

Histone-like protein: a novel method for measuring stress in fish

David W. Robinette*, Edward J. Noga**

North Carolina State University, College of Veterinary Medicine, 4700 Hillsborough Street, Raleigh, North Carolina 27606, USA

ABSTRACT: We assessed the effect of chronic stress using a group of potent, broad-spectrum antimicrobial polypeptides, called histone-like proteins (HLPs), which appear to be an important component of non-specific immunity in channel catfish *Ictalurus punctatus* skin. An enzyme-linked immunosorbent assay (ELISA) was developed to measure the predominant HLP (HLP-1) in channel catfish skin. Catfish were then exposed to a chronic stress consisting of overcrowding and elevated ammonia. Healthy unstressed fish had consistently high HLP-1 levels, but fish that had been stressed for 1 wk had significantly depressed HLP-1 levels; HLP-1 levels declined further in fish stressed for 3 or 4 wk. The time-dependent decline in HLP-1 levels was not accompanied by any gross signs of disease. In contrast to HLP-1 levels, antibacterial activity in the skin was significantly greater in fish stressed for 1 wk compared with unstressed fish; in addition, antibacterial activity was the same in fish that were unstressed or stressed for 3 or 4 wk. This suggests that other antibiotics besides HLP-1 may be induced in the skin, especially during early stages of stress, that may compensate for depressed HLP-1 levels. Our results indicate that chronic stress has a significant suppressive effect on HLP-1 levels in channel catfish skin. The reduction of HLP-1 in the absence of clinical signs of disease, combined with evidence that its levels are not affected by the acute stressors of capture or sampling, suggests that HLP levels may be a promising indicator for monitoring fish health.

KEY WORDS: Endobiotics · Non-specific immunity · Diagnostic stress test · ELISA

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INTRODUCTION

Aquaculture is an intensive process in which fish are reared under very high stocking densities, such that the potential for catastrophic disease outbreaks poses a serious economic threat. Avoiding disease epidemics is one of the major goals of a successful aquaculture enterprise, and this typically involves the reduction of 'stress' in the population. The physiological and behavioral responses to stress are essentially adaptive in that they have evolved as a mechanism to allow the organism to attempt to maintain homeostasis under potentially harmful conditions. But, under conditions of chronic or severely acute stress, these responses may

become deleterious to the health of the fish. Such conditions may often be found in intensive aquaculture operations in which fish are kept at high densities and are not capable of escaping the stressor(s) (Pickering 1981, Wedemeyer 1996).

The important relationship between stress and disease resistance in fish is becoming increasingly apparent. Stress induces a variety of complex changes in fish physiology, including defense mechanisms, which may compromise the ability to resist disease (Snieszko 1974, Ellis 1982, Wendelaar Bonga 1997). A number of stressors commonly associated with aquaculture can impact the piscine immune system, including crowding (Klinger et al. 1983), handling (Ellsaesser & Clem 1986), temperature fluctuation (Clem et al. 1984, Miller & Clem 1984), and 'poor water quality' (Smart 1981, Schwedler et al. 1985). Stress is a major predisposing factor for infectious disease epidemics in commercial aquaculture (Meyer 1970, Walters & Plumb 1980,

*Present address: US Environmental Protection Agency, National Exposure Research Laboratory, ERC Mail Drop 44, Research Triangle Park, North Carolina 27711, USA

**Corresponding author. E-mail: ed_noga@ncsu.edu

Barton 1997). Correlations between stress-associated immunosuppression and susceptibility of fish to infectious disease have been demonstrated in various fish species, such as rainbow trout *Oncorhynchus mykiss* (Angelidis et al. 1987), chinook salmon *Oncorhynchus tshawytscha* (Maule et al. 1989), and carp *Cyprinus carpio* (Yin et al. 1995).

A number of indicators have been used to study the effects of stress on the immune responses of fish. These methods can generally be divided into 3 categories (Anderson 1990): (1) non-specific assays that do not involve antigenic stimulation (e.g., hematocrit and leukocrit) (Ellsaesser & Clem 1986, Pickering & Pottinger 1987); (2) non-specific or specific functional indicators, which may or may not use antigenic stimulation (e.g., chemiluminescent assay of phagocytic oxidative burst potential; T and B cell mitogenesis assays) (Stave & Roberson 1985, Ellsaesser & Clem 1986); and (3) indicators that involve immunization of the fish and subsequent assay of the specific immune response (e.g., antibody assays and pathogen challenge) (Hetrick et al. 1979, Cipriano et al. 1985, Smith et al. 1992, Cobb et al. 1998).

The importance of non-specific immune responses as a first-line of protection against infectious disease in fish is increasingly recognized. Within this armamentarium in channel catfish *Ictalurus punctatus* skin are potent, broad-spectrum antimicrobial polypeptides. These antibiotics, which we have named histone-like proteins (HLPs), display a broad spectrum of activity against bacteria (*Aeromonas*, *Vibrio*) and water molds (*Saprolegnia*) (Robinette et al. 1998). Stress commonly results in development of opportunistic infections of the skin (Noga et al. 1994). Previous observations in our laboratory revealed that channel catfish subjected to stress associated with transport from the farm to the laboratory, followed by overcrowding in aquaria having inadequate biological filtration, had depressed levels of skin-associated antibacterial activity, and consequently exhibited poor yield of HLPs. The present experiments were performed to determine if a change in the levels of HLP or its associated antimicrobial activity occurred in fish subjected to a more controlled chronic stress.

MATERIALS AND METHODS

Exposure to chronic stress and collection of samples. Experimental fish: Channel catfish measuring from 10 to 15 cm in total length and approximately 25 to 45 g in weight were obtained from a local supplier (Blue Ridge Fish Hatcheries, Kearnerville, NC). Prior to the experiment, fish were acclimated in a 450 l aquarium at 3 ppt salinity and 18°C. Fish were closely

monitored in this acclimation aquarium to ensure that they exhibited no outward signs of disease. Total ammonia-nitrogen and nitrite-nitrogen levels in the holding tank were monitored daily for 1 wk after introduction of the fish, and approximately weekly thereafter, to insure that the water in the tank was properly cycled. Total ammonia-nitrogen and nitrite-nitrogen levels remained low (<0.025 and <0.10 mg l⁻¹ respectively). Dissolved oxygen was at saturation.

Experimental stressor: A significant stressor in channel catfish aquaculture is crowding (Klinger et al. 1983), which can cause a serious decline in water quality (Schwedler et al. 1985). Thus, our fish were confined in fresh water in three 8 l polypropylene tanks (6 fish per tank) at a stocking density of 25 g fish l⁻¹, which represented an increase in density of about 10-fold greater than the holding aquarium. Crowding conditions of this density and duration suppress non-specific immunity and increase susceptibility to experimental infection in carp (Yin et al. 1995).

