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**Comparison of the Immune Response of Striped
Bass and Hybrid Bass**

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ABSTRACT

We developed methods for examining the pathophysiological response of striped bass and hybrid bass to various forms of stress. This involved development of techniques for the measurement of lysozyme, mitogen blastogenesis, mixed lymphocyte reaction, and oxidative burst, which are important general indicators of systemic immune function. We also examined local immune defenses (epithelial integrity), as well as homeostatic indicators in blood, including osmotic balance and glucose. Acute stress resulted in significant perturbations in a number of parameters, including glucose, electrolytes, osmolality, lysozyme, and mixed lymphocyte reaction. Most significantly, acute confinement stress resulted in severe damage to the epidermal epithelium, as indicated by the rapid (within 2 hr) development of erosions and ulcerations on various fins. There were significant differences in the resting levels of some immune functions between striped bass and hybrid bass, including response to mitogens in the leukocyte blastogenesis test. Our studies also revealed that there were significant differences in how striped bass and hybrid bass respond to stress, with striped bass being much more severely affected by stress than the hybrid. This was reflected in more severe changes in glucose, cortisol dynamics, and plasma lysozyme. Most significantly, striped bass developed more severe idiopathic skin ulceration after stress, which may be a major reason why this fish is so prone to develop opportunistic bacterial and fungal infections after stress.

Hybrid bass injected with equine serum albumin developed a typical humoral immune response, with peak antibody production 28 days after primary immunization. Fish that were exposed to a chronic stress after a primary immunization showed almost complete inhibition of antibody production.

OBJECTIVES OF THE ORIGINAL RESEARCH PROPOSAL

The major objective of this research was to compare the immune response of striped bass (Morone saxatilis) and hybrid bass (M. saxatilis x M. chrysops), including lysozyme levels, phagocyte function, lymphocyte blastogenic response, and antibody production. Another objective was to determine how immunity was perturbed by stress.

Most of the objectives were achieved. The main deviation from the above objectives was that we were not able to examine the effect of immunization on the protective immune response. As originally planned, we started our work by trying to establish a disease model using different strains of Vibrio damsela (formerly Pasteurella piscicida) that we isolated from fish during an outbreak of pasteurellosis of hybrid bass in Israel. In addition, we tested the pathogenicity of additional strains isolated from IOLR in Eilat and the Nir-David Laboratory. We were unable to develop a consistent and reproducible model that could be successfully established in combination with a well-controlled stress induction model. However, work described in

this project showed that stress may be used to establish disease and protective immunization models.

We performed additional studies which although not planned in the original proposal, provided important information on the pathophysiology of stress in Morone and how this response differs between striped bass and hybrid bass. This included examination of osmotic balance (osmolality, electrolytes) and local immunity (skin integrity). This additional research lead to some important insights into why disease epidemics may occur after stressful events (See EVALUATION OF RESEARCH ACHIEVEMENTS).

RESEARCH SUB-PROJECT I

WHOLE BLOOD ASSAY FOR EXAMINING LYMPHOCYTE BLASTOGENESIS
OF PERCICHTHYID BASS (MORONE).

INTRODUCTION

Mitogen-stimulated lymphocyte blastogenesis is widely used as an indicator of immune competence in vertebrates, including fish. Incorporation of radiolabelled nucleic acid precursors is the major method for testing the functional status of fish peripheral blood lymphocytes (PBL). Lymphocytes are usually separated from other cell types by density gradient centrifugation using separation media such as Ficoll (Luft et al 1991) or Percoll (Reitan and Thuvander 1991) gradients. However, this technique is labor-intensive and time-consuming (Wemme et al 1992). A simplified alternative to density gradient centrifugation is direct centrifugation of whole blood under low speed to obtain the buffy-coat (Etlinger et al 1976). An even simpler method is the use of whole blood culture. Whole blood cultures have been performed with human (Frankenburg 1988; Bloemena et al 1989), cat (Lappin et al 1992), opossum (Brozek et al 1992), calf (Whitbread 1986), and turkey (Sharma and Belzer 1992) blood samples. In this paper, we describe the adaptation of whole blood culture to studying mitogen blastogenesis in fish, using hybrid bass (Morone saxatilis x M. chrysops) as a model.

We also provide preliminary evidence for its usefulness in striped bass (M. saxatilis). These two commonly cultured fish strains vary considerably in their resistance to disease (Noga et al 1994). The immunological mechanisms responsible for this difference in resistance are unclear.

MATERIALS AND METHODS

Animals

Hybrid bass (striped bass [Morone saxatilis] female x white bass [M. chrysops] male) were spawned at the Pamlico Aquaculture Center, Aurora, NC. Striped bass (Roanoke River source) were spawned at the Edenton National Fish Hatchery, Edenton, NC. Hybrid bass (about 3 years old, with an average body weight of 570 g) and striped bass (about 2 years old, with an average body weight of 420 g) were kept in tanks supplied with aerated, flow-through, fresh well water at a temperature of 20°C. Fish were fed a commercial pelleted feed twice daily, and were kept on a photoperiod of 12 h light:12 h dark.

Sampling

Fish were sedated with about 21 mg/l of tricaine methanesulfonate. Blood samples were collected from the caudal sinus into heparinized syringes and needles (22-G). For density

gradient centrifugation, the blood was immediately diluted 1:3 with RPMI-1640 (GIBCO) in sterile centrifuge tubes. All blood samples were transported to the laboratory within 30 minutes. Spleens were removed after the fish were euthanised with MS-222. The fish were exsanguinated by caudal sinus puncture to reduce the number of erythrocytes in the spleen. A ventral incision was made and the spleen was carefully removed with forceps. Each spleen was placed into 4 ml of A-L medium (Luft et al 1991) (GIBCO) in a sterilized plastic trough.

Leucocyte Separations

Gradient separation was performed using the procedures of Tillitt et al (1988) with the following modifications: 6 ml samples of diluted whole blood were carefully layered over 3 ml of Histopaque-1077 (Sigma) in a 15-ml polystyrene tube (Falcon). The tubes were centrifuged at 700 x g in a swinging-bucket rotor for 30 minutes at room temperature. The leucocytes at the interface were aspirated with a fine tip transfer pipette (Fisher).

Leucocytes were released from the spleen by teasing with scissors and forceps. Tissue debris was then removed from the leucocyte suspension.

Cells in the leucocyte suspensions were enumerated using a hemocytometer and cell viability was concurrently determined by trypan blue (Sigma) dye exclusion (Philips 1973). Cell

concentrations were adjusted with A-L medium to the desired concentrations. Suspensions from density gradient separation contained less than 5% erythrocytes based on visual examination. The original spleen elution contained roughly 40% erythrocytes. After adjustment, the suspensions from density gradient separation of blood contained 5×10^5 cells/ml and those from spleen homogenates contained 5×10^7 cells/ml.

Dilution of Whole Blood

Smears of whole blood samples were stained with modified Wright's stain (Diff-Quick staining kit, Baxter). The total cell concentration was then enumerated with a hemocytometer after a 1:10,000 dilution. Samples were then diluted 1:10 with A-L medium in sterile plastic troughs.

Mitogens

Lyophilized mitogens were purchased from Sigma Chemical Co., St. Louis, MO. Concanavalin A (ConA, Lot # 12H9408), phytohemagglutinin-P (PHA-P, Lot # 122H9401), lipopolysaccharide (LPS, Lot # 112H4011, from Escherichia coli 055:B5), and pokeweed mitogen (PWM, Lot # 81H9585) were rehydrated with A-L medium to a concentration of 200 ug/ml (ConA, PHA-P, and PWM) or 1000 ug/ml (LPS), and stored at -20°C .

