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# Effects of dietary supplementation of a purified nucleotide mixture on immune function and disease and stress resistance in channel catfish, *Ictalurus punctatus*

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## Abstract

Juvenile channel catfish (14.4 g average initial weight) were fed diets supplemented with a purified nucleotide mixture for 8 weeks. The mixture consisted of five nucleotides supplied on an equal basis as disodium salts at combined concentrations of 0 (control), 0.1%, 0.3%, 0.9% or 2.7% of diet. Addition of nucleotides to diet produced a dose-dependent reduction in survival of channel catfish to *Edwardsiella ictaluri*. Although the reasons are unclear, the high levels of nucleotides supplemented in this study may have contributed to the decrease in disease resistance, and this decrease appeared to be unrelated to the innate immune (unaffected) and specific antibody (enhanced) responses. Stress resistance increased with a corresponding amelioration of the immunosuppressive effects of the stress response on non-specific immunity (lysozyme and bactericidal activity) as nucleotides increased in diet. Use of exogenous nucleotides as a prophylactic treatment before culture-related stress exposure may prove beneficial by decreasing the immunosuppressive effects of stress but not in prevention of ESC.

**Keywords:** cortisol, *Edwardsiella ictaluri*, enteric septicaemia, immunity

## Introduction

Enteric septicaemia (ESC) of channel catfish (*Ictalurus punctatus*), caused by *Edwardsiella ictaluri* (Hawke 1979), is one of the leading causes of economic loss in the catfish industry and is responsible for approximately US$60 million in lost revenue each year *r* (Shoemaker, Klesius & Evans 2002). Increases in infectivity and mortality of channel catfish from ESC have largely been attributed to stress-related reductions in immune function (Klesius, Shoemaker & Evans 2003; Sink & Strange 2004). Restriction of feeding, antibiotics and vaccination are currently used to varying degree to control ESC. However, each of these treatment methods has its drawbacks, including cost (antibiotics and vaccination), antibiotic resistance, increased labour (vaccination) and lack of effectiveness and growth reduction (feed restriction). The addition of immunostimulants to fish diets has been suggested as a cost-effective alternative for prophylactic treatment against disease outbreaks in intensive aquaculture (Sakai 1999). Feeding channel catfish diets supplemented with immunostimulants before exposure to stressful practices or at times of increased disease susceptibility may prove effective in preventing infection from *E. ictaluri* and other pathogens. Although most research on dietary immunostimulants in fish has focused on yeast and yeast subcomponents, such as β-glucans, recent evidence suggests that addition of nucleotides to fish diets can have positive benefits on growth, immune function and disease and stress resistance (Li & Gatlin 2006).

Purine (e.g. adenine, guanine and hypoxanthine) or pyrimidine (uracil, thymine and cytosine) bases are combined with a ribose or 2′-deoxyribose sugar and one or more phosphate groups to form

 

nucleotides (Li & Gatlin 2006). The nucleotide pool in vertebrates is derived from three potential sources: *de novo* synthesis, the salvage pathway or diet (Low, Wadsworth, Burrells & Secombes 2003). However, the processes to produce purines and pyrimidines by *de novo* synthesis and the salvage pathway are thought to be energetically costly. In mammals, activities of the salvage and *de novo* pathways vary considerably among various tissue types and can be significantly influenced by metabolic needs and physiological functions (Li & Gatlin 2006). These pathways are suspected to also operate similarly in fish. Nucleotides supplied through the diet may optimize the functions of rapidly dividing tissues, such as those comprising the immune system, which cannot synthesize nucleotides and therefore must depend on exogenous sources (Carver & Walker 1995).

Dietary nucleotides have shown promise in enhancing immune response and disease resistance in tilapia (Ramadan, Afifi, Moustafa & Samy 1994), Atlantic salmon (*Salmo salar*), coho salmon (*Oncorhynchus kisutch*) and rainbow trout (*Oncorhynchus mykiss*) (Burrells, Williams, Southgate & Wadsworth 2001), common carp (Sakai, Taniguchi, Mamoto, Ogawa & Tabata 2001) and hybrid striped bass (Li, Lewis & Gatlin 2004). Supplementation of nucleotides in diets has also increased stress tolerance in Atlantic salmon (Burrells, Williams & Forno 2001) and rainbow trout (Leonardi, Sandino & Klempau 2003) and may have the added benefits of enhancing immune function and disease resistance (Leonardi *et al.* 2003). Hypothesized reasons associated with beneficial effects of nucleotides include dietary provision of physiologically required levels of nucleotides due to limited synthetic capacity of certain tissues (e.g. lymphoid tissue), inadequate energetic expenditure for *de novo* synthesis, immunoendocrine interactions and modulation of gene expression patterns (Li & Gatlin 2006).

Nucleotides have been generally considered non-essential nutrients, because deficiency signs have not been observed (Carver & Walker 1995; Li & Gatlin 2006; Lin, Wang & Shiau 2009). It is known that tissues such as intestinal mucosa, haematopoietic cells, lymphocytes and the brain of terrestrial mammals have limited capacity for *de novo* synthesis of nucleotides and depend on the salvage pathway (Yamauchi, Hales, Robinson, Niehoff, Ramesh, Pellis & Kulkarni 2002). Fish are exposed to numerous stressors in intensive and semi-intensive fish culture, which can cause immunosuppression, reduced growth and increased susceptibility to disease (Anderson 1996). Exposure to stress places additional demands on available nucleotides and an additional exogenous supply of nucleotides provided by dietary supplementation may help to counter the immunosuppressive effects of stress (Low *et al.* 2003).

Currently, there are numerous gaps in existing knowledge about supplementation of nucleotides in diets of fish and its effects on physiology and immunity. Research has not been conducted in most species of fish, including channel catfish. Most studies have utilized commercial nucleotide products derived from yeast, which contain other components, such as trace elements and polysaccharides, which can also influence immunity and other physiological functions. Our objective was to determine the effects of dietary supplementation of purified nucleotides as a 1:1:1:1:1 mixture of inosine monosphophate (IMP), adenosine monosphophate (AMP), guanosine monosphophate (GMP), uridine monosphophate (UMP) and cytidine monosphophate (CMP) in diets of juvenile catfish on immune function and disease resistance. In addition, we endeavoured to evaluate the effects of dietary nucleotides on the stress response and whether dietary supplementation had an ameliorating effect on accompanying immunosuppressive effects.

