The Effect of Ochratoxin A on Antimicrobial Polypeptide Expression and Resistance to Water Mold Infection in Channel Catfish (*Ictalurus punctatus*)

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Abstract

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Mycotoxin contamination of agricultural commodities poses a serious risk to animal health, including aquaculture species. Ochratoxin A (OA) is the most immunotoxic ochratoxin, yet little is known about its effect on immune function in fish. Antimicrobial polypeptides (AMPPs) are one of the most potent, innate, host defense factors, yet very little is known about what types of chronic stressors affect their expression. Among the most prevalent and potent AMPPs in fish are histone-like proteins (HLPs). In this study, fish were fed 2, 4, or 8 mg OA/kg diet. Skin antibacterial activity and HLP-1 levels were measured on Days 0, 28 and 56. Feeding 2, 4 or 8 mg OA/kg diet resulted in significant growth depression, but higher levels (4 or 8 mg OA/kg diet) resulted in lowering feed intake (FI) and impaired feed conversion ratio. In addition, feeding 8 mg OA/kg diet increased susceptibility to experimental water mold (*Saprolegnia*) challenge, suggesting that OA toxicity might contribute to some saprolegnosis outbreaks. However, there were no changes in AMPP expression in any treatment group. Our data suggests that the increased disease susceptibility of channel catfish due to OA is probably due to mechanisms other than a direct effect on antimicrobial polypeptide expression.

Keywords: Antimicrobial peptides, Ochratoxin A, Water mold

1. Introduction

Mycotoxins are secondary fungal metabolites, which contaminate various types of feed commodities such as corn, wheat, cottonseed meal, peanuts, and soybean meal [1, 2]. Ochratoxin A (OA), produced by *Penicilliumand* and *Aspergillus* species, especially *Aspergillus ochraceous*, is one of the most important mycotoxins [3] and is associated with many signs such as reduced growth rate, feed efficiency ratio, reproductive performance, resistance to infectious diseases. It also causes damage to liver and other organs of fish and farm animals [4, 5]

OA mediates its toxic effect by acting on cellular respiratory enzymes through competitive inhibition of ATPase, succinate dehydrogenase, and cytochrome C oxidase in mitochondria. Moreover, because of its phenylalanine moiety, it competitively inhibits phenylalonyl-tRNA synthase, thus disrupting protein synthesis. In addition, cellular damage is caused by hydroxyl radical formation and lipid peroxidation [6]. OA induces oxidative damage in vivo [7] and in vitro [8] that eventually, lead to mitochondrial dysfunction, apoptosis and DNA damage [9].

OA is toxic to fish, but susceptibility varies considerably among species. Sea bass (*Dicentrarchus labrax* L.), are highly sensitive, having a 96 h LC_{50} of 9.23 mg OA/kg diet [10]. The LD_{50} for rainbow trout (*Oncorhynchus mykiss*) by injection is 4.76 mg OA/kg [11]. Channel catfish are much more resistant, tolerating as high as 4 mg OA/kg diet for at least 8 weeks without mortalities, and 8 mg OA/kg diet with 80% survival [12].

However, the sublethal effects of OA intoxication are much more economically damaging [13]. OA is well-known to increase disease susceptibilty in homeotherms [14]. Pigs fed 3 mg OA/kg diet for up to three weeks spontaneously contracted salmonellosis, with *Salmonella choleraesuis* found in the faeces and liver, and deaths between days 15 and 17. Fewer pigs were affected at a lower dose (1 mg OA/kg diet) and none was affected in the control group [15]. Broiler chickens fed 2 mg OA/kg diet and challenged after 14 days with *Escherichia coli* O_{78} had increased mortality (by 21.4%) compared with chicks inoculated with *E. coli* alone [16]. Eleven-day-old chickens orally challenged with *Salmonella typhimurium* for 2 consecutive days (3 mg OA/kg) had increased *S. typhimurium* in both their duodenal and cecal contents [17].

OA also increases disease susceptibility to *Edwardsiella ictaluri*, one of the most important pathogens affecting channel catfish (*Ictalurus punctatus*) [18]. Channel catfish fed 2.0 or 4.0 mg OA/kg diet for 6 weeks and then challenged by immersion with a highly virulent isolate of *Edwardsiella ictaluri* had significantly greater mortality (80%) than the control (68%). This result together with the less weight gain in both doses compared to control, indicate that feeding intoxicated diet could be implicated in higher susceptibility to the infection.

One of the most prevalent innate defenses in animals is antimicrobial polypeptides (AMPPs) [19]. Studies that have shown that depressed AMPP levels can greatly increase disease susceptibility. For example, recurrent bacterial infections occur in cases where there is a deficiency of α-defensins in neutrophils. Morbus Kostmann