Assay Procedures

One hundred microliters of mitogen dilutions were placed into wells of a 96-well microtiter plate (Falcon). Then, 100 μ l of cell suspension were added to each well. There were 5×10^4 cells in each well for samples prepared by density gradient separation and 5×10^6 cells in each well having spleen homogenate. Duplicate cultures were treated with each mitogen concentration.

Cultures were incubated in an atmosphere of 95% air and 5% CO₂ in Modular Incubation Chambers (Billups-Rothenburg, Inc.). For whole blood culture, incubation conditions were studied by varying the incubation period (3, 5 and 7 days), and the incubation temperature (17°C, 24°C and 30°C). All other leucocyte samples were incubated for 3 days at 24°C. Lymphocyte proliferation was measured by the incorporation of [methyl-³H]-thymidine (ICN, Irvine, CA, specific activity 6.7 Ci/ μ mol) into DNA. ³H-thymidine (0.8 μ Ci) in 20 μ l of A-L medium was added to each well 16 h prior to harvesting the cells.

Cells were harvested with a multiple, semi-automatic cell harvester (Bellco Glass, Inc. Vineland, NJ) under 23 inches of Hg vacuum, and rinsed with distilled water followed by 95% alcohol. Cell debris and macromolecules incorporating ³H-thymidine-labeled DNA were collected on fiberglass filter disks (Cambridge Technology, Cambridge, MA). Filter disks were dried and placed in liquid scintillation vials (7 ml) and 4 ml scintillation fluid

(Ecoscint O, National Diagnostics, Atlanta, GA) was added to each vial. The radioactivity was quantitated by liquid scintillation counting in a beta counter (1219 Rackbeta, LKB). Results were expressed as counts per minute (cpm) or a stimulation index (SI), which was calculated according to the following formula: $SI = \text{cpm of stimulated culture} / \text{cpm of non-stimulated control culture}$.

Statistical Analysis

For comparing the mitogen-stimulated responses between different Morone strains, different incubation periods and different temperatures, multiple analysis of variance (ANOVA) plus Tukey's multiple comparisons (Neter et al 1990) was performed with the Statistical Analysis System (SAS; SAS Institute Inc., Cary, NC), and $p < 0.05$ was set as the limit for statistical significance. Means, standard deviations (S.D.), standard errors of mean (S.E.M), and the coefficients of variation (CV) were calculated using Microsoft Excel (Microsoft Corporation). To reduce the complexity of figures, error bars are not shown in some figures. In these cases, they were quite large and overlapping.

RESULTS

Optimal Conditions for Mitogen Stimulation Using Whole blood

Effect of Incubation Period

Whole blood samples from three hybrid bass were stimulated with serially diluted ConA, PHA-P, LPS, and PWM. Duplicate samples from each fish were incubated for 3 days, 5 days, or 7 days. Samples stimulated with ConA showed significantly higher SI after 3 days' incubation than those after 5 or 7 days' incubation ($p < 0.05$) (Fig. I-1a). No significant difference was found between 5 days' and 7 days' incubation. For PHA-P, there were no significant differences among incubation periods (Fig. I-1b). For LPS-stimulated cultures, peak SI appeared after 5 days' incubation, but the overall response of 5 days' incubation was not significantly different from that of 3 days' incubation. However, the SI of 7 days' incubation was significantly lower than that of 5 days' incubation (Fig. I-1c). For PWM, the SI of 5 days' incubation was significantly higher than that of 3 days' or 7 days' incubation. No significant difference was found between 3 days' and 7 days' incubation (Fig. I-1d).

Effect of Incubation Temperature

Whole blood samples from three hybrid bass were stimulated

with serially diluted ConA, PHA-P, LPS, and PWM. Duplicate samples from each fish were incubated at 17°C, 24°C or 30°C. For ConA-stimulated cultures, peak SI appeared at 24°C, but the difference between 24°C and 30°C was not significant; the SI at 17°C was significantly lower than that at 30°C (Fig. I-2a). For PHA-P, the response at 17°C was significantly reduced compared to that at 24°C or 30°C; the SI at 24°C and 30°C did not show any significant difference (Fig. I-2b). For LPS, the response was abolished at 30°C; the highest SI appeared at 24°C, but the responses at 17°C and 24°C were not significantly different (Fig. I-2c). For PWM, a significant difference was found between all pairwise comparisons of 17°C, 24°C and 30°C; peak SI appeared at 24°C; at 17°C the response was greatly reduced (Fig. I-2d).

Optimal Mitogen Concentrations

The optimal concentration of ConA was 12.5 ug/ml after 3 days' incubation at 24°C (Figs. I-1a, I-2a and I-4a). The optimal concentrations of PHA-P were 12.5 ug/ml after 3 days' incubation at 30°C, 25 ug/ml after 3 or 7 days' incubation at 24°C, and 50 ug/ml after 5 days' incubation at 24°C (Figs. I-1b and I-2b). So, the optimal concentration of PHA-P varied with culture conditions. The optimal concentrations of LPS were 250 ug/ml after 3 or 5 days' incubation at 24°C or 5 days' incubation at 17°C (Figs. I-1c, I-2c). The optimal concentrations of PWM were 1.56 ug/ml after 7 days' incubation at 24°C, 12.5 ug/ml after 3

days' incubation at 24°C, or 25 ug/ml after 5 days' incubation at 30°C (Figs. I-1d, I-2d); the peak SI was obtained at the highest concentration of PWM (200 ug/ml) used after 5 days' incubation at 24°C (Figs. I-1d, I-2d). Like PHA-P, the optimal dose of PHA-P varied with culture conditions.

Comparison of Cultures of Whole Blood with Histopaque-Separated Leucocytes and Splenocytes

Gradient-purified hybrid bass leucocytes were extremely sensitive to a change in the ConA concentration from 0.1 to 0.39 ug ConA/ml (Fig. I-3). The responsiveness decreased gradually from 3.13 to 100 ug ConA/ml. The optimal ConA concentration was much lower than that reported for rainbow trout (Oncorhynchus mykiss) (Tillitt et al 1988).

Under similar conditions, splenocytes from hybrid bass (containing about 40% erythrocytes), had an optimal response using 3.13 ug/ml of ConA (Fig. I-4b). This ConA concentration was more than eight-fold higher than that of Histopaque-separated leucocytes, but was lower than that of whole blood (containing more than 99% erythrocytes) (Figs. I-1a, I-2a, and I-4a). Thus, the purity of the leucocyte preparation was negatively correlated with the optimal ConA concentration.

Comparison of Blastogenesis of Whole Blood and Splenocytes from Striped Bass and Hybrid Bass

The SI of striped bass whole blood was significantly higher than that of hybrid bass (Fig. I-4a). In contrast, the SI of hybrid bass splenocytes was significantly higher than that of striped bass (Fig. I-4b).

DISCUSSION

Use of a Defined Medium for Lymphocyte Stimulation

Our results substantiate the findings of Luft et al (1991) who found that mitogen blastogenesis of fish lymphocytes can be carried out in the defined medium A-L.

Density Gradient Centrifugation Versus Whole Blood Culture

For mitogen blastogenesis experiments, blood is usually processed to obtain partially purified lymphocytes. A well-established technique for partial purification is density gradient separation with Ficoll-sodium diatrizoate (Tillitt et al 1988; Luft et al 1991). This technique is difficult to perform with large numbers of blood samples and is relatively expensive and time-consuming. Moreover, the separation procedure can give rise to selective depletion or enrichment of certain

subpopulations of lymphocytes (Romeu et al 1992; Mansour et al 1990) or monocytes, or can even lead to preactivation (Bloemena et al 1989; Frankenburg 1988). Conventional culture techniques are performed with a standardized number of lymphocytes. Therefore, diminished responsiveness due to lymphocytopenia is not reflected by this method (Bloemena et al 1989).