Water in experimental tanks was continually aerated by use of an airstone. Fifty percent of the water in the tanks was replaced approximately every 24 h to prevent the buildup of ammonia to lethal levels. Tanks were covered at all times, except during water changes, to minimize external disturbances to the fish. At regular intervals (1, 3, or 4 wk), all 6 fish from 1 tank were individually removed and skin samples were collected as described below. Six unstressed control fish were initially sampled in an identical manner, except that they were taken directly from the 450 l holding aquarium and immediately processed.

Preparation of skin samples for testing: Fish were individually anesthetized with tricaine until deeply sedated and then maintained on ice throughout processing. A sterile scalpel was used to gently scrape the epidermis from the dermis as described previously (Robinette et al. 1998), along each side of the fish in an area 1 cm × 8 cm from the operculum to the base of the caudal fin. Skin scrapings were placed directly into 600 µl of ice cold 1% glacial acetic acid in a microfuge tube. This procedure yielded a similar volume of tissue extract for each fish. The suspension was heated in a boiling water bath for 5 min to inactivate endogenous proteases, and allowed to cool to room temperature. The material was then thoroughly homogenized on ice using a Potter-Elvehjem homogenizer. The resulting acid extract was centrifuged at 15 000 × g for 10 min at 10°C, and the supernatant was decanted and an aliquot was immediately analyzed for antibacterial activity using the radial diffusion assay described below. An additional aliquot was removed and the protein concentration was determined using the Micro BCA Assay® (Pierce Chemical Co., Rockford, IL) using purified calf histone H2B (Boehringer-Mannheim Bio-

chemicals, Indianapolis, IN) as a standard. This standardized skin extract was also used to measure HLP-1 levels via enzyme-linked immunosorbent assay (ELISA) (see below).

Measurement of antibacterial activity. The antibacterial activity of skin extracts from catfish stressed by various periods of confinement was assessed using the radial diffusion assay as described by Hultmark et al. (1982). Briefly, an overnight culture (18 to 20 h) of *Escherichia coli* D31 grown at 37°C in trypticase soy broth with 1% NaCl was washed 3 times with cold, phosphate-buffered saline (pH 7) after centrifuging at 1000 × *g* for 10 min at 4°C. The bacterial suspension was adjusted to an optical density (OD₅₇₀) of 0.1 (10⁸ CFU ml⁻¹). One ml of the bacterial suspension and 1 ml of streptomycin sulfate (10 mg ml⁻¹, Sigma Chemical Co., St. Louis, MO; final concentration 100 µg ml⁻¹) was added to an autoclaved agarose medium (1.57 g of low electroendosmosis (EEO) agarose, 0.5 g of NaCl, 20.7 ml of 5× Luria-Bertani broth, 20.7 ml of 1 M phosphate buffer pH 6.7, and 58.6 ml of dH₂O) that was cooled in a water bath to 45 to 48°C. Ten milliliters of the *E. coli* D31 agarose was poured into each sterile Petri dish, which was used immediately or stored at 4°C until use. For radial diffusion assays, sample was pipetted into a 2 mm diameter well in an *E. coli* D31 agarose plate. After diffusion of the sample into the agarose, the plate was incubated at 37°C for 18 h, at which time clearing zone diameters were measured by calipers to the nearest 0.1 mm. Radial diffusion assay clearing zone diameters were converted to nmol equivalents (referred to as 'e') of HLP-1 by reference to a standard curve of serially diluted HPLC-purified HLP-1. Antibacterial activity units (U) were then defined as 100 × nmol equivalents (e).

ELISA to HLP-1. We have previously identified 3 HLPs in channel catfish skin, HLP-1, HLP-2 and HLP-3 (Robinette et al. 1998). Since HLP-1 is present in the highest concentration and has the most activity against the widest range of aquatic pathogens (Robinette et al. 1998), we chose to develop an ELISA to this antibiotic. Pure HLP-1 was produced as described previously (Robinette et al. 1998). Briefly, fish were euthanized in tricaine and tissue was placed in 1% acetic acid, boiled, and then the acid-soluble extract was clarified by centrifugation. The extract was placed on a carboxymethylcellulose ion-exchange column (CM-52, Whatman) and the sample was eluted using a gradient of ammonium acetate (pH 5.15). All fractions were tested for antibacterial activity using an antibacterial assay, where samples were spotted onto a plate of our test bacterium *Escherichia coli* D31 (Zasloff 1987). Active fractions (i.e., fractions having antibacterial activity) were pooled, lyophilized, loaded onto a reverse-phase, C₄ HPLC column (PolyLC, Columbia, MD) and eluted

using an acetonitrile gradient in 0.10% trifluoroacetic acid. The active fractions were then pooled. This procedure yields HLP-1 with over 95% purity.

ELISA development using anti-calf H2B antibodies: HLP-1 has a very high N-terminus sequence homology to histone H2B (Robinette et al. 1998). While more information is needed, this finding, along with the molecular weight of HLP-1, suggests that HLP-1 may be a member of the histone H2B family (Robinette et al. 1998). However, until we have determined the entire sequence of HLP-1, we have conservatively chosen to refer to this molecule as histone-like. Because of the high N-terminus sequence homology of HLP-1 to histone H2B, we explored the possibility of using an antibody to H2B to measure HLP-1. The applicability of anti-histone H2B antibody to detecting HLP-1 was confirmed by examining the cross-reactivity of the antibody to equivalent concentrations of HPLC-purified HLP-1 and histone H2B.

Two-fold serial dilutions of calf histone H2B or HPLC-purified HLP-1 in PBS (0.01 M Na₂HPO₄/NaH₂PO₄, pH 7.2 plus 0.14 M NaCl), starting at 2.5 µg ml⁻¹, were added to duplicate wells (50 µl well⁻¹) of a 96-well microtiter ELISA plate (Corning Laboratory Products, Corning, NY). After adding all samples, the plate was sealed and incubated at room temperature overnight. After overnight coating, the plate was washed 3 times by filling each well with PBS and decanting after 30 s. One hundred microliters of blocking buffer (PBS + 1 mg ml⁻¹ enzyme immunoassay grade gelatin [Bio-Rad, Richmond, CA]) was added to all wells and incubated for 2 h at room temperature; then the plate was washed 3 times as described above. Fifty microliters of affinity-purified sheep anti-calf histone H2B (Fitzgerald Industries, Concord, MA) diluted 1:1000 in blocking buffer plus 0.05% Tween 20 (Sigma Chemical Co., St. Louis, MO) were then added to each well and the plate was incubated for 1.5 h at room temperature. The primary antibody was then decanted and the plate was washed 3 times with PBS. Fifty microliters of horseradish peroxidase-conjugated rabbit anti-sheep IgG (Bethyl Laboratories, Montgomery, TX) diluted 1:1000 in blocking buffer plus 0.05% Tween 20 was then added to all wells and the plate was incubated for 1.5 h at room temperature. All wells were then washed 3 times with PBS and 100 µl of freshly prepared TMB peroxidase substrate/peroxide solution (3, 3', 5, 5' tetramethylbenzidine reagent, Kirkegaard and Perry Laboratories, Gaithersburg, MD) was added to all wells to allow color development. After 5 min, 100 µl of 1 M phosphoric acid was added to each well to stop color development. Absorbance was measured at 450 nm using an automated program on a model BT 2000 MicroKinetics[®] microplate reader (Fisher Scientific, Raleigh, NC). Background, non-specific binding was

assessed from blank-corrected OD values of wells which received all reagents except test sample.