94 syndrome, which is a severe congenital neutropenia in humans that is typified by low
95 concentrations of AMPPs in the mouth, results in recurrent oral infections [20].
96 Depressed levels of histatins, AMPPs in human oral mucosa, have been associated with
97 an increased risk of developing human immunodeficiency virus (HIV) infection [21].
98 Furthermore, Furci et al. [22] showed that α-defensin-5 (HD5) is a potent and broad-
99 spectrum inhibitor of biologically diverse HIV-1 strains, through their interaction with
100 the major HIV-1 envelope glycoprotein, gp120, and with its primary cellular receptor,
101 CD4 interfering with their reciprocal binding. In the same context, immunomodulatory
102 peptide (IDR-1002) enhanced in vitro chemokine induction activities and stronger in
103 vivo anti-infective properties were confirmed. Additionally, it showed protection against
104 the Gram-negative bacterial pathogen *Escherichia coli* [23].
105
106 Among the most common AMPPs in fish are histone-like proteins (HLPs),
107 which have high homology to core nuclear histones. Originally isolated from the skin of
108 channel catfish (*Ictalurus punctatus*) [24], HLPs were subsequently identified in skin,
109 gill and/or spleen of hybrid striped bass (*Morone saxatilis* male x *M. chrysops* female)
110 and rainbow trout (*Oncorhynchus mykiss*) [25, 26]. HLP-1 is the most prevalent and
111 potent HLP, with broad-spectrum activity against bacteria, parasites and water molds
112 [24, 26]. HLPs are also the predominant AMPP in normal healthy channel catfish [24].
113
114 Water molds (Oomycetes), one of the most important pathogens of freshwater
115 fish **[27],** are classical opportunists that typically cause outbreaks when fish are exposed
116 to some type of stress, such as adverse water temperature, poor water quality, handling
117 or crowding. These factors may compromise immunity, increasing susceptibility to

infection **[28, 29].** Members of the genus *Saprolegnia* are the most common and important water molds and thus the disease is often termed saprolegnosis. Saprolegniosis is associated with ‘‘winter kill’’ a syndrome in channel catfish in the southeastern United States that is associated with rapid temperature drop (below 15°C) in fall and winter **[30, 31].**

There have been no studies that have examined the effect of OA or any other mycotoxin on the expression of AMPPs in any animal. Thus, to better understand possible mechanisms underlying OA immunotoxicity, we assessed the effect of OA on the expression of AMPPs in channel catfish. We also determined if there was any relationship between AMPP tissue levels and resistance to challenge with the water mold *Saprolegnia*.

2. Materials and Methods

2.1. Experimental Fish

Channel catfish were obtained from a local producer and transported to North Carolina State University. Fish were acclimated in a 380 liter fiberglass aquarium at 24ºC for 60 days prior to beginning the experiment. During that time, fish were fed ad lib with a 2 mm pellet (40% crude protein, 10% crude fat, and 4% crude fiber, Zeigler Bros, Inc., Gardners, PA). Ammonia, nitrite, and pH were monitored weekly. Two weeks before initiating the experiment, fish were sedated with 60 mgL^{-1} buffered tricane and transferred to the experimental aquaria.

142 *2.2. Experimental Design*

143

144 Fifteen fish (7 month old and 17-21cm length) were placed in each of twelve,
145 60-liter freshwater aquaria (total N = 180). All aquaria were connected to a central
146 filtration system having a conditioned biofilter (biocubes and bead filter [Aquadyne,
147 Koi Camp Aquariology, Loganville, GA]) and a titanium heater (Process Technology
148 Co., Mentor, OH). Fish began feeding normally almost immediately and were fed close
149 to apparent satiation twice daily. After 7 days, the temperature was increased from 24°C
150 to 29°C over 7 days. After 14 d at 29°C (day 0), all fish were weighed, the mean weight
151 of all fish on day 0 was 55 ±15 g and the day 0 sampling was performed. At day 1, all
152 fish were switched to semi-purified diets (Table 1) prepared as described previously
153 (Manning et al. 2003), and having one of four concentrations of OA (0, 2.0, 4.0, or 8.0
154 mg OA/ kg diet). "The experiment was conducted with a protocol approved by the
155 North Carolina State University Institutional Animal Care and Use Committee.
156 Triplicate aquaria were assigned to one of four treatments. During the experiment, 75%
157 water changes were performed thrice weekly, and water quality was measured
158 (ammonia, nitrite, nitrate, pH, and dissolved oxygen) twice weekly via water quality test
159 kits (Aquarium Pharmaceuticals, Inc.).

160

161 *2.3. Sample Collection*

162

163 Three fish from each aquarium were sampled at days 0, 28 and 56. Each
164 aquarium was sampled one at a time; the three fish were sedated with a low dose of
165 buffered tricane; (**MS-222, ARGENT),** (30 mg/L tricane + 60 mg/L sodium

bicarbonate) and each fish was then euthanized one at a time in a separate container having a high dose of buffered tricane (200 mg/L tricane + 400 mg/L sodium bicarbonate). Each fish was weighed and measured to **calculate the condition factor, feed intake (FI), and feed conversion ratio (FCR) according to the following formulae:**

K (K = W (g) x 10^2 /L^3 (cm)

Feed intake (FI) (g /fish) =Dry feed intake (g)/Number of fish

Feed conversion ratio (FCR) (%) = Feed intake (g)/ Gain in body mass (g)

A skin scraping and a gill clip were then taken and examined immediately for pathogens under a light microscope. A skin sample was then collected for AMPP levels. Skin was processed for measuring AMPPs as previously described [25, 32]. Briefly, 50 µL of tissue was collected to yield a total volume of 200 µL in 150 µL of 1% acetic acid (1:4 dilution of tissue). After boiling for 5 min, the sample was homogenized and then centrifuged at 14,000 x g for 10 min at 4°C. The resulting extract (supernatant) was used to measure antibacterial activity and HLP-1.

2.4. Bacterial Culture

Blood from representative fish in each treatment (2 fish per aquarium) was collected from the caudal vasculature with a 1 cc syringe having a 23 GA needle. A drop was placed on a Columbia blood agar plate and then spread with a sterile swab (Mini-tip Culturette, Becton Dickinson, Franklin Lakes, NJ). Culture plates were incubated at room temperature and observed daily for 14 d.

