an estimated digestible energy level of 3.5 kcal/g (Table 2). All diets were kept isonitrogenous and isocaloric, and satisfied and/or exceeded all known nutrient requirements of hybrid striped bass established in the NRC (2011). The basal diet without nucleotide supplementation was used as the negative control in this study. adenosine 5'-monophosphate (AMP), uridine 5'-monophosphate (UMP), cytidine 5'-monophosphate (CMP), guanosine 5'-monophosphate (GMP), and inosine 5'-monophosphate (IMP) disodium salts were obtained from Chem-Impex International (Wood Dale, Illinois, USA). Each nucleotide (AMP, UMP, CMP, GMP, or IMP) was added to the basal diet at 0.5% of dry weight at the expense of cellulose. This concentration was selected based the limited studies on other fish species which have recommended single purified nucleotide dosages between 0.1 and 0.6% of dry weight (Lin et al., 2009; Song et al., 2012; Hossain et al., 2016b; Hossain et al., 2017; Zhang et al., 2019). The nucleotide products in this study from Chem-Impex have not been evaluated in previous research; therefore, another positive control diet used in this study was AMP obtained from Sigma-Aldrich (St. Louis, Missouri, USA) that was added to the basal diet at 0.5% of dry weight. All dry ingredients were mixed in a V-mixer (Blendmaster Lab Blender; Patterson-Kelly, Stroudsburg, PA, USA) after which oil and water were sequentially mixed in an industrial mixer (Model A-200; Hobart, Troy, OH,)

Then, the mixture pressure pelleted using a commercial meat grinder attachment (Model A-200; Hobart, Troy, OH, USA) with 3-mm die plate. Then, the pellets were air-dried and broken into appropriate sizes to match the mouth gape of the fish. The pellets were stored at -20℃ until fed.

Table 2. Formulation and analyzed composition of the basal diet.[1]

Ingredients	%
Menhaden fishmeal[2]	14.70
Soybean meal[3]	58.33
Menhaden oil[4]	6.03
Vitamin premix[5,6]	3.00
Mineral premix[5,6]	4.00
Dextrinized starch[6]	9.00
Carboxymethyl cellulose[6]	2.00
Glycine[7]	1.00
Taurine[7]	0.43
DL-Methonine[7]	0.36
Cellulose[8]	1.10
Agar[9]	0.05
Analyzed composition, g/100 g[1]	
Crude protein	44.0
Crude lipid	10.2
Ash	10.9

[1] Dry-matter basis, means of two replicate analyses.
[2] Special Select, Omega Protein, Abbeville, Louisiana (crude protein [CP] = 680.3 g/kg; lipid = 117.9 g/kg on a dry-matter basis).
[3] Producers Cooperative Association, Bryan, Texas (CP = 514.3 g/kg; lipid = 38.4 g/kg on a dry-matter basis).
[4] Omega Protein, Reedville, Virginia
[5] Moon and Gatlin (1991).
[6] MP Biomedicals, Solon, Ohio.
[7] Ajinomoto North America, Inc.
[8] U.S. Biochemical Corp., Cleveland, Ohio.
[9] Sigma Aldrich Co., St. Louis, Missouri.

II.2.2 Facilities, feeding trial, and feeding regime

Juvenile hybrid striped bass (*Morone chrysops* x *M. saxatilis*) were acquired from Keo Fish Farms, Keo, Arkansas, USA. The feeding trial was conducted at the Texas A&M University Aquacultural Research and Teaching Facility. Animal care and experimental protocols were permitted by the Institutional Animal Care and Use Committee at Texas A&M University. The study was conducted in a recirculation system consisting of 110-L glass aquaria with a settling chamber, biofilter, UV sterilizer and sand filter. Salinity was generated by mixing Red Sea Salt (Red Sea, Houston, Texas) with well water. Water temperature was maintained at 27 ± 0.6℃ by conditioning ambient air. Other water quality parameters such as dissolved oxygen, total ammonia nitrogen, and nitrite were monitored throughout the experiment and were maintained at acceptable ranges for hybrid striped bass culture. Water temperature and dissolved oxygen were measured using a YSI ProODO meter (YSI Inc., Yellow Springs, OH, USA), and total ammonia nitrogen (TAN) and nitrite were measured spectrophotometrically (Hach Inc, Loveland, CO, USA). The photoperiod was set at 12h light-12h dark by controlling lights on automatic timers.

The fishes were acclimated to the system for 2 weeks prior to initiation of the feeding trial. Groups of 14 hybrid striped bass fingerlings with an average initial weight of 5.6 ± 0.1 g were assigned randomly to each of 21 aquaria (7 treatments × 3 replicates × 14 individuals per aquarium). All diets were fed twice daily at a rate approaching apparent satiation with pre-weighed rations. Feeding rate was initially 6% of total body weight per day and progressively reduced equally for all diets overtime to keep a level

close to apparent satiation without overfeeding. Fish in each aquarium were group-weighed every week and feed rations were adjusted accordingly. At the end of 9 weeks, the experiment was terminated and the fish fasted for approximately 15h prior to obtaining terminal samples.

II.2.3 Sample collection and compositional analysis

At termination of the feeding trial, percentage weight gain (WG) = ([g final weight − initial weight]/g initial weight) × 100; condition factor (K) = (g final weight × 100)/total length^3; feed intake (FI) = g dry feed consumed /fish; feed efficiency ratio (FER) = g weight gain /g dry feed offered; and survival = (final no. of organisms/initial no. of organisms) × 100 were computed. Then, three fish per aquarium were randomly selected and euthanized with an overdose of tricainemethane sulphonate (300 mg/L) (MS-222, Western Chemical Inc, Ferndale, WA, USA) to measure the following body condition indices: hepatosomatic index (HSI) = g liver weight /100 g body weight; intraperitoneal fat (IPF) ratio = g IPF weight /100 g body weight; and muscle ratio = g fillet weight /100 g body weight. Additionally, three fish per aquarium also were collected and homogenized as a composite sample. These composite samples were analyzed for proximate composition using the following established methods: the Dumas protocol for crude protein (6.25 × N) (AOAC, 2005), chloroform: methanol extraction for crude lipid (Folch et al., 1957), and heating samples at 650 °C in the muffle furnace for 4 h for ash (AOAC, 1990). Protein conversion efficiency (([(g final

body weight × % final body protein) − (g initial body weight × % initial body protein)] / (protein intake (g)]) × 100) also was computed.

II.2.4 Sample collection and analysis for immune parameters

For blood and plasma analysis, three fish per replicate aquarium (n = 9 fish/treatment group) were bled from the caudal vasculature with heparinized 1-mL syringes. Blood neutrophil oxidative radical production was measured as described by Siwicki et al. (1994) whereby 50-µL aliquots of whole blood were mixed in a 96-well microtiter flat-bottom microplate (Falcon, Le Pont de Claix, France) with nitroblue tetrazolium (NBT, Sigma-Aldrich) solution for 30 min, after which formazan granules were suspended using N-N dimethylformamide (Sigma-Aldrich), and read in the spectrophotometer at 545 nm. Plasma was separated from whole blood by centrifugation at 3000 × *g* for 10 min and stored at -80°C prior to analysis. Plasma lysozyme activity was determined using a turbidimetric reduction assay described by Jørgensen et al. (1993) by using *Micrococcus lysodeikticus* (Sigma-Aldrich) suspension in 6.1 pH phosphate-buffered saline (PBS, Sigma-Aldrich). Other non-specific immune parameters such as total immunoglobulin (Ig) and total plasma protein were also determined as described by Siwicki et al. (1994).

For phagocyte isolation, head kidney and spleen from three fish per replicate aquarium were aseptically excised using scalpels and forceps, pooled and stored in 15 ml of cold Leibovitz cell culture medium (L-15, Corning, NY, USA) supplemented with 2% fetal bovine serum (FBS, Sigma-Aldrich), 10 units mL^{-1} of heparin

(Akron Biotechnology, Boca Raton, Florida), and 100 units mL^{-1} of penicillin and streptomycin (Sigma-Aldrich). The techniques established by Secombes (1990) and modified by Sealey and Gatlin (2002) was used to isolate the phagocytes. The isolated phagocytes were counted and their viability measured via trypan blue exclusion as described in Yamamoto et al. (2018). All isolated phagocytes had more than 95% survivability and the concentration was adjusted to 2×10^7 cells/ml by adding 0.1% L-15. Respiratory burst activity of phagocytes was measured by extracellular superoxide anion production as suggested by Secombes (1990). The concentration of extracellular O^{2-} produced was calculated as follows: extracellular superoxide anion nmol of anion superoxide O^{2-} = ([Δ Absorbance after 45 min×100] ÷ 6.3) Pick and Mizel (1981).

For specific immune functions, the lymphocytes were isolated from head kidney and spleen dissected from three fish per replicate aquarium following the procedures suggested by Secombes (1990) with minor modification as described by Carvalho et al. (2018). Briefly, the pooled tissue was mechanically disaggregated using a glass Potter-Elvehjem tissue grinder and filtered through a 100-mm nylon mesh. Then cell suspensions were diluted in 5 mL PBS and then layered over 4 mL of Lymphoprep™ (Cosmo Bio, USA). The layered cell suspension was centrifuged at 350 × *g* for 25 min at room temperature, and lymphocytes were recovered from the interface. The isolated lymphocytes were enumerated and their viability (≥95%) measured via trypan blue exclusion as described by Pohlenz et al. (2012). The final lymphocyte concentration for all replicate samples was adjusted to 2.5×10^6 cells mL^{-1} by adding 0.1% L-15.

The proliferation of lymphocytes upon stimulation of a non-specific mitogen, lipopolysaccharide solution (LPS, Sigma Aldrich, 1 mg mL^{-1}) was assessed using a colorimetric assay based on the tetrazolium salt MTT (3-(4,5-dimethyl-2thiazolyl)-2,5-diphenyl tetrazolium bromide) as recommended by Mosmann (1983). Finally, the lymphocyte proliferation capacity was computed and presented as stimulation index (SI = ABS stimulated cells ÷ ABS non-stimulated [control] cells).

II.2.5 Statistical analysis

Data were evaluated for normality using the Shapiro–Wilk test and differences among treatment means were analyzed using one-way ANOVA with significance set at $P<0.05$. The post hoc Duncan's multiple range test was used to identify mean differences. All statistical procedures were performed using Statistical Analysis System, version 9.4 (SAS Institute, North Carolina, USA)

II.3 Results

II.3.1 Growth performance

At the end of 9 weeks of feeding, the hybrid striped bass exhibited weight gain (WG) values ranging from 700 to 785% of initial weight, with significant ($P\leq 0.05$) differences observed among some treatments (Table 3). The highest WG was observed in fish fed the AMP Sigma diet but was not different from the AMP diet from Chem-Impex International. The other dietary treatments produced WG values that were not different from the basal diet based on the post-hoc test. The same trend was seen for

FER values which ranged from 0.86 to 0.89 which were close to statistically significant at P=0.052 (Table 3). Significant differences (P=0.016) was seen in FI but none of the nucleotide-supplement diets were significantly different from that of fish fed the basal diet (Table 3). Over the 9 weeks, fish survival was 100% except for fish fed with CMP dietary treatment had 2 dead fish due to the aerator in one of the replicate tank stop working for couple hours (Table 3).

Table 3. Growth performance of hybrid striped bass fed diets with different nucleotides for 9 weeks.[1]

Diet designation	Weight gain[2] (%)	FER	Survival (%)	Muscle ratio (%)	HSI (%)	IPF ratio (%)	K	FI (%)
Basal	716^{c}	0.86	100	37.6	1.66	4.20	1.38	47.9^{ab}
AMP[3]	760^{ab}	0.89	100	37.7	1.47	4.31	1.36	48.6^{ab}
UMP[4]	716^{c}	0.86	100	37.5	1.48	3.59	1.37	46.5^{b}
CMP[5]	727^{bc}	0.87	95.2	38.3	1.42	3.61	1.38	47.0^{b}
GMP[6]	731^{bc}	0.87	100	37.5	1.50	4.57	1.40	46.6^{b}
IMP[7]	700^{c}	0.86	100	38.1	1.38	4.26	1.36	46.6^{b}
AMP Sigma[8]	785^{a}	0.89	100	38.2	1.37	3.90	1.41	49.7^{a}
Pr > F[9]	0.003	0.052	0.463	0.968	0.309	0.081	0.780	0.016
Pooled SE[10]	11.9	0.01	1.80	0.75	0.09	0.24	0.03	0.62

[1] Means of three replicates groups (n=3). Values within the same column with different letters are significantly different (P < 0.05). FER = feed efficiency ratio; HSI = hepatosomatic index; IPF = intraperitoneal fat; K = Fulton's condition factor; FI = feed intake.
[2] Gain in weight of fish initially averaging 5.6 ± 0.1 g/fish.
[3] Adenosine 5'-monophosphate (AMP), Chem-Implex International, Wood Dale, Illinois.
[4] Uridine 5'-monophosphate (UMP), Chem-Implex International, Wood Dale, Illinois.
[5] Cytidine 5'-monophosphate (CMP), Chem-Implex International, Wood Dale, Illinois.
[6] Guanosine 5'-monophosphate (GMP), Chem-Implex International, Wood Dale, Illinois.
[7] Inosine 5'-monophosphate (IMP), Chem-Implex International, Wood Dale, Illinois.
[8] Adenosine 5'-monophosphate (AMP Sigma), Sigma Aldrich Co., St. Louis, Missouri.
[9] Probability associated with the F statistic.
[10] Pooled standard error.

No significant differences were observed for condition indices including muscle ratio, HSI, IPF ratio and K factor (Table 3). In addition, no differences were observed in whole-body proximate composition and protein conversion efficiency of fish fed the various experimental diets (Table 4). Fish had no signs of ill health but towards the end of week 7, tiny red spots were noted on some fish all dietary treatments indicative of a mild *Mycobacterium* infection.

Table 4. Whole-body proximate composition[1] and protein conversion efficiency of hybrid striped bass at the end of the 9-week feeding trial.

Diet designation	Moisture	Protein	Lipid	Ash	Protein conversion efficiency
	%	%	%	%	%
Reference	67.2	19.4	9.02	5.03	38.3
AMP	67.5	18.8	9.11	5.20	37.6
UMP	67.8	18.8	8.66	5.07	37.5
CMP	68.1	19.1	8.37	5.14	38.0
GMP	66.8	18.8	9.85	5.13	37.4
IMP	68.0	18.8	9.05	5.00	37.3
AMP Sigma	68.3	18.5	8.55	4.90	37.7
Pr > F[2]	0.4746	0.3417	0.2238	0.9153	0.8401
Pooled SE[3]	0.54	0.24	0.39	0.18	0.56

[1]Fresh-weight basis.

[2] Probability associated with the F statistic.

[3]Pooled standard error.

Table 5. Innate immune responses of hybrid striped bass fed diets with different dietary nucleotides for 9 weeks.[1]

Diet designation	NBT[2]	Superoxide anion extra-cellular (nmol/well)	Lysozyme (units/ml)	Total Protein (mg/ml)	Total Immunoglobulin (mg/ml)
Reference	0.66[b]	2.77[c]	644	42.1	24.3
AMP	0.65[b]	3.00[c]	593	42.6	23.9
UMP	0.65[b]	2.87[c]	589	37.3	20.1
CMP	0.65[b]	3.10[bc]	693	43.2	25.0
GMP	0.65[b]	2.77[c]	585	40.2	21.7
IMP	0.65[b]	3.93[a]	585	39.6	22.6
AMP Sigma	0.69[a]	3.77[ab]	614	43.2	26.0
Pr > F[3]	0.0437	0.0148	0.4677	0.0562	0.0601
Pooled SE[4]	0.01	0.24	40.7	1.35	1.16

[1] Values are mean of three group of three fish (n=9). Values within the same column with different letters are significantly different ($P < 0.05$).

[2] Whole blood neutrophil oxidative radical production. Mean at OD 545.

[3] Probability associated with the F statistic.

[4] Pooled standard error.

II.3.2 Immune parameters

The blood neutrophil oxidative radical production values were significantly ($P \leq 0.05$) different with the highest values observed in fish fed the AMP Sigma diet (Table 5). Dietary IMP also enhanced the capacity of phagocytes to generate extracellular superoxide anion and was significantly higher than all dietary treatments except for the AMP Sigma diet (Table 5). No statistical differences due to nucleotide supplementation were observed for other innate immune parameters such as plasma lysozyme, total plasma protein and total immunoglobulin (Table 5). For the adaptive immune responses, lymphocyte proliferation prompted by the presence of lipopolysaccharides was significantly ($P \leq 0.05$) different among dietary treatments (Fig. 1). The highest simulation index was shown in fish fed the AMP Sigma diet with a value of 1.68 although none of the nucleotide-supplemented diets had values from that of fish fed the basal (Fig. 1).

II.4 Discussion

It is evident in the present study that exogenous supply of AMP nucleotide at 0.5% of dry weight significantly improved percentage weight gain (WG) of hybrid striped bass compared to the basal diet without supplementation. Similar findings also were reported in that supplementing 0.2% and 0.15% of AMP to *Pagrus major* and *Epinephelus malabaricus*, respectively, enhanced the growth rate of those fish species (Lin et al., 2009; Hossain et al., 2016a).

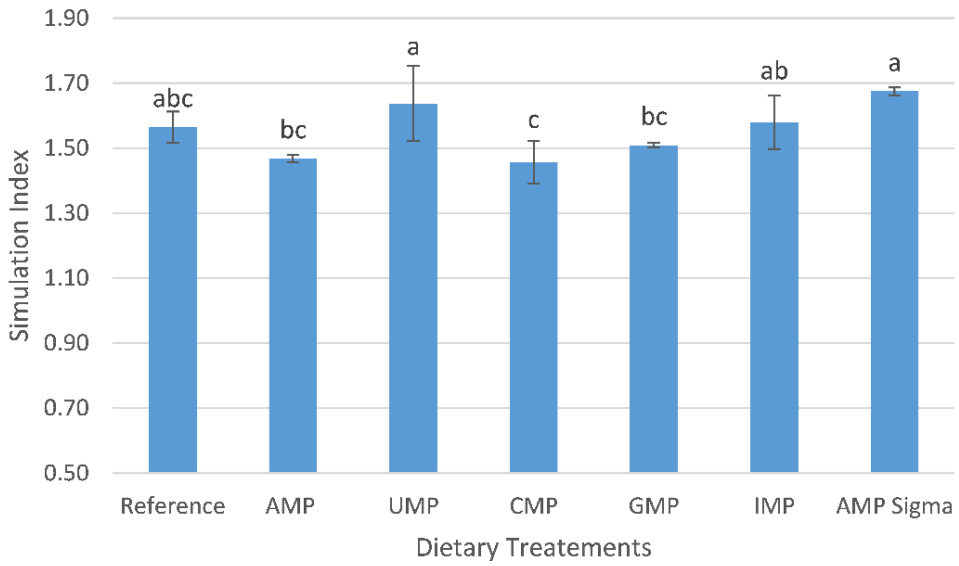


Figure 1. Stimulation index of hybrid striped bass lymphocytes isolated from head kidney and spleen of fish fed the various dietary treatments for 9 weeks. Values indicate the proliferation after stimulation with mitogen lipopolysaccharide from *E. coli* O26:B6.

