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Exploring potential synergistic effects between dietary adenosine and inosine monophosphate on growth performance and acute stress-induced immune responses of hybrid striped bass *Morone chrysops* × *Morone saxatilis*

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Abstract

Two experiments were carried out to evaluate the effects of dietary adenosine monophosphate (AMP) and inosine monophosphate (IMP) on growth performance and acute stress-induced immune response of hybrid striped bass (HSB). Nine isonitrogenous and isolipidic diets were prepared including a basal diet and eight treatments consisting of singular or combined additions of 0.5% AMP and 0.5% IMP, and combinations of 0.25% AMP and 0.25% IMP from each of two suppliers, Sigma-Aldrich and Chem-Impex. The first experiment included two 9-weeks feeding trials in which triplicate groups of HSB (~9.7 g) were fed the experimental diets. Dietary 0.5% IMP (C-Impex) showed significantly higher stimulation index of B-lymphocyte proliferation compared with other dietary treatments. However, no significant synergistic effects between AMP and IMP supplementation were observed in production performance. In a subsequent experiment, HSB (~59.3 g) were fed the diets described previously for 2 weeks and subjected to a standardized acute stress challenge. Fish fed some of the dietary nucleotide treatments had significant enhancement of innate immunity at 0.5 and 12 hr poststress challenge compared with those fed the basal diet. Dietary 0.5% AMP (Chem-Impex) provided the best capability for enhancing innate immunity during poststress and resistance to stress-induced immunosuppression.

KEYWORDS

acute stress, adenosine monophosphate, immune modulation, inosine monophosphate, physiology, purine nucleotides

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 & Sullivan, 2006). These fish are cultured for food, sports fishing, and stock enhancement programs (Garber & Sullivan, 2006; Schramm et al., 1991; Smith, Jenkins, & Haggerty, 1986). 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 hybrid striped bass farming (Davis & Griffin, 2004) are linked to acute stress responses (Reubush & Heath, 1997) and may increase disease susceptibility and mortality (Davis & Griffin, 2004; Noga, Botts, Yang, & Avtalion, 1998). There are numerous studies reporting detrimental effects of handling and hauling stress in fish (Carmichael, Wedemeyer, McCraren, & Millard, 1983; Noga et al., 1998; Wedemeyer, 1976; Weirich, Tomasso, & Smith, 1992). 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, Beitinger, & Wooten, 1984; Randall & Tsui, 2002; Santos, Schrama, Mamauag, Rombout, & Verreth, 2010; Weirich et al., 1992).

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

Numerous studies have extensively reported the relationship between nutrition, dietary supplements and fish immunity (Hassaan et al., 2020; Kiron, 2012; Li & Gatlin, 2006; Yamamoto et al., 2020). 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, Williams, & Forno, 2001; de Cruz, Yamamoto, Castillo, & Gatlin, 2020; de Cruz, Yamamoto, Ju, et al., 2020; Guo et al., 2019; Reda, Selim, Mahmoud, & El-Araby, 2018; Welker, Lim, Yildirim-Aksoy, & Klesius, 2011; Zhang et al., 2019). 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; Li, Zhao, & Gatlin, 2015). Furthermore, based on various studies in which soybean meal (Gallagher, 1994) and other protein feedstuffs (de Cruz, Lubrano, & Gatlin, 2018; Gaylord, Rawles, & Gatlin, 2004; Perez-Velazquez et al., 2019; Rawles et al., 2006) have been substituted for fishmeal in the diet of hybrid striped, there are greater opportunities for purine nucleotides to be a limiting nutrient.

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. Also, this study attempted to characterize acute stress-induced innate immune responses of hybrid striped bass and the potential dietary effects of purine nucleotides (AMP and IMP) to modulate innate immunity.

2 | MATERIALS AND METHODS

Experiment 1 and 3 encompassing three different feeding trials were conducted to evaluate the effects of AMP and IMP either singularly or in combination on hybrid striped bass. In addition, a preliminary study (experiment 2) was conducted to evaluate the effects of acute stress response on the haematological and innate immunological parameters of hybrid striped bass.

2.1 | Basal diet

The basal diet used in all the experiments was formulated predominantly from dehulled soybean meal and approximately 150 g/kg menhaden fishmeal inclusion to contain 400 g/kg protein, 100 g/kg lipid and an estimated digestible energy level of 14.6 MJ/kg (Table 1). As such, the basal diet was designed to be limiting in purine nucleotides due to the high inclusion of soybean meal (Kojima, 1974; Li et al., 2015). The experimental diets were formulated to be isonitrogenous, isolipidic, isocaloric and to meet or exceed all established nutrient requirements reported for 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 reported previously in published material from our laboratory (de Cruz et al., 2018).

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 permitted by the Institutional Animal Care and Use Committee at Texas A&M University (IACUC 2018-0388). The juvenile hybrid striped bass (*M. chrysops* × *M. saxatilis*) used in all the experiments were acquired 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 air stones. Water quality parameters were kept at suitable ranges for hybrid striped bass culture (average ± *SD*); water temperature = 27.7 ± 0.14°C, dissolved oxygen = 7.5 ± 0.36 mg/L, total ammonia nitrogen

TABLE 1 Ingredients and analysed composition of the basal diet

Ingredients[a]	g/kg
Menhaden fishmeal[b]	147.0
Soybean meal[c]	553.0
Menhaden oil[d]	60.3
Vitamin premix[e]	30.0
Mineral premix[e]	40.0
Dextrinized starch[f]	90.0
Calcium phosphate dibasic[f]	10.0
Carboxymethyl cellulose[f]	20.0
Glycine[g]	10.0
Taurine[g]	4.3
DL-Methonine[g]	4.0
Cellulose[f]	30.9
Purified nucleotide[h]	0–10.0
Agar[i]	0.5
Analysed composition, g/100 g[j]	
Crude protein	406.4
Crude lipid	93.9
Ash	103.4

[a]Dry-matter basis.
[b]Special Select, Omega Protein (crude protein [CP] = 645 g/kg; lipid = 114 g/kg on a dry-matter basis).
[c]Producers Cooperative Association (CP = 515.4 g/kg; lipid = 36.7 g/kg on a dry-matter basis).
[d]Omega Protein.
[e]Moon and Gatlin (1991).
[f]MP Biomedicals.
[g]Ajinomoto North America, Inc..
[h]Supplementation of purified nucleotides to the basal diet at the expense of cellulose.
[i]Sigma-Aldrich Co.
[j]Means of two replicate determinations.