Analysis of semi-purified HLP-1 by ELISA: In preliminary studies, we explored the possibility of measuring HLP-1 levels in skin extracts using both indirect and sandwich ELISA formats. However, all of the methods which we tested yielded relatively high background to signal (authors' unpubl. data). In order to improve the sensitivity of the assay, we examined the effect of pre-treating the samples by running them over a cation exchange column. This procedure both concentrates basic molecules (such as HLP-1) and removes anionic contaminants.

Catfish skin extract was prepared for ELISA analysis with a single step partial purification using carboxymethylcellulose cation-exchange sep pak cartridges (CM sep pak) (Accell Plus CM Sep Pak; Waters Corp., Milford, MA). A CM sep pak was conditioned with 5 ml of 1.0 M AA (1 M ammonium acetate adjusted to pH 5.15 with glacial acetic acid) followed by 5 ml of 0.1 M AA. Skin extract was then diluted with an equal volume of 0.1 M AA and applied to the cartridge at a flow rate of about 1 ml min⁻¹. The cartridge was then washed with 5 ml of 0.1 M AA at 1 ml min⁻¹. The flow-through and wash, containing unbound material, was pooled and stored on ice. The bound HLP-1 was then step-eluted with 5 ml of 1.0 M AA. The flow through/wash and the eluted, semi-purified HLP-1 were immediately frozen in liquid nitrogen and lyophilized.

The flow-through/wash was redissolved in 0.1 M AA at 50× the original concentration and assayed for antibacterial activity to determine if all activity was bound to the cartridge. The lyophilized eluate (containing the semi-purified HLP-1) was redissolved in 100 µl of PBS. Duplicate 2.5 µl aliquots of the sample were both diluted with 47.5 µl of PBS and added to duplicate wells of a 96-well ELISA microtiter plate. The ELISA was carried out on these samples as described above. Background, non-specific binding was assessed from blank-corrected OD values of wells that received all reagents except skin eluate samples (negative control). In order to control for plate-to-plate variation, the mean, blank-corrected optical densities of the samples were used to calculate ELISA values (EVs) according to the method of Hornitzky & Searson (1986), where:

$$EV = \frac{(\text{OD sample}) - (\text{OD negative control})}{(\text{OD positive control}) - (\text{OD negative control})} \times 100$$

A standard curve using calf histone H2B was run in each ELISA. The positive control value for calculating EVs was taken from the standard curve's OD for 0.313 µg ml⁻¹ histone H2B. This concentration of H2B consistently represented the approximate mid-point of the linear region of the H2B standard curve as determined in preliminary experiments.

To determine the reproducibility of the assay for a single sample, tissue extracts from 6 fish were pooled and then extracted and tested at three separate times for HLP-1 levels. To determine the reproducibility of the assay among fish, CM sep pak semi-purified HLP-1 samples were prepared from the skin of 8 healthy catfish in 4 independent assays (2 fish per assay).

To determine the possible effect of the acute stressors of chasing, netting and capturing the fish on measurement of either antibacterial activity or HLP-1 levels, we compared the antibacterial activity levels and HLP-1 levels among fish sequentially sampled within all 4 time periods.

Analysis of semi-purified HLP-1 by analytical RP-HPLC. Analytical reverse-phase HPLC of representative samples was performed to compare the elution profile of CM sep pak semi-purified HLP-1 samples from stressed and unstressed fish which differed significantly in ELISA response. One sample of semi-purified HLP-1 from 1 individual in the unstressed control group and 1 individual from the group stressed for 4 wk, which demonstrated EVs closest to the mean for each of these 2 respective groups, were chosen for HPLC analysis. Twenty-five microliters of each sample were diluted in 1 ml of 0.1% trifluoroacetic acid (TFA) in water and injected onto a butyl C₄ reverse-phase column and chromatographed using buffers and gradient conditions as previously described (Robinette et al. 1998). The elution profile was monitored at 214 nm. Fractions were collected across the entire elution, lyophilized, and reconstituted in 10 µl of 0.1 M AA, and 2 µl were assayed for antibacterial activity against *Escherichia coli* D31 using the spot assay (Robinette et al. 1998). The remainder was diluted with 30 µl of SDS-PAGE sample buffer for electrophoretic analysis. Electrophoresis (Laemmli 1970) was conducted using the Mini-Protean II system according to manufacturer's instructions.

Statistical analyses. Calculations of means, standard deviations and analyses of variance (ANOVA) of the differences between means were carried out using Microsoft Excel versions 5.0 and 2000. One-factor ANOVA and Duncan's multiple range test for equal or unequal sample sizes (Bernstein & Bernstein 1999) were used to evaluate the differences in means for HLP-1 ELISA values and antibacterial activity. Linear regression analyses and coefficients of variation were performed using Lotus SmartSuite 1-2-3 Release 9.

RESULTS

ELISA directed against HLP-1

The biochemical/antigenic similarity between histone H2B and the catfish skin antimicrobial protein

HLP-1 was exploited in the ELISA. Affinity-purified antibody against histone H2B was commercially available, which facilitated the development of an immunoassay based on shared antigenic properties of the molecules. The applicability of anti-histone H2B antibody for use in these experiments was confirmed by examining the cross-reactivity of both proteins to the antibody. Serial 2-fold dilutions of calf histone H2B and RP-HPLC-purified HLP-1 exhibited highly similar dose-responses to a standard concentration of sheep anti-calf histone H2B (Fig. 1A).

For calculating EVs for sample comparison among plates, the blank-corrected OD value for $0.313 \mu\text{g ml}^{-1}$ from the histone H2B standard curve was the positive control. This concentration represented the approximate mid-point of the linear region of the H2B standard curve (Fig. 1A). Histone H2B was chosen as the positive control standard due to its similarity to HLP-1 and commercial availability.