191 *2.5. Total Antibacterial Activity*

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193 Total antibacterial activity was measured using the radial diffusion assay as
194 described previously [26]. Briefly, *Escherichia coli* D31 was propagated overnight in
195 trypticase soy broth with 1% NaCl and then washed in PBS (pH 7) and diluted until to
196 an OD_{570} of 0.10 with a Vitek colorimeter (Hach, Loveland, CO). One mL of the
197 bacterial suspension and 1 mL of streptomycin sulfate (10 mg/mL stock) was added to
198 an autoclaved agarose medium (1.57 g of low electroendosmosis agarose, 0.5 g of NaCl,
199 20.7 mL of 5X Luria-Bertani broth, 20.7 mL of 1 M phosphate buffer pH 6.7, and 58.6
200 ml of deionized water) that was cooled in a water bath to 45-48°C. Ten milliliters of the
201 suspension was poured into each sterile Petri dish, which was used immediately or
202 stored at 4°C until use. Samples were pipetted into 2.5 mm diameter wells punched in
203 the agarose plate. After diffusion of the sample into the agarose, the plate was incubated
204 at 37°C for 18 h, at which time clearing zone diameters were measured by calipers to
205 the nearest 0.1 mm. The radial diffusion assay clearing zone diameters were then
206 converted to Units of activity by reference to a standard curve prepared by serially
207 diluting calf histone H2B (Roche Diagnostics Co, Basel, Switzerland). The 1000 µg/mL
208 concentration of calf histone H2B was considered to be 100 Units of activity. All values
209 were multiplied by 10 to report the tissue concentrations (as μgmL^{-1} equivalents of calf
210 histone H2B).

211

212 *2.6. Anti-Edwardsiella ictaluri Activity*

213

We have previously discovered that other AMPPs are present in channel catfish skin besides HLPs [33]. These AMPPs appear to be mainly small polypeptides (<10 kDa). We have recently discovered that some of these AMPPs have potent activity against *Edwardsiella ictaluri* (A Ullal and E Noga, North Carolina State University, Unpublished Data). We subsequently developed a method for selectively detecting this activity (see below), which involves concentrating the activity via reverse-phase chromatography (using a Sep-Pak [Waters]) followed by measuring the activity using a pathogenic isolate of *Edwardsiella ictaluri*. *Edwardsiella ictaluri* is not sensitive to HLPs [24]; thus, using this bacterium in the assay allows one to exclude measurement of antibacterial activity due to HLPs.

Pooled skin extracts were tested from fish sampled at Day 0 and Day 56 in the 0 and 8 mg OA/ kg diet treatments. Each sample (25 µL) was loaded onto a Sep-Pak Plus C_8 cartridge (Waters #036775), which was previously washed with 3 mL of acidified water (0.1% trifluoroacetic acid); the activity was then eluted with 2 mL of 80% acetonitrile (ACN) in 0.1% trifluoroacetic acid. The effluents was lyophilized and resuspended in 10 µL of 0.01% HAc, and screened for antibacterial activity against *Edwardsiella ictaluri* using the ultrasensitive assay as described in Seo et al. [34]. Clearing zone diameters were then converted to Units of activity by reference to a standard curve prepared by serially diluting synthetic piscidin 1, an AMPP which is known to have potent activity against *E. ictaluri* (J Stephenson and E Noga, Unpublished Data). All values were then divided by 2.5 to account for the 10-fold concentration of the extract; the values were then multiplied by 10 to report the tissue concentrations as µg/ml equivalents of piscidin 1.

238 *2.7. Quantification of HLP-1*

239

240 HLP-1 was measured via ELISA according to Noga et al. [35]. Briefly,
241 microtiter plates were coated for one h at room temperature using primary antibodies
242 that were homologous to the N-terminus and C-terminus of HLP-1 (anti-PDPA and anti-
243 VSEG antibodies). After a washing step, the plates were incubated with Stabilcoat
244 (Surmodics, Inc., Eden Prairie, MN) for 20 min to eliminate nonspecific binding and
245 then stored at 4ºC until use. Immediately before use, plates were washed with 0.05 M
246 tris-buffered saline with 0.05% Tween 20. The pooled skin tissue extracts were diluted
247 to the desired concentration (1:100) in 0.05 M tris-buffered saline with 0.05% Tween 20
248 and 1% bovine serum albumin (BSA) (Sigma #T6789, St. Louis, MO). All samples
249 were added in duplicate to the ELISA plate and incubated for 1 h. The plate was washed
250 again before the secondary antibody (affinity-purified, peroxidase-labeled antibody to
251 calf histone H2B) was added and incubated for 1 h. The plate was then washed and
252 TMB peroxidase substrate was added and incubated for 15 min. The reaction was
253 terminated with 3 M phosphoric acid. Absorbance was measured at 450 nm with an
254 ELISA plate reader (KCjunior, Bio-Tek Instruments, Inc., Winooski, VT). A standard
255 curve was prepared using a serial dilution of calf histone H2B, and the positive control
256 was prepared from a synthetic antigen created by linking the N-terminus and C-terminus
257 peptide antigens (used to prepare the coating antibodies) to BSA.

258

259 Tissue concentration of HLP-1 was calculated by the software (KCjunior, Bio-
260 Tek Instruments, Inc., Winooski, VT) at 450 nm absorbance based on the optical
261 density (OD) of the 4 parameter curve-fitting standards. Samples with an OD $\geq$ 3 were

diluted twofold and re-ran. All values were multiplied by 4 to account for the 1:4 dilution of tissue in 1% acetic acid (50 µL of tissue in 150 µL of 1% acetic acid) during sample collection. Tissue concentrations were reported as µg/mL equivalents of calf histone H2B.

