Effect of handling stress on susceptibility of channel catfish *Ictalurus punctatus* to *Ichthyophthirius multifiliis* and channel catfish virus infection

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Received 16 March 2002; received in revised form 22 July 2002; accepted 24 July 2002

Abstract

A quantitative bioassay employing immersion exposure was developed for the infection of channel catfish *Ictalurus punctatus* with the protozoan parasite *Ichthyophthirius multifiliis*, commonly referred to as ich. This bioassay as well as waterborne challenge of channel catfish with channel catfish virus (CCV) was used to investigate the effect of confinement stress on the sensitivity of the fish to exposure of these pathogens. Infestation by ich was shown to be proportional to the density of infective theronts in the exposure tank and low-water crowding stress was shown to increase susceptibility of catfish to infection. Mortality from CCV was related to the virus exposure dose; however, low-water crowding stress did not affect mortality. Increased susceptibility, due to crowding stress of naïve channel catfish to *I. multifiliis* but not to CCV, suggests a difference in the defence mechanisms. Stress-induced increased susceptibility to *I. multifiliis* may be due to a suppression of an innate protection mechanism. The lack of effect of stress on CCV mortality may be due to protection afforded by an inducible system which was not affected by the stressor, or the lethal effects of the virus were too fast for the stress to change susceptibility in fish exposed to CCV for the first time.

Published by Elsevier Science B.V.

Keywords: Channel catfish; Ich; CCV; Stress

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1. Introduction

Ichthyophthirius multifiliis is an obligate protozoan parasite that infects the external surfaces of freshwater fish, penetrates the epithelium, and causes a disease known as ichthyophthiriasis. Both the parasite and the disease are commonly referred to as ich. Infections of ich occur during the spring and fall when the water temperatures are between 18 and 25 °C. Ich is capable of killing large numbers of fish in a short time. Approximately 4% of annual losses to farmers of channel catfish are attributed to protozoan diseases and ich is the most commonly reported protozoan pathogen (Durborow et al., 1998). The relative susceptibility to ich has been difficult to quantitate in fish, particularly by immersion exposure. Channel catfish virus (CCV) causes an acute haemorrhagic disease in channel catfish fry and fingerlings (Buck, 1990; Plumb, 1977). This herpesvirus infects by entrance through the gills and causes lytic destruction in most tissues. Most problems with CCV are found in small fingerling fish and are most common when the temperature is 28 to 30 °C. Outbreaks of ich and CCV are thought to be more likely when fish are stressed; however, the mechanism of protection with respect to these two pathogens is likely different (Ellis, 1981). The methods of infection are different since trophonts of I. multifiliis burrow through the mucus and penetrate the epithelium whereas CCV enters the body through the gills.

Aquacultural conditions and practices, including exposure of fish to poor water quality (Tomasso et al., 1981a,b), exposure to xenobiotic toxic molecules (Schlenk et al., 1999; Griffin et al., 1999), and handling (Davis et al., 1993), induce stress which is characterized by hormonal changes and metabolic and osmoionic disturbances. A number of physiological responses, including increases in plasma cortisol and glucose and decreases in plasma electrolytes, have been used to characterize the degree of stress the animal experienced. An increase in plasma cortisol is thought to be particularly important due to the immunosuppressive action of this hormone (Tripp et al., 1987; Maule et al., 1989). Channel catfish have both non-specific and specific systems for protection against pathogenic organisms. The mechanisms of protection from external parasites and from virus infection are likely to be different and the protection may also differ between an initial and a second exposure. The studies presented here were done to determine the sensitivity of channel catfish to initial ich and CCV exposures and to determine if the sensitivity was altered in fish stressed by confinement prior to exposure to the pathogens.