We developed a whole blood culture technique which was very simple and reproducible. Using this method, the peak SIs were always significantly higher than their controls. One possible disadvantage of the whole blood blastogenesis assay is that considerable variation exists in the number of lymphocytes in whole blood samples from different animals, which might contribute to a large variation in response. However, in many of our experiments, the between-fish variation was not excessively high (Table 1), taking into account the fact that ^3H -quantitated blastogenesis is usually associated with quite large variation. In cases where large variation did occur, it may not be due to the variation in lymphocyte counts since Leroux et al (1985) found that lymphocyte numbers did not correlate with the proliferative response in healthy individuals. The blastogenesis of whole blood is a reflection of the cooperation of all blood cells as well as the interaction between cells and plasma components, rather than only the leucocytes in the blood. Conversely, blastogenesis using separated leucocytes is an indicator of the intrinsic response of the leucocytes and their interaction. It will be valuable to further combine these two

methods to investigate different functions of lymphocytes and their accessory cells or components.

It should also be noted that not all cell harvesters can be used with this technique. In preliminary experiments, we found that the PHDTM cell harvester (Cambridge Technology, Inc.), which only produces 15 mm Hg vacuum, was not sufficient to effectively harvest the samples.

Effects of Different Mitogens

Relatively high SIs occurred in ConA and PWM-stimulated samples (Figs. 1d, 2d and 4a); PHA-P also stimulated PBL, inducing a relatively low SI (Figs. I-1b and I-2b). LPS was an effective mitogen only when extremely high concentrations were used (Figs. I-1c and I-2c). ConA is designated as a T-cell mitogen, which induces a high level of activation of CD8 cells in mammals. This activity may reflect the induction of T-suppressor cell activity by ConA. PWM activates only a low proportion of CD8 cells but stimulates predominantly CD4 cells in mammals, possibly reflecting the activation of T-helper cells in inducing immunoglobulin production (Schauer et al 1989). Like ConA, PHA-P is also a T-cell mitogen but with a different carbohydrate specificity (Tizard 1992). LPS is the classical B-cell mitogen (Tillitt et al 1988). While all of these definitions and descriptions are based upon more well-defined cell types in mammals, the strong responses of striped bass and hybrid bass

leucocytes to classical mitogens is good indirect evidence for lymphocyte heterogeneity in the two bass strains, and suggests T-cell heterogeneity.

Effects of Incubation Period

At 24°C, the optimal incubation periods for each mitogen were different. For best results, PWM-stimulated cells needed a longer culture period compared to those treated with ConA or PHA-P. Similar results were observed for the chicken lymphocyte transformation test (Barta et al 1992). LPS-treated cells resembled those treated with PWM, but the increase in SI was not as obvious as with PWM. After 7 days, the samples partially (LPS and PWM) or completely (ConA) lost the ability to respond as measured by ³H-thymidine incorporation (Figs. I-1a, I-1c, and I-1d), but the samples were still sensitive to PHA-P after 7 days' incubation (Fig. 1b). Viability examination with trypan blue dye exclusion after 7 days' incubation showed that most of the cells were still alive. It is possible that the nutrients in the culture medium were exhausted, or cell growth was stopped by inhibitors released from the cells.

Effects of Temperature

Incubation temperature is the most important variable affecting mitogen blastogenesis of fish lymphocytes (Faulmann et

al 1983). In general, a 24°C incubation temperature was best for all mitogens (Figs. I-2a, I-2b, I-2c, and I-2d). For hybrid bass, low temperature (17°C) reduced the response of ConA and PWM-stimulated cultures and abolished the response stimulated by PHA-P. Conversely, low temperature (17°C) significantly increased the stimulation by LPS. Stimulation by LPS was increased significantly at low temperature (17°C) in other experiments with different fish (Wang and Noga, Unpublished data). High temperature (30°C) reduced the stimulation by PWM to a lesser extent than low temperature (17°C), and low temperature (17°C) did not abolish the stimulation by ConA. PHA-P stimulation was not reduced by high temperature, but high temperature abolished the stimulation by LPS. Low temperature increased the ConA or PWM concentrations needed to reach the maximum response. These results agree with the hypothesis by Faulmann et al (1983), who found that low temperature immunosuppression in channel catfish may result from a preferential effect on T-like cells. In our studies, low-temperature abolished or reduced the response of T-like cells. In contrast, low temperature did not reduce the response of B-like cells significantly (Fig. 2c). In other experiments with different fish, higher SIs were found at 17°C than at 24°C (data not shown). It is tempting to speculate that low-temperature immunosuppression on T-like cells in Morone may result from a preferential effect on T helper-like (CD4) cells which are sensitive to PWM, since the effect of low temperature inhibition on T suppressor-like cells (CD8) (Fig. 2a), which are

sensitive to ConA, is less pronounced than the effect on T helper-like cells (CD4).

Optimal ConA Concentration for Different Lymphocyte Preparations

The optimal ConA concentration for whole blood cells was higher than that for splenocytes, whose optimal concentration was in turn higher than that for leucocytes separated by density gradient centrifugation (Figs. I-3, I-4a and I-4b). Competitive binding of ConA might explain the differences in optimal ConA concentrations for different leucocyte preparations. In whole blood, ConA may bind to plasma components and cells other than lymphocytes, which may cause a decrease in the amount of ConA available to bind to lymphocytes. This competitive binding might be overcome by increasing the concentration of ConA. For splenocytes, the chance of competitive binding of ConA should drop dramatically compared to whole blood since only about 40% of the cells were erythrocytes. For leucocytes separated by density gradient centrifugation, the competitive binding should be further decreased.

PBL Versus Splenocytes in Striped Bass and Hybrid Bass

Both PBL and splenocytes responded to ConA, but the responses of the two Morone strains varied with the tissue tested. The responses of striped bass PBL were significantly

higher than those of hybrid bass (Fig. I-4a) even though the hybrid bass had higher total cell counts, but the responses of striped bass splenocytes were significantly lower than those of hybrid bass with the same number of splenocytes. The total cell number for each PBL sample was counted but not adjusted, because we wanted to measure the PBL as a whole system. On average, there were over ten-fold more total cells in hybrid bass PBL than in striped bass PBL.

Published data on the spleen of striped bass or hybrid bass are limited (Groman 1982), and detailed investigations are lacking. Plaque-forming techniques have demonstrated that antibody-producing cells occur in the spleen of various teleosts (Fange & Nilsson 1985). Ontogenetic studies in salmon and trout suggest that the spleen does not play an important role during the development of the immune system (Van Muiswinkel et al 1991). This suggests that species differences may exist (Fange & Nilsson 1985). Because the function of Morone splenic leucocytes is poorly studied, the differences in blastogenesis between PBL and splenocytes requires further investigation.

In this pproject, we describe a whole blood culture assay using PBL of Morone. The culture conditions were optimized with PBL of hybrid bass and compared well with traditional assay methods. Preliminary experiments showed that whole blood culture also worked for striped bass. The data suggested that there may be significant differences in immune function between these genetically related groups.

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Table I-1. Mean counts per minute (cpm), standard deviation (S.D.), stimulation index (SI), and coefficient of variation (CV) for whole blood blastogenesis with hybrid bass PBL using serially diluted ConA.