2.8. Water Mold Challenge

Zoospores for challenge were prepared according to Udomkusonsri and Noga [28]. Briefly, *Saprolegnia* sp. (Isolate #97-2010A) from a diseased channel catfish was cultured on glucose yeast extract (GY) agar [36] at 19ºC for 2 days. Agar with mycelia was then aseptically cut into 8×8 mm squares and placed into a petri-dish having 30 mL GY broth. After 2 days, the agar remenants were removed and the mycelium was washed three times with sterile sporulation medium [MSM (modified Griffin's sporulation medium) [37]]. The mycelium was then incubated in MSM at 19ºC for 24 h to induce zoospore formation. **About 300 mL of zoospore suspension was collected from about 10 petri dishes containing sterile MSM and placed in a plastic aquarium bag for inoculation into the challenge aquarium.**

On day 58 of the feeding trial, the remaining fish were challenged with zoospores. Nine fish from each group (0, 2.0, 4.0, and 8.0 mg OA/ kg diet) were placed in separate plastic cages (48 cm in diameter, 38 cm deep) in a 1100 liter closed system fiberglass aquarium at 29ºC; over ~12 hr, the temperature was dropped to 11ºC by un-plugging the heaters and placing an external chiller (Aqua Logic, Inc, San Diego, CA) in the water. When the water temperature reached 11ºC, the plastic bag containing the

zoospore suspension was placed in the aquarium for ~30 min to acclimate the zoospore suspension to the aquarium temperature. The suspension was then mixed with 25% aquarium water for 25 min, followed by pouring the suspension into the aquarium. The challenged fish were examined thrice daily for typical signs of water mold infection (e.g., cottony white growth on skin and fins). Moribund or dead fish were immediately removed. Skin scrapings and gill and fin biopsies of all removed fish were examined; water mold infection was confirmed via identification of broad, aseptate hyphae, sporangia and encysted zoospores with light microscopy.

2.9. Statistical Analyses

All data were analyzed using SPSS version 17.0 (SPSS Inc, Chicago, Illinois). The effect of experimental variables (OA feed rate, aquarium replicate, sampling day) was tested by analysis of variance (ANOVA). A significant difference was accepted if $P < 0.05$. The Kruskal-Wallis test, the nonparametric version of ANOVA, was used to analyze the the effect of treatment on survival time after water mold challenge. In addition, a Wilcoxon test was used for each pair of treatments, adjusting for multiple comparisons using Tukey's method for pairwise comparisons. Values were considered significant if $P < 0.05$.

3. Results

3.1. Necropsy Examinations

There were no gross external lesions in any fish during the entire period of OA exposure (8 weeks); also, no pathogens were detected in skin or gill biopsies at any time. Blood cultures had a small number of bacterial colonies in some fish of each of the four treatment groups (ranging from 1 to 26 colonies). No predominant colony type was observed and in the sample with the largest number of colonies, three colony types were present.

3.2. Growth Parameters

At Day 28, the 8 mg OA/kg diet treatment group weighed significantly less than all other groups (Fig. 1A). By Day 56, all treatment groups (2, 4 and 8 mg OA/ kg diet) weighed significantly less than the control. Changes in condition factor (K) were less consistent; the 8 mg OA/kg diet treatment was significantly lower than the control at day 28, but was not different at day 56 (Fig. 1B). Feed intake (FI) was significantly lower in treatment groups compared to the control, with an apparent dose-related trend with increasing OA dose. This same trend was also evident with feed conversion ratio (FCR) (Fig. 2) suggesting poor feed utilization.

3.3. AMPP Levels

There were no differences in the total antibacterial activity among any treatment groups at any of the sampling times (Fig. 3). This was also reflected in the lack of obvious changes measured in pooled extracts when measuring HLP-1 levels (Table 2).

333 There was no detectable activity in these same samples against *E. ictaluri* (data not
334 shown).
335
336

337 *3.4. Susceptibility to Water Mold Challenge*

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339 Water mold infection was first grossly visible on Day 4 post-challenge, where,
340 in the 8 mg OA/ kg diet treatment, one fish was dead and all others were heavily
341 infected. Affected fish had typical signs of water mold infection, with cotton-like
342 growths on the body and fins. By Day 8, fish in all groups were dead except one in the
343 control. **There was a significant effect of treatment on survival time (Kruskal-**
344 **Wallis test, p=0.0074). The 8 mg OA/kg diet group had a significantly shorter**
345 **survival time than all three other treatments (*p<0.05*) as determined using**
346 **Wilcoxon test for each pair of treatments (Fig. 4)**.

347

348 **4. Discussion**

349

350 OA is well-known to be immunosuppressive to vertebrates. OA reduces the size
351 of immune organs, such as thymus, spleen, and lymph nodes, and the number of
352 immune cells within tissues. It also causes profound alterations in the function of
353 mononuclear leukocytes [38], affecting production of reactive oxygen species,
354 chemotaxis, phagocytosis and cytokine synthesis [39].