2. Materials and methods

2.1. Validation and development of a bioassay for exposure to ich

An active culture of I. multifiliis was maintained by placing uninfected fish in 60-l aquaria with infected fish. The original infection came from a large fingerling channel catfish from stocks held at the Harry K. Dupree-Stuttgart National Aquaculture Research Center. The culture was renewed periodically when a loss of virulence was observed after several generations of the parasite. For quantitative studies, newly exposed fish were
observed until mature trophonts were evident. Mature trophonts were gently dislodged from infected fish with a soft bristle brush into a container of aerated fresh water. Trophonts were held for 22 h at the acclimation temperature (22° to 24 °C) during which time the trophonts developed into tomites and then released as the infective stage called theronts. Theront concentration (or density) was determined by counting the number in 10 separate 2.5-μl volumes with a dissecting microscope at 10 × magnification, calculating the average, and multiplying by 400 to determine the number of theronts per milliliter. Challenge exposures were prepared by adding a volume of this stock preparation needed to obtain the desired number of theronts/liter of water. Water flow was stopped for 15 min during exposure, then flow was resumed at a rate of 0.5 l/min.

Exposed fish were visually inspected daily until mature trophonts were observed. The fish were then killed by blunt trauma to the head or pithing and the fish were placed in 10% formalin. Trophont density was determined by averaging the number of trophonts counted in four randomly selected cm² areas with a template and reported as the number of trophonts per cm². Body locations for trophont counts excluded the fins, head and belly. Infections were fairly uniform over the body.

Preliminary exposure tests were carried out to determine the effect of anesthesia with MS222 (Argent Chemical Laboratories, Redmond, WA) compared to blunt trauma or pithing on the temporal stability of the counts. Ten fish, of approximately 100 g, were exposed to 10,000 theronts/l and trophonts were allowed to develop for 6 days. Trophonts on three non-fixed fish were counted immediately, and the remaining fish were killed by blunt trauma or pithed and placed in 10% formalin. Trophonts on three fish were counted immediately, returned to formalin and recounted 4 and 8 days later. Fresh counts were compared to fixed counts. Trophonts on four fish were counted for the first time on the eighth day after fixation.

The relationship between theront density and subsequent trophont development was determined in ten 16-l aquaria with 20 °C flowing well water (0.5 l/min). Each tank was stocked with eight fish and acclimated for 7 days. The fish were fed a 36% protein commercial feed (Farmer’s Choice, ARKAT feeds, Dumas, AR) at 1% of the body weight each day. Fish in duplicate tanks were exposed to 0, 1.25, 2.50, 5.00 or 10.00 kilotheronts/l. After 7 days, the fish were killed by blunt trauma and fixed in formalin, and trophonts counted.

2.2. Confinement-induced stress and infection by ich

The effect of stress on susceptibility to I. multifiliis was determined in eighteen 16-l aerated aquaria with 24 °C flowing well water (0.5 l/min). Each tank was stocked with 12 fish (49.4 ± 14 g, mean ± SD) and acclimated for 7 days. Fish were fed at approximately 1% body weight every other day. Nine tanks of fish were exposed to 2500 tomites/l for 15 min; of these, three tanks were exposed without low-water stress. A low-water crowding stress was created in the other tanks by lowering the standpipe to reduce the tank volume to 4 l. Fish remained submerged but were unable to maintain their normal orientation. Fish in three tanks were stressed for 2 h and those in the remaining three tanks for 6 h. Stress treatments were started prior to the parasite exposure and were coordinated so that all treatments were simultaneously exposed to the parasite at the same age of develop-
ment. The second set of nine aquaria was used to determine the physiological changes due to the low-water confinement stress. Six fish were removed from each tank, anesthetized with MS222, and a blood sample was taken from the caudal vessels with a heparinized syringe. The remaining six fish in each aquaria were sampled after 2 or 6 h of low-water confinement. Blood samples were centrifuged and the plasma frozen for later analysis.