(TAN) = 0.12 ± 0.04 mg/L, nitrite nitrogen = 0.07 ± 0.06 mg/L, salinity = 3.0 ± 0.18 g/L and pH = 8.09 ± 0.19. The photoperiod in the cultured system was set to 12 light-12 hr dark by fluorescent lights regulated by automatic timers.

2.3 | Experiment 1

Two concurrent feeding trials (each of 9-weeks duration) were conducted using nucleotide products (Purity >98%) from either Sigma-Aldrich or Chem-Impex International. The nucleotide supplements, AMP and IMP disodium salt, were 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, Sigma-Aldrich (Sig.) and Chem-Impex International (C. Impex) were prepared with the individual supplementation of AMP at 0.5%, IMP at 0.5% and the combined supplementation of AMP at 0.5% and IMP at 0.5% to the basal diet at the expense of cellulose. The concentration of 0.5% was targeted based on studies with other species that recommended single purified nucleotide dosages between 0.1% and 0.6% of dry weight (Hossain, Koshio, Ishikawa, Yokoyama, & Sony, 2016; Lin, Wang, & Shiau, 2009; Song, Lim, & Lee, 2012; Zhang et al., 2019). In addition, two more dietary treatments were prepared by supplementing both AMP and IMP each at 0.25% of diet, each from either Sig. or C. Impex. 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 totalled up to 1% nucleotide supplementation; therefore, the lower dose (0.5%) combination of AMP and IMP could validate if there was any sign of purine toxicity involved with the high dose of purine nucleotide.

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 in section 2.2. 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. Fish were fed twice daily with preweighed rations, and feeding rate was gradually reduced equally for all diets overtime to maintain 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, production 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 *N* of fish/initial *N* of fish) × 100. Six fish were euthanized from each replicate aquarium with an overdose (300 mg/L) of tricaine methanesulphonate (MS-222, Western Chemical Inc). 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/g body weight × 100; and intraperitoneal fat (IPF) ratio = g IPF weight/g body weight × 100. 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, Lees, & Stanley, 1957), and heating samples at 650°C in the muffle furnace for 4 hr for ash (AOAC, 2005). The protein conversion efficiency was calculated with the following equation: (([(g final body weight × % final body protein) − (g initial body weight × % initial body protein)]/(protein intake (g)]) × 100).

2.4 | Experiment 2

This experiment was conducted to compare the haematological and innate immunological parameters of hybrid striped bass that were acutely stressed compared with a group of fish that was not subjected

to any stressors (control). Prior to initiating the stress challenge protocol (SCP), 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 (Table 1). The fish were fasted for approximately 15 hr 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). The experimental stressor was air exposure for 1 min by completely netting out all the fish from the aquarium in a single pass and suspending them in the air over the aquarium. For the acute stress challenge, groups of six replicate fish were sampled at 0 (prechallenge), 0.5, 1, 2, 6, 12 and 24 hr postchallenge (air exposure). 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 min. At each sampling time point, the six fish from each treatment were collected and immediately sedated with MS-222 (150 mg/L) (Trushenski, Bowker, Gause, & Mulligan, 2012). Blood samples were collected to determine plasma cortisol, blood glucose, haematocrit, lysozyme and anti-protease. Details of sample collection and analytical procedures are described in section 2.6.

2.5 | Experiment 3

Juvenile hybrid striped bass from the feeding trials associated with experiment 1 was 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 aquaria associated with the recirculating system and conditioned for an additional 2 weeks. Details of diets and culture conditions were mentioned in section 2.3. Fish were withheld from feeding approximately 15 hr prior to the day of sampling. Based on results from experiment 2, three time points were chosen to investigate acute stress-induced haematological parameters. For the acute stress challenge response, groups of six replicate fish from each dietary treatment were sampled at 0 (prechallenge), 0.5 and 12 hr postchallenge to investigate the effects of the dietary nucleotide supplements as potential immune-modulating nutrients during stress. Fish were subjected to the same SCP used in experiment 2. Blood samples were collected at each time point for all dietary treatments to determine haematological parameters: haematocrit, anti-protease, lysozyme and total immunoglobulin. Details of sample collection and analytical procedures are described in section 2.6.

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 analysed for glucose using a handheld glucose metre (Accu-Check guide®, Roche), and haematocrit was acquired by centrifugation of capillary glass tubes (Drummond Scientific). The handheld glucose metre was validated to be a suitable and reliable method to measure glucose in fish where relative measurements were acceptable (Beecham, Small, & Minchew, 2006). The remaining blood sample was centrifugated at 10,000 *g* for 10 min and 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). Plasma lysozyme activity was determined according to the turbidimetric reduction assay (Jørgensen, Sharp, Secombes, & Robertsen, 1993) by using *Micrococcus lysodeikticus* (Sigma-Aldrich), where 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 measured as described by Siwicki, Anderson, and Rumsey (1994) in which bovine serum albumin solution was used as a protein standard. Plasma anti-protease activity was determined by a modification of the method reported by Ellis (1990) and was calculated as the percentage of inhibition of trypsin activity compared to a reference sample with 100% digestion of isolated trypsin (Magnadóttir et al., 1999).