Preliminary experiments revealed that crude skin extract was not suitable for use in the immunoassay due to substantial cross-reactivity with the conjugated

secondary antibody. During development of a purification method for the catfish skin antibacterial activity, Robinette et al. (1998) noted that most of the crude skin extract protein applied to cation-exchange CM52 columns did not bind to the column. However, the antibacterial activity was strongly retained by the column and could be eluted by high ionic strength buffer. This property was exploited for eliminating interference in the crude skin extract for use in the ELISA. In contrast to crude skin extract, the semi-purified HLP-1 eluted from the sep pak cartridge was not highly cross-reactive with peroxidase conjugated rabbit anti-sheep antibody in controls in which the sheep anti-calf histone H2B primary antibody was omitted (not shown). Analysis of CM sep pak flow-through/wash from unstressed fish by the radial diffusion assay revealed no detectable antibacterial activity, indicating activity had bound to the cartridge. To determine if CM sep pak semi-purified HLP-1 was suitable for use in ELISA, an assay was conducted comparing the dilution curves of histone H2B and CM sep pak semi-purified HLP-1 (Fig. 1B). The semi-purified HLP-1 produced a dilution response in ELISA very similar to that of pure calf histone H2B, indicating that this semi-purified material produces a much better signal:noise ratio than the crude skin extract.

Preliminary analysis of CM sep pak semi-purified HLP-1 samples prepared from the skin of 8 healthy catfish examined in 4 independent assays produced EVs of greater than 90, with a mean EV (\pm SD) of $99 (\pm 9)$, and a range of 90.3 to 117. The coefficient of variation (CV) of these samples was 9.1%. These results indicated that healthy, unstressed fish produced a consistent ELISA response. Similarly, a pooled sample of skin extract from a separate group of 6 clinically normal fish produced a highly consistent response when tested three independent times by ELISA. The EVs for these samples were 90, 95 and 101 (CV = 5.7%).

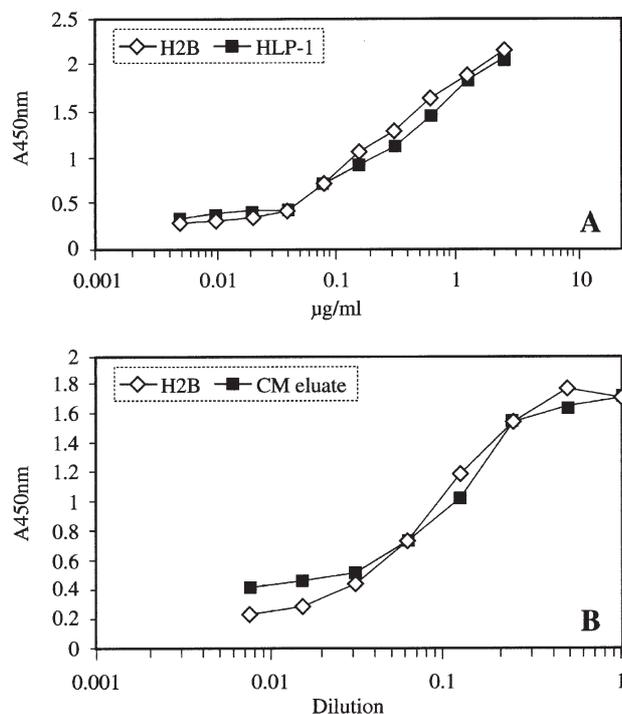


Fig. 1. (A) Dose-response comparison of HPLC-purified HLP-1 and calf histone H2B from 2.5 to $0.005 \mu\text{g ml}^{-1}$ using a standard dilution (1:1000) of sheep anti-histone H2B antibody. Each data point represents the mean from 2 experiments. $N = 2$ values per data point. (B) Comparison of 2-fold serial dilution curves by ELISA of calf histone H2B and CM sep pak semi-purified HLP-1. Serial 2-fold dilutions of histone H2B (final concentration $5 \mu\text{g ml}^{-1}$) or CM sep pak semi-purified HLP-1 were coated onto the microtiter plate wells and analyzed by ELISA

Comparison of stressed versus unstressed fish by ELISA

Catfish subjected to chronic stress for up to 4 wk showed no gross signs of disease as evidenced by the absence of skin lesions, skin discoloration, or behavioral abnormalities. Ammonia levels in the tanks rose rapidly, reached total ammonia nitrogen levels $\geq 1 \text{ mg l}^{-1}$ by 24 h after initiation of the experiment, and generally remained between 0.5 to 1 mg l^{-1} with daily 50% water changes, but did not cause mortality or obvious morbidity. All fish refused feed when offered 24 h after initiation of the experiment and onward throughout the duration of the experiment. No feeding was observed when food was offered each day; uneaten food was still visible in the tanks the next day.

While fish did not appear sick, ANOVA indicated that there was a significant difference ($p < 0.0001$) among the mean EVs. Duncan's multiple range test at $\alpha = 0.05$ was then performed to determine which EVs differed; this analysis showed that the mean EV of fish stressed for 1 wk (EV = 87.2) was significantly depressed compared to that of the unstressed controls (EV = 118.2) (Fig. 2). An even greater depression of HLP-1 levels was observed after 3 wk of stress (EV = 51.2), with an EV which was 57% lower than that of control fish. While the mean EV of fish subjected to 4 wk of stress (EV = 50.5) was statistically the same as the 3 wk group, it was also significantly lower than both the unstressed control fish and the fish stressed for 1 wk ($p < 0.05$).

Antibacterial activity of stressed versus unstressed fish

ANOVA indicated that there was a significant difference ($p < 0.0001$) among the mean antibacterial activities of various groups. Duncan's multiple range test at $\alpha = 0.05$ was then performed to determine which treatments differed; this analysis showed that the mean antibacterial activity of fish confined for 1 wk was significantly higher (3 times higher) than that of the unstressed control group (Table 1). Average activity of fish confined for 3 and 4 wk was not different from that of unstressed fish via Duncan's multiple range test. ANOVA indicated that there was a significant difference ($p < 0.01$) among the mean protein concentrations of the various groups. Duncan's multiple range test at $\alpha = 0.05$ was then performed to determine which treatments differed; this analysis showed that the mean protein concentration of extracts from fish confined for 1 wk was lower than that of the control group and fish stressed for 4 wk (Table 1).