355

356 Feeding 4 mg OA/kg for 10 weeks caused lymphoid depletion in the thymus of
357 turkeys and was accompanied by reduced T-lymphocyte-mediated delayed cutaneous
358 hypersensitivity responses [40]. In other studies, OA inhibited natural killer (NK) cell
359 activity and increased the growth of tumor cells in mice. Ochratoxin had little direct
360 effect on NK cells, but did impair the induction of interferon. Because interferon is

361 necessary for NK cell activity, the suppression of NK cell activity was attributed to a
362 decrease of endogenous interferon levels in the ochratoxin-treated mice [41]. The effect
363 of 0.5 or 2.0 mg OA/kg feed on broiler chicks revealed significant reductions in cell-
364 mediated immunity, as indicated by diminished skin sensitivity, graft versus host
365 reactions and T-lymphocyte counts. Total lymphocyte counts, total serum protein,
366 serum albumin and serum globulin levels were significantly depressed on day 21.
367 Phagocytosis was inhibited, and the number of splenic macrophages was drastically
368 reduced in both intoxicated groups, as well as the weights of the thymus, bursa of
369 Fabricius and spleen [42]. Feeding Nile tilapia on AFB1 contaminated diet (0.2 mg/kg
370 diet) showed up-regulation of CYP1A and down-regulation of SOD, IL-1β and TGF- β
371 after 16 weeks exposure [43]. OA (0.4 and 0.6 mg/kg diet) fed to Nile tilapia for 90
372 days, revealed reduction in RBCs, Hb and Hct values. Besides, a significant decrease in
373 plasma total protein, total lipids, AST and lactate dehydrogenase along with significant
374 increase in the glucose concentration and ALT levels [44]. Furthermore, Zheng et al.
375 [45] confirmed the association between the OA induced toxicity and zinc, they showed
376 that OA exposure was associated with inhibition of cell proliferation, decrease in the
377 intracellular zinc concentration, induction of ROS production, decreases of SOD
378 activity and DNA strand breaks, DNA oxidation and hypomethylation. But, zinc
379 supplementation alleviated OA induced such toxicity.
380
381 **To our knowledge, we are the first to examine the effect of any mycotoxin**
382 **on expression of any AMPP. However, Fujiwara et al. [46] reported that another**
383 **food-borne toxin, 2-amino-1-methyl-6 phenylimidazo[4,5-b]pyridine (PhIP), one of**
384 **the most abundant heterocyclic amines produced while cooking meat and fish,**

385 **induces an overexpression of α-defensin genes by Paneth cells in rats developing**
386 **colon cancers**. A synthetic lytic peptide (D4E1) gene, were expressed in transgenic
387 hybrid poplar (*Populus tremula* L. × *Populus alba* L.) against bacterial disease. One
388 transgenic poplar line, Tr23, was the highest transcript accumulated for the D4E1 gene,
389 and exhibited a significant relief in symptoms caused by *A. tumefaciens* and *X. populi*
390 [47]. Recently, Badea et al. [48] cloned antimicrobial peptides genes, MsrA2 and 10R
391 and showed that T3 generation genetically modified (GM) plants had a 53% reduction
392 in *Fusarium* damaged kernels and some lines also had a 59% reduction in powdery
393 mildew susceptibility compared with the non-GM control.

394 HLP-1 levels at Day 0 for both control and treatment groups in the pooled skin
395 extracts were similar (12 and 10.8 µg/mL, respectively; Table 2); at Day 56, both
396 groups showed nominally higher HLP-1 levels (15.6 and 25.6 µg/mL, respectively;
397 Table. 2). The latter levels are within concentrations that are inhibitory or lethal to some
398 important pathogens such as the protozoan ectoparasite, *Amyloodinium ocellatum* (lethal
399 at 12.5 µg/mL) [25] and the bacterium *Aeromonas hydrophila* (inhibitory at 25 µg/mL)
400 [24]. However, our resting levels of antibacterial activity for skin extracts at both Day 0
401 and 56 for both groups were low (≤ 0.5 U = 5 µg/mL, Fig. 3). This is in contrast to our
402 previous study [33], where there was much higher antibacterial activity in control
403 channel catfish (5.7 U = 57 µg/mL). However, some of the activity measured in the
404 Ullal et al. [33] study might have been due to the upregulation of certain AMPPs related
405 to hemoglobin, due to the possible presence of a subclinical ectoparasite infestation on
406 some fish. Thus, the resting levels that we measured in the present study might more
407 closely reflect the true baseline levels in channel catfish skin.
408

409 Nonetheless, our data did not show any effect of OA on antimicrobial
410 polypeptide expression. Total antibacterial activity was not significantly different
411 among treatment groups. This was supported by the measurments of HLP-1 in pooled
412 tissue extracts, which did not reveal any apparent trends among OA feed levels. HLP-1
413 is the major AMPP in unstimulated channel catfish skin and gills [24, 33]. Similar
414 results were seen in measurements of anti-*E. ictaluri* activity. These data suggest that
415 OA does not affect AMPP levels in channel catfish. Our data also suggests that the
416 increased susceptibility to edwardsiellosis observed with 8 mg OA/ kg diet in the feed
417 [18] is not due to depressed AMPP levels but rather to some other form of
418 immunosuppression. OA has been found to affect intestinal epithelial cells and alter
419 both intestinal barriers and selected transport functions. Moreover, OA selectively
420 affects the protein content of plasma membrane microdomains, inducing perturbation of
421 microdomain composition, which may account for the decrease of transepithelial
422 resistance and thus destabilizing the tight junctions of intestinal epithelial cells,
423 facilitating its own absorption [49]. **Thus, OA might primarily target an intrinsic**
424 **component of intestinal immunity (its barrier function) rather than having a direct**
425 **effect on extrinsic components of immunity such as AMPPs. However, the**
426 **immunosuppressant effect of OA was still evidenced by increased fish**
427 **susceptibility to *Saprolegnia* challenge.**
428
429 Manning et al. [50] found that in channel catfish fed OA for 8 weeks, survival
430 was high for fish fed diets containing 0–4 mg OA/kg feed, but fish fed the diet
431 containing 8.0 mg OA/kg had significantly lower survival. At 8 weeks,
432 histopathological examination showed a significant increase in the severity of liver and