Also, head kidney leukocytes were isolated by density gradient sedimentation to measure 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 was aseptically excised using scalpels and forceps, pooled and stored in 15 ml of cold Leibovitz cell culture medium (L-15, Corning Inc.) containing 2% foetal bovine serum (FBS, Sigma-Aldrich), 10 units/ml of heparin (Akron Biotechnology), and 100 units/ml of penicillin and streptomycin (Sigma-Aldrich). Techniques established by Secombes (1990) and modified by Sealey and Gatlin (2002) were used to isolate the phagocytes. Respiratory burst activity of phagocytes was measured by intracellular and extracellular superoxide anion production as described by Secombes (1990). Concentration of intracellular O_2^- produced was determined by reading the absorbance at 620 nm. 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 by Secombes (1990) with minor modification as suggested by Carvalho, Yamamoto, Barros, and Gatlin (2018) was used to isolate the lymphocytes. The proliferation of lymphocytes upon stimulation with the non-specific mitogen lipopolysaccharide solution (LPS, Sigma-Aldrich, 1 mg/ml) 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).

2.7 | Statistical analysis

2.7.1 | Experiment 1

Data were evaluated for normality using the Shapiro–Wilk test and the synergistic effects between AMP and IMP were analysed using

two-way ANOVA with significance set at $p < .05$ for both feeding trials. The same data were analysed with the additional dietary treatments (AMP and IMP each at 0.25% of diet) for both feeding trials using one-way ANOVA analysis. The post hoc Tukey HSD test was used to test the differences among means. All statistical procedures for experiment 1 were performed using JMP Pro, version 14 (SAS Institute).

2.7.2 | Experiments 2 and 3

Repeated measures analysis of variance (RM-ANOVA) was used for both experiments in this study using SAS software version 9.4 (SAS Institute). 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 as follows:

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

where, Y_{ijk}: the investigated responses at kth hour on the jth fish assigned to the ith treatment effect; $\boldsymbol{\alpha}_i$: treatment 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 as follows

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

where, Y_{ijk}: the investigated responses at kth hour on the jth fish assigned to the ith 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.

Data analyses for experiments 2 and 3 were computed by RM-ANOVA within the PROC MIXED framework of the Statistical Analysis System, version 9.4 (SAS Institute) to determine significant differences ($\alpha \leq 0.05$). Contrast pair-wise comparisons were carried out only if a statistically significant interaction was identified; otherwise, the overall significant main treatment effects were discussed.

3 | RESULTS

3.1 | Experiment 1

At the end of the two, concurrent 9-week feeding trials, no significant differences in growth performance of hybrid striped bass were observed for the main effects or interactions between AMP and IMP from either supplier (Tables 2 and 3). 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 (Tables 2 and 3). Over both of the 9-week feeding trials, fish survival was 100% for all dietary treatments (Tables 2 and 3). No statistical differences were detected for whole-body proximate composition or protein conversion efficiency among the various AMP and IMP Sig. dietary treatments (Table 4). However, for protein deposition in the whole-body, there were significant differences in the interaction between AMP and IMP C. Impex dietary treatments (Table 5). 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 combinations 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 (Tables 4 and 5). 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 6 and 7). Lymphocyte proliferation prompted by the presence of LPS showed a significant interaction between AMP and IMP from Sigma-Aldrich; however, none of the treatments were significantly different compared with fish fed the basal diet (Table 6). Nevertheless, the highest stimulation 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 7).

3.2 | Experiment 2

All haematological parameters examined showed statistically significant interaction between treatments (stressed fish vs. control fish) and time (pre- and poststress; Figure 1). The primary stress response of plasma cortisol was evoked by air exposure for 1 min and was statistically significant at time point 0.5 hr poststress compared with the control group, and then, completely recovery within 1 hr (Figure 1a). Similarly, the secondary stress response of blood glucose had a significantly higher peak from time 0.5 to 2 hr poststress compared with the baseline peak of the control group, and made a complete recovery afterwards (Figure 1b). For the haematocrit response, it was noted that the packed cell volume increased at time 0.5 hr poststress but was not significantly different from the control group (Figure 1c). It was also observed in the stressed group at 12 and 24 hr poststress, that the packed cell volume was significantly lower than the control group (Figure 1c). Plasma anti-protease activity was affected in the group of fish subjected to acute stress with significant immune suppression observed at time points 1, 2 and 12 hr poststress, and then recovery at 24 hr poststress (Figure 1e). The plasma lysozyme activity in both groups of fish showed a fluctuating trend within the 24-hr period; however, the plasma lysozyme activity of the stressed fish group was only significantly lower than the control group at 12 hr poststress (Figure 1d).

TABLE 2 Dietary effects of AMP[a] and IMP[b] from Sigma-Aldrich on the growth performance of hybrid striped bass at the end of 9 weeks

Dietary treatments	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 (0.5%)	493	0.76	1.70	100	2.00	4.12
IMP (0.5%)	520	0.81	1.68	100	1.80	4.04
AMP (0.5%) + IMP (0.5%)	530	0.81	1.44	100	1.97	4.47
PSE[c]	23.6	0.03	0.25	–	0.05	0.25
Two-way ANOVA *P*[d]						
AMP	0.65	0.74	0.63	–	0.03	0.73
IMP	0.14	0.12	0.59	–	0.25	0.97
AMP × IMP	0.98	0.92	0.65	–	0.53	0.20
Basal	481	0.75	1.70	100	1.90	4.38
AMP (0.5%)	493	0.76	1.70	100	2.00	4.12
IMP (0.5%)	520	0.81	1.68	100	1.80	4.04
AMP (0.5%) + IMP (0.5%)	530	0.81	1.44	100	1.97	4.47
AMP (0.25%) + IMP (0.25%)	491	0.76	1.42	100	1.96	4.29
PSE	24.5	0.04	0.22	–	0.05	0.24
One-way ANOVA *P*[e]	0.60	0.61	0.79	–	0.09	0.70

[a]Adenosine monophosphate (AMP Sig.), Sigma-Aldrich.

[b]Inosine monophosphate (IMP Sig.), Sigma-Aldrich.

[c]Pooled standard error.