Con A (ug/ml)	Fish 1 (cpm)	Fish 2	Fish 3	Fish 4	Mean±S.D.	SI	CV
0	462	1999	660	817	984± 692	1.0	70%
0.39	704	1700	2530	3007	1985± 1010	2.0	51%
0.78	582	2090	1387	1567	1406± 625	1.4	44%
1.56	1284	2341	957	2262	1711± 695	1.7	41%
3.13	4055	1549	1089	1726	2104± 1327	2.1	63%
6.25	44993	15912	5299	14081	20071±17248	20.4	86%
12.5	47482	44301	12115	20771	31167±17414	31.7	56%
25.0	25730	34450	13988	31963	26533± 9132	27.0	34%
50.0	37261	20228	4471	21850	20954±13403	21.3	64%
100.0	9331	9347	5279	9144	8279± 2003	8.4	24%

Figure I-1. SIs of whole blood samples from three hybrid bass that were stimulated with serially diluted ConA (a), PHA-P (b), LPS (c), or PWM (d) and incubated at 24°C for 3 days, 5 days, and 7 days, respectively, before ³H-thymidine labelling. Significant ($p < 0.05$) differences among treatment times are indicated with different letters (A, B). Treatments having the same letters (e.g., "AB" vs. "B") were not significantly different. SIs labelled with an "*" were significantly different from their negative control (no mitogen).

Figure I-2. SIs of whole blood samples from three hybrid bass that were stimulated with serially diluted mitogen and incubated for 3 days (ConA or PHA-P) or 5 days (LPS or PWM) at 17°C, 24°C and 30°C, respectively, before ³H-thymidine labelling. The effect of different temperatures was significant ($p < 0.05$) for all mitogens. Significant ($p < 0.05$) differences among treatment temperatures are indicated with different letters (A, B, or C). Treatments having the same letters (e.g., "AB" vs. "B") were not significantly different. SIs labelled with an "*" were significantly different from their negative control (no mitogen).

Figure I-3. SIs of leucocyte samples from three hybrid bass that were separated by density gradient centrifugation and stimulated with serially diluted ConA at 24°C for 3 days. Bar=S.E.M.

Figure I-4. a. SIs of whole blood samples from hybrid bass and striped bass that were stimulated with serially diluted ConA at 24°C for 3 days. Bar=S.E.M. b. SIs of splenocytes from hybrid bass and striped bass that were stimulated with serially diluted ConA at 24°C for 3 days. Bar=S.E.M.

Fig. I-1a

Fig. I-1b

Fig. I-1c

Fig. I-1d

Fig. I-2a

Fig. I-2b

Fig. I-2c

Fig. I-2d

Fig. I-3

Fig. I-4a

Fig. I-4b

RESEARCH SUB-PROJECT II

EFFECT OF ACUTE HYPOXIC STRESS ON IMMUNE FUNCTION

INTRODUCTION

Stress is a physiological response of the organism, induced by extreme levels of environmental chemical, physical and social factors (reviewed by Ellis, 1981; Pickering, 1981). Overcrowding, low levels of dissolved oxygen, handling elevated ammonia and nitrite concentrations which are often the products of intensive aquaculture and recirculating systems, were reported as stress inducers which affect the homeostatic regulation in fish (Wedemeyer and Mcleay, 1981; Thomas and Roberson, 1991; Tomasso, 1994; Yin et al, 1995). Increased plasma glucose, corticosteroid hormones, adrenaline and catecholamines were reported to be consequence of stress in different fishes, and their elevated concentrations have been linked to the impairment of the immune and circulatory functions, and consequently to disease resistance (Wahlquist and Nilsson, 1980; Tomasso et al, 1981; Nilsson, 1983; Mazik et al, 1991; Thomas and Roberson, 1991; Jeney et al, 1992; Tomasso, 1994 and Yin et al, 1995).

Overcrowding, low levels of dissolved oxygen, mainly when associated to excess of ammonia and nitrite, particularly in pond and recirculating aquaculture systems, were inculped as

immunosuppressors because of their stressory effects on cultured animals and of their prevalent correlation with epizootic outbreaks thus being a major cause of losses (reviewed by Tomasso, 1994).

It is well known that striped bass (Morone saxatilis) is easily affected by different stressing agents whereas its hybrid with the white bass (M. chrysops) is rather resistant (Kerby 1986). In the present work, some experimental stress procedures such as temperature modulations, anoxia and handling/bleeding were investigated in this hybrid in order to define a simple and accurate laboratory controlled stress model leading to measurable alterations of some physiological and immunological functions.

MATERIALS AND METHODS/RESULTS/DISCUSSION

Modulatory Effects of Different Stressing Procedures on Blood Glucose

The blood was checked for glucose using a glucose monitoring instrument (Accutrend GC, Boehringer Mannheim, Germany) and the above external symptoms of stress were recorded.

Anoxic Stress

Water was autoclaved for 20 min, cooled to $20 \pm 1^\circ\text{C}$ and poured into a twenty liter aquarium. Three healthy fish were bled (0.2

ml), moved to this water and watched until the appearance of external signs of stress (tegument darkness and slow movement) that occurred 90 minutes later. Then the fish were bled as above and blood glucose tested. Results showed a rapid elevation in the glucose level, starting from 52 ± 3.6 mg/ml before being exposed to stress, to 326 ± 20.8 mg/ml, after being stressed for 1.5 hours, only (Table II-1).

Temperature Stress

A group of 4 fish, after being acclimatized to $20 \pm 1^\circ\text{C}$, were abruptly moved to 13°C . Blood glucose was checked before stress and 24 hours after the beginning of the experiment. Results showed an increase from 54.25 mg/ml before stress to 338 ± 101.7 mg/ml after a shift to low temperature (Table II-1).

Handling and/or Bleeding Stress

Handling stress consist on the physical disturbance and exposition to air intoxication caused by handling the fish out of the water. This handling effect was tested in groups of 3-4 fish kept into 40x30x30 plastic floating cages at $20 \pm 1^\circ\text{C}$. The cages were pulled out of water, every 35 or 60 min for 60 sec during 4 or 7 hours, depending on the experiment. Sample of 0.2 ml blood were withdrawn without heparin from the caudal vessels of the fish before and after stress.

Linear Correlation between Stress Intensity and Glucose Level (Experiment 1)

Every 35 min, during a period of 4 hours, the fish (a group of 3) were handled out of water for 60 sec (this period includes a mean bleeding time of 40.1 ± 4.2 sec). A linear increase in glucose level, starting from 48.7 ± 4 to 333.14 ± 14 mg/ml $\pm$ SD, was recorded during the test period (Fig. II-1 and Table II-1, exp. 1). This linearity shows a direct correlation between stress intensity and glucose level. All the fish displayed darkness of teguments hyperventilation and slow movements.

Extreme Limits of Bleeding/Handling Stress

In order to test the extreme limit of bleeding/handling stress, a group of 4 fish were bled before the experiment and every 60 minutes they were held out of the water for 60 seconds during 7 hours as detailed above. The fish were bled a second time at the end of the experiment. Results showed a mean of 54.8 ± 9.3 mg/ml glucose before stress and 391.8 ± 32.8 mg/ml glucose at the end of the experiment. This seems to be the highest glucose level that could be reached, because stress periods higher than 7 hours resulted in the death of the fish. The external signs of stress in this group were more pronounced: mainly tegument darkness, hyperventilation and swimming at the water surface. Death started sporadically after a few hours

until all fish died within the next 36 hours, probably due to a secondary water mold infection (Table II-1, exp. 2-a).

Bleeding Influence on Stress Intensity

The above experiment was repeated as before but without the first bleeding at time 0. The glucose level (354.0 ± 56.8 mg/ml) at the end of this experiment was not significantly different from its former value ($p=0.150$) and similar external signs of stress were observed in the two groups (Table II-1, exp. 2-b). Stressing effect of the bleeding at time 0 alone was not significantly expressed after 7 hours when glucose levels at times 0 and 7 hours were compared ($p=0.161$), (Table II-1, exp. 2-c).