Antibacterial activity of CM sep pak semi-purified HLP-1 samples was also examined using the radial dif-

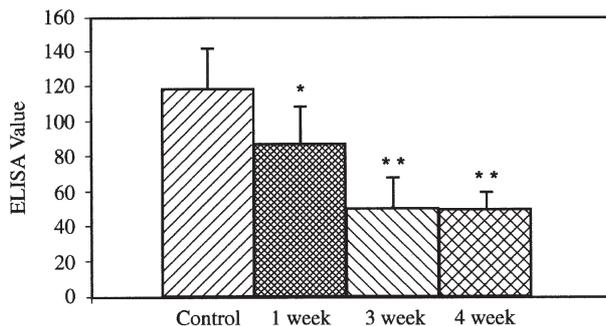


Fig. 2. HLP-1 ELISA values of fish subjected to chronic stress for 0 (control), 1, 3 or 4 wk. Times having different numbers of asterisks (*) are significantly different from each other

fusion assay. Although the CM sep pak eluate was 6 times more concentrated than the original skin extract, only samples from control (unstressed) fish demonstrated measurable clearing zones (Table 2). In samples from fish confined for 1 wk, only trace activity was detectable, and no bacterial inhibition was detectable in samples from fish confined for 3 or 4 wk.

Test group	Extract protein (mg ml ⁻¹)	Clearing (mm)	Activity (U)
Control	3.3 (± 0.4) ^a	5.4	2.05 (± 0.14) ^a
1 wk	2.5 (± 0.5) ^b	8.2	6.16 (± 0.73) ^b
3 wk	ND	5.4	2.03 (± 0.15) ^a
4 wk	3.6 (± 0.7) ^a	4.6	1.65 (± 0.24) ^a

ANOVA indicated that there was a significant difference ($p < 0.01$) among the mean protein concentration of CM sep pak semi-purified HLP samples of the various groups. Duncan's multiple range test at $\alpha = 0.05$ was then performed to determine which treatments differed; this analysis showed that the mean protein concentration of extracts from fish confined for 3 or 4 wk was significantly lower than that of the unstressed controls (Table 2). These results suggest that

fusion assay. Although the CM sep pak eluate was 6 times more concentrated than the original skin extract, only samples from control (unstressed) fish demonstrated measurable clearing zones (Table 2). In samples from fish confined for 1 wk, only trace activity was detectable, and no bacterial inhibition was detectable in samples from fish confined for 3 or 4 wk.

ANOVA indicated that there was a significant difference ($p < 0.01$) among the mean protein concentration of CM sep pak semi-purified HLP-1 prepared from catfish skin extracts of unstressed (control) fish, and from fish subjected to various periods of stress. For each experimental group, N = 6 fish, except Week 3, where samples from 3 fish were lost during processing. Values in parentheses represent 1 standard deviation of the mean. Values with different letter superscripts are significantly different from each other. T: trace clearing, estimated at less than 25%, insufficient for determination of the clearing zone diameter. NC: no clearing zone present

Test group	Extract protein (mg ml ⁻¹)	Clearing zone (mm)
Control	4.6 (± 1.2) ^a	5.3
1 wk	3.4 (± 1.3) ^{a,b}	T
3 wk	1.9 (± 0.4) ^b	NC
4 wk	2.0 (± 1.2) ^b	NC

there was a lower abundance of basic material binding to the CM sep pak cation-exchange cartridge in samples from fish subjected to the longer duration confinement periods.

Effect of time of collection within a test group on antibacterial activity and HLP-1 levels

There was no relationship between the rank order in which a fish was sampled from a test group and either the individual's antibacterial activity or its HLP-1 levels (Figs. 3 & 4). Thus, there was no apparent change in these parameters from when the first fish was sampled (less than 3 min after initially disturbing the tank) to the last fish (from 90 to 120 min after disturbing the tank).

Analytical RP-HPLC

Results of RP-HPLC analysis of semi-purified HLP-1 from 1 fish from the unstressed control group and 1 from the group stressed for 4 wk are shown in Fig. 5. The sample from the unstressed control fish resolved into several prominent peaks (Fig. 5A). Antibacterial activity co-eluted with fractions encompassing an incompletely resolved peak in the center of the chromatogram with an absorbance (A_{214}) of 0.117. The antibacterial activity eluted at 45 to 50% acetonitrile concentration, which is the typical retention time for HLP-1 (Robinette et al. 1998). No other fractions had

activity. The SDS-PAGE of pooled fractions containing antibacterial activity revealed several proteins, including a band which co-migrated with purified HLP-1. No bands co-migrating with HLP-1 were observed in other peak fractions, indicating that the band co-migrating with HLP-1 in fractions containing antibacterial activity was likely to be HLP-1.

The elution profile of the representative sample from a fish stressed for 4 wk revealed that all peaks were much less prominent (Fig. 5B). The general pattern of elution remained the same, however, indicating that lower relative amounts of protein were present in this sample compared to the sample from an unstressed fish, an observation which is supported by protein measurements (Table 2). The A_{214} of the peak corresponding to that which contained antibacterial activity in Fig. 5A was only 0.053, less than half of the absorbance intensity of the equivalent peak from the unstressed fish. This also indicated that substantially less material was present in the CM sep pak eluate from stressed fish. No bands were visible on SDS-PAGE with Coomassie staining, probably due to insufficient protein content for detection with this stain. Antibacterial activity was also not detectable in fractions from this chromatogram.

DISCUSSION

The HLP-1 ELISA was reproducible and gave highly consistent values in randomly sampled, presumptively