[d]Value expressed as means of three replicates groups ($n = 3$). In case of significant interaction ($p < .05$), Tukey's HSD test was performed and values with the different letters within the same column are significantly different.

[e]Value expressed as means of three replicates groups ($n = 3$). Same data were analysed in one-way ANOVA with additional AMP (0.25%) × IMP (0.25%) dietary treatment. In case of statistically significant ($p < .05$), Tukey's HSD test was performed and values with the different letters within the same column are significantly different.

3.3 | Experiment 3

It was observed that dietary nucleotide supplementation modulated haematocrit and innate immune responses during acute stress (Table 8). Fish fed all the dietary nucleotides had significantly higher haematocrit values compared with those fed the basal diet at 0 hr prestress. 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 hr poststress. At 12 hr poststress, fish fed the other nucleotide treatments had significantly higher packed cell volume compared with those fed the basal diet. The plasma antiprotease 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 with those fed the basal diet at 0.5 hr poststress. Likewise, the plasma lysozyme activity was noted to be enhanced at 0.5 hr poststress in fish fed the AMP C. Impex (0.5%) + IMP C. Impex (0.5%) diet compared with those fed the basal diet. There also was a significant enhancement of lysozyme activity observed at 12 hr poststress in fish fed the AMP C. Impex (0.5%) dietary treatment compared with those fed the basal diet.

4 | DISCUSSION

The findings of the present study showed that dietary supplementation of 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 with those fed the basal diets (Tables 2 and 3). 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; Li et al., 2015). Limiting dietary purine nucleotide may not necessarily lead to reduced weight gain or other signs of deficiency as typically observed with many nutrients. This is because nucleotides can be synthesized de novo or obtained through the salvage pathway, unless the organism is under stressful

TABLE 3 Dietary effects of AMP[a] and IMP[b] from Chem-Impex on the growth performance of hybrid striped bass at the end of 9 weeks

Dietary treatments	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 (0.5%)	532	0.81	1.43	100	1.97	4.56
IMP (0.5%)	507	0.78	1.41	100	1.91	4.28
AMP (0.5%) + IMP (0.5%)	530	0.81	1.40	100	2.01	4.09
PSE[c]	23.5	0.03	0.12	–	0.08	0.30
Two-way ANOVA *P*[d]						
AMP	0.15	0.20	0.26	–	0.29	0.98
IMP	0.62	0.64	0.21	–	0.75	0.37
AMP × IMP	0.56	0.68	0.29	–	0.91	0.56
Basal	481	0.75	1.70	100	1.90	4.38
AMP (0.5%)	532	0.81	1.43	100	1.97	4.56
IMP (0.5%)	507	0.78	1.41	100	1.91	4.28
AMP (0.5%) + IMP (0.5%)	530	0.81	1.40	100	2.01	4.09
AMP (0.25%) + IMP (0.25%)	506	0.77	1.46	100	2.01	4.70
PSE	24.4	0.03	0.11	–	0.08	0.27
One-way ANOVA *P*[e]	0.60	0.67	0.32	–	0.77	0.56

[a]Adenosine monophosphate (AMP C. Impex), Chem-Impex International.

[b]Inosine monophosphate (IMP C. Impex), Chem-Impex International.

[c]Pooled standard error.

[d]Value expressed as means of three replicates groups ($n = 3$). In case of significant interaction ($p < .05$), Tukey's HSD test was performed and values with the different letters within the same column are significantly different.

[e]Value expressed as means of three replicates groups ($n = 3$). Same data were analysed in one-way ANOVA with additional AMP (0.25%) × IMP (0.25%) dietary treatment. In case of statistically significant ($p < .05$), Tukey's HSD test was performed and values with the different letters within the same column are significantly different.

conditions which may interfere with their endogenous synthetic capacity (Carver & Walker, 1995; Gil, 2002). Throughout the present feeding trials, the fish were in excellent health condition with 100% survival for all dietary treatments. Besides that, the weight gain and feed efficiency ratio responses among dietary treatments were not statistically significant. This raises the hypothesis that if the fish were not subjected to a stressful condition, then no interference would be observed for the endogenous synthesis of nucleotides using amino acids as precursors. If this hypothesis is correct, then supplementing nucleotides in the diet may not necessarily result in large differences in weight gain. This hypothesis would corroborate the correlation found in a previous study from our laboratory in which supplementing 0.5% commercial nucleotide product (Ascogen P®, Canadian Bio-Systems Inc.) in the diet of juvenile hybrid striped bass did not result in statistically different growth performance after 8 weeks of feeding. However, weight gain was numerically greater when the fish were challenged with the pathogen *Streptococcus iniae* over a 6-week period (Li, Lewis, & Gatlin, 2004). Another possible explanation for the lack of growth differences in the present study is that purine nucleotides were not a limiting factor in the basal diet, even with high inclusion of soybean meal. This condition apparently was substantiated by an earlier report that showed no significant differences were detected in growth performance of hybrid striped bass fed a nucleotide-rich diet (100% fishmeal contributing to CP) compared with a high-plant protein diet in which 75% of the CP was contributed by soybean meal (Gallagher, 1994). Similarly, a recent study has reported that supplementing IMP in a high-soybean-meal diet did not improve growth performance of gibel carp compared with those fed the basal diet (Zhang et al., 2019). However, in the same study, the authors suggested the lack of significant differences observed in growth performance may have been related to the formulation of the basal diet, which had a high level of fishmeal (Zhang et al., 2019). Moreover, few studies have reported that supplementing purine nucleotides in semi-purified (Hossain et al., 2016a, 2016b) and purified diets (Lin et al., 2009) improved growth performance of *Pagrus major* and *Epinephelus malabaricus*, respectively. Thus, these

TABLE 4 Dietary effects of AMP[a] and IMP[b] from Sigma-Aldrich on whole-body proximate (g/kg fresh weight basis) of hybrid striped bass at the end of 9 weeks