## Materials and methods

### Experimental fish

Juvenile channel catfish (NWAC 103) raised from yolk-sac fry to juveniles on a commercial fry diet were acclimated to laboratory conditions and fed the basal experimental diet without added nucleotides for approximately 4 weeks. At the end of the acclimation period, 30 fish of approximately 14.4 $\pm$ 0.2 g (mean SEM) were randomly selected and stocked in each of twenty 57 L aquaria. The aquaria were supplied with flow-through, dechlorinated city water heated with a central water heater at an initial rate of 0.5 L min$^{-1}$ and increased gradually to about 0.8 L min$^{-1}$ by the fifth week of the trial. Water flow rates were checked and adjusted twice daily to ensure proper water exchange. The water was continuously aerated with compressed air diffused through air stones, and the photoperiod was maintained on a 12:12 h light/dark schedule. Dissolved oxygen and temperature in three randomly chosen aquaria were measured daily using an YSI model 58 Oxygen Meter (Yellow Spring Instrument, Yellow Spring, OH, USA) (Use of trade name or commercial products is solely for purpose of

providing specific information and does not imply endorsement by the USDA.). During the trial, water temperature averaged 27.4 ± 0.2 °C, and dissolved oxygen averaged 5.1 ± 0.4 mg mL$^{-1}$. Care and handling of fish were in accordance with guidelines established by the Institutional Animal Care and Use Committee at the USDA, ARS Aquatic Animal Health Research Unit, Auburn, AL, USA and the 'Guidelines for the Use of Fishes in Research' of the American Fisheries Society, Bethesda, MD, USA (http://www.fisheries.org/afs/docs/policy.guidelines 2004.pdf).

## Feed and feeding

A nutritionally complete, practical basal diet formulated to contain approximately 32% crude protein, 5.6% crude lipid and 2877 kcal of digestible energy kg$^{-1}$ based on feedstuff values reported in 26 National Research Council (1993) (Table 1) was supplemented with a mixture of nucleotide disodium salts (AMP: adenosine-5′-monophosphate; CMP: cytidine-5′-monophosphate; UMP: uridine-5′-monophosphate; IMP: inosine-5′-monophosphate; GMP: guanosine-5′-monophosphate) (United States Biochemical, Cleveland, OH, USA) at the expense of corn meal at a combined concentration of 0%, 0.1%, 0.3%, 0.9% or 2.7% of diet. Each diet was mixed thoroughly and processed into 3-mm-diameter pellets as described by Lim, Sealey and Klesius (1996). Diets were dried at room temperature (23 °C) to a moisture content of approximately 10%, ground in a S-500 Disc Mill (Glen Mills, Glenmill, NJ, USA), and sieved with a No. 14 US standard sieve. The particles retained in the sieve were stored at − 20 °C in sealed plastic bags until used. Determined crude protein content of the basal diet was 32.7 ± 0.3%. Each dietary treatment was randomly assigned to four aquaria. Fish were fed to apparent satiation twice daily (between 07:30–08:30 and 15:00–16:00 hours) for 8 weeks. At the end of the feeding trial, fish were sampled to measure haematological and immunological parameters, and six fish were transferred from each aquarium to a new set of aquaria for the stress challenge (for a detailed explanation, see below). The number of fish remaining in the original aquaria was adjusted to 22 and used in the *E. ictaluri* challenge outlined below. No feeding was done on disease challenge or sampling days. All the aquaria were cleaned thoroughly once every other week. On cleaning days, fish were fed only in the afternoon.

**Table 1** Composition of basal diet

| Ingredient | Per cent in diet |
|---|---|
| Menhaden fish meal | 8.0 |
| Soybean meal | 45.0 |
| Corn meal | 25.0 |
| Wheat middlings | 14.0 |
| Carboxymethyl cellulose | 3.0 |
| Menhaden fish oil | 3.0 |
| Dicalcium phosphate | 1.0 |
| Mineral pre-mix* | 0.5 |
| Vitamin pre-mix† | 0.5 |
| Nucleotide mix‡ | 0–2.7 |

*Mineral pre-mix (mg kg$^{-1}$ diet unless otherwise stated): cobalt chloride hexahydrate, 0.2; zinc sulphate heptahydrate, 659.6; iron sulphate pentahydrate, 199.0; manganese sulphate monohydrate, 77.0; copper chloride, 4.7; potassium iodide, 6.5; and sodium selenite, 0.2.

†Vitamin pre-mix (mg kg$^{-1}$ diet unless otherwise stated): vitamin A-acetate, 4000 IU; vitamin D3, 2000 IU; vitamin K, 10; a-tocopherol acetate, 50; thiamin, 10; riboflavin, 12; pyridoxine, 10; pantothenic acid, 32; nicotinic acid, 80; folic acid, 5; biotin, 0.2; cyanocobalamine, 0.01; choline chloride, 400; L-ascorbyl acid-2-polyphosphate (15% vitamin C activity), 75.

‡Added to diets at the expense of corn meal. Supplemented as disodium salts: cytidine-5′-monophosphate (CMP), uridine-5′-monophosphate (UMP), adenosine-5′-monophosphate (AMP), guanosine-5′-monophosphate (GMP), inosine-5′-monophosphate (IMP).

## Growth measurements

Fish in each aquarium were counted and group-weighed after 4 weeks feeding of experimental diets and again after 8 weeks, following 24 h of feed deprivation. Weight measurements and fish counts were used for estimation of weight gain, feed efficiency ratio (FER; g wet weight gain g dry feed intake$^{-1}$) and survival.