Influence of Handling Stress on Plasma and Tissue Lysozyme Activity

Calibration of Plasma Lysozyme Activity

Commercial lysozyme and a heat killed suspension of Staphylococcus aureus (Sigma) were used to perform a standard calibration curve as previously reported (Ellis et al. 1981). Briefly, lysozyme activity was checked in 1% agarose (Sigma) plates containing a suspension of 10^9 bacteria/ml. Wells of 3 mm in diameter were perforated in a 2.5 mm thick layer and 9 μ l of serum or tissue extract were added to the wells. Tissue extracts

were prepared by homogenizing tissue samples 1:4 (W/V) in phosphate buffer pH 6.2, 0.05M followed by centrifugation at 9861 g for 15 min. The plates were incubated at 20°C for 48h and the lysis diameter, D (= well diameter subtracted from total diameter) was measured.

A calibration curve performed with commercial lysozyme showed a linear activity between log lysozyme concentration (in ug/ml) and D in mm (Fig II-2a). The serum lysozyme activity was similarly tested and found as well linear with the D values (Fig. II-2a) in both stressed and non stressed groups of 3 fish each. The lysozyme activity in different plasma dilutions of stressed fish was significantly lower than in the non stressed fish (e.g. $p=0.01$, 0.002 and 0.04 for dilutions 1:1, 1:8 and 1:6, respectively) (Fig. II-2b).

Lysozyme Activity in Tissues

Lysozyme activity was as well checked in different tissues (spleen, anterior and posterior nephroses, and bloods of normal and stressed groups of fish ($n=4-6$) as detailed in Table II-2. While a significant decrease in enzyme activity ($p=0.0002$ and 0.0003 tested 7 and 24 h after the beginning of the stress experiment, respectively) could be confirmed, the enzyme concentration in all organs which showed no significant decrease (as judged by D value) after 7h, ($p>0.2$) increased considerably ($p<0.05$) after 24 h in spleen and posterior kidney but not in

anterior kidney.

Lymphocyte Proliferation in Normal versus Stressed Fish

Lymphocyte Proliferation of Periferal Blood Lymphocytes (PBL)

The PBL of 3 fish were separated ($5-10 \times 10^6$ mononuclear cells/ml) from blood of each fish on a Ficoll bed (Phamacia), seeded in triplicate in culture plates (Nunc, Denmark), PHA-stimulated and cultured as previously reported (Rosenberg-Wiser and Avtalion 1982). This culture was optimized for striped bass as follows: 1) the addition of 2% pooled bass serum (PBS) was found more efficient ($p=0.0002$) than 2% of pooled carp serum (PCS) which is currently in use for carp PBL cultures and higher but marginally significant ($p=0.09$), than 4% of this serum (Table 3). The addition of 2% PBS to cultures was used in the next experiments 2) the kinetics of PBL proliferation was performed at daily intervals and cultures were labeled with 0.4 uci H^3 -thymidine/well for 18 hours. Results pointed on days 4-5 as optimal for cell culture harvesting (Fig. II-3).

Influence of Anoxia Stress on PBL Proliferation

A group of fish, tagged 1-15, were used in this experiment. Fish 1-8 were submitted to anoxia stress as defined in experiment 2 (Table II-1) and fish 9-19 served as normal controls. H^3 -

thymidin uptake versus blood glucose level were checked in the two groups. Lymphocyte proliferation varied in normal fish from 39584 ± 1411 to 73105 ± 4325 cpm and in stressed fish from $80.5 \pm .52$ to 2064 ± 94 cpm. In contrast, blood glucose level showed an inversed profile where glucose values varied from 48 to 62 mg/ml in the normal fish and 600 to 280 mg/ml in the stressed fish (Fig. II-4).

Mixed Lymphocyte Reaction (MLR)

Two way MLR and stimulation index calculation were performed with a group of fish tagged 1-10 as previously reported (Caspi and Avtalion, 1984), but in the presence of 2% PBS. Fish 1-5 were submitted to stress as above (Table II-1, Exp. 2), and fish 5-10 served as normal controls. Fish of both groups were tested in couples (Table II-4). As expected, due to allogeneic divergence, results showed individual variations within couples and highly significant differences ($p=0.0002$) between the groups (2898 ± 1389 and 315.1 ± 185.2 in normal and stressed groups, respectively). Differences between groups were as well reflected in their stimulation index (SI) averages: 2.37 ± 1.4 in stressed versus 4.59 ± 1.4 in normal fish ($p=0.012$) pointing on the strong effect of stress on cellular immunity.

Antibody Production

Kinetics Study

Three fish were immunized against equine serum albumin (ESA) in emulsion with complete Freund's adjuvant, by intramuscular injection of 0.2 ml emulsion containing 0.2 mg antigen. The fish received a similar second injection on day 42. Serum specific antibody activity was checked once a week until the end of the experiment using ELISA titration as previously reported (Mor and Avtalion, 1990). Results showed primary response visualized after day 7 and peaking on day 28 ($\text{Log}_2=15.2 \pm 1.6$), and secondary peak ($\text{Log}_2=19 \pm 0.5$) occurring on day 49. Antibody degradation rate (half-life) of 10.1 days could be calculated from the slope occurring after the second peak (Fig. II-5).

Influence of Stress on Antibody Production

The main aim of this experiment was to check when during the process of antibody formation at the first 7 days the fish are the more susceptible to stress. For this purpose, 4 groups (A-D) of 3 fish each were subjected to light stress (60 min anoxia, applied twice with an interval of 4 hours), at days 1, 3, 6 or 9 after immunization, respectively. Another group (E) was subjected to chronic stress, namely the same schedule of stress but applied every one or two days during 21 days. Group F served as immunized

and non stressed control. Results showed that stress given 24 hours after immunization had the highest inhibitory effect on antibody production ($\text{Log}_2=5\pm 1.4$) in comparison to group F ($\text{Log}_2=13.8\pm 1.0$). A lesser decrease in antibody level ($\text{Log}_2=9.7\pm 1.5$ and 9.3 ± 1.2) could be seen in the groups stressed on days 6 and 9, respectively. Group E showed practically no antibody activity ($\text{Log}_2 < 2$, is considered as background), thus reflecting the complete inhibitory effect of chronic stress (Fig. II-6).

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Table II-1. Modulatory effect on blood glucose of different stressing processes.

	Glucose level, mg/ml $\pm$ SD		Student t-test p
	Time 0	4 hours	
1 Handling* or/and bleeding			
Exp. 1 (n=3)			
Handling and bleeding (every 35 min)	48.7 $\pm$ 4.0	333.14 $\pm$ 14.0	
Exp.2 (n=4)	Time 0	7 hours	
a. Handling every 60 min and bleeding at time 0	54.8 $\pm$ 9.3	391.8 $\pm$ 32.8	0.150
b. Handling every 60 min without bleeding at time 0	-	354.0 $\pm$ 56.8	
c. Bleeding at 0 time, only	47 $\pm$ 5.6	54 $\pm$ 10.4	0.161
	Time 0	1.5 hours	
2 Deaerated water (n=3)	52 $\pm$ 3.6	326 $\pm$ 20.8	
	Time 0	24 hours	
3 Temperature shift (from 20 to 13°C)	54.25 $\pm$ 7.8	338.0 $\pm$ 101.7	

*Handling = physical disturbance and exposition to air intoxication.

Table II-2. Lysozyme activity in tissues of stressed versus non stressed fish.

	Spleen		Posterior kidney		Anterior kidney		Blood	
	D	μ g/ml	D	μ g/ml	D	μ g/ml	D	μ g/ml
Non stressed ctrl	10.7	0.32	19.2	100	12	0.8	22.75	1100
SD (n=6)	$\pm$ 1.2		$\pm$ 1.47		$\pm$ 0.41		$\pm$ 0.88	
Stressed, after 7h	11.7	0.68	20.1	210	11.3	0.4	19.9	180
SD (n=6)	$\pm$ 0.82		$\pm$ 0.92		$\pm$ 0.83		$\pm$ 0.66	
p	0.203		0.335		0.303		0.0002	
Stressed, after 24h	13.5	1.13	22.6	1060	12.75	1.3	16.6	40
SD (n=4)	$\pm$ 1.47		$\pm$ 1.25		$\pm$ 0.96		0.75	
p	0.036		0.013		0.339		0.0003	

Table II-3. Optimization of serum supplement.