Dietary treatments	Moisture g/kg	Protein g/kg	Lipid g/kg	Ash g/kg	Protein Conversion Efficiency %
Basal	679.1	179.1	95.8	47.9	33.4
AMP (0.5%)	673.6	181.2	108.3	43.6	34.2
IMP (0.5%)	669.8	179.8	107.1	50.0	35.8
AMP (0.5%) + IMP (0.5%)	674.1	180.3	103.6	47.7	35.6
PSE[c]	5.50	2.72	4.95	2.17	1.54
Two-way ANOVA P[d]					
AMP	0.91	0.65	0.39	0.16	0.84
IMP	0.43	0.96	0.52	0.19	0.26
AMP × IMP	0.39	0.77	0.14	0.67	0.76
Basal	679.1	179.1	95.8	47.9	33.4
AMP (0.5%)	673.6	181.2	108.3	43.6	34.2
IMP (0.5%)	669.8	179.8	107.1	50.0	35.8
AMP (0.5%) + IMP (0.5%)	674.1	180.3	103.6	47.7	35.6
AMP (0.25%) + IMP (0.25%)	681.3	181.9	985.4	46.0	34.8
PSE	5.29	2.80	4.85	2.10	1.79
One-way ANOVA P[e]	0.57	0.96	0.35	0.33	0.88

[a]Adenosine monophosphate (AMP Sig.), Sigma-Aldrich.

[b]Inosine monophosphate (IMP Sig.), Sigma-Aldrich.

[c]Pooled standard error.

[d]Value expressed as means of three replicates groups ($n = 3$). In case of significant interaction ($p < .05$), Tukey's HSD test was performed and values with the different letters within the same column are significantly different.

[e]Value expressed as means of three replicates groups ($n = 3$). Same data were analysed in one-way ANOVA with additional AMP (0.25%) × IMP (0.25%) dietary treatment. In case of statistically significant ($p < .05$), Tukey's HSD test was performed and values with the different letters within the same column are significantly different.

refined formulations could make purine nucleotides very limiting in the diet, corroborating the earlier claims that differences in growth performance may be possibly observed only if the diets were very limiting in nucleotides.

For specific immune responses evaluated in the present study, the increase of non-specific mitogenic response (LPS-stimulated) provided by dietary IMP C. Impex was significantly higher than that of fish fed the basal diet (Table 8). Similarly, Leonardi and Klempau (2003) reported that rainbow trout fed dietary nucleotides (Optimûn, Chemoforma) had significantly higher B-lymphocyte proliferation of LPS-stimulated cells compared with fish fed the basal diet. Moreover, the supplementation of the commercial dietary nucleotide (Rovimax Nx, DSM nutritional products) presented higher stimulation index of lymphocytes with LPS in hybrid tilapia; although, it was not statistically different from fish fed the control diet (Shiau, Gabaudan, & Lin, 2015). Kulkarni et al. (1989) illustrated that dietary nucleotides could stimulate the maturation of lymphoid cells. They postulated that when mammals were fed a nucleotide-free diet, a greater percentage of terminal deoxynucleotidyl transferase enzyme (identified as an index of immaturity for lymphocytes) was observed when compared to those fed a nucleotide-rich diet. As such, those authors claimed that maturation of lymphoid cells could exert a predominant effect upon the initial phase of antigen processing and lymphocyte proliferation. Therefore, in the present study, it was hypothesized that enhancement of mitogenic response was possibly due to the predominant effect of fish fed the IMP C. Impex diet. In addition, Carver (1999) suggested that the exogenous supply of nucleotides in the diet could contribute to the pool of nucleotides available to stimulate lymphocytes, which would rapidly turn over and consequently increase the nucleotide requirements.

TABLE 5 Dietary effects of AMP[1] and IMP[2] from Chem-Impex International on whole-body proximate (g/kg fresh weight basis) composition of hybrid striped bass at the end of 9 weeks

Dietary treatments	Moisture g/kg	Protein g/kg	Lipid g/kg	Ash g/kg	Protein Conversion Efficiency %
Basal	679.1	179.1[ab]	95.8	47.9	33.0.4
AMP (0.5%)	672.2	184.8[a]	101.3	43.7	36.8
IMP (0.5%)	674.9	180.1[ab]	104.8	44.9	34.1
AMP (0.5%) + IMP (0.5%)	673.5	174.7[b]	104.5	46.5	33.9
PSE[3]	2.70	2.19	2.18	2.38	1.61
Two-way ANOVA P[4]					
AMP	0.16	0.86	0.26	0.59	0.34
IMP	0.60	0.05	0.02	0.97	0.49
AMP × IMP	0.33	0.02	0.22	0.26	0.29
Basal	679.1	179.1AB	95.8	47.9	33.4
AMP (0.5%)	672.2	184.8[A]	101.3	43.7	36.8
IMP (0.5%)	674.9	180.1[AB]	104.8	44.9	34.1
AMP (0.5%) + IMP (0.5%)	673.5	174.7[B]	104.5	46.5	33.9
AMP (0.25%) + IMP (0.25%)	675.7	181.8[AB]	101.3	48.4	34.0
PSE	2.67	2.06	2.06	2.39	1.59
One-way ANOVA P[5]	0.47	0.03	0.07	0.62	0.59

[1]Adenosine monophosphate (AMP C. Impex), Chem-Impex International.

[2]Inosine monophosphate (IMP C. Impex), Chem-Impex International.

[3]Pooled standard error.

[4]Value expressed as means of three replicates groups ($n = 3$). In case of significant interaction ($p < .05$), Tukey's HSD test was performed and values with the different letters within the same column are significantly different.

[5]Value expressed as means of three replicates groups ($n = 3$). Same data were analysed in one-way ANOVA with additional AMP (0.25%) × IMP (0.25%) dietary treatment. In case of statistically significant ($p < .05$), Tukey's HSD test was performed and values with the different letters within the same column are significantly different.