## Haematological and immunological assays

After the termination of the feeding trial, blood was sampled from the caudal vasculature of three anaesthetized (100 mg L$^{-1}$ tricaine methanesulfonate or MS-222; Argent Chemical Laboratories, Redmond, WA, USA) fish per aquarium with an air-dried, heparinized (500 U sodium heparinate mL$^{-1}$) tuberculin syringe. After blood samples were collected, fish were euthanized with 200 mg L$^{-1}$ MS-222. Haematocrit of each fish was determined in duplicate using a microhaematocrit method (Brown 1988). Red and white blood cell counts were performed in duplicate for each sample by diluting whole blood and counting in a Spencer Bright Line haemacytometer as

described by Barros, Lim and Klesius (2002). Haemoglobin was analysed using a kit from Pointe Scientific (Canton, MI, USA), and values were adjusted by cyanomethaemoglobin correction factor for channel catfish described by Larsen (1964). The remaining whole blood was centrifuged at 1000 *g* for 10 min, and plasma was stored frozen at −80 °C for subsequent assays of bactericidal, lysozyme and spontaneous haemolytic complement ($SH_{50}$) activities. Plasma lysozyme activity was determined by the method of Litwack (1955) as modified by Sankaran and Gurnani (1972). The assay is based on lysis of lysozyme-sensitive Gram-positive bacterium *Micrococcus lysodeikticus* (Sigma, St Louis, MO, USA) by lysozyme present in plasma. A suspension of 0.25 mg mL$^{-1}$ freeze-dried *M. lysodeikticus* was prepared immediately before use by dissolving in sodium phosphate buffer (0.04 M $Na_2HPO_4$, pH 6.0). Plasma (10 μL well$^{-1}$ in duplicate) was placed in a microtitre plate, and 250 μL of bacterial cell suspension was added to each well. Hen egg white lysozyme was used as an external standard. The initial and final (after 20 min incubation at 35 °C) absorbances of the samples were measured at 450 nm. The rate of reduction in absorbance of samples was converted to lysozyme concentration (μg mL$^{-1}$) using a standard curve.

Spontaneous haemolytic complement activity ($SH_{50}$) was calculated using the method reported by Sunyer and Tort (1995). Sheep red blood cells (SRBC) in Alsever's solution (Remel, Lanexa, KS, USA) were washed four times in cold phosphate-buffered saline (PBS+) solution (0.85% PBS, 0.1% gelatin, 0.15 mM $CaCl_2$, 0.5 mM $MgCl_2$) and adjusted to $5 \times 10^7$ cells mL$^{-1}$ in cold PBS+. Twenty-five microlitres of channel catfish plasma to be tested for complement activity was serially diluted in PBS+ in round-bottom microtitre plates, and 25 μL of SRBC was added to each well. Distilled water was substituted for channel catfish plasma in one column to provide 100% haemolysis for a positive control. An additional 100 μL of PBS+ was added to all wells to increase well volume and aid in ease of pipette transfer of lysates. A second plate was assayed in tandem, except no SRBC were added. Values from this plate were subtracted from corresponding complement activity samples and used to account for any spontaneous haemolysis of channel catfish red blood cells during blood sampling and handling. The plates were incubated at room temperature (23 °C) for 1 h. After incubation, plates were centrifuged at 200 *g*, and the supernatant pipetted into a new microplate. Haemolysis was evaluated spectrophotometrically at 570 nm and converted to per cent haemolysis based on distilled water controls. The 50% lysis point ($SH_{50}$) was calculated by linear regression of each serum sample and expressed as the log 10 dilution.

Bactericidal activity was determined as described by Kampen, Tollersrud and Lund (2005) with modifications for plasma. Twenty-microlitres of plasma sample or Hank's Balanced Salt Solution (Gibco Laboratories, Grand Island, NY, USA) for positive controls was added to duplicate wells of a round-bottom 96-well microtitre plate. Twenty-microlitres of a 24 h culture of *E. ictaluri* adjusted to 0.5 optical density at 540 nm was added to sample wells. Plates were incubated at room temperature (23 °C) for 2.5 h on a platform shaker (46 cm × 46 cm) at 9.3 *g*. After incubation, plates were centrifuged at 2000 *g* for 10 min, and the supernatant discarded. The remaining pellets were resuspended in 50 μL of brain–heart infusion broth (BHI) (Difco Laboratories, Sparks, MD, USA) and incubated for 3 h at room temperature on a platform shaker at 9.3 *g*. To each well, 25 μL of 3-(4, 5 dimethyl thiazolyl-2)-2, 5-diphenyl tetrazolium bromide (2.5 mg mL$^{-1}$) (Sigma) was added and incubated for 10 min to allow the formation of formazan. Plates were again centrifuged at 2000 *g* for 10 min, the supernatant discarded and the precipitate dissolved in 200 μL of dimethyl sulfoxide. The absorbance of the dissolved formazan was read at 560 nm. Bactericidal activity was calculated as the decrease in number of viable *E. ictaluri* cells by subtracting the absorbance of samples from that of controls and reported as absorbance units.

### Stress resistance

At the termination of the feeding trial, six fish from each aquarium were transferred to a new set of aquaria. Fish were maintained on their respective experimental diets and acclimated for 3 days after transfer. After the acclimation period, whole blood was collected from three catfish as described previously to obtain plasma for baseline measurement of stress indices (cortisol, glucose and lactate) and immune function ($SH_{50}$, bactericidal and lysozyme activities) parameters. Immediately after baseline sampling, water depth in aquaria was reduced from approximately 26.5 to 6.5 cm by inserting a shortened stand-pipe. Fish were exposed to low-water conditions for 30 min. Aeration and water inflow were maintained during the exposure period. Blood was again sampled from three fish at the end of the 30 min as previously described. Post-stress plasma

was used to determine the effect of low-water stress on immunological and stress parameters. Cortisol was measured using an ELISA kit manufactured by DRG International (Mountainside, NJ, USA). Glucose and lactate concentrations were determined by kits (Pointe Scientific) following the manufacturer's instructions. Immune function parameters were measured as described previously.