The FER values in the present study were almost significant at P=0.052 and have a strong correlation with WG indicating that hybrid striped bass growth rate may be attributed to better feed conversion to body mass when fish were fed the AMP-supplemented diets. Another observation that should be highlighted in terms of growth performance in the present study was the feed intake of fish fed the basal diet was not significantly different from other dietary treatments even though the AMP treatment had significantly higher WG values than fish fed the basal diet. Hence, this contradicts findings that the exogenous supply of AMP to *Pagrus major* improved growth rate due to greater feed intake (Hossain et al., 2016a).

However, it is also well established that the effects of dietary nucleotides have not always been consistently observed among different fish species (Li and Gatlin III, 2006). According to Lin et al. (2009), supplementing single nucleotides such as IMP, GMP, UMP, and CMP at 0.15% significantly improved WG and FER of the grouper; however, this report also contradicts present study (Table 3). One possible explanation is that the diet used in that particular study was a purified nucleotide-free diet making nucleotides a very limiting nutrient. Consequently, this study raises the hypothesis that nucleotide supplemented in practical fish diet versus purified or semi-purified diet could exhibit different effects on growth performance of the fish being evaluated. This also called into question if nucleotide content was limiting in present study with hybrid striped bass (Table 2). According to Gallagher (1994), there was no significant difference in WG for hybrid striped bass when 75% fishmeal was replaced with soybean meal; thus, these findings also elucidates that growth difference was not observed between nucleotide rich diet (100% fishmeal) and 75% soybean meal inclusion level diet. Interestingly, a recent study has shown that a nucleotide-supplemented diet significantly ($P<0.05$) improved specific growth rate of turbot compared to a low-fishmeal reference diet, but not compared with a high-fishmeal reference diet (Meng et al., 2017). Carver and Walker (1995) stated that absent of nucleotide exogenous supply in the diet does not lead to a classic clinical deficiency syndrome becuase this nutrient can be synthesized *de novo* or obtained through salvage pathway; however, conditions under certain disease states, periods of limited nutrient intake or rapid growth, and the presence of regulatory or developmental factors may interfere with the endogenous

synthesis capacity. From these facts and figures, the improved growth rate shown in this study from the AMP-supplemented diet is very likely to be correlated with the health status of the fish during the feeding trial.

Ramadan et al. (1994) reported that dietary nucleotide heightened B-lymphocyte and T-lymphocyte activities through increased antibody levels. It is also well established that mammals fed a nucleotide-free diet showed a higher percentage of terminal deoxynucleotidyl transferase enzyme (known as an index of immaturity for lymphocytes) in thymus and spleen compared to those fed dietary nucleotides (Kulkarni et al., 1989). This further suggests that dietary nucleotides could stimulate the maturation of lymphoid cells (Kulkarni et al., 1989). In present study, it appears that dietary AMP Sigma and UMP had the highest B-lymphocyte proliferation (Fig.1) but none of the treatments was significantly different from the basal diet. According to Leonardi and Klempau (2003), fish infected with infectious pancreatic necrosis virus and fed dietary nucleotides for 60 days had significantly higher B lymphocyte proliferation from LPS-stimulated cells compared to those fed the basal diet without nucleotide supplement; however, when the experiment was extended for 120 days no significance was observed. It is important to note that several studies have reported higher lymphocyte count when nucleotide was supplemented in the diet (Tahmasebi-Kohyani et al., 2012; Reda et al., 2018). Taking all this into consideration, further detailed investigation in this area is needed.

Previous work from our laboratory (Li et al., 2004) demonstrated that supplementing Ascogen P® at 0.5% of diet to hybrid striped bass did not significantly enhance growth performance or innate immunity over an 8-week period feeding; however, improvements in WG, innate immune parameters, and survival were observed in fish fed the nucleotide-supplemented diet when infected with *Streptococcus iniae* over a 6-week feeding experiment. These findings illuminate that WG improvement seen in hybrid striped bass fed the AMP supplemented diet in present study may be due to immunostimulatory and disease resistance effects because at the end of week 7 the fish showed clinical signs of a mild *Mycobacterium* infection. Under these circumstances, it is believed that the hybrid striped bass was immunostimulated in present study due to presence of opportunistic pathogen in the recirculating system. Similarly, one of the earliest studies by Burrells et al. (2001b) reported that improvement of weight gain was seen in Atlantic salmon that were fed a nucleotide-supplemented diet during challenge infection against *Aeromonas salmonicida* after vaccination.

According to Akira et al. (2006), microorganisms that invade a vertebrate host are initially recognized by the innate immune system through germline-encoded pattern-recognition receptors (PRRs). These classes of PRRs which include Toll-like receptors and cytoplasmic receptors, recognize distinct microbial components and directly activate immune cells. The macrophage is a phagocytic cell that plays an important role especially throughout the early phase of infection; when stimulated by pathogens, macrophages produce reactive oxygen species (ROS) via generation of superoxide anions, hydrogen peroxide, and chloramines to kill pathogens (Ellis, 1999).

Macrophages are also able to engulf pathogens (phagocytosis) and release an impressive number of inflammatory cytokines which have been studied extensively through stimulation of non-specific mitogens such as lipopolysaccharides (Cavaillon, 1994). A recent study by Zhang et al. (2019) stated that significant improvement in immune response, phagocytic activity, and disease resistance was observed when the gibel carp that were fed an IMP-supplemented diet and exposed to a bacterial challenge against *Aeromonas hydrophila*. They further elucidate that supplementation of IMP in a high-soybean-meal diet significantly upregulated three pro-inflammatory cytokines; namely IL-1β, TNF-α1, and IL-8 (in spleen and kidney) compared to before and after infection. In present study, the ROS produced extracellularly (Table 5) were significantly higher in phagocytes isolated from fish fed the AMP Sigma and IMP dietary treatments compared to the basal diet. Additionally, fish fed the AMP Sigma diet also had higher blood neutrophil oxidative radical production as indicated by nitroblue tetrazolium (NBT) activity compared to the other dietary treatments. This finding appears to be in line with previous work (Sakai et al., 2001) with common carp; wherein, oral administration of nucleotides resulted in improved respiratory burst and NBT responses in kidney phagocytic cells. Nevertheless, the present study appears to be inconsistent with regard to the AMP treatment from Chem-Impex in that those fish had the lowest respiratory burst and NBT which contrasts with the AMP Sigma treatment effects (Table 5). Furthermore, other researchers have reported the lack of statistical significance on the phagocytic activity of hybrid striped bass (Li et al., 2004) and rainbow trout (Burrells et al., 2001a) that were fed commercial nucleotide products.

Macrophage and neutrophils are also known to be the main source of lysozyme (Murray and Fletcher, 1976). A number of studies have reported that nucleotide-supplemented diets can improve lysozyme activity (Song et al., 2012; Hossain et al., 2016a; Zhang et al., 2019); however, no significant differences were observed in present study and several other published works (Li et al., 2004; Cheng et al., 2011; Welker et al., 2011). This is further explained in the review article by Forlenza et al. (2011) that despite common conservation of effector functions typical of innate activated macrophages, the differences between fish species are undeniable based on a substantial amount of evidence that has been gathered. In addition, over two decades of studies by many researchers have acknowledged that the effects of dietary nucleotides on fish health and growth performance are influenced by factors such as nucleotide type, dosage, and administration time. Furthermore, the present study showed that the effects of AMP from different manufacturer elicited different results in terms of immune parameters but similar findings with regard to growth performance.

In conclusion, the present study showed that exogenous supply of AMP at 0.5% of dry diet may have a positive influence on growth performance and innate immune response o hybrid striped bass. Further investigation is needed on determining the optimal dosage of AMP nucleotide and also further characterize the underlying physiological mechanisms of AMP supplementation in for hybrid striped bass fed practical diets containing very high soybean.

CHAPTER III

EXPLORING POTENTIAL SYNERGISTIC EFFECTS BETWEEN DIETARY ADENOSINE AND INOSINE MONOPHOSPHATE ON GROWTH PERFORMANCE AND ACUTE STRESS-INDUCED IMMUNE RESPONSES OF HYBRID STRIPED BASS

III.1 Introduction

The sunshine bass is produced by crossing the female white bass (*Morone chrysops*) and male striped bass (*Morone saxatilis*), and they are the most common hybrid striped bass commercially cultured in the United States (Garber and Sullivan, 2006). These fish are cultured for food, sports fishing, and stock enhancement programs (Smith et al., 1986; Schramm Jr. et al., 1991; Garber and Sullivan, 2006). According to Davis and Griffin (2004) in a commercial aquaculture setting for hybrid striped bass, the fish are regularly subjected to handling stressors such as seining, grading, confinement and transporting multiple times before reaching their final markets The frequent handling events that occur in a hybrid striped bass farming (Davis and Griffin, 2004) are linked to acute stress responses (Reubush and Heath, 1997) and may increase disease susceptibility and mortality (Noga et al., 1998; Davis and Griffin, 2004). There are numerous studies reporting detrimental effects of handling and hauling stress in fish (Wedemeyer, 1976; Carmichael et al., 1983; Weirich et al., 1992; Noga et al., 1998).

Besides that, other stressors potentially present in the aquatic environment such as low dissolved oxygen, extreme temperature, high levels of ammonia and nitrites are

well documented and also may be associated with chronic stress if fish are exposed for a prolonged period of time (Huey et al., 1984; Weirich et al., 1992; Randall and Tsui, 2002; Brinkman et al., 2009; Santos et al., 2010).

Stress may be defined as a change in biological condition beyond the normal resting state that challenges homeostasis and, consequently, presents a threat to the fish's health (Barton and Iwama, 1991). Mazeaud et al. (1977) further explained that for teleost fish the primary reaction after perception of stressors, also known as the adaptive response, enables fish to cope with stressful conditions by induction of a neuroendocrine cascade, which includes a rapid change in plasma catecholamines (adrenaline and noradrenaline) and corticosteroids (cortisol). Moreover, secondary responses involve various biochemical and physiological effects involving cardiovascular, respiratory, osmoregulatory, immunological responses which are mediated to a great extent by stress hormones (Schreck and Tort, 2016). When these responses are prolonged for a period of time, they shift from adaptive to maladaptive, resulting in impairment of fish growth, resistance to disease, reproduction, and eventually survival (Barton et al., 1987; Barton and Iwama, 1991).

Nucleotides are monomers of nucleic acids that are building blocks of deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) and have numerous physiological functions. Two decades of research have shown that dietary nucleotide supplementation to fish generally results in positive influences on growth performance, immunity, disease resistance, osmoregulation, stress tolerance and survival in various fish species (Burrells et al., 2001; Welker et al., 2011; Reda et al., 2018; Guo et al.,

2019; Zhang et al., 2019). More recently, the use of combinations or single purified nucleotide supplements instead of commercial nucleotide products in fish diets have heightened interest in the potential positive effects of these compounds (Guo et al., 2019; Tie et al., 2019; Zhang et al., 2019). Nonetheless, numerous reports have noted that the effects of dietary nucleotides have not always been consistently observed among different fish species (Li and Gatlin III, 2006). It is well established that seafood products have high concentrations of purine compounds that include nucleotides, nucleosides, and nucleic acids (Kojima, 1974). As such, with fishmeal and other marine protein feedstuffs being increasingly replaced with alternative feedstuffs such as soybean meal which have much lower concentrations of these purine compounds, the levels of nucleotides in aquafeeds may be reduced (Kojima, 1974; NRC, 2011). Therefore, based on various studies in which soybean meal (Gallagher, 1994) and other protein feedstuffs (Gaylord et al., 2004; Rawles et al., 2006b; de Cruz et al., 2018; Perez-Velazquez et al., 2019) are being substituted for fishmeal in the diet of hybrid striped, there are greater opportunities for purine nucleotides to be limiting.

Therefore, the focus of this study was to investigate the potential synergistic effects of two prominent nucleotides, adenosine 5'-monophosphate (AMP) and inosine 5'-monophosphate (IMP), both singularly and in combination, on growth performance and innate and adaptive immunity of hybrid striped bass. It was also of interest to explore the mechanisms of acute stress-induced innate immune system modulation in hybrid striped bass, and the potential dietary effects of purine nucleotides (AMP and IMP) prior to and after stress exposure.

III.2 Materials and methods

Three separate experiments encompassing four different feeding trials were conducted to evaluate the effects of AMP and IMP either singularly or in combination on hybrid striped bass.

Table 6. Ingredients and analyzed composition of the basal diet.

Ingredients	g/100 g
Menhaden fishmeal[2]	14.70
Soybean meal[3]	55.30
Menhaden oil[4]	6.03
Vitamin premix[5,6]	3.00
Mineral premix[5,6]	4.00
Dextrinized starch[6]	9.00
Calcium phosphate dibasic[6]	1.00
Carboxymethyl cellulose[6]	2.00
Glycine[7]	1.00
Taurine[7]	0.43
DL-Methonine[7]	0.40
Cellulose[8]	3.06
Agar[9]	0.05
Analyzed composition, g/100 g[-1, 10]	
Crude protein	40.6
Crude lipid	9.4
Ash	10.3

[1] Dry-matter basis
[2] Special Select, Omega Protein, Abbeville, Louisiana (crude protein [CP] = 645 g/kg; lipid = 114 g/kg on a dry-matter basis).
[3] Producers Cooperative Association, Bryan, Texas (CP = 515.4 g/kg; lipid = 36.7 g/kg on a dry-matter basis).
[4] Omega Protein, Reedville, Virginia
[5] Moon and Gatlin (1991).
[6] MP Biomedicals, Solon, Ohio.
[7] Ajinomoto North America, Inc.
[8] U.S. Biochemical Corp., Cleveland, Ohio.
[9] Sigma Aldrich Co., St. Louis, Missouri.
[10] Means of two replicate determinations.

III.2.1 Basal diet

The basal diet used in all experiments was formulated principally from dehulled soybean meal and a small amount of menhaden fishmeal to contain 40% protein, 9.4% lipid and an estimated digestible energy level of 3.5 kcal g^{-1} (Table 6). As such, the basal diet was designed to be limiting in purine nucleotides due to high inclusion of soybean meal (Kojima, 1974; NRC, 2011). The experimental diets were formulated to be isonitrogenous, isolipid, isocaloric,and meet or exceed all established nutrient requirements of hybrid striped bass (NRC, 2011). Specific dietary treatments evaluated in three separate experiments are described in detail below. Each diet was made into 3-mm sinking pellets. The procedures for diet manufacture and storage were as previously described by de Cruz et al. (2018).

III.2.2 Fish and culture system

All the feeding trials were conducted at the Texas A&M University Aquacultural Research and Teaching Facility. The animal care and experimental protocols were approved by the Institutional Animal Care and Use Committee at Texas A&M University. The juvenile hybrid striped bass (*Morone chrysops* x *M. saxatilis*) used in the experiments were obtained from Keo Fish Farms, Keo, Arkansas, USA. All the experiments were conducted in a recirculation system consisting of 110-L glass aquaria connected to a settling chamber, biofilter, UV sterilizer, and sand filter. Oxygen was maintained near air saturation by diffusing air from a blower through stones. Water quality parameters were kept at a suitable range for hybrid striped bass culture; water

temperature = 27.7 ± 0.14 ˚C, dissolved oxygen = 7.5 ± 0.36 mg L^{-1}, total ammonia nitrogen (TAN) = 0.12 ± 0.04 mg L^{-1}, nitrite nitrogen = 0.07 ± 0.06 mg L^{-1}, salinity = 3.0 ± 0.18 g L^{-1}, and pH = 8.09 ± 0.19. The photoperiod in the cultured system was set at 12h light-12h dark by fluorescent lights regulated by automatic timers.

III.2.3 Experiment 1

The synergistic effect of AMP and IMP were investigated in a 2 × 2 factorial design. Two concurrent feeding trials (each of 9 weeks duration) were conducted using nucleotide products (Purity > 98%) from either Sigma-Aldrich or Chem-Impex International (Wood Dale, Illinois, USA). The nucleotide supplement, AMP and IMP disodium salt, was coated with 1% agar solution to limit nutrient leaching in water. The coated nucleotides were frozen solid at -20°C and the moisture was removed completely by freeze drying. Then dried coated nucleotides were finely ground and added to the diet along with the other dry ingredients. The same basal diet was used in both feeding trials. Three experimental diets from each supplier were prepared with the individual supplementation of 0.5% AMP, 0.5% IMP and the combined supplementation of AMP at 0.5% and IMP at 0.5% to the basal diet at the expense of the cellulose. This concentration of 0.5% was selected based on other studies with other species that recommended single purified nucleotide dosages between 0.1 and 0.6% of dry weight (Lin et al., 2009; Song et al., 2012; Hossain et al., 2016b; Hossain et al., 2017; Zhang et al., 2019). In addition, two more dietary treatments were prepared by supplementing both AMP and IMP each at 0.25 % of diet. One diet of these diets was prepared with

nucleotides from Sigma-Aldrich and the other from Chem-Impex International. Primarily, these additional treatments were investigated due to the concern of purine toxicity with the combination of 0.5% of AMP and 0.5% of IMP that totaled up to 1% supplementation; therefore, the concentration was reduced to 0.25% at each diet so that it could demonstrate if there was any sign of purine toxicity involved.

Groups of 16 hybrid striped bass fingerlings with an average initial weight of 9.7 ± 0.2 g were assigned randomly to each aquarium in the recirculating system mentioned below in section 2.5. Each diet was fed to fish in three randomly assigned replicate groups, and the feeding rate was initially set at 5% of total body weight per day which approached apparent satiation. The fish were fed twice daily with pre-weighed rations, and feeding rate was gradually reduced equally for all diets over time to keep a level close to apparent satiation. Fish were group-weighed each week, and feed rations were adjusted accordingly. At the end of each 9-week period, growth performance was measured as follows; weight gain (WG) = ([g final weight − initial weight]/g initial weight) × 100; condition factor (K) = (g final weight × 100)/total length^3; feed efficiency ratio (FER) = g weight gain /g dry feed offered; and survival = (final no. of organisms/initial no. of organisms) × 100. Six fish were euthanized from each replicate aquarium with an overdose (>300 mg/L) of tricainemethane sulphonate (MS-222, Western Chemical Inc, Washington, USA). Three fish were used to measure body condition indices and another three fish were homogenized as a composite sample for analysis of proximate composition. The body condition indices were measured as follows: hepatosomatic index (HSI) = g liver weight /100 g body weight; and

intraperitoneal fat (IPF) ratio = g IPF weight /100 g body weight. The proximate composition was measured following established methods: the Dumas protocol for crude protein (6.25 × N) (AOAC, 2005), chloroform: methanol extraction for crude lipid (Folch et al., 1957), and heating samples at 650 °C in the muffle furnace for 4 h for ash (AOAC, 1990). The protein conversion efficiency was computed with the following equation: ((([(g final body weight × % final body protein) − (g initial body weight × % initial body protein)] / (protein intake (g)]) × 100).