433 posterior kidney lesions among catfish fed diets containing 2.0 mg OA/kg or above.
434 These lesions were characterized as enlarged melanomacrophage centers, which
435 replaced normal hepatopancreatic or posterior kidney cells. Exocrine pancreatic cells,
436 that normally surround the hepatic portal veins, were decreased or absent in fish fed 1.0
437 mg OA/kg diet or greater. Hematocrit was significantly lower for catfish fed 8 mg
438 OA/kg of feed, but there were no significant differences in white blood cell (WBC)
439 counts at any dietary levels of OA.
440
441 We found that OA significantly reduced weight gain and feed efficiency in a
442 dose-dependent manner. After 56 days (8 weeks), body weight was significantly lower
443 in fish fed 2, 4, or 8 mg $OAkg^{-1}$ diet. This is consistent with the findings of Manning et
444 al. [50], where, after 8 weeks, there was a significant reduction in weight gain in
445 channel catfish fed diets containing 1, 2, 4, or 8 mg OA/kg. In a separate study, catfish
446 fed 2.0 or 4.0 mg OA/kg diet for 6 weeks also weighed significantly less than the
447 control fish [18].
448
449 In the current study, feed intake (FI) and FCR were impaired particulary, in fish
450 fed 4 or 8 mg OA/kg of feed after 28 days and 56 days. This is in line with the findings
451 of Saad [51], who reported impaired FCR of *O.niloticus* treated with OA. Similar results
452 were seen by Srour [5], who found reduction of feed and nutrient utilization of *O.*
453 *niloticus* fed OA contaminated diet. Manning et al. [50] also found poorer FCR in fish
454 fed 4 or 8 mg OA/kg of feed. In same context, Srour [5], found a reduction in growth
455 performance parameters in Nile tilapia exposed to OA at 4.8, 9.6 and 14.4 mg/kg diet.
456 Similarliy, Farag [52] found depression in weight, gain and specific growth rate (SGR)

457 of *O. niloticus* and *Cyprinus carpio* fed on diet contaminated with 7 mg of OA/kg diet.
458 Feeding Nile tilapia on OA contaminated diet at low and high doses (0.8 and 0.16
459 mg/kg fish), respectively, led to a significant reduction in final body weight, body gain,
460 average daily gain (ADG) and SGR in a dose dependent manner [53]. This may be
461 attributed to different mechanism of OA such as, inhibition of protein synthesis [54],
462 induction of ROS with consequent damage of nucleic acids, protein and lipids [55].
463 Thus, it is reasonable to expect that OA would cause growth retardation.
464
465 The condition factor (K) of the 8 mg OA/kg diet treatment was significantly
466 lower than the control at day 28, but K was not different at day 56, and that of the
467 control fish was similar to what has been reported for channel catfish [56]. Our results
468 were corroborated with Abdallah et al. [53] who reported that K was not affected with
469 OA treatment in Nile tilapia at lower (0.8 mg/kg fish) and higher (0.16 mg/kg fish)
470 doses. Thus, condition factor changes were less consistent and did not reflect the lower
471 innate immune status of the fish group fed the highest tested dose 8 mg OA/ kg diet.
472
473 Despite the lack of effect on AMPP levels, exposure of channel catfish to
474 *Saprolegnia* significantly increased mortality in fish that were fed a diet containing the
475 highest level of OA (8 mg OA/ kg diet). OA exerts suppression of the immune response
476 renders animals more susceptible to pathogen with subsequent increasing in the severity
477 of infection and aggravating the OA toxicity [57]. Our results are consistent with
478 previous studies showing that 4 mg OA/kg feed increases susceptibility of channel
479 catfish to *Edwardsiella ictaluri* challenge causing significant higher mortalities (80%)
480 versus the control (68%) [18]. Previous studies conducted in homeotherms, showed that

481 OA increased susceptibility of rabbits to *Pasteurella multocida* associated with higher
482 mortalities [58]. OA increased chicks' susceptibility to *Eimeria acervulina* leading to
483 sever duodenal coccidiosis [59]. In the present study, impairment of the protein
484 metabolism one of the possible causes to lowering animal resistance, along with
485 suppression of the immune response [60] can ultimately enhance the susceptibility to
486 various infections. Recent surveys of the occurrence of mycotoxin-contaminated feed or
487 feed ingredients are not extensive; however, an evaluation of OA contamination of feed
488 grains or animal feeds conducted in the USA indicates that levels of OA of up to16
489 mg/kg were found in surveys and up to 90 mg/kg in feeds from OA case studies [61];
490 thus, high doses can be found naturally in fish feed and pose a risk for animals and
491 humans as well. Thus, OA might be a contributing factor to some saprolegnosis
492 outbreaks in channel catfish.

493 **Conclusions**

494

495 In summary, channel catfish exposed to moderate to high dietary levels of
496 ochratoxin A (2-8 mg OA/ kg diet) exhibited a significant decrease in growth rate and
497 significant increase in feed conversion ratio, supporting findings in prior studies.
498 However, only very high levels (8 mg OA/ kg diet) had any effect on the susceptibility
499 to water mold infection and this effect appeared relatively small. This increased
500 susceptibility did not appear to be associated with depressed AMPP levels, suggesting
501 that other aspects of impaired immune function are responsible for this greater
502 susceptibility.