### Disease challenge

*Edwardsiella ictaluri* (AL-93-75) from an outbreak of ESC was used to challenge channel catfish by immersion. A pre-challenge trial was conducted 14 days before the experimental challenge that determined $5 \times 10^6$ *E. ictaluri* colony-forming units (CFU) $mL^{-1}$ was needed to produce the target mortality of 20% (or 80% survival) by immersion (data not shown). For the experimental disease challenge trial, frozen stock-culture of *E. ictaluri* was grown in BHI broth for 24 h at 27 °C, and the concentration of the culture after 24 h growth was estimated at $1 \times 10^{10}$ CFU $mL^{-1}$ by spread plate technique of the *E. ictaluri* culture serially diluted in BHI broth. Twenty-two channel catfish were challenged in each aquarium by addition of the *E. ictaluri* culture at a rate to produce $5 \times 10^6$ CFU $mL^{-1}$. During immersion challenge, water volume in aquaria was reduced by one-half, and water flow but not aeration was halted for 1 h. The final challenge concentration was approximately $5.1 \times 10^6$ CFU $mL^{-1}$, determined using a spiral autoplater and Qcount (Spiral Biotech, Norwood, MA, USA) after challenge. Fish continued to be fed twice daily with the experimental diets, and mortality was recorded twice a day for 21 days. At the end of the challenge trial (day 22), blood was sampled from the caudal vasculature of four surviving fish in each aquarium by syringe, allowed to clot for 4 h at 4 °C, and centrifuged at 1000 *g*. Serum was collected and stored frozen at − 80 °C for subsequent determination of post-challenge agglutinating antibody titre against *E. ictaluri*. Necropsies were performed and anterior kidney tissue from dead fish was cultured to confirm death due to *E. ictaluri* (Klesius 1992). Plasma collected at the termination of the feeding trials for use in immune assays ($n = 3$ fish per aquarium) was used to determine pre-challenge antibody titres – all sampled fish were negative for *E. ictaluri*.

Agglutinating antibody titre against *E. ictaluri* (AL-93-75) in pre- and post-challenge serum was determined by modifying the method of Chen and Light (1994). *Edwardsiella ictaluri* was grown for 24 h in BHI broth at 28 °C and killed in 1% formalin. The cells were centrifuged at 3000 *g* for 15 min, and the resulting pellet was suspended and washed three times in sterile PBS. The bacterial concentration was adjusted to an optical density of 0.8 at 540 nm. Fifteen microlitres of sterile PBS was pipetted into each well of a 96-round-bottomed microtitre plate, and then 15 μL of plasma was added to the first well of each row and mixed. Two-fold serial dilutions were then made by adding 15 μL of diluted plasma into the remaining wells. An equal volume (15 μL) of bacterial suspension was added to each well making the initial dilution of the plasma 1:4. Positive plasma from *E. ictaluri*-infected fish and negative (PBS) were used as assay controls. The plates were covered with plastic film and incubated at room temperature for 16 h. The agglutination end point was established as the last dilution where cell agglutination was visible.

### Statistical analysis

Nucleotide concentration and stress treatment (baseline or low-water stress) were fixed effects in this study. The effects of diet on immune parameters, growth, haematology and disease resistance were analysed by one-way analysis of variance (ANOVA) using statistical analysis software (SAS) (Cary, NC, USA). The effects of stress and diet on stress indices and immune function after stress exposure were measured with two-way ANOVA. The relationship between level of dietary nucleotide and post-challenge survival was also evaluated by linear regression. To determine differences between individual treatment means, the Waller–Duncan *k*-ratio *t*-test (Waller & Duncan 1969) was used. If an interaction effect was significant ($P < 0.05$), then each factor was analysed within each level of the other factor using the SLICE option of the LSMeans and orthogonal contrasts (simple effects analysis) in SAS and differences between treatment means are shown in figures rather than tables. A significance level of $\alpha = 0.05$ was used for statistical analyses. Measured values from individual fish were averaged for each aquarium (experimental unit) for use in statistical analyses.

## Results

Nucleotide supplementation had a significant effect on post-*E. ictaluri* challenge survival and agglutination antibody titre to *E. ictaluri* but not bactericidal,

**Table 2** Mean (± SE) values and statistical analysis for immune function parameters and survival to *Edwardsiella ictaluri* challenge* in channel catfish fed nucleotide supplemented diets†

| | Survival (%)‡ | Ab titre (log 10)§ | BA¶ | $SH_{50}$ (U mL$^{-1}$) | Lysozyme (μg mL$^{-1}$) |
|---|---|---|---|---|---|
| Nucleotide (%) | | | | | |
| 0 | 88.8 ± 4.2$^{a}$ | 1.98 ± 0.06$^{a}$ | 0.341 ± 0.011 | 7.69 ± 2.87 | 7.50 ± 0.64 |
| 0.1 | 85.4 ± 4.6$^{a}$ | 2.35 ± 0.11$^{b}$ | 0.451 ± 0.117 | 8.27 ± 2.94 | 7.38 ± 0.46 |
| 0.3 | 80.4 ± 2.2$^{ab}$ | 2.43 ± 0.13$^{b}$ | 0.447 ± 0.113 | 8.25 ± 1.43 | 7.27 ± 0.45 |
| 0.9 | 79.3 ± 3.3$^{ab}$ | 2.15 ± 0.16$^{b}$ | 0.429 ± 0.082 | 7.69 ± 1.33 | 7.13 ± 0.39 |
| 2.7 | 72.2 ± 3.2$^{b}$ | 2.05 ± 0.03$^{a}$ | 0.428 ± 0.049 | 8.67 ± 2.20 | 7.16 ± 0.18 |
| Average | 81.2 ± 2.0 | 2.19 ± 0.06 | 0.419 ± 0.029 | 8.11 ± 0.98 | 7.29 ± 0.17 |
| ANOVA | | | | | |
| *F*-value | 3.283 | 3.158 | 0.545 | 0.086 | 0.327 |
| *P*-value | 0.04 | 0.05 | 0.71 | 0.98 | 0.86 |

*Survival and antibody titre were measured 21-day post-immersion challenge.
†Means within a column with different superscript letters are significantly different.
‡Per cent cumulative survival after challenge with *E. ictaluri*.
§Agglutination antibody titre (log 10) to *E. ictaluri*.
¶Plasma BA activity reported as absorbance units.