Another report by Low, Wadsworth, Burrells, and Secombes (2003) stated that exogenous supply of nucleotides resulted in an increase in the humoral immune response by upregulating the IgM gene expression; they further hypothesized that the 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 in the circulating blood when nucleotides were supplemented in the diet (Barros et al., 2015; Reda et al., 2018; Tahmasebi-Kohyani, Keyvanshokooh, Nematollahi, Mahmoudi, & Pasha-Zanoosi, 2012).

In the present study, all dietary nucleotide treatments demonstrated significantly higher values of haematocrit than the basal dietary treatment prior to stress challenge (Table 8). Similar findings were reported where haematocrit values were higher for juvenile red sea bream fed an AMP-supplemented diet (Hossain et al., 2016a). The fluctuations in haematocrit values after fish were subjected to acute stress may be due to changes in erythrocyte numbers, erythrocyte swelling or hemodilution from osmoregulatory disturbance (Iwama, Morgan, & Barton, 1995). It is also reported that catecholamines have direct effects on erythrocytes of marine teleosts, which could be elevated in plasma during stress, consequently changing the erythrocyte volume (Ling & Wells, 1985). Moreover, in experiment 3, the haematocrit response was reduced at 0.5 hr poststress and all the nucleotide dietary treatments made a complete recovery at 12 hr postacute-stress challenge, yet the basal dietary treatment failed to completely recover in experiment 2 and 3 (Figure 1c; Table 8). Based on the facts and figures, there are possibilities that purine nucleotides may play a role in restoring hematopoiesis, by increasing the number of erythrocytes in the blood, or preventing haemolysis when fish are exposed to the stressor, or possibly by

TABLE 6 Dietary effects of AMP[1] and IMP[2] from Sigma-Aldrich on immune responses of hybrid striped bass at the end of 9 weeks

Dietary treatments	Superoxide anion extracellular nmol O_2^-/ml	Superoxide anion intracellular[3]	Plasma lysozyme U/ml	Total plasma protein mg/ml	Total immunoglobulin mg/ml	Plasma anti-protease %	LPS[4]
Basal	1.87	0.57	313	46.3	23.2	32.7	2.31^{ab}
AMP (0.5%)	1.26	0.58	341	49.0	24.2	31.6	2.09^{b}
IMP (0.5%)	1.44	0.54	320	47.1	23.7	30.0	2.20^{b}
AMP (0.5%) + IMP (0.5%)	1.76	0.60	350	49.1	26.4	28.2	2.58^{a}
PSE[5]	0.38	0.06	20.2	2.30	2.08	2.57	0.07
Two-way ANOVA P[6]							
AMP	0.71	0.55	0.19	0.33	0.41	0.58	0.30
IMP	0.93	0.91	0.69	0.85	0.52	0.26	0.03
AMP × IMP	0.26	0.58	0.96	086	0.68	0.87	0.003
Basal	1.87	0.57	313	46.3	23.2	32.7	2.31^{ABC}
AMP (0.5%)	1.26	0.58	341	49.0	24.2	31.6	2.09^{C}
IMP (0.5%)	1.44	0.54	320	47.1	23.7	30.0	2.20^{BC}
AMP (0.5%) + IMP (0.5%)	1.76	0.60	350	49.1	26.4	28.2	2.58^{A}
AMP (0.25%) + IMP (0.25%)	1.75	0.63	327	46.4	24.1	27.3	2.42^{AB}
PSE	0.46	0.07	18.4	2.20	2.00	2.44	0.07
One-way ANOVA P[7]	0.87	0.87	0.63	0.80	0.82	0.52	0.003

[1]Adenosine monophosphate (AMP Sig.), Sigma-Aldrich Co.

[2]Inosine monophosphate (IMP Sig.), Sigma-Aldrich Co.

[3]Measurement absorbance at 620 nm.

[4]Stimulation index (SI) of head kidney lymphocytes. Values indicate the proliferation after stimulation with mitogen (LPS, lipopolysaccharide from *Escherichia coli* O26:B6). Stimulation index = O.D. 570 nm of leukocyte wells with test mitogen/mean O.D. 570 nm of leukocyte wells without mitogen.

[5]Pooled standard error.

[6]Value expressed as means of three replicates groups ($n = 3$). In case of significant interaction ($p < .05$), Tukey's HSD test was performed and values with the different letters within the same column are significantly different.

[7]Value expressed as means of three replicates groups ($n = 3$). Same data were analysed in one-way ANOVA with additional AMP (0.25%) × IMP (0.25%) dietary treatment. In case of statistically significant ($p < .05$), Tukey's HSD test was performed and values with the different letters within the same column are significantly different.

promoting the swelling of erythrocytes in response to hypoxia after the fish were acutely stressed with air exposure. For example, a recent study reported that zebrafish had hypoxia-induced increases in haemoglobin O_2 affinity via a decrease in erythrocyte adenosine triphosphate after erythrocyte swelling (Cadiz, Bundgaard, Malte, & Fago, 2019).

Although numerous studies have reported that dietary nucleotide supplementation improved innate immunity of fish (Hossain et al., 2016a; Song et al., 2012; Tahmasebi-Kohyani et al., 2012; Zhao et al., 2015), no such effects were observed in the present study. However, this lack of enhancement 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, Gatlin, & Neill, 2007). A hypothesis raised in the present study was if the endogenous supply of nucleotides plays an important role in modulation of innate immunity during stressful conditions. 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 hr poststress, and these responses made a complete recovery at 24 hr poststress (Figure 1d,e). In experiment 3, all the innate immune parameters showed no differences prior to the stress challenge (Table 8), and these findings

TABLE 7 Dietary effects of AMP[1] and IMP[2] from Chem-Impex International on immune parameters of hybrid striped bass at the end of 9 weeks