2.3. Analytical procedures

Plasma cortisol concentrations were analyzed by radioimmunoassay (RIA) using the BioChem ImmunoSystems Cortisol Bridge kit (# 14394, Polymedco, Cortlandt Manor, NY). This kit has not been previously validated for channel catfish. Recovery estimates were performed with channel catfish plasma from unstressed fish spiked with five replicates of 5, 50, and 500 ng/ml cortisol standards added to one-half of the volume for the unstressed plasma to give 2.5, 25, 250 ng/ml plus one half the cortisol concentration of unstressed fish. Concentrations of cortisol measured in the unstressed plasma and the spiked samples were 4.5, 11.4, 58.7 and 542 ng/ml respectively. Dilution experiments were done by 1:1 and 1:3 (plasma:0 standard) dilutions of plasma from stressed channel catfish. Concentrations of cortisol measured from diluted samples were 35.9 (undiluted), 18.0, and 9.9 ng/ml, respectively. Within assay variation was determined on 10 replicate determinations of a plasma pool from unstressed fish and fish stressed in a net for 2 h. The mean and standard deviation of the unstressed fish were 4.5 \pm 0.25 and 35.9 \pm 1.02 from the stressed fish with a coefficient of variation of 5.5% and 2.8%, respectively.

Plasma glucose was determined by the hexokinase reaction (Sigma Diagnostics No.115-A, St. Louis, MO). The coefficient of variation for analysis over 11 consecutive days of a normal serum pool was 3.2% as reported by the manufacturer. Plasma chloride was measured with a Corning 925 chloride analyzer.

2.4. Confinement-induced stress and mortality due to CCV

The effect of stress on susceptibility of channel catfish to CCV was determined with channel catfish fry. Randomly selected fish approximately 4 weeks old (0.864 \pm 0.360 g, mean \pm SD, \(n=40\)) were stocked into each of thirty-six 16-l aquaria supplied with heated well water held at 28–29 °C and fed at approximately 5% of the body weight per day for 4 days. On the fifth day, the fish were challenged with 0, 3.3 \times 10^4, 3.3 \times 10^5 or 3.3 \times 10^6 plaque forming units (PFU) of CCV. Water flow was halted, the volume of the tanks was reduced to about 400 ml and the appropriate dose of a CCV culture was delivered into the tank, and exposure was continued for 30 min. Six tanks were exposed to each of the concentrations of CCV. Fish in three tanks in each of the four treatment groups were stressed by low-water exposure. Three tanks in each treatment group were exposed to low water confinement stress for 0, 2 or 6 h before exposure to the virus. Each virus treatment and each stress treatment was done in triplicate. Mortality was recorded each day for 14 days with day one as the day of exposure.
2.5. Statistical analysis

The relationship between exposure to theronts and trophont development was analyzed by linear regression. Ich infestation, mortality due to CCV, and plasma characteristics were analyzed by analysis of variance followed by Tukey’s multiple range test when significance \( P<0.05 \) was indicated (Statistix for Windows, 1996).

3. Results

3.1. Bioassay for exposure to ich

When fish exhibiting trophonts were anesthetized with MS222 before being placed in formalin, the trophonts were shed from the fish. Shedding did not occur after blunt trauma or pithing; therefore, blunt trauma was used for all subsequent experiments. Trophont counts were very consistent regardless of the time after fixation, which demonstrates that counts were not affected by time in the fixative (Table 1). It should be noted that rough

<table>
<thead>
<tr>
<th>Fish No.</th>
<th>Trophont infestation (number/cm²)</th>
<th>Day trophont count was made relative to fixation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>18</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>12.75</td>
<td>4</td>
</tr>
<tr>
<td>2</td>
<td>14.5</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>14</td>
<td>4</td>
</tr>
<tr>
<td>3</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>4.5</td>
<td>4</td>
</tr>
<tr>
<td>4</td>
<td>14.75</td>
<td>4</td>
</tr>
<tr>
<td>4</td>
<td>14.5</td>
<td>8</td>
</tr>
<tr>
<td>5</td>
<td>8.5</td>
<td>4</td>
</tr>
<tr>
<td>5</td>
<td>8.75</td>
<td>8</td>
</tr>
<tr>
<td>6</td>
<td>14</td>
<td>4</td>
</tr>
<tr>
<td>6</td>
<td>16.25</td>
<td>8</td>
</tr>
<tr>
<td>7</td>
<td>16.25</td>
<td>8</td>
</tr>
<tr>
<td>8</td>
<td>15</td>
<td>8</td>
</tr>
<tr>
<td>9</td>
<td>16.25</td>
<td>8</td>
</tr>
</tbody>
</table>