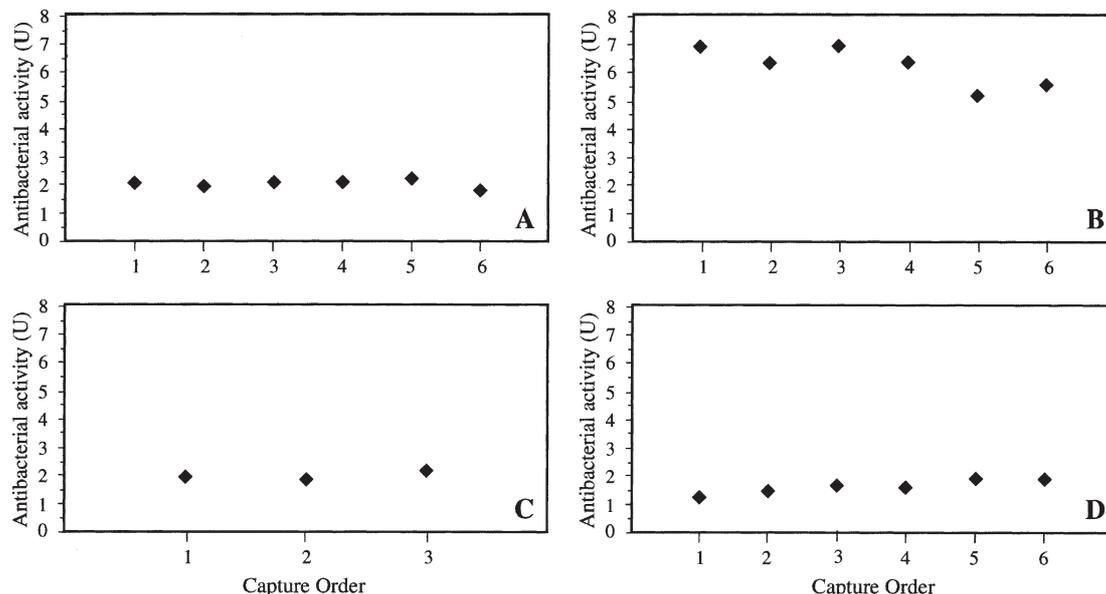


Fig. 3. Relationship between the rank order of sample collection and antibacterial activity measured in the sample. Each data point represents a single fish. Fish 1 was collected within 3 min of disturbing the tank. Subsequent fish were sequentially sampled about every 15 to 20 min. (A) Unstressed control; (B) stressed 1 wk; (C) stressed 3 wk; (D) stressed 4 wk

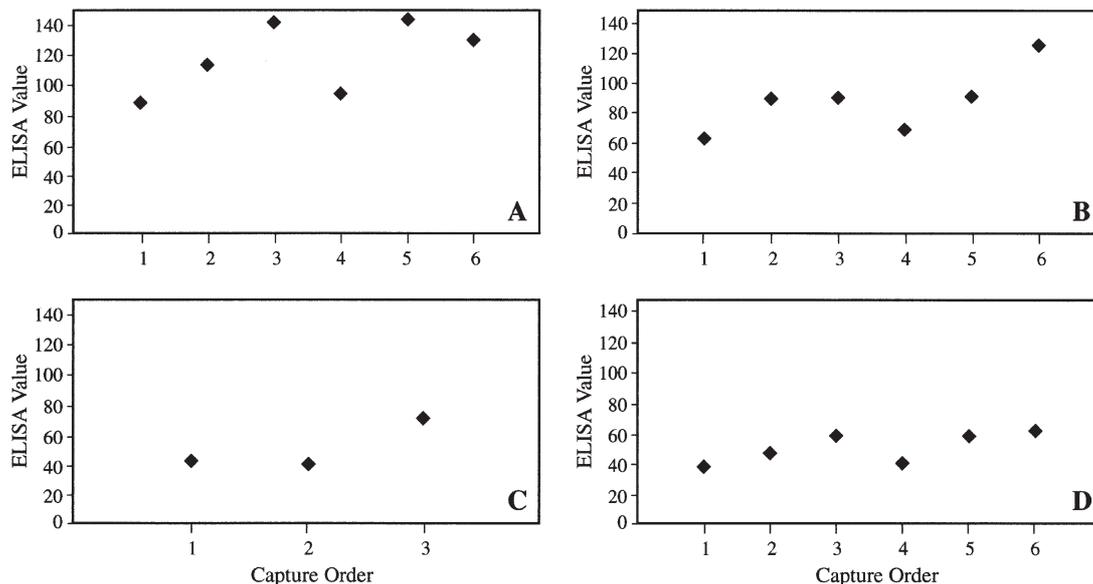


Fig. 4. Relationship between the rank order of sample collection and the ELISA value measured in the sample. Each data point represents a single fish. Fish 1 was collected within 3 min of disturbing the tank. Subsequent fish were sequentially sampled about every 15 to 20 min. (A) Unstressed control; (B) stressed 1 wk; (C) stressed 3 wk; (D) stressed 4 wk

healthy channel catfish. One possible shortcoming of this test is that there may be cross-reactivity with related molecules. However, the commercial antibody used in our study is specific for histone H2B (P. Williams, Fitzgerald Industries, Concord, MA, USA) and closely cross-reacts with HLP-1 (Fig. 1). While HLP-3 is closely related to HLP-1 in N-terminus amino acid sequence and is present in skin cell extracts (Robinette et al. 1998), it is a relatively minor component compared to HLP-1 (Robinette et al. 1998).

Levels of HLP-1 measured with ELISA were significantly lower in the skin of fish that had been subjected

to crowding and poor water quality for 1 wk, and declined further in fish stressed for 3 to 4 wk. The RP-HPLC profiles (Fig. 5) of CM sep pak eluates from unstressed fish and fish stressed for 4 wk support these ELISA data, indicating that stressed fish have lower HLP-1 levels. The progressively decreasing EVs after 1, 3 and 4 wk of stress were not accompanied by any mortalities or gross signs that would indicate the onset of infection, such as skin lesions or behavioral abnormalities. This indicates that the early change in HLP-1 response may provide a useful warning of declining health status prior to development of clinical disease.

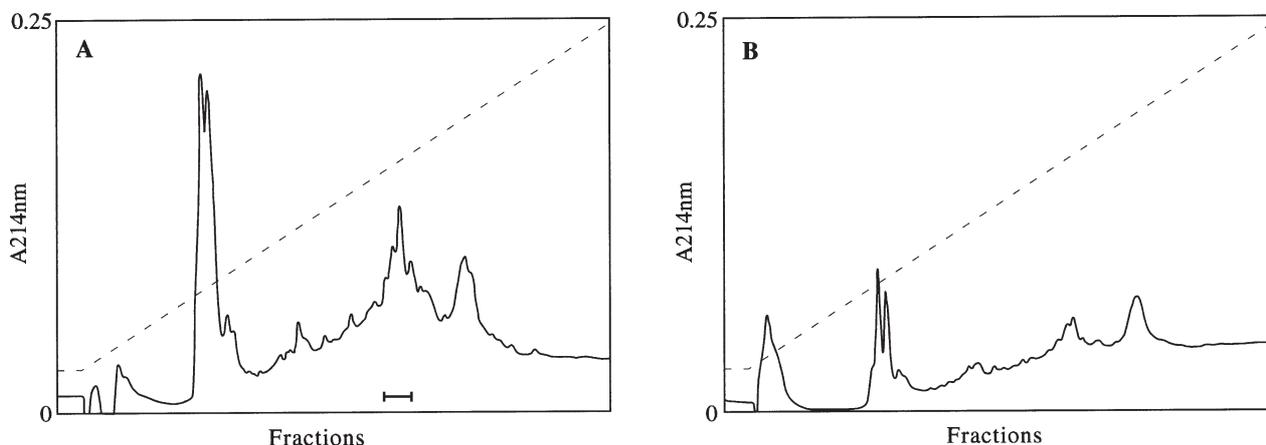


Fig. 5. Analytical reverse phase HPLC of CM sep pak eluates. (A) Sample from unstressed control fish. Continuous solid line represents the optical density of the eluate. Short horizontal line (I-I) indicates the eluted fractions which had antibacterial activity. Dashed line (---) represents the acetonitrile/0.1% TFA gradient. (B) Sample from a fish subjected to 4 wk of stress. Note the lack of any detectable antibacterial activity