Dietary treatments	Superoxide anion extracellular nmol O_2^-/ml	Superoxide anion intracellular[3]	Plasma lysozyme U/ml	Total plasma protein mg/ml	Total immunoglobulin mg/ml	Plasma anti-protease %	LPS[4]
Basal	1.87	0.57	313	46.3	23.2	32.7	2.31^{ab}
AMP (0.5%)	1.92	0.65	363	48.5	24.2	30.8	2.41^{ab}
IMP (0.5%)	1.54	0.54	331	48.1	22.3	23.7	2.80^{a}
AMP (0.5%) + IMP (0.5%)	1.68	0.64	350	49.6	25.2	31.5	2.17^{b}
PSE[5]	0.60	0.09	33.0	2.28	1.62	3.58	0.11
Two-way ANOVA P[6]							
AMP	0.87	0.33	0.33	0.42	0.26	0.44	0.04
IMP	0.64	0.76	0.93	0.54	0.96	0.28	0.30
AMP × IMP	0.93	0.88	0.64	0.88	0.55	0.21	0.01
Basal	1.87	0.57	313	46.3	23.2	32.7	2.31^{B}
AMP (0.5%)	1.92	0.65	363	48.5	24.2	30.8	2.41^{AB}
IMP (0.5%)	1.54	0.54	331	48.1	22.3	23.7	2.80^{A}
AMP (0.5%) + IMP (0.5%)	1.68	0.64	350	49.6	25.2	31.5	2.17^{B}
AMP (0.25%) + IMP (0.25%)	1.23	0.57	407	50.0	26.0	34.9	2.30^{B}
PSE	0.55	0.08	29.6	2.55	2.04	3.37	0.09
One-way ANOVA P[7]	0.90	0.82	0.29	0.84	0.72	0.26	0.01

[1]Adenosine monophosphate (AMP C.Impex), Chem-Impex International.

[2]Inosine monophosphate (IMP C. Impex), Chem-Impex International.

[3]Measurement absorbance at 620 nm.

[4]Stimulation index (SI) of head kidney lymphocytes. Values indicate the proliferation after stimulation with mitogen (LPS, lipopolysaccharide from *Escherichia coli* O26:B6). Stimulation index = O.D. 570 nm of leukocyte wells with test mitogen/mean O.D. 570 nm of leukocyte wells without mitogen.

[5]Pooled standard error.

[6]Value expressed as means of three replicates groups ($n = 3$). In case of significant interaction ($p < .05$), Tukey's HSD test was performed and values with the different letters within the same column are significantly different.

[7]Value expressed as means of three replicates groups ($n = 3$). Same data were analysed in one-way ANOVA with additional AMP (0.25%) × IMP (0.25%) dietary treatment. In case of statistically significant ($p < .05$), Tukey's HSD test was performed and values with the different letters within the same column are significantly different.

were highly correlated with experiment 1 results in which no significant differences in innate immunity were observed (Tables 6 and 7). However, at 0.5 and 12 hr poststress, it was intriguing to observe that some of the dietary nucleotide treatments activated some of the innate immune responses which were significantly higher than observed for fish fed the basal diet (Table 8). These responses indicate that dietary nucleotide supplementation plays an important role in modulating innate immunity during stressful conditions. It is well established that the ability of an organism to maintain homoeostasis 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 B-cell proliferation and the innate immunity of hybrid striped bass subjected to the air exposure stressor were influenced by the dietary supplementation of nucleotides. For instance, fish fed with diet supplemented with AMP C. Impex demonstrated significantly higher plasma lysozyme activity, and total immunoglobulin at 12 hr poststress compared with fish fed the basal diet. This suggests that the exogenous supply of AMP