III.2.4 Experiment 2

This experiment was conducted to compare the hematological parameters of hybrid striped bass that were acutely stressed compared to a group of fish that was not subjected to any stressors (control). The experimental stressor in this experiment was air exposure for 1 minute by completely netting out all the fish from the aquarium in a single pass and suspending them in the air over the aquarium. Prior to initiating the stress-challenge protocol, groups of six hybrid striped bass juvenile with an average initial weight of 52.6 ± 1.72 g were assigned randomly to each aquarium in the recirculating system and conditioned for 2 weeks with the basal diet. The fish were fasted for approximately 15 h from last feeding prior to the sampling day. The experimental unit for this experiment was the individual fish (n=6) nested within the treatments (stressed fish and control). For the acute stress challenge, groups of six replicate fish were sampled at 0 (pre-challenge), 0.5, 1, 2, 6, 12, and 24 h post-challenge (air exposure) to investigate the effects of acute stress on plasma cortisol, blood glucose,

hematocrit, and innate immunity of hybrid striped bass. Fish in the control group were sampled at the same times as the stressed fish group. Fish at each time point were sampled in less than 4 minutes. At each sampling time point, the six fish from each treatment were collected and immediately sedated with MS-222 (150 mg L^{-1}) as recommended by Trushenski et al. (2012). The blood samples were collected to determine hematocrit, plasma cortisol, blood glucose, lysozyme, and anti-protease. The details of the sample collection and analytical procedures are described below in section 2.6.

III.2.5 Experiment 3

Juvenile hybrid striped bass from the feeding trials associated with experiment 1 were used in this experiment after being fed their respective dietary treatments for 9 weeks. The experimental unit for this study was the individual fish (n=6) nested within the dietary treatments. Similar to experiment 2, groups of six hybrid striped bass juveniles with an average weight of 59.3 ± 4.0 g were maintained on their respective treatments in aquarium associated with the recirculating system and conditioned for an additional 2 weeks. The details of the diets and culture conditions were mentioned in section 2.3. The fish were withheld from feeding approximately 15 h prior to the day of sampling. Based on the result from experiment 2, three time points were chosen to investigate acute stress- induce immunosuppression and post-stress innate immune system recovery. For the acute stress challenge response, groups of six replicate fish from each dietary treatment were sampled at 0 (pre-challenge), 0.5, and 12 h post-

challenge to investigate the effects of the dietary nucleotide supplements as potential immune-modulating nutrients during stress. As mentioned in experiment 2, the fish were subjected to the same stress challenge protocol. The blood samples were collected at each time point for all the dietary treatments to determine the hematological and innate immunity parameters: hematocrit, anti-protease, lysozyme, and total immunoglobulin. The details of the sample collection and analytical procedures are described in section 2.6.

III.2.6 Sample collection and analytical procedures for immune and stress parameters

Fish were bled from the caudal vasculature with heparinized 1-mL syringes with 26-gauge needles. A portion of the blood sample was analyzed for glucose using a handheld glucose meter (Accu-Check guide®, Roche, Basel, Switzerland), and hematocrit was acquired by centrifugation of capillary glass tubes (Drummond Scientific, Pennsylvania, USA). The remaining blood sample was centrifugation at 3000 × *g* for 10 min and the plasma was stored in multiple aliquots at -80°C prior to analysis. Plasma cortisol levels were measured using an enzyme-linked immunosorbent assay (ELISA) kit (DRG International, New Jersey, USA).

Plasma lysozyme activity was determined according to the turbidimetric reduction assay (Jørgensen et al., 1993) by using *Micrococcus lysodeikticus* (Sigma-Aldrich, Missouri, USA), and the lysozyme activity unit was defined as the amount of enzyme producing a reduction in absorbance of 0.001 min^{-1}. Total immunoglobulin and total plasma protein were determined as described by Siwicki et al. (1994). The plasma

anti-protease activity was determined by a modification of the method described by Ellis (1990) and were calculated as the percentage inhibition of trypsin activity compared to a reference sample (without plasma).

Head kidney leukocytes also were isolated by density gradient sedimentation to determine the respiratory burst activity of phagocytes, and lymphocyte proliferation upon non-specific mitogen stimulation. Briefly, the head kidney from a group of three fish per replicate tank were aseptically excised using scalpels and forceps, pooled and stored in 15 ml of cold Leibovitz cell culture medium (L-15, Corning, NY, USA) containing 2% fetal bovine serum (FBS, Sigma-Aldrich), 10 units mL^{-1} of heparin (Akron Biotechnology, Boca Raton, Florida), and 100 units mL^{-1} of penicillin and streptomycin (Sigma-Aldrich). The techniques established by Secombes (1990) and modified by Sealey and Gatlin (2002) was used to isolate the phagocytes.

Respiratory burst activity of phagocytes was measured by intracellular and extracellular superoxide anion production as suggested by Secombes (1990). The concentration of intracellular O^{2-} produced was determined by reading the absorbance at 620 nm. The concentration of extracellular O^{2-} produced was calculated as described by Pick and Mizel (1981) as superoxide O^{2-} = ([Δ Absorbance after 45 min×100] ÷ 6.3). The technique established Secombes (1990) with minor modification as described by Carvalho et al. (2018) was used to isolated the lymphocytes. The proliferation of lymphocytes upon stimulation with the non-specific mitogen lipopolysaccharide solution (LPS, Sigma Aldrich, 1 mg mL^{-1}) was assessed using a colorimetric assay as recommended by Mosmann (1983). The lymphocyte proliferation capacity was computed and presented as stimulation index (SI = ABS stimulated cells ÷ ABS non-stimulated [control] cells).

III.2.7 Statistical analysis

Data were evaluated for normality using the Shapiro–Wilk test and differences among treatment means were analyzed using ANOVA with significance set at $P < 0.05$ for experiment 1. Post hoc Tukey HSD test was used to test the mean differences.

Repeated measure analysis of variance (RM-ANOVA) was the most suitable analysis for experiment 2 and 3 in this study. The RM-ANOVA was unequally spaced in time (distance between each time periods); therefore, an appropriate covariance structure was selected for the analysis.

The full model developed for experiment 2 responses was;

$Y_{ijk} = \boldsymbol{\beta}_0 + \boldsymbol{\alpha}_i + \boldsymbol{\gamma}_k + (\boldsymbol{\alpha\gamma})_{ik} + \boldsymbol{\varepsilon}_{ijk}$ where,

Y_{ijk}: the investigated responses at k^{th} hour on the j^{th} fish assigned to the i^{th} treatment effect

$\boldsymbol{\alpha}_i$: treatmenth effect (i=1, 2)

$\boldsymbol{\beta}_0$ is the overall intercept;

$\boldsymbol{\gamma}_k$: hour effect (k=1, 2, 3, 4, 5, 6, 7)

$(\boldsymbol{\alpha\gamma})_{ik}$: treatment *hour effect

$\boldsymbol{\varepsilon}_{ijk}$ denotes the error term

The full model developed for experiment 3 responses was;

$Y_{ijk} = \boldsymbol{\beta}_0 + \boldsymbol{\alpha}_i + \boldsymbol{\gamma}_k + (\boldsymbol{\alpha\gamma})_{ik} + \boldsymbol{\varepsilon}_{ijk}$ where,

Y_{ijk}: the investigated responses at k^{th} hour on the j^{th} fish assigned to the i^{th} dietary treatment effect

$\boldsymbol{\alpha}_i$: dietary treatment effect (i=1, 2, 3, 4, 5, 6, 7, 8, 9)

$\boldsymbol{\beta}_0$ is the overall intercept;

$\boldsymbol{\gamma}_k$: hour effect (k=1, 2, 3)

$(\boldsymbol{\alpha\gamma})_{ik}$: dietary treatment effect *hour effect

$\boldsymbol{\varepsilon}_{ijk}$ denotes the error term

Table 7. Evaluation of potential synergistic effects of dietary AMP and IMP from Sigma-Aldrich on the growth performance of hybrid striped bass at the end of 9 weeks.[1]

Dietary treatment level (%)		Weight gain	Feed Efficiency Ratio	Fulton's Condition Factor	Survival	Hepatosomatic index	Intraperitoneal fat ratio
AMP Sig.[2]	IMP Sig.[3]	%			%	%	%
0	0	481	0.75	1.70	100	1.90	4.38
0.5	0	493	0.76	1.70	100	2.00	4.12
0	0.5	520	0.81	1.68	100	1.80	4.04
0.5	0.5	530	0.81	1.44	100	1.97	4.47
Pooled SE[4]		23.6	0.03	0.25	-	0.05	0.25
2 factor ANOVA (P value)							
AMP		0.6503	0.7485	0.6348	-	0.0323	0.7357
IMP		0.1469	0.1231	0.5900	-	0.2542	0.9792
AMP × IMP		0.9808	0.9247	06532	-	0.5361	0.2036

[1] Value expressed as means of three replicates groups (n=3). In case of significant interaction ($P < 0.05$), Tukey's HSD test was performed to compare the differences among dietary treatment means.
[2]Adenosine monophosphate (AMP Sig.), Sigma Aldrich Co., St. Louis, Missouri.
[3] Inosine monophosphate (IMP Sig.), Sigma Aldrich Co., St. Louis, Missouri.
[4]Pooled standard error

All data were analyzed in both experiments 2 and 3 by RM-ANOVA within the PROC MIXED framework of the Statistical Analysis System, version 9.4 (SAS Institute, North Carolina, USA) to determine significance of differences which was set at < 0.05. Only if significant interaction was detected between treatment effect and time, were pair-wise comparisons carried out.

III.3 Results

III.3.1 Experiment 1

At the end of the two, concurrent 9-week feeding trials, no significant difference in growth performance of hybrid striped bass was observed in the main effects and interactions between AMP and IMP from either supplier (Table 7, Table 8). Similarly,

no significant differences were observed among all dietary treatments from both suppliers with the two additional dietary treatment combinations of 0.25 % AMP and 0.25 % IMP from Sig. and C. Impex (Table 9). Over the 9 week feeding trial, fish survival was 100% for all dietary treatments (Tables 7, 8 and 9).

Table 8. Evaluation of potential synergistic effects of dietary AMP and IMP from Chem-Impex Int on the growth performance of hybrid striped bass at the end of 9 weeks.[1]

Dietary treatment level (%)		Weight gain	Feed Efficiency Ratio	Fulton's Condition Factor	Survival	Hepatosomatic index	Intraperitoneal fat ratio
AMP C. Impex[2]	IMP C. Impex[3]	%			%	%	%
0	0	481	0.75	1.70	100	1.90	4.38
0.5	0	532	0.81	1.43	100	1.97	4.56
0	0.5	507	0.78	1.41	100	1.91	4.28
0.5	0.5	532	0.81	1.40	100	2.01	4.09
Pooled SE[4]		23.5	0.03	0.12	-	0.08	0.30
2 factor ANOVA (P value)							
AMP		0.1547	0.2045	0.2604	-	0.2991	0.9871
IMP		0.6262	0.6414	0.2134	-	0.7524	0.3709
AMP × IMP		0.5698	0.6858	0.2925	-	0.9160	0.5624

[1] Value expressed as means of three replicates groups (n=3). In case of significant interaction ($P < 0.05$), Tukey's HSD test was performed to compare the differences among dietary treatment means.
[2]Adenosine monophosphate (AMP C. Impex), Chem-Impex International, Wood Dale, Illinois.
[3] Inosine monophosphate (IMP C. Impex), Chem-Impex International, Wood Dale, Illinois.
[4]Pooled standard error

Table 9. Effects of dietary nucleotide treatments on growth performance of hybrid striped bass at the end of 9 weeks.[1]

Diet[1,2,3,4,5]	Weight gain	Feed Efficiency Ratio	Fulton's Condition Factor	Survival	Hepatosomatic index	Intraperitoneal fat ratio
	%			%	%	%
Basal	481	0.75	1.70	100	1.90	4.38
AMP Sig. (0.5%)	493	0.76	1.70	100	2.00	4.12
IMP Sig. (0.5%)	520	0.81	1.68	100	1.80	4.04
AMP (0.5%) + IMP (0.5%) Sig.	530	0.81	1.44	100	1.97	4.47
AMP (0.25%) + IMP (0.25%) Sig.	492	0.76	1.42	100	1.96	4.29
AMP C. Impex (0.5%)	532	0.81	1.43	100	1.97	4.56
IMP C. Impex (0.5%)	507	0.78	1.41	100	1.91	4.28
AMP (0.5%) + IMP (0.5%) C. Impex	530	0.81	1.40	100	2.01	4.09
AMP (0.25%) + IMP (0.25%) C. Impex	506	0.77	1.46	100	2.02	4.70
Pooled SE[6]	23.4	0.03	0.17	-	0.07	0.25
Pr > F[7]	0.7226	0.7865	0.7248	-	0.4827	0.6079

[1] Means of three replicates groups (n=3). Values within the same column with different letters are significantly different ($P < 0.05$).
[2]Adenosine monophosphate (AMP Sig.), Sigma Aldrich Co., St. Louis, Missouri.
[3] Inosine monophosphate (IMP Sig.), Sigma Aldrich Co., St. Louis, Missouri.
[4]Adenosine monophosphate (AMP C. Impex), Chem-Impex International, Wood Dale, Illinois.
[5] Inosine monophosphate (IMP C. Impex), Chem-Impex International, Wood Dale, Illinois.
[6]Pooled standard error.
[7]Significance probability associated with the F-statistic.

No statistical differences were detected for whole-body proximate composition or protein conversion efficiency among the various AMP and IMP Sig. dietary treatments (Table 10). However, for protein deposition in the whole-body, there were significant differences in the interaction between AMP and IMP C. Impex dietary treatments (Table 11). The highest protein deposition was seen in fish fed the AMP C. Impex diet at 18.5% which was significantly higher than those fed the combination of AMP and IMP C. Impex. Nevertheless, it is important to note that none of the nucleotide-supplemented diets yielded body composition values that were significantly different from those of fish fed the basal diet (Table 11).

Table 10. Evaluation of potential synergistic effects of dietary AMP and IMP from Sigma-Aldrich on whole-body proximate of hybrid striped bass at the end of 9 weeks.[1]

Dietary treatment level (%)		Moisture	Protein	Lipid	Ash	Protein Conversion Efficiency
AMP Sig.[2]	IMP Sig.[3]	%	%	%	%	%
0	0	67.9	17.9	9.58	4.79	33.4
0.5	0	67.4	18.1	10.8	4.36	34.2
0	0.5	67.0	18.0	10.7	5.00	35.8
0.5	0.5	67.4	18.0	10.4	4.77	35.6
Pooled SE[4]		0.55	0.27	0.50	0.22	1.54
2 factor ANOVA (P value)						
AMP		0.9117	0.6517	0.3935	0.1659	0.8403
IMP		0.4392	0.9670	0.5216	0.1913	0.2641
AMP × IMP		0.3988	0.7759	0.1421	0.6783	0.7645

[1] Value expressed as means of three replicates groups (n=3). In case of significant interaction ($P < 0.05$), Tukey's HSD test was performed to compare the differences among dietary treatment means.
[2]Adenosine monophosphate (AMP Sig.), Sigma Aldrich Co., St. Louis, Missouri.
[3] Inosine monophosphate (IMP Sig.), Sigma Aldrich Co., St. Louis, Missouri.
[4]Pooled standard error

Table 11. Evaluation of potential synergistic effects of dietary AMP and IMP from Chem-Impex International on whole-body proximate composition of hybrid striped bass at the end of 9 weeks.[1]

Dietary treatment level (%)		Moisture	Protein	Lipid	Ash	Protein Conversion Efficiency
AMP C. Impex[2]	IMP C. Impex[3]	%	%	%	%	%
0	0	67.9	17.9[ab]	9.58	4.79	33..4
0.5	0	67.5	18.5[a]	10.1	4.38	36.8
0	0.5	67.2	18.0[ab]	10.5	4.49	34.1
0.5	0.5	67.3	17.4[b]	10.5	4.65	33.9
Pooled SE[4]		0.27	0.22	0.22	0.24	1.61
2 factor ANOVA (P value)						
AMP		0.1597	0.8616	0.2606	0.5979	0.3433
IMP		0.6011	0.0491	0.0228	0.9715	0.4937
AMP × IMP		0.3389	0.0244	0.222	0.2590	0.2989

[.1] Value expressed as means of three replicates groups (n=3). In case of significant interaction ($P < 0.05$), Tukey's HSD test was performed to compare the differences among dietary treatment means.
[2]Adenosine monophosphate (AMP C. Impex), Chem-Impex International, Wood Dale, Illinois.
[3] Inosine monophosphate (IMP C. Impex), Chem-Impex International, Wood Dale, Illinois.
[4]Pooled standard error

No statistical difference was observed in the one-way ANOVA for whole-body proximate composition of fish fed all of the dietary treatments (Table 12). Likewise, no statistical significance was seen in any innate immune parameters such as plasma lysozyme, total plasma protein, total immunoglobulin, plasma anti-protease, or intracellular and extracellular respiratory burst (Tables 13, 14, and 15).

Table 12. Effects of dietary nucleotide treatments on the whole-body proximate composition of hybrid striped bass at the end of 9 weeks.[1]

Diet[1,2,3,4,5]	Moisture	Protein	Lipid	Ash	Protein Conversion Efficiency
	%	%	%	%	%
Basal	67.9	17.9	9.58	4.79	33.4
AMP Sig. (0.5%)	67.4	18.1	10.8	4.36	34.2
IMP Sig. (0.5%)	67.0	18.0	10.7	5.00	35.8
AMP (0.5%) + IMP (0.5%) Sig.	67.4	18.0	10.4	4.77	35.6
AMP (0.25%) + IMP (0.25%) Sig.	68.1	18.2	9.85	4.60	34.8
AMP C. Impex (0.5%)	67.2	18.5	10.13	4.37	36.8
IMP C. Impex (0.5%)	67.5	18.0	10.48	4.49	34.1
AMP (0.5%) + IMP (0.5%) C. Impex	67.3	17.4	10.45	4.65	33.9
AMP (0.25%) + IMP (0.25%) C. Impex	67.6	18.2	10.13	4.84	34.0
Pooled SE[6]	0.44	0.26	0.38	0.22	1.64
Pr > F[7]	0.7349	0.2786	0.4067	0.4635	0.8709

[1] Means of three replicates groups (n=3). Values within the same column with different letters are significantly different ($P < 0.05$).
[2]Adenosine monophosphate (AMP Sig.), Sigma Aldrich Co., St. Louis, Missouri.
[3] Inosine monophosphate (IMP Sig.), Sigma Aldrich Co., St. Louis, Missouri.
[4]Adenosine monophosphate (AMP C.Impex), Chem-Impex International, Wood Dale, Illinois.
[5] Inosine monophosphate (IMP C.Impex), Chem-Impex International, Wood Dale, Illinois.
[6]Pooled standard error.
[7]Significance probability associated with the F-statistic

Table 13. Evaluation of potential synergistic effects of dietary AMP and IMP from Sigma-Aldrich on immune responses of hybrid striped bass at the end of 9 weeks.[1]

Dietary treatment level %		Superoxide anion extra-cellular	Superoxide anion intra-cellular	Plasma lysozyme	Total plasma protein	Total immunoglobulin	Plasma anti-protease	LPS[5,6]
AMP Sig.[2]	IMP Sig.[3]	nmol O_2^-	O.D. at 620 nm	units/ml	mg/ml	mg/ml	%	
0	0	1.87	0.57	313	46.3	23.2	32.7	2.31[ab]
0.5	0	1.26	0.58	341	49.0	24.2	31.6	2.09[b]
0	0.5	1.44	0.54	320	47.1	23.7	30.0	2.20[b]
0.5	0.5	1.76	0.60	350	49.1	26.4	28.2	2.58[a]
Pooled SE[4]		0.38	0.06	20.2	2.30	2.08	2.57	0.07
2 factor ANOVA (P value)								
AMP		0.7169	0.5553	0.1923	0.3320	0.4094	0.5887	0.3032
IMP		0.9306	0.9177	0.6902	0.8565	0.5207	0.2696	0.0343
AMP × IMP		0.2616	0.5790	0.9645	08686	0.6867	0.8784	0.0033

[1] Value expressed as means of three replicates groups (n=3). In case of significant interaction ($P < 0.05$), Tukey's HSD test was performed to compare the differences among dietary treatment means.
[2]Adenosine monophosphate (AMP Sig.), Sigma Aldrich Co., St. Louis, Missouri.
[3] Inosine monophosphate (IMP Sig.), Sigma Aldrich Co., St. Louis, Missouri.
[4] Pooled standard error
[5] Stimulation index (SI) of head kidney lymphocytes. Values indicate the proliferation after stimulation with mitogen (LPS, lipopolysaccharide from *E. coli* O26:B6).
[6] Stimulation index = O.D. 570 nm of leukocyte wells with test mitogen/mean O.D. 570 nm of leukocyte wells without mitogen.