Yin et al. (1995) also examined the effect of crowding for up to 30 d on various non-specific immune parameters in carp. Non-specific immune responses (serum lysozyme activity, bactericidal complement activity) and resistance to experimental challenge with *Aeromonas hydrophila* were significantly reduced after 1 wk of confinement, and remained low through termination of the experiment at Day 30, although no mortality was observed during confinement. While non-specific defenses may be suppressed, fish exposed to chronic stresses such as long-term crowding may have a certain capacity to adapt to these conditions and resist infectious disease (Yin et al. 1995). However, the demonstration of a rapid decline in a putatively protective response such as HLP-1, which displays broad-spectrum inhibition of a wide array of fish pathogens (Robinette et al. 1998), may play an important role in enhancing the probability that an infectious disease epidemic may occur in a fish population.

The specific stressor(s) responsible for the decrease in HLP-1 within our study is not known, as both crowding and ammonia are stressful. Anorexia might also contribute to a change in HLP-1. Cessation of feeding is a behavioral response of channel catfish to stress (MacMillan 1985). And starvation can adversely affect the immune response or immunity of fish. African catfish *Clarias gariepinus* which were deprived of food for 87 d and then injected with *Yersinia ruckeri* antigen had much lower hemagglutinating antibody titers than those of well-fed fish (Henken et al. 1987). Fasting channel catfish for 10 d significantly increases their susceptibility to challenge with *Flexibacter columnare* (Klesius et al. 1999).

Exposing channel catfish to chronic confinement and sub-optimal water quality causes an initial increase in skin-associated antibacterial activity, which then significantly decreases (returning to baseline) as the period of stress lengthens. The significant increase in antibacterial activity after 1 wk of stress did not correlate with ELISA measurements in which the HLP-1 signal of these fish was significantly depressed. The increased antibacterial activity may be due to induction of additional antibiotic(s), which may act to enhance antibacterial defenses in the skin as an adaptation response to the stress. Maule et al. (1989) observed that survival of chinook salmon after an experimental challenge with *Vibrio anguillarum* was higher when the challenge occurred 24 h after transport stress rather than immediately post-transport or 8 d post-transport, suggesting that some stressors may induce a transient non-specific immune stimulation which is followed by a longer term immunosuppression.

The antibacterial activity in CM sep pak eluates was lower than expected. Although eluates were concentrated 6-fold greater than the crude skin extracts, their

antibacterial activity was weak after 1 wk of stress, and not detectable with 3 wk of stress. Hultmark et al. (1982) observed that there is much lower antibacterial activity in semi-purified antibiotic samples compared to crude extracts. This is most likely caused mainly by 2 factors. One is the fact that there can be multiple antibiotics with different ionic characteristics in crude extracts. For example, Lemaitre et al. (1996) found potent, anionic, polypeptide antibiotics in the skin of carp. Such antibiotics would not bind to a cation exchange column; thus, their activity would be lost in the analysis. These observations support our previous hypothesis that one or more antibiotics other than HLP-1 may be induced during the earlier, more acute stages of our stress. A second possible reason for the lower recovery of antibacterial activity in semi-purified material is that multiple antibiotic mixtures appear to act synergistically when present together and conversely, appear weaker when chromatographically separated (Frohm et al. 1996). We have already shown that channel catfish skin has at least 3 antibiotics (Robinette et al. 1998).

An important question to be addressed is the relationship between HLP levels, antibacterial activity and susceptibility to infectious disease. The results reported here suggest that chronic stress has a deleterious effect on HLP-1 levels in channel catfish skin. The degree to which this influences disease resistance has not yet been determined. Nonetheless, the fact that significant suppression of this activity results from chronically stressful crowding and sub-optimal water quality, as is often found in intensive aquaculture, and the fact that we have identified similar antibiotics in other commercially important species such as rainbow trout and hybrid striped bass (*Morone saxatilis* × *M. chrysops*) (Noga et al. 2001), suggest that HLPs may be a promising indicator for monitoring the health of many fish. Such an indicator could be a valuable tool for discerning the effects of stress on non-specific immunity and disease resistance, as well as evaluating the effectiveness of strategies designed to enhance defense mechanisms of aquaculture species. Interestingly, other investigators have recently found that glucocorticoid hormones associated with stress repress the expression of antimicrobial peptides in amphibians (Simmaco et al. 1997). Insights into the genetic signals controlling up- or down-regulation of polypeptide antibiotics, including the function of transcription factors such as NF- κ B, is an important goal in understanding their mechanism of action.

A major problem with assessing stress in real-world situations is that the process of capturing and sampling the fish can cause a change in many immune and endocrinological parameters, hindering their usefulness as biomarkers of stress. One example is blood cor-

tisol, which may rapidly rise as soon as 2 to 3 min or less after an acute stress, such as netting or disturbance. We have presented some evidence that HLP-1 levels are not affected by such acute stresses, as there was no significant relationship between time of capture and HLP-1 levels. This suggests that changes in HLP-1 may be a viable means of assessing health in both wild and aquaculture populations.