Serum	CPM $\pm$ SD	Stud. t-test
PCS 2%	8286.7 $\pm$ 6136	p =
		0.0002
PBS 2%	41269.7 $\pm$ 12856	
		0.09
PBS 4%	35020.0 $\pm$ 7692	

Table II-4. Two-way MLR.

Stressed fish						Non-stressed fish					
cpm $\pm$ SD						cpm $\pm$ SD					
crossed fish	MLR	self cult.	self	SI		crossed fish	MLR	self cult.	self cult.	SI	
1	2	199 $\pm$ 21	192 $\pm$?	142 $\pm$ 12	1.19	6	7	5353 $\pm$ 215	218 $\pm$ 263	1174 $\pm$ 432	7.69
1	3	233 $\pm$ 63		151 $\pm$ 1	1.35	6	8	2414 $\pm$ 271		504 $\pm$ 39	6.68
1	4	238 $\pm$ 0		84 $\pm$ 12	1.72	6	9	1438 $\pm$ 124		752 $\pm$ 274	2.96
1	5	250 $\pm$ 48		104 $\pm$ 0	1.75	6	10	1704 $\pm$ 132		200 $\pm$ 35	8.15
2	3	329 $\pm$ 125	142 $\pm$ 0	151 $\pm$ 1	2.24	7	8	3201 $\pm$ 261	1174 $\pm$ 4	504 $\pm$ 39	3.81
2	4	536 $\pm$ 0		84 $\pm$ 12	4.7	7	9	4203 $\pm$ 499		752 $\pm$ 274	4.36
2	5	272 $\pm$ 0		104 $\pm$ 0	2.21	7	10	4588 $\pm$ 1142		200 $\pm$ 35	6.67
3	4	746 $\pm$ 56	151 $\pm$ 1	84 $\pm$ 12	6.3	8	9	2612 $\pm$ 348	504 $\pm$ 39	752 $\pm$ 274	4.15
3	5	142 $\pm$		104 $\pm$ 0	1.11	8	10	1508 $\pm$ 345		200 $\pm$ 35	4.28
4	5	206 $\pm$ 101	84 $\pm$ 12	104 $\pm$ 0	2.19	9	10	1969 $\pm$ 470	752 $\pm$ 274	200 $\pm$ 35	4.13
Mean		315			2.37			2898			4.6

*, p=0.012; **, p=0.0002

Figure II-1. Effect of various periods of stress on blood glucose concentrations in hybrid bass.

Figure II-2. a. Standard curve for the lysozyme radial diffusion assay using commercial lysozyme. b. Plasma lysozyme concentration in stressed and unstressed hybrid bass using the radial diffusion assay.

Figure II-3. Effect of incubation time on mitogen blastogenesis response in hybrid bass peripheral blood lymphocytes.

Figure II-4. Effect of anoxic stress in hybrid bass on blood glucose and mitogen blastogenesis responses.

Figure II-5. Primary and secondary serum antibody response of hybrid bass to immunization (arrows).

Figure II-6. Effect of stress on serum antibody titers in hybrid bass.

Fig. II-1

Fig. II-2a

Fig. II-2b

Fig. II-3

Fig. II-4

Fig. II-5

Fig. II-6

RESEARCH SUB-PROJECT III

PATHOPHYSIOLOGY OF ACUTE STRESS IN STRIPED BASS AND HYBRID BASS

INTRODUCTION

Acute stress is often accompanied by the development of an infectious disease epidemic in fish culture. These epidemics are typically associated with various opportunistic infections, especially certain bacteria and fungi. The opportunistic nature of these pathogens suggests that certain forms of acute stress are accompanied by severe immunocompromise. A number of systemic changes in physiology and immune function have been reported in fish exposed to various forms of acute stress. Since the skin is often a primary target organ for these opportunistic infections, it is likely that local as well as systemic immunosuppression may occur.

The striped bass (Morone saxatilis) is considered to be one of the most sensitive fish species to environmental stress (Kerby 1986). The closely related hybrid bass is considered to be less stress-prone. In previous studies, we observed that striped bass exposed to an acute, short-term (45 min) confinement stress rapidly developed opportunistic microbial infections on the fins in less than 24 hr after the stress (Noga et al 1994). The purpose of the present paper was to characterize the

pathophysiological changes associated with acute confinement stress to begin to elucidate the mechanisms responsible for this very rapid immunosuppression. We also wished to determine the relative resistance of striped bass versus hybrid bass to this stress.

MATERIALS AND METHODS

Test Fish

Striped bass (Morone saxatilis, Roanoke River strain) and hybrid bass (M. saxatilis female x M. chrysops male) were maintained in a flow-through freshwater system. Fish used for experiments were of a similar age and size. Fish were fed a commercial pelleted feed twice daily.

Confinement System

All test fish were acclimated to a 10,000-liter holding tank with flow-through well water for at least 2 months prior to the initiation of the experiment. At the start of the experiment, 10-12 fish of each strain were quickly removed from the holding aquarium and placed into individual 20-liter glass test aquaria having 10 liters of water. An anesthetic overdose (200 mg/l tricaine buffered with 400 mg/l sodium bicarbonate) was added to fish in 11 of the test aquaria (5 hybrid and 6 striped bass) and

the anesthetized fish were sampled as described below. The remaining 5-6 fish of each strain were left in their test aquaria, which were each supplied with a constant flow (2 liters/min) of well water. During the confinement, dissolved oxygen was 6.8-7.5 mg/l, temperature was $19 \pm 1^{\circ}\text{C}$, pH was 6.65-6.87, unionized ammonia was <0.001 mg/l and nitrite was <0.10 mg/l. These conditions were the same as those in the holding tank. After 2 hr of confinement, an anesthetic overdose was added to all test aquaria and fish were sampled as described below.

Sampling Regimen

All fish in the unstressed group were bled via the caudal vessels within 3 min of being removed from the 10,000-liter holding tank using a heparinized, 22 GA, 1" needle and 3 cc syringe. Immediately after a fish was bled, a culture was taken from the caudal fin and spread on a Columbia blood agar plate using a Mini-tip Culturette (Marion Scientific, Kansas City, Missouri). After sampling for bacteria, representative areas of the body (ventrum and flank) and fins (caudal, dorsal, anal, left pectoral) were fixed in 10% neutral buffered formalin. The right pectoral fin was fixed in McDowell and Trump's fluid (McDowell and Trump 1975).

Evaluation Procedures

Microbiology

Blood agar plates were incubated at room temperature and the total number of bacterial colonies was counted after several days in order to estimate the number of bacteria present.

Histopathology

For light microscopy, tissues were decalcified in EDTA, embedded in paraffin, and processed routinely for light microscopy. Sections were stained with hematoxylin and eosin. All slides were evaluated blindly for lesions, based upon a scoring scale of 1-5 with 1 being minimal damage, 2 being slight/mild damage, 3 being moderate damage, 4 being moderately severe damage and 5 being severe/high damage.

Lysozyme Assay

The blood was centrifuged at 13,500 x g for 10 min and the plasma was stored at -70°C until tested for lysozyme and clinical chemistry. Lysozyme was measured using the turbidometric assay. This method was run as described by Parry et al (1965) with modifications. Briefly, Micrococcus luteus was dissolved in 0.045 M phosphate buffered saline (PBS) at pH 6.35. The final

concentration of M. luteus was 300 ug/ml. Substrate suspension (985 ul) was mixed with 15 ul of fish plasma samples in a cuvette (Fisher). Hen egg white lysozyme (HEWL, Sigma #L-6876) solution in PBS (pH 7.3) was used as a positive control. Each cuvette was briefly vortexed and the decrease in absorbance (ΔA) per minute was recorded with a spectrophotometer at 600 nm at 25°C. The optical density (OD) at the 30th second and 90th second were used for calculating enzyme activity.