Table 14. Evaluation of potential synergistic effects of dietary AMP and IMP from Chem-Impex International on immune parameters of hybrid striped bass at the end of 9 weeks.[1]

Dietary treatment level %		Superoxide anion extra-cellular	Superoxide anion intra-cellular	Plasma lysozyme	Total plasma protein	Total immunoglobulin	Plasma anti-protease	LPS[5,6]
AMP C.Impex[2]	IMP C.Impex[3]	nmol O_2^-	O.D. at 620 nm	units/ml	mg/ml	mg/ml	%	
0	0	1.87	0.57	313	46.3	23.2	32.7	2.31[ab]
0.5	0	1.92	0.65	363	48.5	24.2	30.8	2.41[ab]
0	0.5	1.54	0.54	331	48.1	22.3	23.7	2.80[a]
0.5	0.5	1.68	0.64	350	49.6	25.2	31.5	2.17[b]
Pooled SE[4]		0.60	0.09	33.0	2.28	1.62	3.58	0.11
2 factor ANOVA (P value)								
AMP		0.8716	0.3352	0.3299	0.4239	0.2655	0.4423	0.0391
IMP		0.6461	0.7698	0.9350	0.5402	0.9667	0.2841	0.3038
AMP × IMP		0.9361	0.8866	0.6463	0.8859	0.5550	0.2157	0.0094

[1] Value expressed as means of three replicates groups (n=3). In case of significant interaction ($P < 0.05$), Tukey's HSD test was performed to compare the differences among dietary treatment means.
[2] Adenosine monophosphate (AMP C.Impex), Chem-Impex International, Wood Dale, Illinois.
[3] Inosine monophosphate (IMP C. Impex), Chem-Impex International, Wood Dale, Illinois.
[4] Pooled standard error
[5] Stimulation index (SI) of head kidney lymphocytes. Values indicate the proliferation after stimulation with mitogen (LPS, lipopolysaccharide from *E. coli* O26:B6).
[6] Stimulation index = O.D. 570 nm of leukocyte wells with test mitogen/mean O.D. 570 nm of leukocyte wells without mitogen.

Table 15. Effects of dietary nucleotide treatments on the immune responses of hybrid striped bass at the end of 9 weeks.[1]

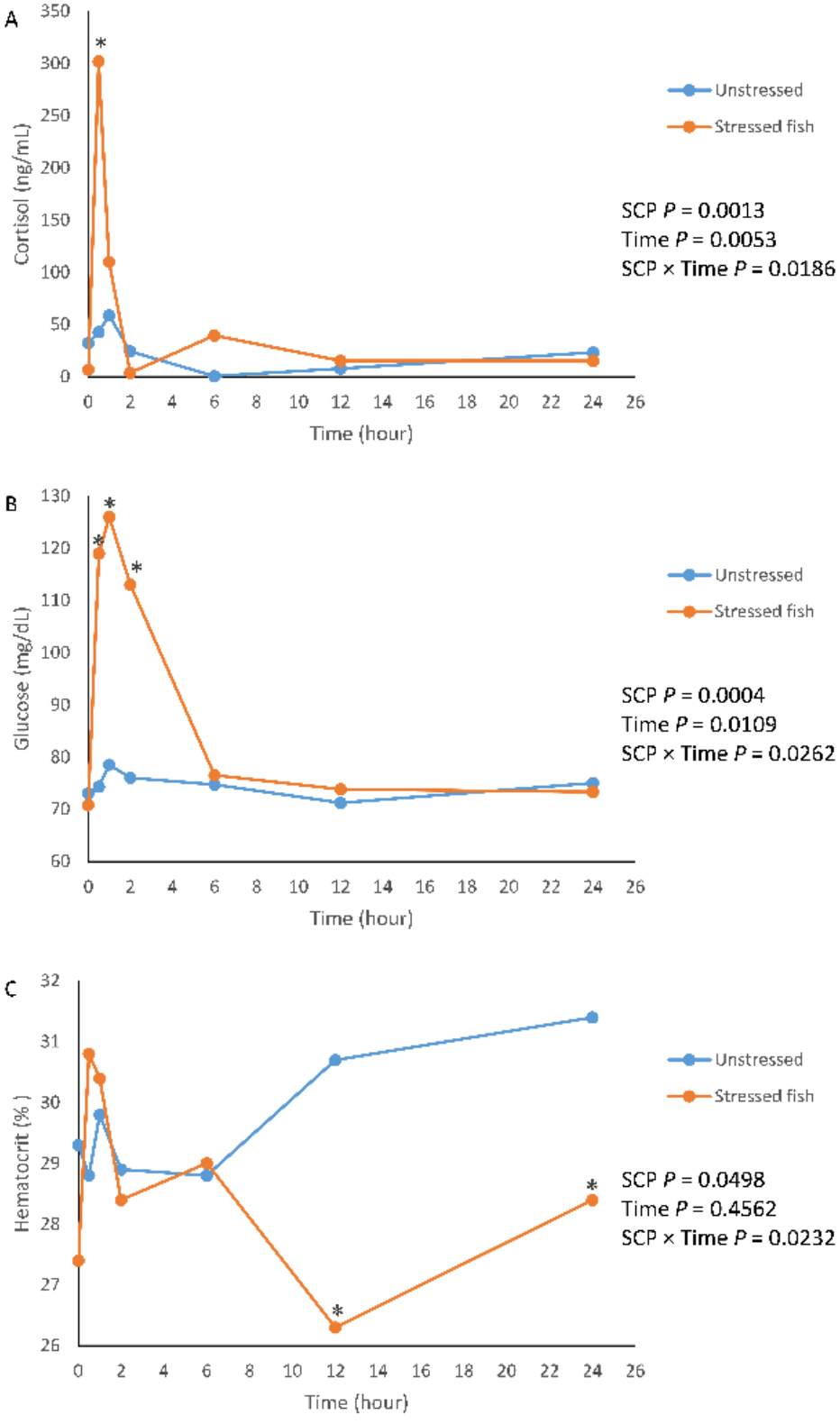
Diet[1,2,3,4,5]	Superoxide anion extra-cellular nmol O_2^-	Superoxide anion intra-cellular O.D. at 620 nm	Plasma lysozyme units/ml	Total plasma protein mg/ml	Total immunoglobulin mg/ml	Plasma anti-protease %	LPS[5,6]
Basal	1.87	0.57	313	46.3	23.2	32.7	2.31^{bc}
AMP Sig. (0.5%)	1.26	0.58	341	49.0	24.2	31.6	2.09^{c}
IMP Sig. (0.5%)	1.44	0.54	320	47.1	23.7	30.0	2.20^{bc}
AMP (0.5%) + IMP (0.5%) Sig.	1.76	0.60	350	49.1	26.4	28.2	2.58^{ab}
AMP (0.25%) + IMP (0.25%) Sig.	1.75	0.63	328	46.4	24.1	27.3	2.42^{abc}
AMP C. Impex (0.5%)	1.92	0.65	363	48.5	24.2	30.8	2.41^{abc}
IMP C. Impex (0.5%)	1.54	0.54	331	48.1	22.3	23.7	2.80^{a}
AMP (0.5%) + IMP (0.5%) C. Impex	1.68	0.64	350	49.6	25.2	31.5	2.17^{bc}
AMP (0.25%) + IMP (0.25%) C. Impex	1.23	0.57	407	50.0	26.0	34.9	2.30^{bc}
Pooled SE	0.51	0.08	24.1	2.42	2.02	3.03	0.09
Pr > F[6]	0.9768	0.9457	0.2746	0.9435	0.8958	0.3652	0.0005

[1] Means of three replicates groups (n=3). Values within the same column with different letters are significantly ($P < 0.05$) different.
[2]Adenosine monophosphate (AMP Sig.), Sigma Aldrich Co., St. Louis, Missouri.
[3] Inosine monophosphate (IMP Sig.), Sigma Aldrich Co., St. Louis, Missouri.
[4]Adenosine monophosphate (AMP C. Impex), Chem-Impex International, Wood Dale, Illinois.
[5] Inosine monophosphate (IMP C. Impex), Chem-Impex International, Wood Dale, Illinois.
[6]Significance probability associated with the F-statistic.

For the adaptive immune responses, lymphocyte proliferation prompted by the presence of lipopolysaccharides showed a significant interaction between AMP and IMP from both suppliers; however, none of the treatments were significantly different compared to fish fed the basal diet (Tables 13, 14). The highest simulation index was shown by fish fed the diet with IMP C. Impex which was significantly different from that of fish fed the basal diet (Table 15).

III.3.2 Experiment 2

All hematological parameters examined showed statistically significant interaction between treatment effect (stressed fish versus control fish) and time (pre and post stress) (Fig. 2). The primary stress response of plasma cortisol was evoked by air exposure for 1 minute, and statistically significant at time point 0.5 h post-stress compared with the control group, and then, made a complete recovery within 1 h (Fig.2A). Similarly, the secondary stress response of blood glucose had a significantly higher peak from time 0.5 h to 2 h post-stress compared to the baseline peak of the control group, and made a complete recovery afterward (Fig. 2B). Plasma anti-protease activity was affected by the group of fish subjected to acute stress with significant immune suppression observed at time points 1 h, 2 h, and 12 h post-stress, and then recovered at 24 h post stress (Fig. 2E). For the hematocrit response, it was noted that the packed cell volume increased at time 0.5 h post-stress but was not significantly different from the control group (Fig. 2C).


A
Cortisol (ng/mL)
Time (hour)
Unstressed
Stressed fish
SCP P = 0.0013
Time P = 0.0053
SCP × Time P = 0.0186
B
Glucose (mg/dL)
Time (hour)
Unstressed
Stressed fish
SCP P = 0.0004
Time P = 0.0109
SCP × Time P = 0.0262
C
Hematocrit (%)
Time (hour)
Unstressed
Stressed fish
SCP P = 0.0498
Time P = 0.4562
SCP × Time P = 0.0232

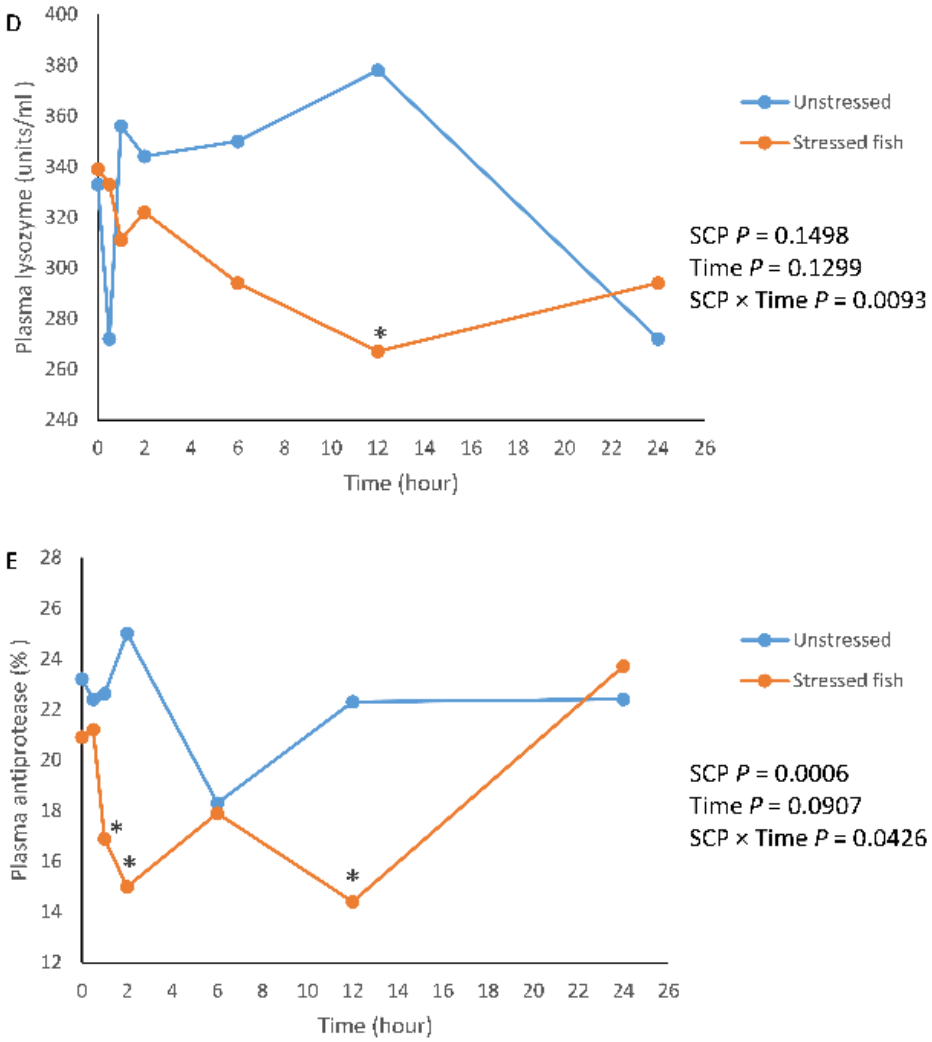


Figure 2. Figure 2 Modulation of plasma cortisol, blood glucose, hematocrit, and innate immunity (A = cortisol, B = glucose, C = hematocrit, D = plasma lysozyme, and E = plasma antiprotease) of hybrid striped bass subjected to the stress challenge protocol (SCP) compared to the unstressed group.

It was also observed in the stressed group at 12 h and 24 h post-stress, that the packed cell volume was significantly lower than control group (Fig. 2C). The plasma lysozyme activity in both groups of fish showed a fluctuating trend within the 24 h period; however, the plasma lysozyme activity of the stressed fish group was only significantly lower than the control group at 12 h post-stress (Fig. 2D).

III.3.3 Experiment 3

It was observed that dietary nucleotide supplementation modulated hematological parameters and innate immune response during acute stress (Table 16). The plasma anti-protease activity was significantly higher for hybrid striped bass fed the IMP C. Impex (0.5%) and AMP C. Impex (0.25%) + IMP C. Impex (0.25%) diets compared to those fed the basal diet at 0.5 h post-stress. Similarly, the plasma lysozyme activity was noted to be enhanced at 0.5 h post-stress in fish fed the AMP C. Impex (0.5%) + IMP C. Impex (0.5%) diet compared to those fed the basal diet. There also was a significant enhancement of lysozyme activity also observed at 12 h post-stress in fish fed the AMP C. Impex (0.5%) dietary treatment compared to those fed the basal diet. Fish fed all the dietary nucleotides had significantly higher hematocrit values compared to those fed the basal diet at 0 h pre-stress. Interestingly, none of the responses of fish fed the dietary nucleotide treatments were significantly different from that of fish fed the basal diet at 0.5 h post-stress based on the hematocrit response. At 12 h post-stress, fish fed the other nucleotide treatments had significantly higher packed cell volume compared to those fed the basal diet.

Table 16. Effects of dietary nucleotide on hematological parameters and innate immune-modulation before and after subjecting hybrid striped bass to acute stress via air exposure for 1 minute.

Innate immune parameters			Dietary treatments							
	Time (hour)	Basal	AMP S.[1] 0.5%	IMP S.[2] 0.5%	AMP&IMP S.[3] 1%	AMP&IMP S.[4] 0.5%	AMP C.[5] 0.5%	IMP C.[6] 0.5%	AMP&IMP C.[7] 1%	AMP&IMP C.[8] 0.5%
Plasma anti-protease (%)										
Dietary treatment $P = 0.0486$	0	19.0	23.1	21.5	21.5	22.4	19.1	20.6	19.3	19.8
Time $P < 0.0001$	0.5	17.3	17.8	16.1	18.5	17.3	19.5	24.2*	19.5	21.7*
Dietary treatment × Time $P = 0.0020$	12	15.4	16.7	16.7	18.2	15.3	20.6	17.4	16.8	16.6
Pooled standard error = 1.26										
Plasma lysozyme (units/ml)										
Dietary treatment $P = 0.006$	0	877.8	905.6	811.1	855.6	822.2	883.3	783.3	855.6	1122*
Time $P < 0.0001$	0.5	861.1	861.1	811.1	983.3	700.0	1045	861.1	1167*	1000
Dietary treatment × Time $P = 0.0008$	12	1006	950.0	933.3	955.6	1172	1233*	955.6	1044	961.1
Pooled standard error = 66.7										
Hematocrit (%)										
Dietary treatment $P < 0.0001$	0	33.3	41.0*	39.8*	39.8*	44.8*	39.5*	40.3*	41.5*	42.7*
Time $P < 0.0001$	0.5	29.5	24.8	28.0	30.3	30.8	30.3	30.7	33.7	31.8
Dietary treatment × Time $P = 0.0002$	12	27.5	32.8*	37.7*	35.5*	40.2*	40.0*	38.2*	39.3*	43.3*
Pooled standard error = 1.50										
Total immunoglobulin (mg/ml)										
Dietary treatment $P = 0.1578$	0	39.3	42.0	38.3	41.2	41.2	37.1	38.0	41.2	40.3
Time $P < 0.0001$	0.5	34.9	38.8	41.1*	35.1	36.2	39.3*	40.1*	39.1*	39.9*
Dietary treatment × Time $P = 0.0030$	12	32.2	34.7	35.1	31.8	37.1	38.3*	32.7	33.2	31.0
Pooled standard error = 1.46										

[2]AMP Sig. (0.5%)
[3]IMP Sig. (0.5%)
[4]AMP (0.5%) + IMP (0.5%) Sig.
[5]AMP (0.25%) + IMP (0.25%) Sig.
[6]AMP C. Impex (0.5%)
[7]IMP C. Impex (0.5%)
[8]AMP (0.5%) + IMP (0.5%) C. Impex
[9]AMP (0.25%) + IMP (0.25%) C. Impex
*Asterisks represent treatment that is significantly different ($P<0.05$) than the basal diet for each immune parameter at each time points (values within the row)

For total immunoglobulin, all the dietary nucleotide treatments from supplier C. Impex and the IMP Sig. (0.5%) diet were significantly higher than that of the basal diet at 0.5 h post-stress. The total immunoglobulin response of fish fed AMP C. Impex (0.5%) at 12 h post-stress was also significantly higher than that of fish fed the basal diet.