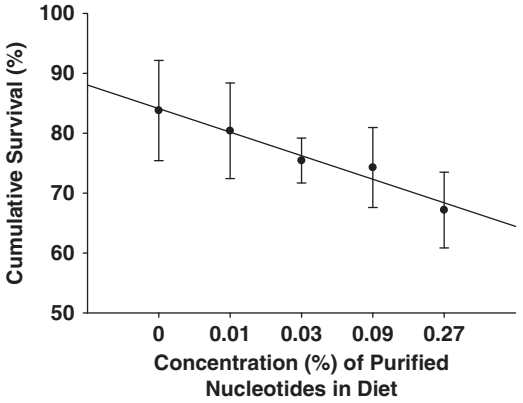


**Figure 1** Mean cumulative survival (% ± SE) 21 days after challenge with *Edwardsiella ictaluri*. The linear response was significant ($R^2 = 0.67$; $P = 0.003$). Catfish fed the 0% control diet had significantly higher survival than catfish fed the 0.9% or 2.7% nucleotide diets.

alternative complement ($SH_{50}$) or lysozyme activities (Table 2). Post-challenge survival had a linear response to dietary nucleotide level (Fig. 1; $R^2 = 0.67$; $P = 0.003$). Survival was highest in the control group and declined with increasing concentration of nucleotide in diet. Catfish fed the control or 0.1% nucleotide diet had survival significantly higher than fish fed the 0.9% or 2.7% diets (Table 2). Survival was slightly higher than the 80% target for the control group; however, average survival across all experimental groups was 81.2%. Antibody titres in catfish fed the control diet or the diet supplemented with 2.7% nucleotide were significantly lower than fish on the 0.1%, 0.3% and 0.9% diets, which were not different from one another. Nucleotide supplementation did not affect weight gain, feed intake, FER or survival (Table 3). No differences in FER or weight gain were noticeable between the dietary groups from 0 to 4 or 4 to 8 weeks as well. Weight gain and FER were highest in catfish fed the 0.1% nucleotide diet and lowest for those fed the 2.7% diet, although the differences were not statistically significant. The effect of nucleotide supplementation on all haematological measures (haematocrit, haemoglobin and total, white and red blood cell counts) was also statistically non-significant (Table 4).

Application of low-water stress had a significant effect on the cortisol, glucose and lactate responses of channel catfish – indices of stress were higher after exposure to low-water stress (Table 5). Furthermore, low-water stress caused a significant decline in bactericidal, alternative complement ($SH_{50}$) and lysozyme activities (Table 5). Diet also had a significant effect on cortisol, bactericidal activity and $SH_{50}$. Cortisol values post stress were significantly lower in catfish fed the 0.9% and 2.7% nucleotide diets compared with the control and 0.1% diets. Bactericidal and $SH_{50}$ activities were highest in fish fed the 0.9% diet compared with the control and the control and 0.1% diets respectively. A non-significant decline in both indices occurred in catfish on the 2.7% diet. Because there was a significant interaction between stress and nucleotide concentration in diet for cortisol, glucose and BA and lysozyme activities, the interactions were further analysed by simple effects testing for these measures. In post-stress channel catfish, cortisol (Fig. 2) concentrations were significantly lower in

**Table 3** Mean (± SE) feed efficiency ratio (FER), weight gain, feed intake and survival and statistical analysis of channel catfish fed nucleotide-supplemented diets

| | FER* | | | Weight gain | Feed intake | |
|---|---|---|---|---|---|---|
| | 0–4 weeks | 4–8 weeks | 0–8 weeks | (g fish $^{-1}$) | (g DM fish $^{-1}$)† | Survival (%)‡ |
| Nucleotide (%) | | | | | | |
| 0 | 0.83 ± 0.07 | 0.77 ± 0.09 | 0.80 ± 0.06 | 15.5 ± 1.4 | 19.2 ± 0.06 | 94.2 ± 1.3 |
| 0.1 | 0.82 ± 0.05 | 0.90 ± 0.16 | 0.86 ± 0.09 | 17.7 ± 2.2 | 20.6 ± 0.11 | 95.0 ± 1.5 |
| 0.3 | 0.74 ± 0.09 | 0.86 ± 0.14 | 0.81 ± 0.12 | 17.0 ± 3.1 | 20.7 ± 0.13 | 95.8 ± 0.8 |
| 0.9 | 0.80 ± 0.03 | 0.86 ± 0.07 | 0.83 ± 0.04 | 16.3 ± 0.9 | 19.5 ± 0.16 | 95.8 ± 0.7 |
| 2.7 | 0.78 ± 0.07 | 0.72 ± 0.08 | 0.75 ± 0.06 | 15.4 ± 1.8 | 20.3 ± 0.03 | 95.0 ± 1.1 |
| Average | 0.79 ± 0.04 | 0.82 ± 0.05 | 0.81 ± 0.03 | 16.4 ± 0.8 | 20.1 ± 0.30 | 95.2 ± 0.5 |
| ANOVA | | | | | | |
| *F*-value | 0.233 | 0.418 | 0.281 | 0.219 | 0.367 | 0.318 |
| *P*-value | 0.92 | 0.79 | 0.89 | 0.92 | 0.83 | 0.86 |

Weight gain, feed intake and survival were calculated for the entire 8 week feeding trial.
*Feed efficiency ratio (g wet weight gain g dry feed intake $^{-1}$).
†Calculated as g dry matter (DM) per g fish wet weight.
‡Cumulative survival (%).

**Table 4** Mean (± SE) haematology values and statistical analysis for channel catfish fed nucleotide-supplemented diets

| | Haematocrit (%) | Haemoglobin (g dL $^{-1}$) | TCC ($10^6$ µL $^{-1}$) | RBC ($10^6$ µL $^{-1}$) | WBC ($10^5$ µL $^{-1}$) |
|---|---|---|---|---|---|
| Nucleotide (%) | | | | | |
| 0 | 35.4 ± 2.6 | 6.7 ± 0.4 | 2.01 ± 0.20 | 1.54 ± 0.19 | 4.67 ± 0.26 |
| 0.1 | 34.8 ± 1.7 | 6.9 ± 0.3 | 2.11 ± 0.13 | 1.56 ± 0.11 | 5.47 ± 0.34 |
| 0.3 | 35.1 ± 1.3 | 6.5 ± 0.2 | 2.07 ± 0.11 | 1.52 ± 0.13 | 5.48 ± 0.55 |
| 0.9 | 35.8 ± 0.7 | 6.8 ± 0.2 | 2.04 ± 0.69 | 1.43 ± 0.11 | 6.10 ± 0.27 |
| 2.7 | 34.1 ± 1.8 | 6.7 ± 0.4 | 2.12 ± 0.12 | 1.50 ± 0.12 | 6.28 ± 0.40 |
| Average | 35.0 ± 0.7 | 6.7 ± 0.1 | 2.07 ± 0.06 | 1.51 ± 0.05 | 5.60 ± 0.22 |
| ANOVA | | | | | |
| *F*-value | 0.126 | 0.182 | 0.130 | 0.154 | 1.890 |
| *P*-value | 0.97 | 0.94 | 0.96 | 0.96 | 0.16 |