503

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Table 1. Ingredient composition of the semipurified basal diet[a].

Ingredient	Amount (g/kg dry mixture)
Casein, vitamin free (USB #12866)[b]	290.0 g
Gelatin	80.0 g
Dexrin (USB #9004-53-9)	360.0 g
Cellulose	106.0g
Fish meal, menhaden	20.0 g
Carboxymethyl cellulose	30.0 g
Corn oil	30.0 g
Menhaden oil (USB #8002-50-4)	30.0 g
Mineral premix[c]	40 g
Vitamin premix[d]	12.5 g
Vitamin C[e]	1.5 g

[a] This prepares 1000 g (1 kg) of diet with a calculated crude protein concentration of 32.7 %. Prepare feed in 2-4 kg batches.

[b] USB: USB Corp., Cleveland, OH

[c] Williams and Briggs mineral premix (Purina Mills Test Diets, Richmond, IN).

[d] Vitamin premix supplies per kg of diet the following: vitamin A, 5500 IU; vitamin D3, 1835 IU; vitamin E, 110 IU; vitamin K, 7.3 mg; thiamin, 8.4 mg; riboflavin, 22 mg; pyridoxine, 18.4 mg; pantothenic acid, 58.7 mg; niacin, 36.7 mg; biotin, 2 mg; folic acid, 3.7 mg; vitamin B12, 0.018 mg; choline, 2327 mg; selenium, 0.1 mg.

[e] L-ascorbyl 2-polyphosphate (Stay-C, Hoffman La Roche, Nutley, NJ, USA, 25% ascorbic acid active)(Manning et al. 2003)

Table 2. HLP-1 levels in pooled skin extracts of channel catfish fed 0 or 8 mg OA/ kg diet. HLP-1 levels were measured at Day 0 and Day 56 of the experiment. Values for both the diluted acidic extract and concentration present in the undiluted tissue are reported; both are expressed in µg/ml equivalents of calf histone H2B.

Time	**Treatment group**			
	0 (mg OA/ kg diet)		**8 (mg OA/ kg diet)**	
	Extract concentration	**Tissue concentration**	**Extract concentration**	**Tissue concentration**
Day 0	3.0	12.0	2.7	10.8
Day 56	3.9	15.6	6.4	25.6

747 ***Figure Legend***

748 **Figure 1.** Weight (A) and condition factor (B) of channel catfish fed either 0, 2, 4, or 8
749 mg OA/ kg diet ochratoxin A. **Data is expressed as the mean of nine fish ±**
750 **SEM. Values with a different letter superscript within a sampling day are**
751 **significantly different between treatment groups (ANOVA, p <0.05).** N = 9
752 fish per treatment group per sampling time.
753
754
755 **Figure 2.** Feed intake (FI) and feed coversion ratio (FCR) of channel catfish fed either
756 0, 2, 4, or 8 mg OA/ kg diet ochratoxin A. **Data is expressed as the mean of**
757 **nine fish ± SEM. Values with a different letter superscript within a**
758 **sampling day are significantly different between treatment groups**
759 **(ANOVA, p <0.05).** N = 9 fish per treatment group per sampling time.
760
761
762 **Figure 3.** Antibacterial activity Units (mean ± SE) in the skin of channel catfish fed
763 either 0, 2, 4, or 8 mg OA/ kg diet ochratoxin A. N = 9 fish per treatment group
764 per sampling time.
765
766 **Fig. 4.** Mortality curves of fish that had been fed various doses of ochratoxin A and
767 challenged with water mold (*Saprolegnia*). **Curves with different letters were**
768 **significantly different between treatment groups (p < 0.05) using the**
769 **Wilcoxon test for each comparison prior to multiple testing correction.**
770 Performing Tukey's method for pairwise comparisons yielded identical results.