III.4 Discussion

The findings in the present study showed that dietary supplementation of the nucleotides AMP and IMP either singularly or in combination did not significantly improve weight gain of juvenile hybrid striped bass although some of the nucleotide treatments did numerically increase the weight gain of fish compared to those fed the basal diets (Table 9). The basal diet in the present study was designed to have limiting amounts of purine nucleotides due to the high inclusion soybean meal (Kojima, 1974; NRC, 2011). Limiting purine nucleotide may not necessarily lead to reduced weight gain or other signs of deficiency as typically observed with many nutrients. Furthermore, nucleotides can be synthesized *de novo* or obtained through the salvage pathway unless the organism is under stressful conditions which may interfere with their endogenous synthesis capacity (Carver and Walker, 1995; Gil, 2002). Throughout the feeding trials, the fish were in excellent health condition with 100% survival for all dietary treatments. This raises the hypothesis that they were not in a stressful condition and thus did not interfere the endogenous synthesis of nucleotides from amino acid precursors. If this hypothesis is correct, then supplementing nucleotides in the diet would not necessarily result in large differences in weight gain. This hypothesis would confirm the correlation

found in a previous study from our laboratory in which supplementing 0.5% commercial nucleotide product (Ascogen P®, Canadian Bio-Systems Inc., Alberta, Canada) in the diet of juvenile hybrid striped bass diet did not result in statistically different growth performance after 8-weeks of feeding. However, differences in weight gain were noted when the fish were infected with *Streptococcus iniae* over a 6-week period (Li et al., 2004). Another possible explanation for the lack of growth differences in the present study is that purine nucleotides were not limiting in the basal diet even with high soybean meal inclusion This condition apparently was substantiated by an earlier report that showed no significant differences in growth performance of hybrid striped bass a nucleotide-rich diet in which fishmeal contributed all the CP compared to a high-plant protein diet in which 75% of the CP was contributed by soybean meal (Gallagher, 1994). A few studies have reported that supplementing purified purine nucleotides has improved growth performance of *Pagrus major* and *Epinephelus malabaricus* (Lin et al., 2009; Hossain et al., 2016a; Hossain et al., 2016b); however, it should be noted that the diet formulation used in those studies was semi-purified (Hossain et al., 2016a; Hossain et al., 2016b) and purified diet (Lin et al., 2009). Thus, these refined formulations could make nucleotides very limiting in the diet. Another recent study reported supplementing IMP in a high-soybean-meal diet improved growth performance of gibel carp compared to those fed the basal diet without supplementation; whereas, no statistical significance was detected between fish fed a high-fishmeal diet and high-fishmeal- diet supplemented with IMP (Zhang et al., 2019).

For specific immune responses evaluated in the present study, the increase in mitogenic responses (LPS-stimulated) induced by dietary IMP C. Impex was significantly higher than that of fish fed the basal diet (Table 15). There also differences in immune responses noted as a function of the nucleotide manufacturer. Similarly, Leonardi and Klempau (2003) reported that rainbow trout fed with dietary nucleotides (Optimûn, Chemoforma, Augst, Switzerland) had significantly higher B lymphocyte proliferation of LPS-stimulated cells compared to fish fed the basal diet prior to viral infection. Similar findings also were noted after the disease challenge with infectious pancreatic necrosis virus. However, dietary nucleotide (Rovimax Nx, DSM nutritional products, Basel, Switzerland) showed higher simulation index of lymphocytes with LPS in hybrid tilapia but it was not statistically different from fish fed the control diet (Shiau et al., 2015). Kulkarni et al. (1989) reported that dietary nucleotides could stimulate the maturation of lymphoid cells in that a higher percentage of immature lymphocytes was seen in mammals that were fed a nucleotide-free diet compared to those fed a diet supplemented with mixtures of nucleotides and nucleosides. Carver (1999) suggested that exogenous supply of nucleotides in the diet would contribute to the pool of nucleotides available to stimulate lymphocytes, which rapidly turn over and consequently have increased nucleotide requirements. Another report by Low et al. (2003) stated that an exogenous supply of nucleotides resulted in an increase in the humoral immune response as seen in upregulating IgM gene expression; they further hypothesized that increasing production of IgM synthesis could potentially amplify the production of functional B lymphocytes. Moreover, this hypothesis is supported by

several studies reporting increased lymphocyte count when nucleotides were supplemented in the diet (Tahmasebi-Kohyani et al., 2012; Barros et al., 2015; Reda et al., 2018).

Although numerous studies have reported that dietary nucleotide supplementation improved innate immunity of fish (Tahmasebi-Kohyani et al., 2011; Song et al., 2012; Zhao et al., 2015; Hossain et al., 2016a), no such effects were observed in the present study. However, this lack of effect on innate immunity is in line with a few studies with Nile tilapia (Barros et al., 2015), channel catfish (Welker et al., 2011), and red drum (Li et al., 2007).

A hypothesis evaluated in the present study with hybrid striped bass was if the endogenous supply of nucleotides plays an important role in modulation of innate immunity during stressful conditions in. In fact, prior to testing this hypothesis in experiment 3, experiment 2 demonstrated that acute stress caused temporary immune-suppression of plasma lysozyme and anti-protease activity which was seen at 12 h post-stress, and these responses made a complete recovery at 24 h post-stress (Fig. 2D and Fig 2E). Moreover, it was observed that the acute-stress induced a suppressive hematocrit response at 0.5 h post-stress which did not completely recovery at 24 h post-stress (Fig. 2C).

In experiment 3, all the innate immune parameters except for hematocrit showed no differences prior to the stress challenge (Table 16), and these findings were highly correlated with experiment 1 results in which no significant differences in innate immunity were observed (Table 15). However, at 0.5 h and 12 h post-stress, it was

intriguing to observe that some of the dietary nucleotide treatments activated some innate immune responses which were significantly different from that of fish fed the basal diet (Table 16). These responses indicate dietary nucleotide supplementation play an important role in modulating innate immunity during stressful conditions.

It is well established that the ability of an organism to maintain homeostasis is critically reliant on bi-directional communication between the neuroendocrine system and the immune system to monitor the environment and allow adaptive responses to psychological and physiological disturbances as well as disease challenges (Engelsma et al., 2002). Our findings indicate the adaptive response of innate immunity in hybrid striped bass subjected to the air exposure stressor was influenced by the supplementation of nucleotides. For example, fish fed with diet supplemented with AMP C. Impex demonstrated significantly enhanced hematocrit, plasma lysozyme, and total immunoglobulin activity at 12 h post stress compared to fish fed the basal diet. This suggests that an exogenous supply of AMP plays the most significant role in enhancing innate immunity during and after exposure to a stressful condition (Table 16).

The potential synergistic effects of combining dietary AMP and IMP supplementation demonstrated that nucleotide concentrations and suppliers exhibited some differences in immunomodulation at post-stress 0.5 h; however, the greatest enhancement of immunity at 12 h post-stress was most apparent in the single addition of AMP C. Impex (Table 16). In fact, experiment 2 demonstrated that the fish were only able to recover their innate immunity at 24 h post-stress (Fig. 2). A recent study also reported that dietary nucleotide supplementation improved the innate immune activity of

gibel carp at 7 h post bacterial challenge with *Aeromonas hydrophila,* and prior to the diseases challenge, no statistical significance was detected between fish fed the nucleotide-supplemented diet and control diet without nucleotide supplement (Zhang et al., 2019). Burrells et al. (2001) stated that stressful events related to various aquaculture practices such as vaccination, handling, and disease exposure may increase the need for dietary nucleotides to provide an exogenous supply for optimal responses of the fish.

The price of purified nucleotide sold in the market varies depending on where it is manufactured and the company's manufacturing procedures. As previously mentioned, some commercial nucleotide products are primarily derived from yeast products. The nucleotide supplement from Sigma and Chem-Impex were laboratory-certified products with guaranteed purity of $\geq$98%. The AMP and IMP products were obtained for approximately $250/25g and $20/25g by Sigma-Aldrich and Chem-Impex, respectively. There are less expensive feed grade high purity adenosine and inosine 5'-monophosphate disodium salt that are sold in the market from China and valued at approximately $20/kilogram though the quality of the products must be verified. It is noteworthy that numerous published studies have recently reported that purified nucleotides manufactured in China have demonstrated positives results on various fish species. It appears that supplementing nucleotide products in this price range to hybrid striped bass diets is economically feasible.

Based on our current findings, the effects of dietary nucleotides on hybrid striped bass immunity were influenced by the type of nucleotide used, the dosage, and also the manufacturer. Existing literature also has noted inconsistency findings that may be

related to species of fish, diet formulation, and administration time. In conclusion, the present study showed that the exogenous supply of AMP C. Impex at 0.5% of diet yielded the best capability of hybrid striped bass to enhance their innate immune responses and resistance stress-induced innate immune system suppression after an air exposure stress challenge. In addition, only the IMP C. Impex supplement yielded the highest B-lymphocyte proliferation which was significantly different from that of fish fed the control diet. Further investigations are being pursued on the effects of incremental dosages of dietary AMP on hybrid striped bass fed practical diets containing very high levels of soybean meal.

CHAPTER IV

ESTABLISHING THE OPTIMAL PURIFIED DIETARY NUCLEOTIDE (ADENOSINE 5'-MONOPHOSPHATE) LEVEL FOR HYBRID STRIPED BASS: EFFECTS ON GROWTH PERFORMANCE, NUTRIENT DIGESTIBILITY, AND IMMUNE MODULATION DURING ACUTE AND CHRONIC STRESS

IV.1 Introduction

Fishmeal is one of the densest protein and energy feed ingredients that has become increasingly more expensive as its demand continues to increase with aquaculture expanding as the fastest growing food-producing sector in the world. Traditionally, diet formulation for carnivorous species such as hybrid striped bass contained relatively high levels of fishmeal although other protein feedstuffs including those of plant origin have been successfully incorporated at relatively high levels to replace considerable amounts of fishmeal (Gallagher, 1994; Rawles et al., 2006a; Rawles et al., 2006b; Rossi Jr et al., 2015; de Cruz et al., 2018; Perez-Velazquez et al., 2019). It is also well established that fishmeal is a rich source of other constituents such as nucleotides, and successful replacement of fishmeal with plant protein feedstuffs such as soybean meal and soy protein concentrate which have much lower concentrations of nucleotides (Kojima, 1974; Li et al., 2015), could make nucleotides limiting in the diet.

Nucleotides are monomers of nucleic acids that are building blocks of deoxyribonucleic acid and ribonucleic acid (RNA), and play significant roles in a number of biochemical processes including storage and transfer genetic information,

mediating energy metabolism, enzymatic regulation, and signal transduction as well as being structural components of coenzymes (Carver and Walker, 1995; Gil, 2002; Steinberg and Kemp, 2009). The various nucleotides can be synthesized endogenously from amino acid precursors; thus, they are not classified as essential nutrients, and limited inclusion in the diet may not necessarily lead to reduced weight gain or other signs of deficiency as typically observed with other nutrients (Carver and Walker, 1995; NRC, 2011). Nevertheless, nucleotides have been classified as conditionally essential, because under certain circumstances such as extraordinary stressful events such as rapid growth, reproduction, disease infection, and injury recovery may limit the organism's endogenous synthetic capability (Carver, 1999). In addition, nucleotides are efficiently recycled from products of cellular turnover in the salvage pathway and also can be obtained exogenously by dietary intake (Grimble and Westwood, 2001). The concentration of nucleotides in feedstuffs mainly depends on the presence of nucleoproteins; therefore, feedstuffs derived from high cell density items such as fish, chicken, and animal organ meats were reported to have higher concentrations of nucleotides compared to other feedstuffs such as barley, corn, and soy protein concentrate (Gil, 2002; Li et al., 2015). In comparison to fishmeal, yeast extract has twice the concentration of purine and pyrimidine bases (Devresse, 2000). Commercial brewer's yeast (*Saccharomyces cerevisiae*), the inactive yeast remaining after the brewing process, has been reported to have 12-20% of purine and pyrimidine nucleoproteins (RNA nitrogen) from the total nitrogen (Rumsey et al., 1992). Thus, brewer's yeast has been used widely for commercial production of supplemental

nucleotides (Ferreira et al., 2010) including AMP (adenosine 5'-monophosphate), GMP (guanine 5'-monophosphate), IMP (inosine 5'-monophosphate), UMP (uracil 5'-monophosphate), and CMP (cytosine 5'-monophosphate). Most of the earlier studies on nucleotide nutrition mainly focused on the dietary effects of commercial nucleotide products on fish growth performance, diseases resistance, and innate and adaptive immune responses (Ramadan et al., 1994; Adamek et al., 1996; Burrells et al., 2001a; Low et al., 2003; Li et al., 2004; Russo et al., 2006). However, one possible limitation to using commercial nucleotides is that they may contain other undisclosed non-nucleotide compounds that may provide additional benefits to the animal. For example, it is well established that brewer's years also contains other constituents such as β-glucans and mannan-oligosaccharides that have proven to be capable of enhancing growth performance, immunity, and intestinal health of fish (Ferreira et al., 2010; Hisano et al., 2018). There is the possibility that some of these compounds may be retained in the commercial oligonucleotide cocktail because disclosed nucleotide content in most of the commercial nucleotide products range between 9 and 35% (Wei et al., 2007; Reda et al., 2018). This concern about the heterogeneity of commercial products (Li et al., 2015) prompted more studies to be focused on known purified single nucleotides or nucleotide mixtures (Li et al., 2007; Lin et al., 2009; Song et al., 2012; Hossain et al., 2017; Guo et al., 2019).

Among the various nucleotides, the nucleoside adenosine is documented as a key mediator of the immune response as adenosine regulates tissue function by activating four G-protein-coupled adenosine receptors which are known to be expressed in immune

cells and modulate all aspects of immune and inflammatory responses (Antonioli et al., 2019). It was also demonstrated in two separate studies that supplementing AMP to the diet of *Pagrus major* and *Epinephelus malabaricus* enhanced their growth rate and immune responses (Lin et al., 2009; Hossain et al., 2016a). Previous studies also have reported a very high dosage of purified mixture nucleotide had a significant ($P<0.05$) increase in mortality of fish after being subjected to stress (Welker et al., 2011) and also a significant reduction in weight gain (Song et al., 2012). Thus, establishing the optimal dietary level of individual nucleotides by dose-response may allow formulation of economically feasible diets to promote the growth performance and overall health of the fish. To date, no studies have evaluated the dose-response of the single nucleotide AMP in hybrid striped bass. Therefore, the focus of this study was to investigate the relationship between dietary doses of AMP and response in terms of growth performance, immunity, and whole-body nutrient deposition in hybrid striped bass. In addition, the present study also investigated the effects of supplementing the optimal dosage of AMP on nutrient digestibility, digestive enzyme activities, acute-stress responses and chronic stress responses of hybrid striped bass.

IV.2 Materials and methods

IV.2.1 Diets and formulations

Five separate feeding trials were conducted to evaluate the dietary effects of AMP on hybrid striped bass. The basal diet for all trials was formulated principally from dehulled soybean meal, soy protein concentrate, and fishmeal to contain 39.1% crude

protein, 10.7% lipid, and estimated digestible energy 2.5 kcal g^{-1} (Table 17). Previous studies have reported that hybrid striped bass performed well with high inclusion of soybean meal and soy protein concentrate, and their growth was not significantly different in comparison to fish fed diet that contained all of its protein from fishmeal (Gallagher, 1994; Rossi Jr et al., 2015). It was presumed the basal diet would be limiting in purine nucleotides due to high inclusion of soybean meal and soy protein concentrate (Kojima, 1974; Li et al., 2015). All the formulated experimental diets were isonitrogenous, isolipid, isocaloric, and met or exceeded all established nutrient requirements of hybrid striped bass (NRC, 2011). The processes for diet production and storage were as previously described by de Cruz et al. (2018). The nucleotide supplement, adenosine 5'-monophosphate disodium salt, was coated with 1% agar solution (Sigma Aldrich Co., St. Louis, Missouri) to limit its solubility in water. After freezing solid at -20°C, moisture was removed completely by freeze drying. Then the coated nucleotide was finely ground so it could be added to the diet along with the other dry ingredients. The dietary treatments for each feeding trial are described below.

IV.2.2 Fish and culture system

All five trials were conducted at the Texas A&M University Aquacultural Research and Teaching Facility. The animal care and experimental protocols were permitted by the Institutional Animal Care and Use Committee at Texas A&M University. Keo Fish Farms (Keo, Arkansas, USA) provided the hybrid striped bass (*Morone chrysops* x *M. saxatilis*) for all the experiments. Four trials (dose-response,

digestive enzyme activities, acute-stress response, and chronic-stress response) and one digestibility trial were conducted in a recirculation system comprising of 110-L glass aquaria and 1200-L circular fiberglass tank, respectively.

The recirculating system was also connected to a settling chamber, biological filter, ultraviolet sterilizer, and sand filter. The photoperiod in the cultured system was kept at 12D:12L cycle. Salinities were generated by mixing Red Sea Salt (Red Sea, Houston, Texas) with well water. Water quality parameters that were maintained suitable for hybrid striped bass culture as follows; water temperature = 27.2 ± 0.12 ˚C, dissolved oxygen = 7.6 ± 0.50 mg L^{-1}, total ammonia nitrogen (TAN) = 0.09 ± 0.08 mg L^{-1}, nitrite nitrogen = 0.05 ± 0.04 mg L^{-1}, salinity = 2.9 ± 0.2 g L^{-1}, and pH = 7.9 ± 0.2. The water quality parameters for the chronic stress challenge trial was mentioned in section 2.7.

Table 17. Ingredients and analyzed composition of the basal diet.

Ingredients[1]	%
Menhaden fishmeal[2]	6.7
Soybean meal[3]	50.1
Soy protein concetrate[4]	9.7
Menhaden oil[5]	9.4
Vitamin premix[6]	3.0
Mineral premix[6]	4.0
Dextrinized starch[7]	10.0
Calcium phosphate dibasic[7]	1.0
Carboxymethyl cellulose[7]	2.0
Glycine[8]	1.0
Taurine[8]	0.5
DL-Methonine[8]	0.5
Cellulose[7]	2.1
Agar[9]	0.1
AMP[10]	0-2.0
Analyzed composition, g/100 g[1]	
Crude protein	39.1
Crude lipid	10.7
Ash	9.7

[1] Dry-matter basis and means of duplicate analyses.
[2] Special Select, Omega Protein, Abbeville, Louisiana (crude protein [CP] = 67.9 %; lipid = 12.0 % on a dry-matter basis).
[3] Producers Cooperative Association, Bryan, Texas (CP = 53.1 %; lipid = 3.3 % on a dry-matter basis).
[4] Solae LLC, St. Louis, Missouri (CP = 70.8 %; lipid = 0.9% on a dry-matter basis.
[5] Omega Protein, Reedville, Virginia
[6] Same as in Moon and Gatlin (1991).
[7] MP Biomedicals, Solon, Ohio.
[8] Ajinomoto North America, Inc.
[9] Sigma Aldrich Co., St. Louis, Missouri.
[10] Adenosine 5'- monophosphate disodium salt (AMP), Chem-Implex International, Wood Dale, Illinois.