The test for all lysozyme samples was run in duplicate. For the assay, 1 unit of lysozyme activity was defined as giving an absorbance change of 0.001 per minute.

Clinical Chemistry

Major electrolytes (Na, K, Cl, TCO_2) and glucose were measured using an automated chemistry analyzer (Monarch Plus Instrumentation Laboratory, Lexington, Massachusetts). Anion gap was calculated as follows: $(\text{Na} + \text{K}) - (\text{Cl} + \text{TCO}_2)$. Osmolality was determined using freezing point depression (Precision System Model 5004, Sudbury, Massachusetts).

Statistical Analysis

Differences between treatment groups were examined using a paired t-test on the Microsoft Excel 5.0 program. Paired samples were first analyzed to determine if the variances were different

and then the appropriate analysis was performed. Values were considered significant at the $P < 0.05$ level.

RESULTS

Response of the Skin to Acute Stress

Striped bass exposed to a 2 hr acute confinement developed severe epithelial damage, especially on the caudal, dorsal and pectoral fins (Figs. III-1,2). Lesions began on the distal edge of the fins and, during the period of confinement, were often observed to progress towards the base of the fin. The affected, distal area was characterized by epithelial degeneration in which the basilar layers of the epidermis contained ballooned cells with nuclear debris. There were varying degrees of epithelial erosion and ulceration, which was most severe at the distal portion of the fins. Ulceration was associated with submucosal edema and necrosis of the remaining stromal tissue and tips of bone in the fin rays. There was also dermal depigmentation with degranulation of melanophores. There was no hemorrhage or thrombosis to suggest any obvious vascular derangement. None of these lesions were seen in control (unstressed) striped bass. No lesions were seen on the skin of the flank or the ventrum, although trichodinid ciliates were occasionally seen on some sections from both stressed and unstressed fish. No trichodinids were seen on any fin sections.

Hybrid bass exposed to the acute confinement developed similar lesions, but the severity of the response was considerably less (Figs. III-2,3). None of these lesions were seen in control (unstressed) hybrid bass. No lesions were seen on the skin of the flank or the ventrum, although trichodinid ciliates were occasionally seen on some sections. No trichodinids were seen on any fin sections.

Bacterial Flora

Semi-quantitative analysis of the bacterial flora of the caudal fin revealed no increase in the number of bacteria present on the fin after the acute stress. Both unstressed and stressed skin had very few bacteria (striped bass unstressed 29 colonies; striped bass stressed 15 colonies; hybrid bass unstressed 11 colonies; hybrid bass stressed 4 colonies) (means of 5-7 fish per group).

Lysozyme Response

Striped bass exposed to confinement exhibited significantly higher lysozyme levels than unstressed striped bass (Table III-1). There was no significant difference between stressed and unstressed hybrid bass.

Clinico-Pathological Response

There were significant changes in electrolyte balance of both stressed striped bass and hybrid bass, including increased osmolality and potassium and decreased plasma chloride (Table III-1). There was a significant increase in plasma glucose.

DISCUSSION

The most striking pathophysiological change associated with the severe, acute, confinement was severe skin damage on the fins. The damage does not appear to be caused by either infectious agents or trauma, but rather appears to be due to an inherent "shock" response. The very rapid development of these lesions (within 2 hr), combined with the absence of any microscopic or culture evidence of infection, rules infection out as a possible cause. The presence of trichodinids was an incidental finding and not even present on areas where lesions developed. The potential influence of trauma is unlikely. Fish were confined individually in glass aquaria and not exposed to any abrasive surfaces during confinement. We closely observed all fish during confinement and often saw a rapid expansion of these lesions, especially on the caudal and pectoral fins. Furthermore, all fins examined were affected to some degree, even the dorsal fin. However, there was no evidence of damage to the

skin on the ventrum, which had at least as great a contact with the aquarium surfaces. In general, striped bass had more severe lesions than hybrid bass, although there was considerable individual variation (Fig. III-2). Hybrid bass are typically much less stress-prone (Noga et al 1994, Kerby 1986).

In previous studies, we have observed a high degree of rapidly developing (within 24 hr) opportunistic infections in bass that were exposed to an acute confinement stress (Noga et al 1994). Harms et al (1996) observed thrombosis and severe electrolyte imbalance in response to a clinical syndrome referred to as "red tail" in captive striped bass. The Harms study exposed fish to multiple episodes of acute confinement stress over a period of 3 days; "red tail" may be a chronic sequela of the acute epithelial necrosis which we observed in our study. Chronic skin ulceration could lead to severe osmotic imbalance, vascular damage and subsequent thrombosis.

The reason for the specificity of the acute confinement response for the fin epithelium rather than the body epithelium is unknown. We have also recently observed severe epithelial damage to striped bass exposed to a toxic algae, Pfiesteria piscicida. The latter results are the first time that such pathology has been observed in fish exposed to a toxin (Noga et al 1996). Paradoxically, in the studies of exposure to Pfiesteria piscicida, we found that skin ulceration affected the body more severely than the fins (Noga et al 1996). We do not yet know if the algae is directly toxic to the skin or if this is

some type of pathophysiological response (Noga et al 1996). Nonetheless, different stressors apparently lead to different and quite dramatic adverse epithelial responses in fish.

The type and length of stress which we used in our study is very similar to that used in many other studies of stress (eg., Pottinger et al 1992, Davis and Parker 1986). These studies have been intended to mimic conditions that are routinely encountered in aquaculture. For example, Strange and Cech (1992) held 500-1,600 mm striped bass in a 20 liter net for up to 35 min to study swimming performance after stress. Carmichael et al (1984) held groups of 1,300-2,300 mm largemouth bass (Micropterus salmoides) in nets that made them close enough to be in constant contact with their tankmates for up to 48 hr.

Acute confinement stress is often used to examine the physiological effects of stress in fish. Among the most well studied of these are stress hormones (eg., cortisol), glucose and electrolyte balance. Depression of plasma electrolyte concentrations (i.e., osmolality, sodium, potassium, chloride) in freshwater fish has been hypothesized to be due to gill osmoregulatory dysfunction. But, the skin is a major barrier to fluid movement and any significant epithelial loss causes rapid changes in electrolytes.

The increase in lysozyme activity in stressed striped bass is similar to the results obtained by Mock and Peters (1990), who found increased plasma lysozyme levels in rainbow trout exposed to a 30 min confinement stress. The clinical chemical response

of both strains of fish to confinement was similar, and included imbalances in glucose, osmoregulation and electrolyte balance. As expected, glucose was significantly elevated after stress. There was also significantly higher osmolality after stress in both striped and hybrid bass. There was also significantly higher plasma potassium in both strains and significantly lower chloride in striped bass. These results are similar to those reported previously by others for both striped bass and hybrid bass (Davis and Parker 1990, Tomasso et al 1980) as well as other fish species (Carmichael et al 1984) after an acute confinement stress.

We have now found that hybrid bass differ in several ways from striped bass in their tolerance of acute confinement stress. This includes plasma cortisol dynamics (Noga et al 1994) and lysozyme levels. In addition, the amount of skin damage is significantly different. While the importance of some of these changes is unclear in terms of their impact on the fish's homeostasis and defensive capabilities, the epithelial ulceration which we observed is certainly a serious insult, which may be severe enough in itself to be life-threatening, as well as leading to lethal secondary microbial infections.