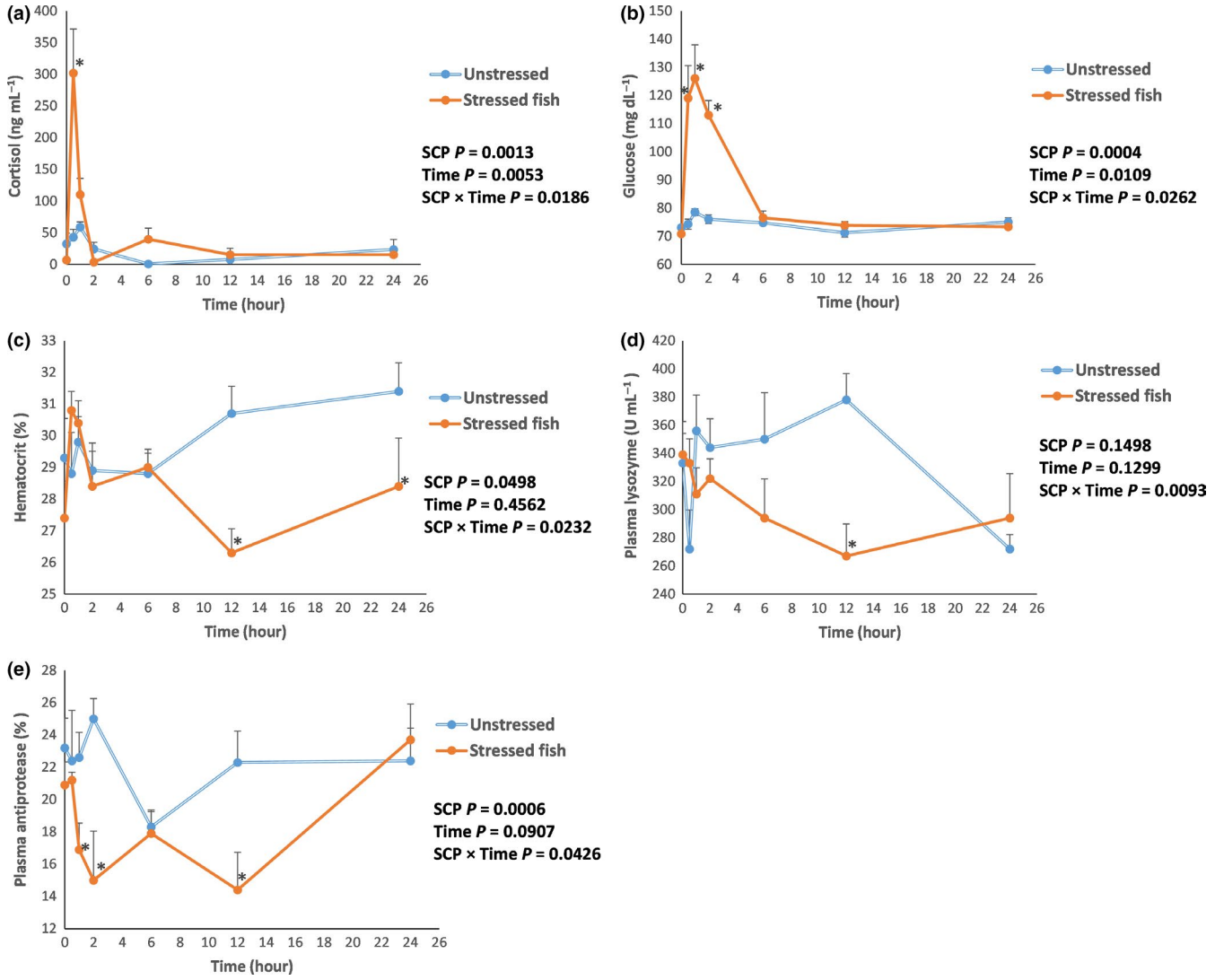

FIGURE 1 Modulation of plasma cortisol, blood glucose, and innate immunity (a = cortisol, b = glucose, c = haematocrit, d = plasma lysozyme, and e = plasma antiprotease) of hybrid striped bass subjected to the stress challenge protocol (SCP) compared with the unstressed group. Data are represented as mean ± standard error ($n = 6$ per sampling time point). Asterisk (*) indicates significant differences between dietary treatments

plays the most significant role in enhancing innate immunity during and after exposure to a stressful condition (Table 8). The potential synergistic effects of combining dietary AMP and IMP supplementation demonstrated that nucleotide concentrations and suppliers exhibited some differences in immunomodulation at 0.5 hr poststress; however, the greatest enhancement of immunity at 12 hr poststress was most apparent in the single addition of AMP C. Impex (Table 8). In fact, experiment 2 demonstrated that the fish were only able to recover their innate immunity at 24 hr poststress (Figure 1). This also is in line with what Burrells, Williams, Southgate, and Wadsworth (2001) observed, in which stressful events related to various aquaculture practices such as vaccination, handling and disease exposure may increase the need for dietary nucleotides to provide optimal responses of the fish.

Based on these 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. This raises a new hypothesis that despite using the same type of nucleotide with the same purity level, the process of producing the nucleotides by the manufacturer may influence their efficacy as dietary supplements.

5 | CONCLUSION

In conclusion, the present study showed that an 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 to stress-induced innate immune system suppression after an air exposure stress challenge. In addition, only the fish fed the IMP C. Impex supplemented diet yielded the highest B-lymphocyte proliferation compared with fish fed the basal diet.

TABLE 8 Effects of dietary nucleotide on innate immunity and haematocrit before and after subjecting hybrid striped bass to acute stress via air exposure for 1 min

	Dietary treatments[1-4]									
Haematological parameter	Time (hour)	Basal	AMP S. 0.5%	IMP S. 0.5%	AMP S. 0.5% + IMP S. 0.5%	AMP S. 0.25% + IMP S. 0.25%	AMP C. 0.5%	IMP C. 0.5%	AMP C. 0.5% + IMP C. 0.5%	AMP S. 0.25% + IMP S. 0.25%
Plasma anti-protease (%)										
Dietary treatment $p = .0486$	0	19.0	23.1	21.5	21.5	22.4	19.1	20.6	19.3	19.8
Time $p < .0001$	0.5	17.3	17.8	16.1	18.5	17.3	19.5	24.2*	19.5	21.7*
Dietary treatment × Time $p = .0020$	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 $p = .006$	0	877.8	905.6	811.1	855.6	822.2	883.3	783.3	855.6	1,122*
Time $p < .0001$	0.5	861.1	861.1	811.1	983.3	700.0	1,045	861.1	1,167*	1,000
Dietary treatment × Time $p = .0008$	12	1,006	950.0	933.3	955.6	1,172	1,233*	955.6	1,044	961.1
Pooled standard error = 66.7										
Total immunoglobulin (mg/ml)										
Dietary treatment $p = .1578$	0	39.3	42.0	38.3	41.2	41.2	37.1	38.0	41.2	40.3
Time $p < .0001$	0.5	34.9	38.8	41.1*	35.1	36.2	39.3*	40.1*	39.1*	39.9*
Dietary treatment × Time $p = .0030$	12	32.2	34.7	35.1	31.8	37.1	38.3*	32.7	33.2	31.0
Pooled standard error = 1.46										
Haematocrit (%)										
Dietary treatment $p < .0001$	0	33.3	41.0*	39.8*	39.8*	44.8*	39.5*	40.3*	41.5*	42.7*
Time $p < .0001$	0.5	29.5	24.8	28.0	30.3	30.8	30.3	30.7	33.7	31.8
Dietary treatment × Time $p = .0002$	12	27.5	32.8*	37.7*	35.5*	40.2*	40.0*	38.2*	39.3*	43.3*
Pooled standard error = 1.50										

[1]Adenosine monophosphate (AMP S.), Sigma-Aldrich Co., St. Louis, MO, USA.

[2]Inosine monophosphate (IMP S.), Sigma-Aldrich Co., St. Louis, MO, USA.

[3]Adenosine monophosphate (AMP C.), Chem-Impex International, Wood Dale, IL, USA.

[4]Inosine monophosphate (IMP C.), Chem-Impex International, Wood Dale, IL, USA.

*Asterisks represent treatment that is significantly different ($p < .05$) than the basal diet for each immune parameter at each time points (values within the row).

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DATA AVAILABILITY STATEMENT

The data that were analysed in the present article are available upon justifiable request to the corresponding author.

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