fish fed the 0.9% and 2.7% nucleotide diets compared with the control and 0.1% nucleotide diets, while glucose concentrations (Fig. 3) were significantly lower in catfish fed the 2.7% nucleotide diet compared with the control, 0.1% and 0.3% nucleotide diets. In general, lysozyme and bactericidal activities were inversely related to stress indices: activities of these measures of non-specific immunity increased as stress parameters decreased in catfish with increasing nucleotide concentration in diet. Lysozyme activity was significantly higher in fish fed the 0.1%, 0.3% or 0.9% diet compared with the control diet after exposure to stress (Fig. 4); in fish fed the 2.7% diet, activity was similar to the other diets. Lysozyme activity in catfish after stress exposure was not significantly reduced compared with baseline levels in the 0.3%, 0.9% and 2.7% nucleotide groups; significant reductions in activity were seen in catfish fed the control and 0.1% diets. Bactericidal activity in catfish fed the 0.9% nucleotide diet was significantly higher than in fish fed the control, 0.1% and 2.7% diets (Fig. 5). Fish fed the 0.1%, 0.3% and 2.7% diets had similar bactericidal activities, which were higher than that of the control diet. Post-stress bactericidal activities were not significantly reduced in channel catfish fed the 0.3% and 0.9% but were in fish fed the control, 0.1% and 2.7% nucleotide diets compared with pre-stress levels.

## Discussion

Generally, increased resistance to infection and enhanced immunity has been attributed to nucleotide supplementation in fish diets. Salmonids fed nucleotide-supplemented diets (0.2% diet of a commercial product giving 0.03% nucleotide in diet) had significantly lower mortality compared with fish fed the control diet after infection with infectious salmon

**Table 5** Mean (± SE) stress and immune function values and statistical analysis for channel catfish fed nucleotide supplemented diets after exposure to low-water stress

| | Cortisol (ng mL$^{-1}$) | Glucose (mg dL$^{-1}$) | Lactate (mg dL$^{-1}$) | BA (U mL$^{-1}$)* | $SH_{50}$(μg mL$^{-1}$) | Lysozyme (μg mL$^{-1}$) |
|---|---|---|---|---|---|---|
| Stress | | | | | | |
| Baseline | 22.5 ± 0.6 | 33.5 ± 2.2 | 34.2 ± 1.45 | 0.416 ± 0.012 | 8.02 ± 0.58 | 7.37 ± 0.09 |
| Post-stress | 78.0 ± 3.7 | 76.6 ± 2.8 | 69.8 ± 5.46 | 0.309 ± 0.030 | 6.01 ± 1.20 | 6.16 ± 0.17 |
| *F*-value | 419.71 | 203.00 | 33.84 | 16.61 | 23.46 | 50.56 |
| *P*-value | <60.001 | <0.001 | <0.001 | 0.001 | 0.006 | <0.001 |
| Nucleotide (%) | | | | | | |
| 0 | 56.0 ± 2.3$^{a}$ | 60.0 ± 3.5 | 57.7 ± 0.29 | 0.272 ± 0.022$^{a}$ | 5.58 ± 1.89$^{a}$ | 6.53 ± 0.07 |
| 0.1 | 56.2 ± 2.5$^{a}$ | 55.4 ± 5.2 | 58.0 ± 0.20 | 0.380 ± 0.071$^{b}$ | 6.04 ± 1.72$^{a}$ | 6.88 ± 0.14 |
| 0.3 | 48.3 ± 3.3$^{ab}$ | 57.1 ± 4.6 | 53.6 ± 0.31 | 0.398 ± 0.039$^{b}$ | 7.68 ± 1.89$^{b}$ | 6.99 ± 0.18 |
| 0.9 | 46.3 ± 3.2$^{b}$ | 48.5 ± 4.5 | 45.6 ± 0.26 | 0.405 ± 0.031$^{b}$ | 8.50 ± 1.01$^{b}$ | 6.79 ± 0.15 |
| 2.7 | 47.4 ± 3.8$^{b}$ | 49.4 ± 5.8 | 45.1 ± 0.23 | 0.359 ± 0.045$^{ab}$ | 6.98 ± 1.54$^{ab}$ | 6.64 ± 0.17 |
| Average | 50.9 ± 2.1 | 54.1 ± 2.4 | 52.0 ± 0.25 | 0.363 ± 0.020 | 6.96 ± 1.21 | 6.77 ± 0.09 |
| ANOVA | | | | | | |
| *F*-value | 3.313 | 1.40 | 0.940 | 3.359 | 3.335 | 0.938 |
| *P*-value | 0.03 | 0.27 | 0.46 | 0.03 | 0.03 | 0.46 |
| ANOVA interaction | | | | | | |
| *F*-value | 4.878 | 2.915 | 0.487 | 3.190 | 0.372 | 3.104 |
| *P*-value | 0.007 | 0.04 | 0.74 | 0.04 | 0.82 | 0.04 |

*Plasma BA activity reported as absorbance units.

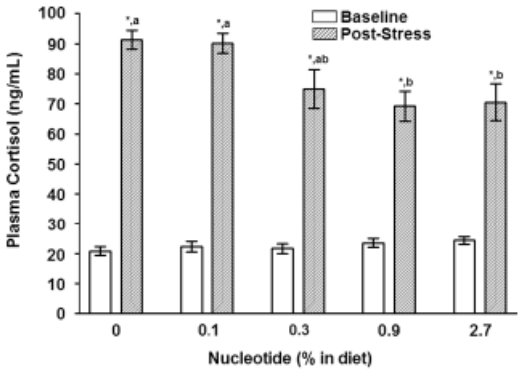


**Figure 2** Mean plasma cortisol (ng mL$^{-1}$ ± SE) of channel catfish fed nucleotide-supplemented diets for 8 weeks. Bars with different letters are significantly different from one another. Significant differences between baseline and post-stress values for the same diet are labeled with an asterisk.