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LITERATURE CITED

- Anderson D (1990) Immunological indicators: effects of environmental stress on immune protection and disease outbreaks. In: Adams M (ed) Biological indicators of stress in fish, Vol 18. American Fisheries Society Symposium, Bethesda, MD, p 38–50
- Angelidis P, Baudin-Laurencin F, Youinou P (1987) Stress in rainbow trout, *Salmo gairdneri*: effects upon phagocyte chemiluminescence, circulating leukocytes, and susceptibility to *Aeromonas salmonicida*. J Fish Biol 31(Suppl A): 113–122
- Barton BA (1997) Stress in finfish: past, present and future—a historical perspective. In: Iwama GK, Pickering AD, Sumpter JP, Schreck CB (eds) Fish stress and health in aquaculture. Soc Exper Biol Seminar Series, Vol 62. Cambridge University Press, New York, p 1–34
- Bernstein S, Bernstein R (1999) Elements of statistics II: Inferential statistics. McGraw-Hill, New York
- Cipriano R, Starliper C, Schachte J (1985) Comparative sensitivities of diagnostic procedures used to detect bacterial kidney disease in salmonid fishes. J Wildl Dis 21:144–148
- Clem L, Faulmann E, Miller N, Ellsaesser D, Lobb C, Cuchens M (1984) Temperature-mediated processes in teleost immunity: differential effects of *in vitro* and *in vivo* temperatures on mitogenic responses of channel catfish lymphocytes. Dev Comp Immunol 8:313–322
- Cobb CS, Levy MG, Noga EJ (1998) Acquired immunity to amyloodiniosis is associated with an antibody response. Dis Aquat Org 34:125–133
- Ellis A (1982) Differences between the immune mechanisms of fish and higher vertebrates. In: Roberts R (ed) Microbial diseases of fish. Academic Press, London, p 1–30
- Ellsaesser D, Clem L (1986) Haematological and immunological changes in channel catfish stressed by handling and transport. J Fish Biol 28:511–521
- Frohm M, Gunne H, Bergman AC, Agerberth B, Bergman T, Boman A, Liden S, Jornvall H, Boman HG (1996) Biochemical and antibacterial analysis of human wound and blister fluid. Eur J Biochem 237:86–92
- Henken AM, Tigchelaar AJ, Van Muiswinkel WB (1987) Effects of feeding level on antibody production in African catfish *Clarias gariepinus* Burchell, after injection of *Yersinia ruckeri* O-antigen. J Fish Dis 11:85–88
- Hetrick F, Knittel M, Fryer J (1979) Increased susceptibility of rainbow trout to infectious hematopoietic necrosis virus after exposure to copper. Appl Environ Microbiol 37: 198–201
- Hornitzky M, Searson J (1986) The relationship between the isolation of *Brucella abortus* and serological status of infected, non-vaccinated cattle. Aust Vet J 63:172–174
- Hultmark D, Engstrom A, Bennich H, Kapur R, Boman H (1982) Insect immunity: isolation of cecropin D and four minor antibacterial components from cecropia pupae. Eur J Biochem 127:207–217
- Klesius P, Lim C, Shoemaker C (1999) Effect of feed deprivation on innate resistance and antibody response to *Flavobacterium columnare* in channel catfish, *Ictalurus punctatus*. Bull Eur Assoc Fish Pathol 19:156–158
- Klinger H, Delventhal H, Hilge V (1983) Water quality and stocking density as stressors of channel catfish (*Ictalurus punctatus* Rafinesque). Aquaculture 30:263–272
- Laemmli U (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680–685
- Lemaitre C, Orange N, Saglio P, Saint N, Gagnon J, Molle G (1996) Characterization and ion exchange activities of novel antibacterial proteins from the skin mucous of carp (*Cyprinus carpio*). Eur J Biochem 240:143–149
- MacMillan J (1985) Infectious diseases. In: Tucker C (ed) Channel catfish culture, Vol 15. Developments in Aquaculture and Fisheries Science, Elsevier, Amsterdam, p 409–496
- Maule A, Tripp R, Kaattari S, Schreck C (1989) Stress alters immune function and disease resistance in chinook salmon (*Oncorhynchus tshawytscha*). J Endocrinol 120: 135–142
- Meyer F (1970) Seasonal fluctuations in the incidence of disease on fish farms. In: Snieszko S (ed) A symposium on diseases of fishes and shellfishes. Special Publication no 5, American Fisheries Society, Washington, DC, p 21–29
- Miller N, Clem L (1984) Temperature-mediated processes in teleost immunity: differential effects of temperature on catfish *in vitro* antibody responses to thymus-dependent and thymus-independent antigens. J Immunol 133:2356–2359
- Noga E, Kirby J, King W, Aucoin D, Geisbrecht F (1994) Quantitative comparison of the stress response of striped bass (*Morone saxatilis*) and hybrid bass (*Morone saxatilis* × *Morone chrysops* and *Morone saxatilis* × *Morone americana*). Am J Vet Res 55:405–409
- Noga EJ, Fan Z, Silphaduang U (2001) Histone-like proteins from fish are lethal to the parasitic dinoflagellate *Amyloodinium ocellatum*. Parasitology (in press)
- Pickering A (1981) Introduction: the concept of biological stress. In: Pickering A (ed) Stress and fish. Academic Press, London, p 1–10
- Pickering A, Pottinger T (1987) Crowding causes prolonged leucopenia in salmonid fish, despite interrenal acclimation. J Fish Biol 30:701–712
- Robinette D, Wada S, Arroll T, Levy M, Miller W, Noga E (1998) Antimicrobial activity in the skin of the channel catfish *Ictalurus punctatus*: characterization of broad-spectrum histone-like antimicrobial proteins. Cell Mol Life Sci 54:467–475
- Schwedler T, Tucker C, Bealeu M (1985) Non-infectious diseases. In: Tucker C (ed) Channel catfish culture, Vol 15. Developments in Aquaculture and Fisheries Science, Elsevier, Amsterdam, p 497–541
- Simmaco M, Boman A, Mangoni ML, Mignogna G, Miele R, Barra L, Boman HG (1997) Effect of glucocorticoids on the synthesis of antimicrobial peptides in amphibian skin. FEBS Lett 416:273–275

- Smart G (1981) Aspects of water quality producing stress in intensive fish culture. In: Pickering A (ed) *Stress and fish*. Academic Press, London, p 277–294
- Smith S, Levy M, Noga E (1992) Development of an enzyme-linked immunosorbent assay (ELISA) for the detection of antibody to the parasitic dinoflagellate *Amyloodinium ocellatum* in *Oreochromis aureus*. *Vet Parasitol* 42: 145–155
- Snieszko S (1974) The effects of environmental stress on outbreaks of infectious diseases of fishes. *J Fish Biol* 6: 197–208
- Stave J, Roberson B (1985) Hydrocortisone suppresses the chemiluminescent response of striped bass phagocytes. *Dev Comp Immunol* 9:77–984
- Walters G, Plumb J (1980) Environmental stress and bacterial infection in channel catfish, *Ictalurus punctatus* Rafinesque. *J Fish Biol* 17:177–185
- Wedemeyer G (1996) *Physiology of fish in intensive culture*. Chapman and Hall, New York
- Wellborn T, Tucker C (1985) An overview of commercial catfish culture. In: Tucker C (ed) *Channel catfish culture*, Vol 15. *Developments in Aquaculture and Fisheries Science*, Elsevier, Amsterdam, p 1–12
- Wendelaar Bonga S (1997) The stress response in fish. *Physiol Rev* 77:591–625
- Yin Z, Lam T, Sin Y (1995) The effects of crowding stress on the non-specific immune response in fancy carp (*Cyprinus carpio* L.). *Fish Shellfish Immunol* 5:519–529
- Zasloff M (1987) Magainins, a class of antimicrobial peptides from *Xenopus* skin: isolation, characterization of two active forms, and partial cDNA sequence of a precursor. *Proc Natl Acad Sci USA* 84:5449–5453

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