The results of these studies indicate that we need to re-examine the pathophysiology of stress-associated skin disease in fish. The skin is the most common target organ for infectious agents in fish and numerous disease complexes, such as "fin rot" and "tail rot", have been described from many species. The

previously accepted hypothesis regarding development of most acute skin ulcers is that an infectious agent (i.e., bacterium, fungus, parasite) initiates the lesion and causes an ulcer to develop. This might be preceded by some type of physical or chemical trauma, which initially damages the skin. Our data indicate that, at least in some cases, the ulceration develops without prior trauma and before any infectious agent invades the skin.

In our preliminary studies of epithelial ulceration, when fish were exposed to water from the holding tank during the acute stress, numerous long, thin, bacterial rods (presumptively a Cytophaga sp.) became attached to areas of ulcerated skin immediately after the confinement. The bacteria originated from floating microcolonies in the holding tank, which were subsequently transferred to the aquaria having the test fish. When experiments were performed using new well water (i.e., water not run through the holding tank), bacteria were never seen on the skin or in the water (Noga, Unpublished Data). This indicates how rapidly the freshly ulcerated areas can be colonized by opportunists and provides direct evidence of how bacterial load in the water may have an important influence on the development of an infectious disease outbreak (Wedemeyer et al 1976).

These results have important implications for stress management in aquaculture, since identifying the physiological basis for the acute epithelial response may lead to ways of

preventing many infectious diseases in aquaculture populations. Inhibiting the physiological signals which lead to this response may reduce morbidity and mortality, especially during stressful events such as confinement, which are often followed by epidemics of opportunistic infections (Noga 1996).

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Table III-1. Clinical chemical values for striped bass and hybrid bass before and after an acute stress.

Group (N)	Glucose (mg/dl)	Osmolality (mOsm/kg)	Na (mEq/l)	K (mEq/l)	Cl (mEq/l)	Lysozyme (U/ml)
SU (6)	100a	335a	180a	3.85a	143a	22a
SS (6)	406b	375b	179a	4.70b	137b	29b
HU (7)	100a	356a	174a	3.30a	144 ^a	33a
HS (5)	333b	387b	176a	5.14b	137b	40a

Test groups: SU - unstressed striped bass, SS = stressed striped bass, HU = unstressed hybrid bass, HS = stressed hybrid bass.

Values with different letters within the same fish strain are significantly different from each other ($P < 0.05$).

Figure III-1. Representative caudal fins of striped bass and hybrid bass exposed to a 2 hour confinement stress. Top left = unstressed striped bass, top right = stressed striped bass, bottom left = unstressed hybrid bass, bottom right = stressed hybrid bass. Note the depigmentation associated with severe ulceration in stressed striped bass fin. Bar = 20 mm.

Figure III-2. Range of responses in the pectoral fins of striped bass (top 6 fins) and hybrid bass (bottom 5 fins) exposed to a 2 hour confinement stress. Note that depigmentation and ulceration varies with individuals. Bar = 15 mm.

Figure III-3. Incidence of fin ulceration in stressed striped bass and hybrid bass. N = 5-6 fish per group.

COOPERATION

During the course of this project, the Israeli and U.S. collaborators developed a close working relationship which resulted in significant sharing of information between the two groups. This not only facilitated the tasks of the present project, but a mutual understanding of the problems facing the respective countries in aquaculture and marine science-related areas was achieved which will facilitate mutually beneficial cooperation in the future.

In October 1995, Dr. Noga visited Dr. Avtalion's laboratory for about one week. During this time, the investigators discussed the results to date of the project and worked on the plans for completion of the remaining phases of the work. During this visit, Dr. Noga also visited I.O.L.R. in Eilat where he gave a seminar and consulted with I.O.L.R. scientists about possible future research directions. This was a follow-up to the meeting "Collaborative Research in Agricultural Biotechnology at Universities and Research Institutes in North Carolina and Israel held on August 1995 and attended by Dr. Noga (N.C.S.U.), Dr. Levy (N.C.S.U.) and Dr. Hillel Gordin (I.O.L.R.), where a formal linkage was established between North Carolina State University and Israeli scientists to further enhance ongoing cooperative research between these respective institutions. At that time, a proposal was written that drew on some of the information learned in the present BARD project to address important bacterial

disease problems affecting the aquaculture industry in the U.S. and Israel. This proposal, a joint effort between Bar-Ilan University (Dr. Avtalion), I.O.L.R. (Drs. H. Gordin, A. Colorni, A. Diamant and W. Knibb), and N.C.S.U. (Drs. Levy, Noga, G. Lewbart and C. Sullivan), is pending.

EVALUATION OF RESEARCH ACHIEVEMENTS

Overall, the results of our research: 1) may explain the increased disease resistance of hybrid bass compared to striped bass, including inherent differences in immune response as well as differences in modulation of that response by stress, 2) provide information needed for the rational development of vaccines for use in Morone and 3) can be used as sensitive tools for health monitoring in both wild and cultured stocks.

During this project, we developed immunodiagnostic reagents for monitoring the health of Morone (striped bass and hybrid striped bass), which are increasingly becoming very commercially valuable species in both the U.S. and Israel. These reagents are readily applicable to health monitoring for many diseases, including bacterial, viral and parasitic infections. The use of such tools is widespread in other areas of veterinary medicine, such as poultry, where they have gained widespread acceptance due to their usefulness in maintaining herd or flock health.

We also characterized Morone immunoglobulin and produced a highly specific antibody in rabbits. We then characterized the

immune response of Morone and showed that fish produced a significant immune response to injection of antigen and was detectable using antibody ELISA. Most importantly, we showed that chronic stress caused severe and virtually complete depression of this antibody response. This has important implications for the use of practical vaccines, since it indicates that stress may totally abrogate the effectiveness of these preparations.

We found that hybrid bass differ in several ways from striped bass in their tolerance of acute stress. This includes plasma cortisol dynamics, resting lysozyme levels, stress lysozyme levels, plasma osmolality and electrolyte balance and glucose.

The most striking pathophysiological change associated with severe, acute, stress is severe skin damage on the fins, which is also more severe in striped bass than in hybrid bass. While the importance of many of these changes is unclear in terms of their impact on the fish's homeostasis, the epithelial ulceration which we observed is certainly a serious insult, which may be severe enough in itself to be life-threatening, as well as leading to lethal secondary microbial infections.

The results of our studies indicate that we need to re-examine the pathophysiology of stress-associated skin disease in fish. The skin is probably the most common target organ for infectious agents in fish and numerous disease complexes, such as "fin rot" and tail rot" have been described from many species.

At times, these lesions appear sterile. However, the accepted hypothesis regarding development of most acute skin ulcers is that an infectious agent (i.e., bacterium, fungus, parasite) initiates the lesion and causes an ulcer to develop. Our data indicate that, at least in some cases, the ulceration develops before any infectious agent invades the skin.

These results have important implications for stress management in aquaculture, since identifying the physiological basis for this response may lead to ways of preventing many infectious diseases in aquaculture populations. Inhibiting the physiological signals which lead to this response may reduce morbidity and mortality, especially during stressful events such as confinement, which are often followed by epidemics of opportunistic infections. Chronic fin rot is a very serious problem in aquaculture. Whether similar mechanisms are responsible for such lesions definitely needs investigation.

CONCLUSIONS

Our studies have shown that striped bass are much more immunologically and physiologically sensitive to stress than the closely related hybrid bass. These data provide information that will be useful in understanding why these commercially and ecologically important fishes become sick. It will also provide the basis for developing methods for monitoring the health of these fish populations and determining when appropriate

interventional strategies (eg., decrease feeding, lower density, prophylactic medical treatment, etc.) will need to be implemented to avert losses from disease. This information is applicable not only to Morone but to other fish species as well.

PUBLICATIONS

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