IV.2.3 Dose-response trial

The optimal dose of AMP was determined in an 8-week feeding trial in which graded levels of AMP were fed. The experimental diets were prepared by supplementing the agar-coated AMP disodium salt (Purity $\geq$ 98%, Chem-Implex International, Wood Dale, Illinois) to the basal diet at six incremental levels (0, 0.25, 0.5, 0.75, 1.5 and 2.0 %) at the expense of cellulose. The hybrid striped bass fingerlings (n=14 per aquarium) with an average weight of 6.0 ± 0.1 g were stocked into each aquarium in the recirculating system described below in section 2.7. Each dietary treatment was randomly assigned to four replicate aquaria (n=4). The feed ration was set to 6% of total body weight per day (initial week) which was close to apparent satiation and distributed in morning and evening feedings. The fish were group-weighed each week, and feed rations were gradually adjusted equally for all dietary treatments over time to maintain a level approaching apparent satiation. The dose-response trial was terminated at the end of the 8-week period and the growth performance was measured as follows: weight gain (WG) = ([g final weight − initial weight]/g initial weight) × 100; feed efficiency ratio (FER) = g weight gain /g dry feed offered; condition factor (K) = (g final weight × 100)/total length^3; and survival = (final no. of fish/initial no. of fish) × 100. From each replicate aquarium, six fish were netted out and euthanized with an overdose (>300 mg/L) of tricainemethane sulphonate (MS-222, Western Chemical Inc, Ferndale, Washington,). Blood samples were collected and body condition indices were obtained from three euthanized fish per aquarium. The body condition indices were calculated with the following equation: hepatosomatic index (HSI) = g liver weight /100 g body

weight; and intraperitoneal fat (IPF) ratio = g IPF weight /100 g body weight. The analytical procedures to determine innate immune responses are described in section 2.8. The other three fish per aquarium were combined and homogenized as a composite sample for determination of whole-body proximate analysis. For the proximate composition analysis, established methods were used as follows: the Dumas protocol for crude protein (6.25 × N) (AOAC, 2005), heating samples at 650 °C in the muffle furnace for 4 h for ash (AOAC, 1990), and chloroform: methanol extraction for crude lipid (Folch et al., 1957). The protein/lipid retention efficiency was computed as follows: (([(g final body weight × % final body protein/lipid) − (g initial body weight × % initial body protein/lipid)] / (protein/lipid intake (g)]) × 100).

IV.2.4 Digestibility trial

Based on the conclusion of the dose-response trial, AMP at 0.5% of dry diet was determined to be the optimal dosage. Therefore, the digestibility trial was conducted to compare the apparent digestibility coefficients (ADCs) of the basal diet compared to a diet supplemented with AMP at 0.5% of diet. Both diets were formulated from the same ingredients (Table 17) and manufactured as described in section 2.1 with the additional of 0.1% yttrium oxide (Y_2O_3, Sigma Aldrich Co.) as an external marker at the expense of cellulose. Groups of 15 hybrid striped bass with an average weight of 452.5 ± 42 g were assigned randomly to each of six, 1200-L fiberglass tanks in a recirculating system configured as described above. The replicate unit was the fiberglass tank (n=3) for each dietary treatment. The fish were fed twice daily to apparent satiation for a 3-week

acclimation period. Following that period, fecal samples were collected 6 h after feeding by the stripping technique (Rawles et al., 2006a). The fecal collection was conducted twice during week 4, and the collections from each replicate tank were pooled to obtain one composite sample for each of the three replicate tanks. The fecal samples were dried overnight at 60˚C and stored at -20°C until further analysis. The proximate composition of the diet and fecal samples were analyzed as described in section 2.3. Fecal and diet sub-samples of were sent to the College of Veterinary Medicine, Texas A&M University for analysis of yttrium oxide. Apparent digestibility coefficients (ADCs) for both diets were calculated using the following formula: ADCs = 100 − (100 × % indicator in feed/% indicator in feces) × (% nutrient in feces/% nutrient in feed) (NRC, 2011).

IV.2.5 Digestive enzyme trial

The objective of this trial was to evaluate the effects of the basal diet and one supplemented with AMP at 0.5% of dry weight (chosen based on results of the dose-response trial) on digestive enzyme activities at two different sampling hours (10 h after feeding and 20 h after feeding) in two different intestinal sections (anterior and posterior). Two groups of juvenile hybrid striped bass were fed with basal and AMP-supplemented diets for 6 weeks in 400-L circular fiberglass tanks configured as a recirculating system prior moving them to the 110-L aquarium system (described in section 2.2). Groups of nine juvenile hybrid striped bass with an average weight 98.1 ± 4.3 g were stocked into each of six aquaria and fed their respective diets for another additional 2 weeks. The fish were fed twice daily with the same procedure described in

section 2.3. The replicate unit was the aquarium (n=2) for each dietary treatment. Three fish were randomly collected from each aquarium at 10 h and 20 h after feeding. The fish were euthanized with an overdose (>300 mg/L) of MS-222 and the intestines with the pyloric caeca were aseptically dissected and flash frozen in liquid nitrogen. The intestines were stored at -80°C prior to extraction.

During enzyme extraction, the intestines were divided into two sections (anterior and posterior). A small piece of intestine (~1g) from each section was collected and homogenized in cold, 50 mM Tris-HCl, 20 mM $CaCl_2$ buffer (~1 ml). The supernatants of intestinal homogenates were stored at -80 °C prior to specific enzyme activity determination. Prior to digestive enzyme activity assessment; the concentration of soluble protein in each intestinal sample was quantified as described by Bradford (1976) (Bio-Rad Laboratories, Hercules, California) using bovine serum albumin as the statndard. Digestive enzymes activity was determined spectrophotometrically for the anterior and posterior intestinal samples from each dietary treatment at both sampling hours. The digestive enzyme activities measured included the following: trypsin using *N*-a-benzoyl-DL-arginine 4-nitroanilide hydrochloride as a substrate (Erlanger et al., 1961); lipase using sodium cholate hydrate and *b*-naphthyl-caprylate as a substrate (Versaw et al., 1989); a-amylase using soluble starch (1%) as a substrate (Vega-Villasante et al., 1993); acid/alkaline phosphatases using 4-nitrophenyl phosphate (2%) as a substrate (Anguiano et al., 2013). The enzymatic activities were expressed as activity units (U) per mg of soluble protein.

IV.2.6 Acute-stress challenge trial

This trial was conducted to investigate the effects of dietary AMP supplementation at 0.5% of diet (chosen based on results of the dose-response trial) compared to the basal diet on hematological parameters when the fish were subjected to acute stress. Prior to initiating the acute-stress challenge, two groups of juvenile hybrid striped bass were fed the basal diet and one supplemented with AMP at 0.5% of diet for 4 weeks in a 400-L circular fiberglass tank recirculating system. Then, groups of six fish with an average weight of 92.9 ± 3.6 g were randomly stocked into each of 20, 110-L aquaria configured as a recirculating system as described in section 2.2 and conditioned on their respective dietary treatments (five aquaria each) for another additional 2 weeks. The fish were fed twice daily according to the same procedures described in section 2.3. Feeding was withheld approximately 15 h before initiation of an acute stress challenge which entailed subjecting the fish to air for 1 minute. The fish from each aquarium were completely netted out in a single pass with a large net and then retained in the net at the surface of the aquarium and exposed to air for 1 minute. The experimental subject for this experiment was the individual fish (n=6) nested within the dietary treatments (basal and 0.5% AMP) and there were five replicate aquaria for each dietary treatment (six fish in one aquarium was sampled at each sampling time). During the acute stress challenge, groups of six replicate fish were sampled at 0 (pre-challenge), 0.5, 1, 12, and 24 h post-challenge to examine the effects of acute stress on plasma cortisol, blood glucose, plasma osmolality and plasma innate immunity of hybrid striped bass. The fish from each dietary treatment at each time point were collected and instantly sedated with MS-

222 (150 mg L^{-1}) as recommended by Trushenski et al. (2012). The blood from all six fish in each aquarium were collected within 4 minutes. The blood sample collection and analytical procedures were described below in section 2.8.

IV.2.7 Chronic stress challenge trial

The focus of this trial was to investigate the effects of dietary AMP supplementation at 0.5% of diet (based on the dose-response trial) on weight gain and hematological parameters after the fish were subjected to osmotic stress for an extended period of time (chronic stress). Juvenile hybrid striped bass associated with the dose-response trial were used in this trial after being fed their respective dietary treatments (basal and 0.5% AMP) for 8 weeks. The fish from all the replicate aquaria were pooled based on dietary treatment, then groups of 12 fish with an average weight of 50.6 ± 0.2 g were assigned to each of 110-L aquaria operated as a recirculating system described in section 2.2. Previous studies in our laboratory demonstrated that culturing hybrid striped bass in full-strength seawater (32 ppt) severely restricted their growth and feeding activity compared to fish cultured in freshwater (0 ppt) and brackish water (7 ppt) (Brown et al., 1992). This was mainly due to osmotic stress, which was further supported by the findings of Weirich et al. (1992) who reported that hybrid striped bass had better survival after being subjected to acute-stress when cultured in brackishwater (8 ppt) which is near isosmotic with the plasma of these fish. Therefore, half strength seawater was used in this trial as a mild stressor. The chronic stress was carried out by increasing the salinity of the water to approximately 15 ppt and the beginning of the trial

which was conducted for 4 weeks. The fish were fed their respective diets according to the procedures described in section 2.3. The replicate unit was the aquarium (n=3) for each dietary treatment. At the end the week 4, three fish per replicate aquarium were sedated with MS-222 and blood was collected to determine the following hematological parameters: cortisol, glucose, osmolality, hematocrit, anti-protease, lysozyme, total protein, and total immunoglobulin. After the blood collection, fish were euthanized with a high dosage of MS-222 and their weight was recorded along with that of the remaining fish in each replicate aquarium to determine weight gain. Three additional fish per aquarium were euthanized to collect the head kidney for leucocyte isolation. The details of the sample collection and analytical procedures are described in section 2.8. Water quality parameters that were kept as follow; water temperature = 26.9 ± 0.5˚C, dissolved oxygen = 7.3 ± 0.6 mg L^{-1}, total ammonia nitrogen (TAN) = 0.06 ± 0.03 mg L^{-1}, nitrite nitrogen = 0.02 ± 0.1 mg L^{-1}, salinity = 15.7 ± 1.5 g L^{-1}, and pH = 8 ± 0.1.

IV.2.8 Sample collection and analytical procedures for immune and stress parameters

Heparinized 1-mL syringes with 26-gauge needles were used to bleed the fish from the caudal vasculature. A sub-sample of blood was examined for glucose using a handheld glucose meter (Accu-Check guide®, Roche, Basel, Switzerland), and hematocrit was determined by centrifugation of capillary glass tubes (Drummond Scientific, Pennsylvania, USA). The remainder of the blood sample was centrifuged at 3000 × *g* for 10 min, and the plasma was stored in several aliquots at -80°C until

analysis. Plasma osmolality was measured using a vapor pressure osmometer (Vapro 5520, Wescor, Inc., Logan, Utah). Plasma cortisol levels were determined by means of an enzyme-linked immunosorbent assay (ELISA) kit (DRG International, Springfield Township, New Jersey).

The plasma innate immune responses were determined as follows: lysozyme activity by turbidimetric reduction assay (Jørgensen et al., 1993) by using *Micrococcus lysodeikticus* (Sigma Aldrich Co.); total immunoglobulin and total protein (Siwicki et al., 1994) by using bovine serum albumin as a standard; anti-protease activity by a modification of the method described by Ellis (1990) and was computed as the percentage inhibition of trypsin activity compared to a reference sample (without plasma).

Head kidney leukocytes were isolated through density gradient sedimentation. Briefly, the head kidney from a group of three euthanized hybrid striped bass per replicate aquarium was aseptically excised using scalpels. The head kidney tissues were pooled and stored in 15 ml of cold Leibovitz cell culture medium (L-15, Corning Inc., Corning, New York) containing 10 units mL^{-1} of heparin (Akron Biotechnology, Boca Raton, Florida), 100 units mL^{-1} of penicillin and streptomycin (Sigma Aldrich Co.), and 2% fetal bovine serum (Sigma Aldrich Co.). The isolation of phagocytes was carried out by the method established by Secombes (1990) and adapted by Sealey and Gatlin (2002). Respiratory burst activity of phagocytes was determined by intracellular and extracellular superoxide anion production as described by Secombes (1990). The absorbance was read at 620 nm to determine the concentration of intracellular O^{2-}

produced. The concentration of extracellular O^{2-} produced was computed by the following equation superoxide O^{2-} = ([Δ Absorbance after 45 min×100] ÷ 6.3) (Pick and Mizel, 1981).

The method established by Secombes (1990) with minor adaptation as described by Carvalho et al. (2018) was used to isolated the lymphocytes. The proliferation of lymphocytes upon stimulation with lipopolysaccharide (1 mg mL^{-1}) solution (LPS, Sigma Aldrich Co.) was measured using a colorimetric assay as suggested by Mosmann (1983). The lymphocyte proliferation capacity upon simulation with the non-specific mitogen was computed and presented as stimulation index (SI = ABS stimulated cells ÷ ABS non-stimulated [control] cells).

IV.2.9 Statistical analysis

All data were assessed for normality using the Shapiro–Wilk test (repeated measure ANOVA doesn't need to meet normality assumption) and $\alpha < 0.05$ was used as significant level for all analysis. Data resulting from the dose-response trial were subjected to orthogonal polynomial contrasts and if statistical significance was detected (linear, quadratic or cubic), the data were subjected to regression analysis to fit the best model. When significant quadratic or cubic responses were noted, the non-linear broken line model was examined as well.

For the trial digestibility, digestive enzyme activity, and chronic stress were subjected to student's t-test to detect significant differences between the basal and AMP 0.5% treatments.

The data resulting from the acute-stress challenge were subjected to repeated measure analysis of variance (RM-ANOVA). The data were analyzed by RM-ANOVA within PROC MIXED (repeated statement) and appropriate covariance structure was selected for the analysis. The differences between dietary treatment were considered significant when p-value ≤ 0.05. Contrast pair-wise comparisons were carried out at each time point between dietary treatments only if statistically significant interaction was detected; otherwise, the overall significant main dietary effects were discussed. All statistical analysis was carried out using the Statistical Analysis System, version 9.4 (SAS Institute, North Carolina, USA).

The full model developed for acute-stress response was;

$Y_{ijk} = \boldsymbol{\beta}_0 + \boldsymbol{\alpha}_i + \boldsymbol{\gamma}_k + (\boldsymbol{\alpha\gamma})_{ik} + \boldsymbol{\varepsilon}_{ijk}$ where,

Y_{ijk}: the investigated responses at k^{th} hour on the j^{th} fish assigned to the i^{th} treatment effect

$\boldsymbol{\alpha}_i$: dietary treatment effect (i=1, 2)

$\boldsymbol{\beta}_0$ is the overall intercept;

$\boldsymbol{\gamma}_k$: hour effect (k=1, 2, 3, 4, 5)

$(\boldsymbol{\alpha\gamma})_{ik}$: dietary treatment * hour effect

$\boldsymbol{\varepsilon}_{ijk}$ denotes the error term

IV.3 Results

At the end of 8-week dose-response trial, incremental dosage on AMP in the diet had a significant relationship on growth performance of hybrid striped bass that was observed in percent weight gain (Fig. 3), survival, and HSI, while no relationship was observed for IPF ratio, K and innate immune responses (Table 18; Table 19). The minimum dietary requirement for AMP was estimated with a two-slope broken line model to be 0.46% of dry diet based on percent weight gain (Fig. 3). A decreasing linear relationship was noted in survival (Table 18); however, numerically the survival was only lower in fish fed the 1.5% AMP and 2% AMP treatments compared to those fed the basal diet (0% AMP). Moreover, the IPF ratio relationship was described as a significant increasing linear model as the dosage of AMP was elevated (Table 18). It was also noted that FER was described in a two-slope broken line model with linear ascending and linear descending portions but the model was not significant (P=0.3113, Table 18). The effects of incremental doses of dietary AMP on whole-body composition was best explained in a significant increasing linear model for whole-body lipid deposition, but no relationship was observed for moisture, protein and ash deposition (Table 20). Besides that, the relationship between increasing dose of AMP to protein retention efficiency was best explained with a two-slope broken line model with a quadratic ascending and linear descending portions in which the requirement for AMP was estimated to be 0.34% of diet. Based on all these findings, the best dosage of AMP was determined to be 0.5% of dry diet.

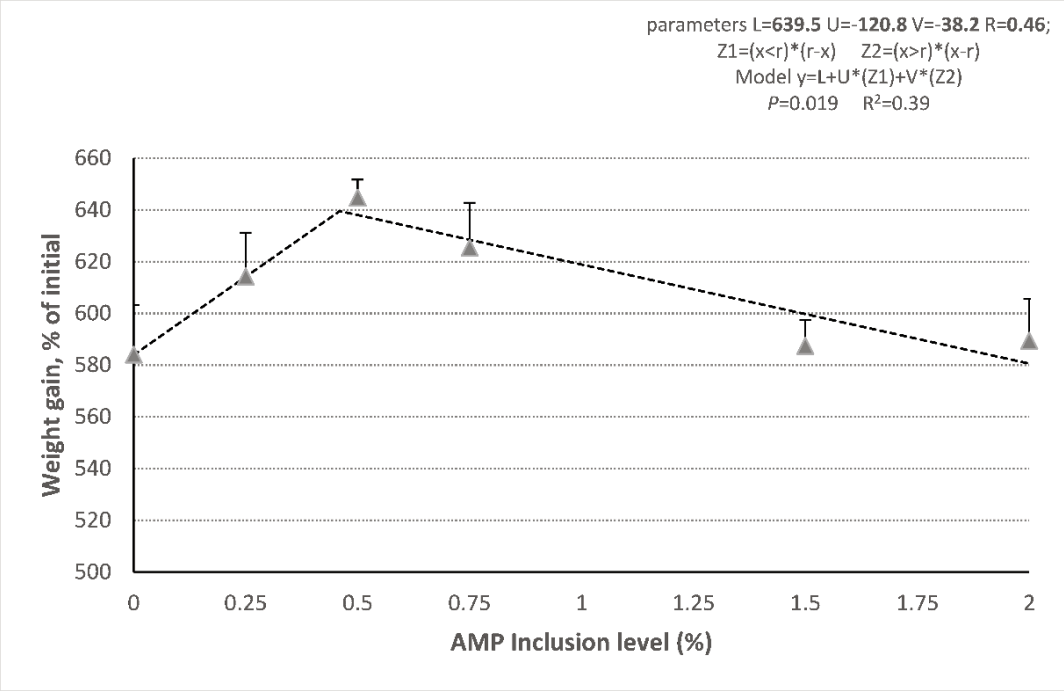


Figure 3. Two-slope broken line-linear ascending and linear descending regression model of hybrid striped bass weight gain in response to increasing levels of dietary adenosine 5'-monophosphate nucleotide. Data are represented as mean ± SE (n=3).

Table 18. Feed efficiency, survival, intraperitoneal fat ratio, hepatosomatic index, and Fulton's condition factor of hybrid striped bass after 8 weeks of feeding the experimental diets.[1]

AMP	FER	Survival	IPF	HSI	K
g/100 g		%	%	%	
0.00	0.82	96.4	5.28	1.50	1.36
0.25	0.83	100.0	4.95	1.53	1.41
0.50	0.86	96.4	5.44	1.50	1.31
0.75	0.83	96.4	5.48	1.63	1.35
1.50	0.79	89.3	5.71	1.84	1.39
2.00	0.83	91.1	5.52	1.76	1.33
PSE (n=4)	0.01	3.01	0.22	0.06	0.04
Orthogonal Contrast					
(Pr>F)[2]					
Linear	0.1839	0.0208	0.0852	0.0001	0.6584
Quadratic	0.8257	0.9667	0.4161	0.3623	0.9271
Cubic	0.0093	0.1972	0.3745	0.0461	0.2892
Regression (n=4)					
Model[3]	2SBL-LL	L	NOS	L	NOS
Pr>F[4]	0.3113	0.0152	-	0.0001	-
R^2	0.16	0.24	-	0.51	-
Requirement	0.43	-	-	-	-

[1] FER = feed efficiency ratio; HSI = hepatosomatic index; IPF = intraperitoneal fat; K = Fulton's condition factor; AMP = Adenosine 5'-monophosphate (AMP); PSE = pooled standard error of treatment means.
[2] If statistical significance detected ($P<0.05$), the model that fits best with the data was selected.
[3] L = linear; NOS = no structure; 2SBL-LL = two slope broken line- linear ascending and linear descending.
[4] Probability associated with the F statistic.