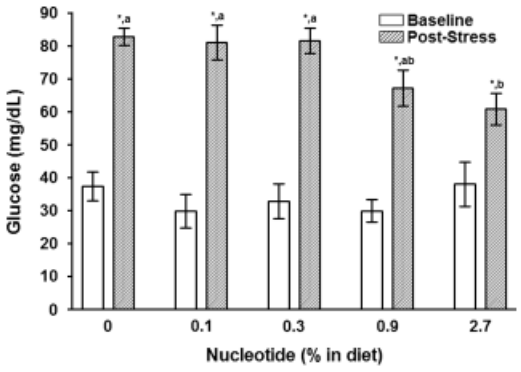


**Figure 3** Mean plasma glucose (mg dL$^{-1}$ ± SE) of channel catfish fed nucleotide-supplemented diets for 8 weeks. Bars with different letters are significantly different from one another. Significant differences between baseline and post-stress values for the same diet are labeled with an asterisk.

anaemia virus (Atlantic salmon), *Vibrio anguillarum* (rainbow trout), *Piscirickettsia salmonis* (coho salmon) or sea lice *Lepeophtheirus salmonis* (Atlantic salmon) (Burrells, Williams, Southgate & Wadsworth 2001). Macrophage activity, the only immune function measured, was lower in fish fed the nucleotide diet. Striped bass fed a commercial nucleotide product containing 0.075% nucleotide at 0.5% of diet exhibited increased blood neutrophil oxidative radical production and survival to *Streptococcus iniae* infection than in fish fed the basal diet (Li *et al.* 2004). Rainbow trout fed a diet supplemented with nucleotide (commercial product containing 0.3% nucleotide at 0.5% of diet) exhibited significantly increased survival to infection with infectious pancreatic necrosis (IPN) virus compared with the control group (Leonardi *et al.* 2003). The exact role of exogenous nucleotides in fish disease resistance and the mechanisms in which nucleotides affect immunity have not been established but are hypothesized to be very diverse (Li & Gatlin 2006). The most widely hypothesized mechanism by which dietary nucleotides might en-

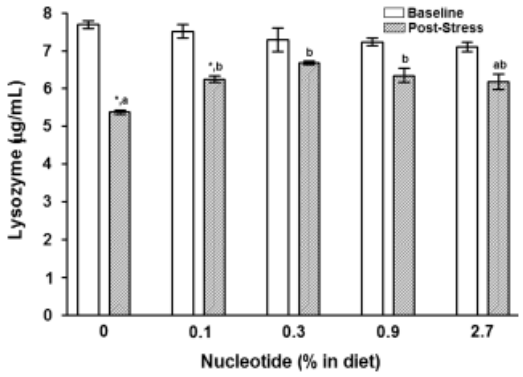


**Figure 4** Lysozyme activity (µg mL$^{-1}$ ± SE) of channel catfish fed nucleotide-supplemented diets for 8 weeks. Bars with different letters are significantly different from one another. Significant differences between baseline and post-stress values for the same diet are labeled with an asterisk.

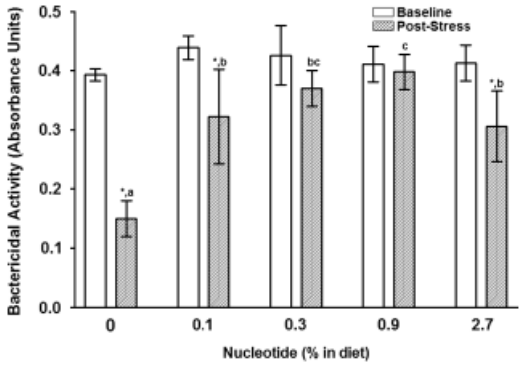


**Figure 5** Mean bactericidal activity (absorbance units ± SE) of channel catfish fed nucleotide supplemented diets for 8 weeks. Bars with different letters are significantly different from one another. Significant differences between baseline and post-stress values for the same diet are labeled with an asterisk.

hance the immune response is that they provide an increased supply of nucleotides at a time when high metabolic demand (such as growth or clonal expansion of immune cells) exceeds *de novo* synthetic capacity (Carver 1994). Strong evidence suggests that nucleotides affect immunity at the molecular level, and nucleotide-mediated changes in immune gene expression have been observed in fish. In turbot, IgM, recombinase activating gene 1 and lysozyme gene expression were up- or down-regulated in response to exogenous nucleotides depending on the tissue examined (Low *et al.* 2003). Other genes, such as transferrin and cytokine gene transforming growth factor-β, were unaffected. Although the majority of reports for the effects of dietary nucleotides on immunity in fish are positive, some studies suggest no effect (Li, Burr, Goff, Whiteman, Davis, Vega, Neill & Gatlin III 2005) and excessive levels of dietary nucleotides can also negatively affect immunity in fish (Burrells, Williams & Forno 2001).

In the present study, non-specific immune parameters were unaffected by addition of nucleotides to catfish diets. Antibody titre to *E. ictaluri* increased significantly with nucleotide supplementation but decline in the response was observed at higher nucleotide concentrations. Survival to *E. ictaluri* infection, on the other hand, declined linearly as nucleotide supplementation increased in the diet and appeared to be unrelated to the measured immune parameters. None of the previous studies have shown the apparent negative, dose-dependent response in resistance to infection that was observed in this study. The two highest levels of nucleotides supplemented in our study, 0.9% and 2.7%, were at least three and nine times greater, respectively, than the highest level used in fish diets of other published studies. Extended feeding duration and/or dietary concentration of nucleotides can have negative side effects in mammals (Rumsey, Winfree & Hughes 1992) and fish (Adamek, Hamackova, Kouril, Vachta & Stibranyiova 1996), including suppression of the immune response (Burrells, Williams, Southgate & Wadsworth 2001). Suppression of immunity by 'immunostimulant overload' has also been documented with other immunostimulants in fish (Couso, Castro, Magarinos, Obach & Lamas 2003). While the exact reasons for nucleotide suppression of immunity and disease resistance are not known, it is possible that negative effects of nucleotide supplementation on immune gene expression contributed to the dose-dependent decrease in disease resistance in the present study, which may have been further exacerbated by excessive levels of supplementation. Furthermore, it is possible that the lower *E. ictaluri* challenge concentration and subsequent higher survival rate we observed influenced the outcome of the disease challenge and immune function results. The survival rate was higher than the 80% target for the control group (88.8%), but average survival approached the target at 81.2% across all experimental groups. A better approach may be to have low and high lethal dose challenges for comparison in future research. In addition, few studies have aimed to determine the underlying causes for enhancement of immune function

resulting from exogenous nucleotides, and further research is needed.