Fig. 1

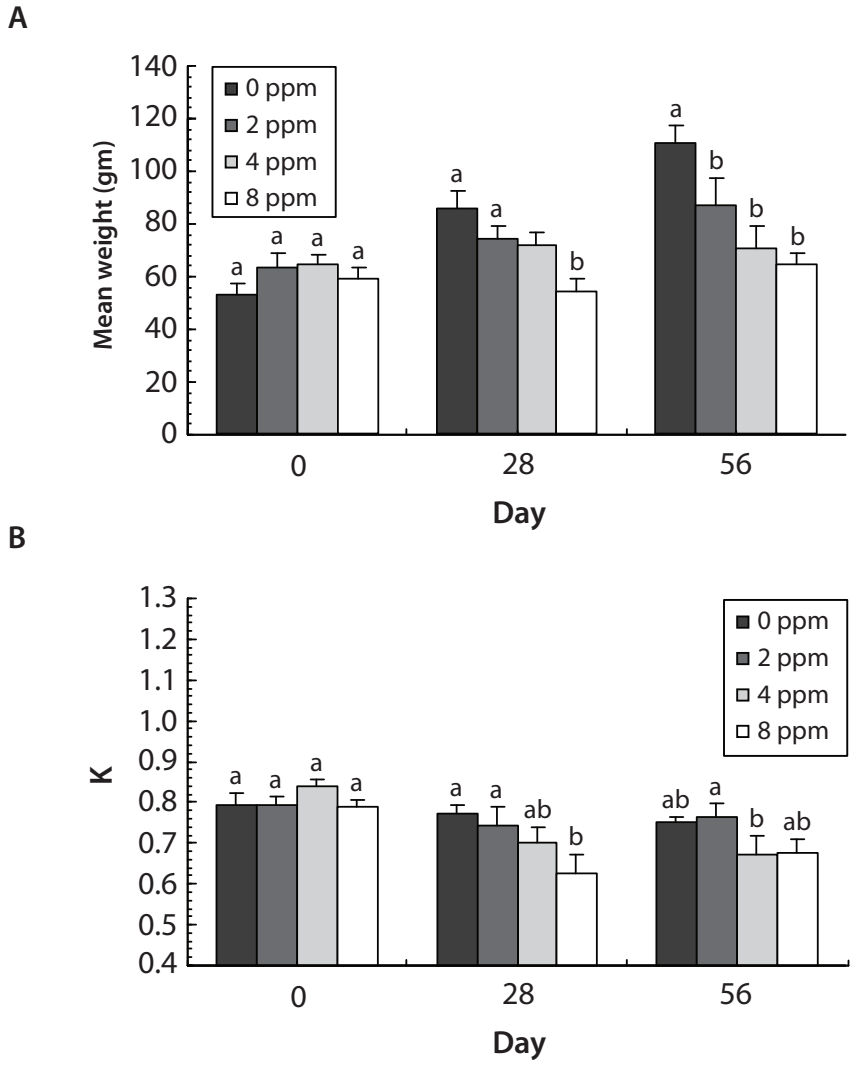
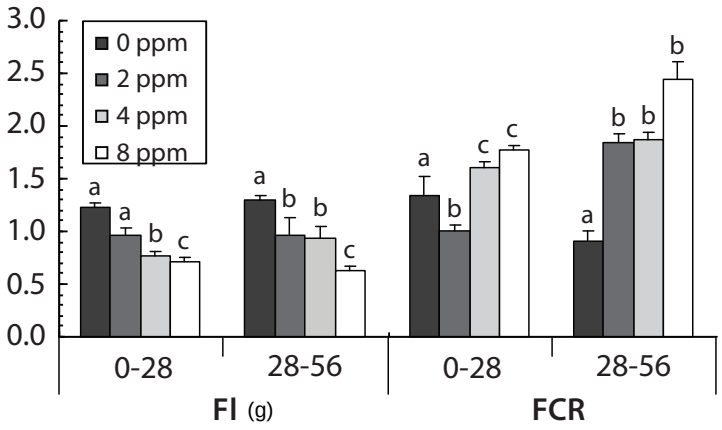


Fig. 2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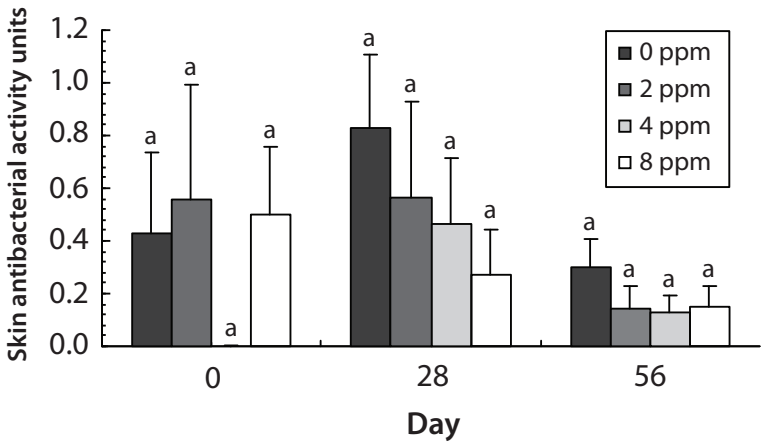
0-28
28-56
FI (g)
FCR

Fig. 3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a
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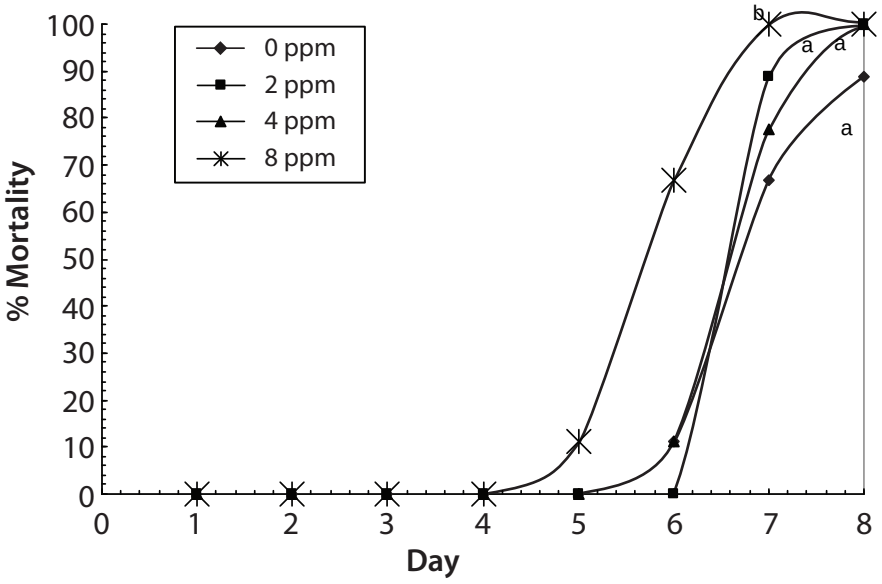
0
28
56
Day

Fig. 4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