Table 19. Innate immune responses of hybrid striped bass after 8 weeks of feeding the experimental diets.

AMP[2]	NBT[1]	Lysozyme activity	Total Plasma Protein	Total Plasma Immunoglobulin	Antiprotease activity
g/100 g		units/ml	mg/ml	mg/ml	%
0.00	0.56	344	34.4	21.8	15.4
0.25	0.57	398	34.6	23.2	14.0
0.50	0.57	350	35.6	24.1	15.4
0.75	0.57	397	31.8	22.3	14.7
1.50	0.57	391	35.7	23.2	16.4
2.00	0.58	352	32.4	21.9	15.1
PSE[3] (n=4)	0.01	25.0	1.32	0.80	0.90
Orthogonal Contrast (Pr>F)[4]					
Linear	0.5659	0.9386	0.4391	0.7205	0.4264
Quadratic	0.8972	0.1687	0.3262	0.1476	0.7964
Cubic	0.7302	0.7816	0.9803	0.7382	0.1716
Regression (n=4)					
Model[5]	NOS	NOS	NOS	NOS	NOS
Pr>F[6]	-	-	-	-	-
R^2	-	-	-	-	-
Requirement	-	-	-	-	-

[1] Whole blood neutrophil oxidative radical production. Mean at OD 545
[2] Adenosine 5'-monophosphate (AMP)
[3] PSE = pooled standard error of treatment means.
[4] If statistical significance detected (P<0.05), the model that fits best with the data was selected.
[5] NOS = no structure.
[6] Probability associated with the F statistic.

Table 20. Whole-body proximate composition[1], protein conversion efficiency, and lipid conversion efficiency of hybrid striped bass at the end of 8 weeks of feeding.[2]

AMP	Moisture	Protein	Lipid	Ash	PRE	LRE
g/100g	%	%	%	%	%	%
0.00	69.3	17.7	9.5	4.3	36.7	72.2
0.25	69.1	17.9	9.8	4.3	38.0	78.0
0.50	68.8	17.7	9.8	4.5	38.5	80.1
0.75	69.3	17.6	9.5	4.4	36.4	74.7
1.50	68.7	17.6	10.7	4.1	33.8	78.5
2.00	68.7	17.6	10.4	4.2	34.4	78.5
PSE (n=4)	0.35	0.19	0.28	0.12	0.81	2.70
Orthogonal Contrast (Pr>F)[3]						
Linear	0.1959	0.3448	0.0042	0.1694	0.0009	0.2807
Quadratic	0.8132	0.7046	0.8513	0.4517	0.6114	0.4096
Cubic	0.9964	0.9838	0.2568	0.0834	0.0193	0.3412
Regression (n=4)						
Model[4]	NOS	NOS	L	NOS	2SBL-QL	NOS
Pr>F[5]	-	-	0.0038	-	0.0053	-
R^2	-	-	0.32	-	0.46	-
Requirement	-	-	-	-	0.34	-

1 Fresh-weight basis

2 PRE = protein retention efficiency; LRE = lipid retention efficiency; AMP = Adenosine 5'-monophosphate; PSE = pooled standard error of treatment means.

3 If statistical significance ($P<0.05$) was detected the model that fits best the data was selected.

4 L = linear; NOS = no structure; 2SBL-QL = two slope broken line-quadratic ascending and linear descending.

5Probability associated with the F statistic.

In the digestibility trial, the ADCs for organic matter and energy were significantly higher in the diet containing AMP at 0.5% of dry weight compared to the basal diet (Table 21). No statistical difference was detected in ADCs of protein, lipid and carbohydrate for fish fed the two diets (Table 21).

Table 21. Calculated percent apparent digestibility coefficients (ADCs) for hybrid striped bass fed a basal diet and one supplemented with AMP at 0.5% of diet.

Diet	ADC (%)				
	Organic matter	Protein	Lipid	Carbohydrate	Energy
Basal	53.1[b]	76.5	69.3	25.1	56.9[b]
AMP[1] 0.5%	56.6[a]	77.7	70.2	30.5	60.0[a]
PSE[2] (n=3)	0.66	0.58	1.66	1.48	0.72
Student's t-test (Pr>t)[3]	0.0199	0.1960	0.7299	0.1033	0.0391

[1] Adenosine 5'-monophosphate (AMP)
[2] PSE = pooled standard error of treatment means. Means of three replicates groups (n=3). Values within the same column with different letters are significantly different ($P < 0.05$).
[3] Probability associated with the t-statistic.

After eight weeks of feeding the basal diet and the one supplemented with AMP at 0.5%, the activities of lipase, amylase, alkaline, and acid phosphatase were not statistically different at both sampling time point (Table 22). However, it was noted that trypsin enzymatic activity was significantly higher in fish fed the AMP 0.5% dietary treatment compared to those fed the basal diet at 10 h after feeding, but only in anterior intestine section. However, no statistical significance was detected at 20 h after feeding (Table 22).

Table 22. Digestive enzyme activities of hybrid striped bass in different digestive tract sections (anterior intestine [AI] and posterior intestine [PI]) at different sampling times (10 and 20 h) after feeding[1].

Digestive enzyme		10 h				20 h			
		Basal diet	AMP[2] 0.5%	P > t[3]	PSE[4]	Basal diet	AMP 0.5%	P > t	PSE
Trypsin	AI	5.17[b]	7.31[a]	0.0113	0.10	3.95	4.11	0.6785	0.20
	PI	5.37	11.09	0.4911	3.94	4.68	7.67	0.5196	2.24
Lipase	AI	5.82	7.07	0.4699	0.81	4.02	5.25	0.0806	0.24
	PI	8.43	12.83	0.5383	3.53	6.38	10.06	0.5276	2.93
Amylase	AI	2.67	3.11	0.4553	0.34	1.71	2.01	0.1761	0.08
	PI	4.58	7.36	0.5670	2.43	3.02	5.32	0.5084	1.70
Alkaline phosphatase	AI	7.49	9.31	0.3571	0.88	5.74	5.81	0.8880	0.32
	PI	8.65	13.06	0.5282	3.41	7.48	9.73	0.5593	1.93
Acid phosphatase	AI	16.27	18.73	0.4497	1.66	14.79	15.17	0.6894	0.57
	PI	15.25	22.50	0.4953	5.06	16.21	19.46	0.6411	3.75

[1] Specific activity= U/mg soluble protein.

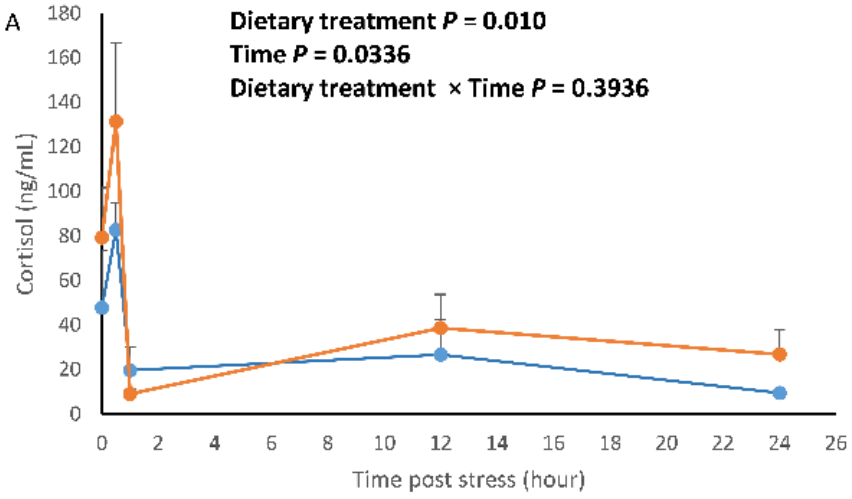
[2] Adenosine 5'-monophosphate (AMP)

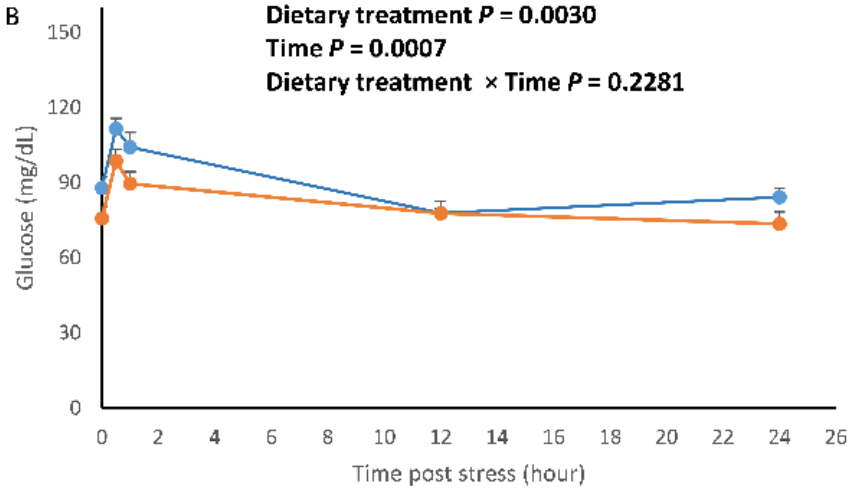
[3] Probability associated with the t-statistic.

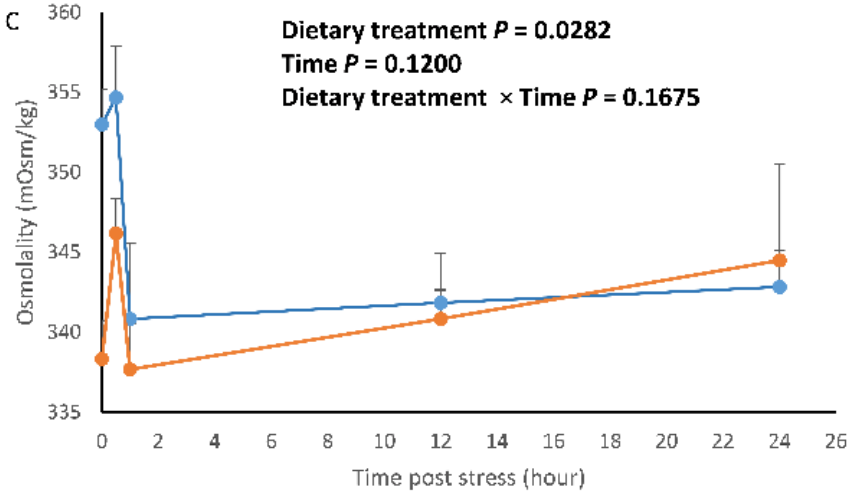
[4] PSE = pooled standard error of treatment means. Means of two replicates groups (n=2). Values within the same column with different letters are significantly different ($P < 0.05$).

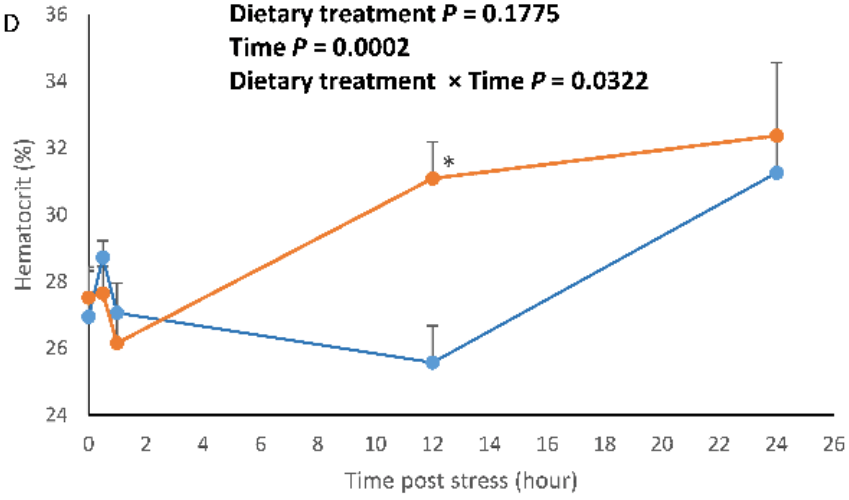
Plasma cortisol as the primary stress response was elevated at 0.5 h after the fish were subjected to the acute stressor but made a complete recovery within 1 h for fish fed both dietary treatments (Fig. 4A). However, it was noted that although there was no significant interaction between dietary treatments and time, there was a main dietary treatment effect of the AMP 0.5% treatment having an overall significantly (P=0.01) higher cortisol level (Fig. 4A). On the other hand, the secondary stress responses of plasam glucose and osmolality did not show any interactions between dietary treatments, but the main effect of dietary AMP at 0.5% resulted in significantly lower glucose (P=0.03) and osmolality (P=0.028) values (Fig. 4B; Fig. 4C).

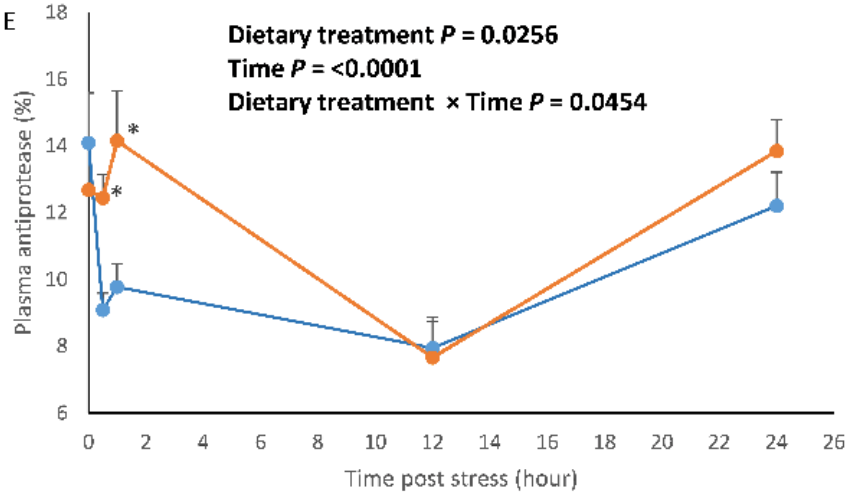
The acute-stress response on hematocrit of hybrid striped bass showed a statistically significant interaction between dietary treatment and time (pre and post stress) (Fig. 4D); it was noted that the hematocrit response made a complete recovery and was significantly higher at 12 h post-stress for fish fed the AMP 0.5% diet compared to those fed the basal diet. After the fish were subjected to acute stress, significant suppression of plasma anti-protease was observed those fed the basal diet at 0.5 h and 1 h post-stress, while those fed the AMP 0.5% diet were significantly enhanced at time point 1 h post-stress (Fig. 4E). In addition, plasma lysozyme activity in both groups of fish showed no significant interaction between dietary treatment and time; however, the main dietary treatment effect of AMP at 0.5% had an overall significantly (P=0.01) greater lysozyme activity (Fig. 4F). No significant differences in dietary main effects and interactions between dietary treatment and time were observed for total protein or total immunoglobulin concentrations (Fig. 4G; Fig. 4H).

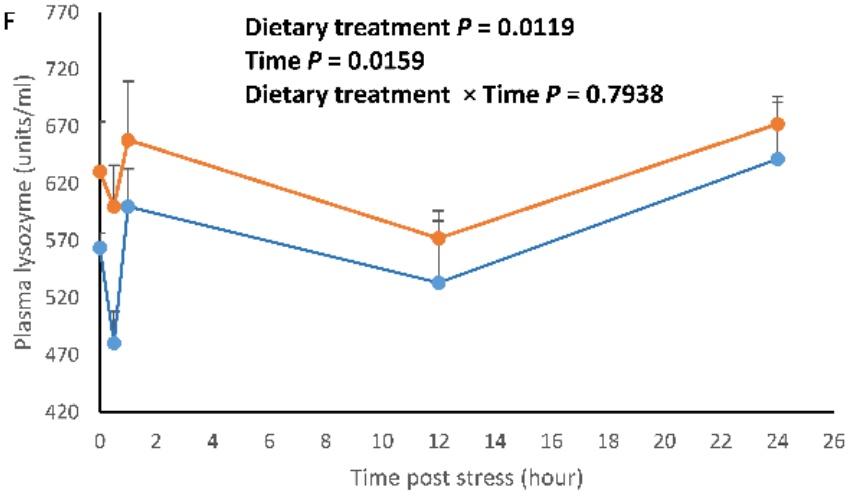

A
Dietary treatment P = 0.010
Time P = 0.0336
Dietary treatment × Time P = 0.3936
Cortisol (ng/mL)
180
160
140
120
100
80
60
40
20
0
0 2 4 6 8 10 12 14 16 18 20 22 24 26
Time post stress (hour)


B
Dietary treatment P = 0.0030
Time P = 0.0007
Dietary treatment × Time P = 0.2281
Glucose (mg/dL)
150
120
90
60
30
0
0 2 4 6 8 10 12 14 16 18 20 22 24 26
Time post stress (hour)


C
Dietary treatment P = 0.0282
Time P = 0.1200
Dietary treatment × Time P = 0.1675
Osmolality (mOsm/kg)
360
355
350
345
340
335
0 2 4 6 8 10 12 14 16 18 20 22 24 26
Time post stress (hour)


D
Dietary treatment P = 0.1775
Time P = 0.0002
Dietary treatment × Time P = 0.0322
Hematocrit (%)
Time post stress (hour)


E
Dietary treatment P = 0.0256
Time P = <0.0001
Dietary treatment × Time P = 0.0454
Plasma antiprotease (%)
Time post stress (hour)


F
Dietary treatment P = 0.0119
Time P = 0.0159
Dietary treatment × Time P = 0.7938
Plasma lysozyme (units/ml)
Time post stress (hour)

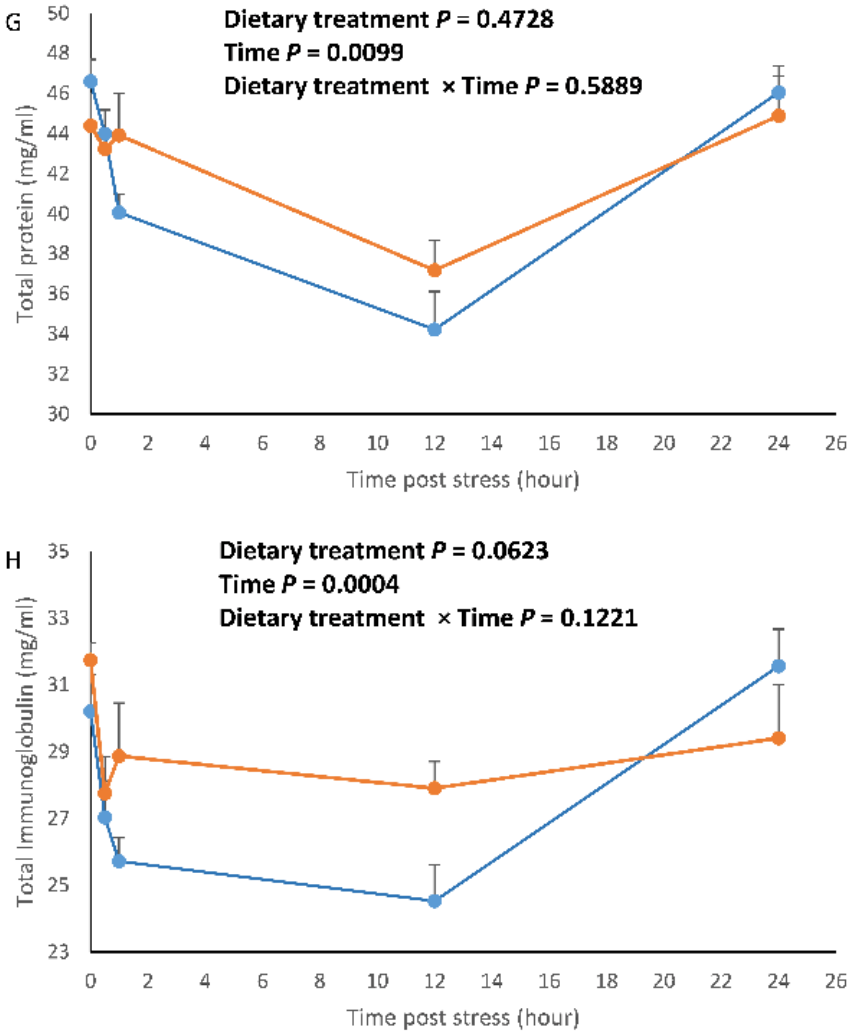


Figure 4. Modulation of plasma cortisol, plasma glucose, plasma osmolality, hematocrit, and innate immunity (A = cortisol, B = glucose, C = osmolality, D = Hematocrit, E = plasma antiprotease, F = plasma lysozyme, G = total protein, and H = total immunoglobulin) of hybrid striped bass in an acute stress challenge protocol. Color indicates different dietary treatments: blue (basal diet) and orange (adenosine monophosphate 0.5%). Data are represented as mean ± standard error (n=6 per sampling time point). Asterisk (*) indicates significant differences between dietary treatments.