Although non-specific immunity was unaffected by dietary nucleotide supplementation, antibody titre to *E. ictaluri* 21 days post challenge increased significantly in fish fed the 0.1% and 0.3% nucleotide diets compared with the control and then declined at higher nucleotide concentrations. Increased antibody production in fish fed nucleotide-supplemented diets has been confirmed in several species of fish. Post-vaccination antibody titres and the mitogenic response of lymphocytes were increased in tilapia fed diets containing a commercial nucleotide supplement (Ramadan et al 1994). In Atlantic salmon, specific antibody production was also enhanced with nucleotide supplementation compared with the basal diet (Burrells, Williams & Forno 2001). Similar responses have been observed in rainbow trout (Low et al. 2003), hybrid striped bass (Li et al. 2004) and grouper (*Epinephelus malabaricus*; non-specific immunoglobulin production) (Lin *et al.* 2009). Toll-like receptors (TLR) in fish have been shown to participate in innate and adaptive immune response of fish (Werling & Jungi 2003). Although the specific functions of TLRs have not been defined in fish (Bilodeau, Peterson & Bosworth 2006), the nucleotides AMP and GMP are TLR-like compounds and may explain why nucleotide supplementation enhances antibody production in fish (Lin *et al.* 2009). The decline in antibody response at higher nucleotide levels may be attributed to negative effects associated with excessive nucleotide supplementation as discussed previously. Based on the results of the current study and results of others, use of dietary nucleotide supplementation in conjunction with vaccination programmes may prove beneficial to the adaptive immune response.

In the majority of studies, commercial nucleotide products have been supplemented in fish diets. These products are derived from yeast and contain other components such as polysaccharides and trace elements (Lin *et al.* 2009), which are also known to provide immunostimulation in fish (Sakai 1999), and only a handful of studies have evaluated the effects of purified nucleotide mixtures added to diets on fish health. In grouper, superoxide anion production of head kidney leucocytes and immunoglobulin concentration are increased when fed a purified nucleotide mixture (0%, 0.05%, 0.1%, 0.15% or 0.2% of diet) (Lin *et al.* 2009). Post-challenge survival from *Vibrio harveyi* infection and neutrophil oxidative radical production in red drum were not influenced by supplementation with a purified nucleotide mixture at 0.03, 0.1 or 0.3% of diet (Li, Gatin III & Nell 2007). In the present study, effects of using a purified nucleotide mixture on immunity were mixed, and disease resistance was negatively affected. In this study, the concentrations of nucleotides supplemented in diets were nine and 13 times higher that the highest concentrations used by Lin *et al.* (2009) and Li *et al.* (2007) respectively. The actual concentrations in the fish meal-based diets of Li *et al.* (2007) were probably higher though, because fish meal has a reported concentration of 2 g $kg^{-1}$ nucleotides (Carver & Walker 1995). In addition, we fed our experimental diets for 8 weeks compared with 8 weeks for Lin *et al.* (2009) and 4 weeks for Li *et al.* (2007), making the total nucleotide intake of catfish in the present study much higher comparatively. It is difficult to determine reasons for the variability of effect between purified nucleotides and commercial nucleotide products supplemented in fish diets on immune function and disease resistance in fish, because so few studies have utilized purified nucleotide mixtures. The majority of studies using commercial nucleotide-containing products have shown positive effects on disease resistance, while we observed a negative effect using a purified nucleotide mixture. The differences may result from extra immunostimulatory components in the commercial products, dietary nucleotide concentration or differences between species.

Stress-related immunosuppression has been identified as one of the leading causes of decreased disease resistance in aquaculture. Supplementation of nucleotides beyond those provided in commercial fish diets may provide beneficial effects in fish exposed to culture-related stressors, such as poor water quality, crowding and handling, which place additional demands on the available nucleotide pool (Li & Gatlin 2006). We observed significant reductions in levels of stress parameters in fish fed nucleotide-supplemented diets after exposure to low-water stress. The stress-related reduction of non-specific immune function (bactericidal and lysozyme activities) was ameliorated as nucleotide concentration increased in diets and plasma cortisol concentrations declined, except at the highest level of dietary nucleotide, which may have been related to negative side effects associated with excessive levels of nucleotide supplementation (Li & Gatlin 2006). Li and Gatlin (2006) suggest that one of the possible mechanisms by which dietary nucleotides influence immune function is by partially offsetting the inhibitory effects of cortisol release associated with stress. In

rainbow trout fed dietary nucleotides, serum cortisol levels were reduced and produced a corresponding increase in resistance to IPN virus (Leonardi *et al.* 2003). However, inhibition of the cortisol response from confinement stress by exogenous nucleotides was not observed in juvenile red drum (Li *et al.* 2005). The authors suggest that the extreme variation in individual fish may have prevented a nucleotide-related reduction in the stress response. Based on the results of the present study, feeding of nucleotide-supplemented diets may prove beneficial as a prophylactic treatment to diminish the severity of the stress response in fish subjected to culture-related stressors with the added benefit of increasing immunity. However, determination of the proper feeding duration and concentration of nucleotides in diet of fish is required.

In the current study, supplementation of a purified nucleotide mixture in channel catfish diets caused a dose-dependent reduction in survival to *E. ictaluri* infection. The specific antibody response was enhanced, but innate immunity was unaffected by dietary nucleotide supplementation. The reasons for the observed decrease in disease resistance and apparent contradiction with innate immune and specific antibody responses are not clear. It is possible that the lower challenge dose and resulting higher survival, as compared with most studies, and the high levels of nucleotides supplemented in diets may have contributed to the decline in disease resistance and variability in immunity in the present study. Stress resistance, however, was increased with a corresponding amelioration of the immunosuppressive effects of the stress response on non-specific immunity as nucleotides increased in diet. The growth performance of channel catfish in this study was slightly lower than that typically observed on channel catfish farms, but this does not change the central findings of the study. The primary purpose of this study was not to determine the effect of nucleotide supplementation on growth. Based on the results of this study, supplementation of nucleotides in diets of channel catfish for prevention of disease cannot be recommended, but use of exogenous nucleotides as a prophylactic treatment before culture-related stress exposure may prove beneficial by decreasing the immunosuppressive effects of stress. There are many gaps in research on the effects of nucleotide supplementation in diets on fish health. As with most immunostimulants, feeding duration, dietary concentration, life stage and species-to-species variation are all variables that need to be addressed.

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