In the chronic stress challenge trial, after four weeks of feeding the experimental diets, no statistical differences were detected for weight gain, plasma cortisol, hematocrit, and plasma osmolality, while blood glucose was significantly higher in fish fed the basal diet compared to those fed the 0.5% AMP diet (Table 23). Innate immune parameters such as total plasma protein, total immunoglobulin, as well as intracellular and extracellular respiratory burst were not statistically different between dietary treatments (Table 24). Nevertheless, plasma lysozyme activity and anti-protease activity were significantly immunosuppressed in fish fed the basal diet (Table 24). For the adaptive immune response, lymphocyte proliferation prompted by the presence of lipopolysaccharides was not significantly different between dietary treatments (Table 24).

Table 23. Growth and hematological responses of hybrid striped bass after high salinity (15.7±1.54 ppt) induced chronic stress at the end of 4 weeks of feeding.

Diet	Weight gain	Cortisol	Glucose	Hematocrit	Osmolality
	%	ng/mL	Mg/dL	%	mOsm/kg
Basal	34.4	131	77.9[a]	29.7	347
AMP[1] 0.5%	39.0	81.3	67.5[b]	30.0	342
PSE[2] (n=3)	2.42	17.0	1.84	1.49	3.55
Student's t-test (Pr>t)[3]	0.2472	0.1081	0.0283	0.8820	0.3983

[1] Adenosine 5'-monophosphate (AMP)
[2] PSE = pooled standard error of treatment means. Means of three replicates groups (n=3). Values within the same column with different letters are significantly different ($P < 0.05$).
[3] Probability associated with the t-statistic

Table 24. Innate and adaptive immunity of hybrid striped bass after high salinity (15.7±1.54 ppt) induce chronic stress at the end of 4 weeks of feeding.

Diet	LPS[1]	Lysozyme activity	Total Plasma Protein	Total Plasma Immunoglobulin	Anti-protease activity	Superoxide anion extra-cellular	Superoxide anion intra-cellular
		units/ml	mg/ml	mg/ml	%	nmol/well	nmol/well
Basal	1.76	866.6[b]	46.2	28.3	11.9[b]	3.95	0.20
AMP[1] 0.5%	1.71	1046[a]	52.4	31.9	15.0[a]	3.64	0.21
PSE[3] (n=3)	0.10	40.2	2.86	1.53	0.56	0.21	0.01
Student's t-test (Pr>t)[4]	0.7302	0.0342	0.2472	0.2029	0.0249	0.3552	0.6901

[1] Stimulation index of hybrid striped bass lymphocytes isolated from head kidney. Values indicate the proliferation after stimulation with mitogen (LPS, lipopolysaccharide from *E. coli* O26:B6).

[2] Adenosine 5'-monophosphate (AMP)

[3] PSE = pooled standard error of treatment means. Means of three replicates groups (n=3). Values within the same column with different letters are significantly different ($P < 0.05$).

[4] Probability associated with the t-statistic.

IV.4 Discussion

The basal diet in all feeding trials in this study (Table 17) wasdesigned to have limiting purine nucleotides due to high inclusion soybean meal and soy protein concentrate (Kojima, 1974; Li et al., 2015). Nevertheless, limiting purine nucleotides may not lead to classic deficiency signs such as suppressed weight gain because nucleotides can be endogenously synthesized. Therefore, it was crucial to ensure that fish in the dose-response trial was fed very close to apparent satiation which stimulated rapid proliferation of cells for growth and consequently resulted in significant incremental dosage effects on weight gain of juvenile hybrid striped bass (Fig. 3). Furthermore, the estimated minimum dosage of AMP that showed maximum weight gain was 0.5% of dry diet, although a slight reduction in weight gain also was observed from concentrations of AMP from 0.75 to 2% of diet; although difference in weight gain between the AMP 2% and basal diets was not so apparent (Fig.3). This raises a hypothesis that supplementing excessive concentrations of AMP might be toxic to fish and counteract the potential growth-enhancing effects. This hypothesis was also supported by findings of Song et al. (2012) who reported dietary levels of IMP from 0.1 to 0.2% of diet resulted in significantly higher final body weight of olive flounder compared to those fed a diet with IMP at 1% of diet although that treatment was not significantly different from that of fish fed the basal diet.

In the present study, it also appeared that the reduction of weight gain was correlated with reduced survival (Table 18) of fish fed the higher concentrations of AMP (1.5-2%). The review by Carver and Walker (1995) elucidated that dietary purines are

mostly catabolized to uric acid with the exception of adenine which is the most extensively re-utilized purine. They also postulated that excessive amounts of adenine may reduce growth of animals; however, such observation was only seen when adenine was fed in the free form and not as a nucleotide or nucleoside. In contrast, the present study demonstrated that a very high concentration of AMP may lead to uric acid toxicity in hybrid striped bass. Furthermore, it was reported in rats that high levels of isolated yeast RNA increased plasma uric acid and causing toxicological effects and disturbances in metabolism of protein, carbohydrate, and lipid (Heaf and Davies, 1976).

It has been hypothesized that supplementing AMP may facilitate dietary protein sparing by conserving energy from *de novo* synthesis of metabolic costly AMP. The lower protein retention values in whole-body tissues of fish fed with basal and high dose of AMP diet (0.75-2%)Table 20) could possibly be due to preferential or inevitable catabolism of amino acid to provide energy (NRC, 2011) that is typically observed in carnivorous fish via gluconeogenesis (Cowey et al., 1977). Rumsey et al. (1992) reported that supplementing high doses of purines did not show a detrimental effect on growth performance of rainbow trout, but the nitrogen retention in the whole-body was significantly reduced due to toxicity. Under these circumstances, one possible explanation for the observed responses in the present trial was the lower dosage of AMP (0.25-0.5%) improved protein retention efficiency by protein sparing while higher dosages of AMP (1.5-2%) may have caused degradation of uric acid that might be toxic to the fish and negated the enhancing effect on weight gain (Table 20).

The follow-up digestibility trial (Table 21) demonstrated that the optimal dose of AMP at 0.5% of diet significantly improved ADCs for energy and organic matter compared to basal diet, and ADC values for the other major nutrient groups were numerically greater for the 0.5% AMP diet. Consequently, it also appears that the hybrid striped bass fed the AMP 0.5% diet were able to significantly utilize organic matter (mainly from carbohydrate) as a source of energy based on the significant increase in energy ADC (Table 21). The linear increase of HSI (Table 18) and linear increase in whole-body lipid deposition (Table 20) also provides more evidence that supplementing AMP increased energy reserves in the liver and enhanced the capacity for synthesizing fatty acids that were eventually deposited in the carcass. A review article by Cosgrove (1998) provided two insights on the mechanism that might be involved in fatty acid synthesis: 1) nucleotides could potentially change the intestinal microbiota that are able to produce long-chain polyunsaturated fatty acids through elongation and desaturation; 2) nucleotides could possibly modulate chain elongation and desaturation in the enterocyte or in the liver. Similar findings also were reported for red sea bream and red drum in which dietary nucleotide supplementation increased deposition of lipid in the whole-body (Li et al., 2005; Hossain et al., 2016a). According to Carver (1999), endogenous synthesis of nucleotides from amino acid precursors uses a great deal of energy in a form of ATP; thus, this study proves that supplementing incremental dose of AMP in the diet may result in energy conservation as seen in the previously mentioned body composition responses (Table 18).

In regard to the effects of nucleotide supplementation on digestive enzymes, it appeared that numerically the secretion of digestives enzyme was higher in fish fed the AMP 0.5% diet compared to those fed the basal diet, but significant differences were only seen in trypsin activity at 10 h after feeding in the anterior intestinal section (Table 22). There are possibilities that improve growth performance of fish may be partially attributed to the enhancement of pancreatic enzyme activities and intestinal enzyme activity (Castillo et al., 2014). Interestingly, Guo et al. (2017) reported that supplementing a purified mixed nucleotide at 0.1% of diet significantly enhanced the intestinal lipase activity which was also correlated with significant increases in the weight gain of zebrafish; however, no statistical differences were detected in the intestinal trypsin and amylase activities. Moreover, one of the concerns pertaining to the present digestive enzyme trial was high variation between replicate samples may have limited the ability to detect significant differences (Table 22); therefore, further investigation on digestive enzymes may be warranted.

Based on the polynomial orthogonal contrast result in the dose-response trial, no significant trends were observed with regard to innate immune responses (Table 19). This finding is in line with studies that have reported supplementing purified nucleotide mixtures in a basal diet showed no statistical significance on innate immunity of channel catfish (Welker et al., 2011) or red drum (Li et al., 2007). However, it is known that effects of immune modulation by nucleotides may be species specific, as there are studies showing that these additives only enhance immunity during stressful conditions

such as disease challenge, and prior to the disease challenge no significant enhancement was observed (Burrells et al., 2001b; Li et al., 2004).

Therefore, a hypothesis was raised to test the efficacy of supplementing the optimal dosage of AMP and its ability to modulate stress response and immunity of hybrid striped bass. As Barton and Iwama (1991) elaborated, stress can challenge homeostasis of fish and as such may pose a threat to their health. Furthermore, Pickering (1989) stated that secretion of corticosteroids in fish may provide an adaptive mechanism to overcome acute stress via gluconeogenesis and osmoregulatory adjustment. Interestingly, findings of the acute stress challenge in the present study showed that the average cortisol secretion was significantly higher in fish fed the diet supplemented with AMP at 0.5% compared to those fed the basal diet. Nevertheless, it is important to note that the highest secretion of cortisol was mainly observed at 0.5 h post stress, and the circulation of cortisol returned to basal levels from 1 h to 24 h post stress (Fig. 4A). Nonetheless, blood glucose (Fig. 4B) and plasma osmolality (Fig. 4C) was significantly lower in fish fed the diet with AMP at 0.5% compared to those fed the basal diet both before and after, suggesting dietary AMP at 0.5% promoted better adaptive responses to overcome acute stress. Lower blood glucose circulation also was reported in red sea bream that were fed dietary AMP (0.1-0.8%) for 56 days (Hossain et al., 2016a). Findings of the present trial elucidated a novel mechanism in which fish fed supplemental AMP had higher secretion of cortisol (58% increase) compared to those fed the basal diet at 0.5 h post-stress (Fig. 4A); however, the high cortisol level did not stimulate excessive gluconeogenesis as manifested by high blood glucose (Fig. 4B).

Suarez and Mommsen (1987) argued that although existing literature interprets increased glucose concentration in response to exogenous cortisol in teleosts, there are possibilities that cortisol exerts its effects through other mechanisms. Findings from the current study further support this claim. These adaptive mechanisms of overcoming acute stress were further substantiated with significant enhancement of plasma anti-protease activity observed 0.5 and 1 h post-stress in fish fed AMP 0.5% of diet while those fed the basal diet were immuno-suppressed (Fig. 4E). Likewise, the increase in hematocrit values at 12 h post stress in fish fed the diet supplemented with AMP (Fig.4D) also indicated immune enhancement. Moreover, it is well established that continuous secretion of high plasma corticosteroids under chronic stress are eventually harmful to the health of the fish and therefore considered maladaptive (Pickering, 1989; Barton and Iwama, 1991). The chronic stress trial (Table 23) suggested that AMP at 0.5% of diet was able to modulate stress response by lowering plasma cortisol level by 58% compared to those fed the basal diet, as well as significantly lowering blood glucose. The ability of dietary AMP to overcome or allow coping with chronic stress is further substantiated with the enhancement of plasma lysozyme and anti-protease activity or could also be interpreted as fish fed the basal diet were immunosuppressed (Table 24). Interestingly, differences in innate immunity were never observed previously in the pre-stress fish (time 0 h) and in the dose-response trial (Fig 4; Table 19) which indicates that under stressful conditions, supplementation of AMP may enhance innate immunity responses and increase resistance to stress-induced immunosuppression. These findings support the hypothesis raised by Yamauchi et al. (2002) that *de novo* synthesis of nucleotides may

not be adequate for optimal functioning, particularly of the immune system under stressful conditions such as sepsis and trauma; therefore, exogenous supply of dietary nucleotides may be of particular significance under those conditions.

Based on all the evidence provided, our study demonstrated that an exogenous supply of AMP at 0.5% of diet enhanced growth response and protein retention efficiency of hybrid striped bass. The highest level of AMP in the diet may have caused negative effects due to uric acid toxicity in the fish. Besides that, supplementing the optimal dosage of AMP (0.5%) in the diet enhanced digestibly coefficients for organic matter and energy. Lastly, AMP at 0.5% of diet showed capabilities to modulated immunity under stressful condition through enhancement of innate immunity and increasing resistance to stress-induced immunosuppression.

CHAPTER V

SUMMARY AND CONCLUSIONS

Fishmeal is rich in nucleotides which have been shown to be very beneficial to carnivorous fish. As world aquaculture continues to expand, fishmeal is being progressively replaced with other alternative feedstuffs such as plant proteins and animal by-products in commercial diets. Many of these ingredients have lower nucleotide concentrations than fishmeal. Therefore, this dissertation compiled a series of feeding trials which evaluated nucleotide nutrition of hybrid striped bass, and consistency documented positive responses to dietary nucleotide supplementation.

In the first feeding trial, the focus was to evaluate the efficacy of supplementing single nucleotides in a basal diet which utilized menhaden fishmeal and soybean meal as protein sources, and contained approximately 40% protein and 10% lipid. Adenosine 5'-monophosphate (AMP), uridine 5'-monophosphate (UMP), cytidine 5'-monophosphate (CMP), guanosine 5'-monophosphate (GMP), and inosine 5'-monophosphate (IMP) were each supplemented at 0.5% of dry weight to the basal diet at the expense of the cellulose and evaluated in a 9-week feeding trial. The findings demonstrated a significant increase in weight gain and innate immunity of fish fed the AMP-supplemented diet compared to the basal diet. Besides that, the dietary treatment IMP and AMP both significantly enhanced the capacity of phagocytes to generate extracellular superoxide anion compared to all other dietary treatments. Therefore, based on the conclusions from this trial, AMP and IMP nucleotides were further investigated in

the next trial. The effects of dietary AMP and IMP, each at 0.5% by weight, were supplemented to the same basal diet, or combinations of both (1%) were evaluated on growth performance of juvenile fish in a 9-week feeding trial. The major concern of using high combination doses of nucleotides (>1%) was uric acid toxicity; therefore, additional combinations of 0.25% AMP and 0.25% IMP were also evaluated in this trial. To determine if different suppliers' products with the same purity may yield different responses, all nucleotide treatments were evaluated with products from two suppliers, Sigma-Aldrich (Sig.) and Chem-Impex International (C. Impex). Besides that, the potential effects on acute stress-induced immunomodulation after an additional 2 weeks of feeding were evaluated. There were no significant effects of AMP or IMP, singularly or in combination, on growth performance, whole-body composition or innate immunity of hybrid striped bass; however, fish fed IMP (C. Impex) had significant enhancement of lymphocyte proliferation upon stimulation with lipopolysaccharides compared to fish fed the basal diet. After an additional 2 weeks, fish fed some of the dietary nucleotides had significant enhancement of innate immunity at 0.5 h and 12 h post stress challenge, imposed by 1-minute air exposure, compared to those fed the basal diet. Supplementation of AMP (C. Impex) at 0.5% of diet provided the greatest enhancement of innate immunity during post-stress. Therefore, based on the results of this trial, the AMP nucleotide treatment was further investigated in a dose-response feeding trial. Thus, an 8-week feeding trial was conducted in which graded levels of AMP (0, 0.25, 0.5, 0.75, 1.5, and 2%) were fed to juvenile hybrid striped bass. The basal diet in this feeding trial was formulated principally from dehulled soybean meal, soy protein

concentrate (SPC), and more limited fishmeal to contain 39.1% crude protein, 10.7% lipid and possibly be more limiting in nucleotides than the basal diet in earlier trials to possibly demonstrate growth-enhancing effects of nucleotide supplementation. In the dose-response trial, the minimum requirement based on the weight gain response was estimated in a two-slope broken-line model to be 0.5% of the diet. Therefore, the AMP diet at 0.5% was evaluated against the basal diet in the remaining four trials. Overall compared to fish fed the basal diet, supplementation of AMP at 0.5% of dry weight demonstrated significantly improved apparent digestibility coefficients for organic matter and energy after 4 weeks of feeding, trypsin enzymatic activity after 8 weeks of feeding, innate immunity as reflected in anti-protease activity at 0.5 and 1 h post-acute stress challenge (air exposure), and elevated plasma lysozyme and anti-protease activity after fish were subjected to chronic stress of high salinity for 4 weeks. Therefore, it was concluded that an exogenous supply of AMP at 0.5% of diet was able to modulate immune responses under stressful conditions and to a limited extent improve growth performance, nutrient digestibility and digestive enzymes of hybrid striped bass.

Based on these findings, it was concluded that adenosine 5'-monophosphate was the most effective nucleotide supplement for hybrid striped bass that was shown to improve growth performance as well as demonstrate immunomodulatory effects during acute and chronic stress. Moreover, it is cautioned that AMP and IMP nucleotides from the two different manufacturers resulted in some slightly different outcomes when supplemented in the hybrid striped bass diet. Therefore, prior to incorporating any particular nucleotide product, preliminary assessment of the effects of the product may

be required. Finally, the findings from this study may encourage feed manufacturers to incorporate AMP in high-plant-protein-based diets for hybrid striped bass.

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