Fish 1, 2, and 3 were counted the first time before they were fixed. All other fish were killed by blunt trauma and fixed in 10% formalin before counting. Trophont counts are presented as the mean of four randomly selected counts per fish.
handling during counting can dislodge the trophonts, so care must be taken if the fish are to be recounted. Trophont infestation was linear and significantly correlated to concentration of the theront exposure (Fig. 1).

3.2. Physiological responses to confinement stress

Physiological characteristics of fish exposed to 2 and 6 h of low-water stress are shown in Table 2. Plasma cortisol concentrations in stressed fish were statistically higher after both time periods than in non-stressed fish. However, concentrations in fish stressed for 2 h were significantly higher than those stressed for 6 h. Plasma glucose concentrations of fish stressed for both time periods were statistically similar to each other and higher than those in fish not exposed to low-water confinement. Plasma chloride levels

Table 2
Plasma cortisol, glucose and chloride concentrations in channel catfish stressed by low-water confinement for 0, 2 or 6 h

<table>
<thead>
<tr>
<th>Duration of stressor (h)</th>
<th>Plasma cortisol (ng/ml)</th>
<th>Plasma glucose (mg/100 ml)</th>
<th>Plasma chloride (mEq/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>14.6 ± 2.8 a</td>
<td>45.9 ± 2.9 a</td>
<td>133.7 ± 1.1 a</td>
</tr>
<tr>
<td>2</td>
<td>64.7 ± 3.4 c</td>
<td>62.2 ± 1.8 b</td>
<td>137.3 ± 1.1 a,b</td>
</tr>
<tr>
<td>6</td>
<td>24.5 ± 1.9 b</td>
<td>63.6 ± 4.2 b</td>
<td>138.0 ± 1.2 b</td>
</tr>
</tbody>
</table>

Values are the mean ± SEM of at least 15 fish. Letters in each column indicate a significant difference (P<0.05) from other groups in that column by Tukey’s multiple range test.
were affected very little by confinement; however, chloride in the group confined for 6 h was significantly higher than the control.

3.3. Ich infection response of fish exposed to confinement stress

Fish exposed to 6 h of crowding stress had significantly higher trophont infections than non-stressed control fish. Fish stressed for only 2 h had an intermediate infection which was not significantly different from either the controls or the 6-h stressed group (Fig. 2).

3.4. Mortality due to exposure to CCV and confinement stress

Mortality following exposure to CCV was related to the density of the viral exposure; however, the response was not linear. The control (0) and low exposure were statistically ($P<0.05$) similar to each other and the two high doses were similar to each other and

Table 3
Cumulative mortality 14 days after exposure of small fingerling channel catfish ($n=6$) to 0, $3.3 \times 10^4$, $3.3 \times 10^5$ or $3.3 \times 10^6$ plaque forming units (pfu) of channel catfish virus

<table>
<thead>
<tr>
<th>Treatment (pfu)</th>
<th>Mortality (% mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>3.3 ± 2.7 a</td>
</tr>
<tr>
<td>$3.3 \times 10^4$</td>
<td>0.9 ± 1.0 a</td>
</tr>
<tr>
<td>$3.3 \times 10^5$</td>
<td>67.0 ± 15.2 b</td>
</tr>
<tr>
<td>$3.3 \times 10^6$</td>
<td>71.5 ± 15.9 b</td>
</tr>
</tbody>
</table>

Letters indicate a significant difference ($P<0.05$) by Tukey’s multiple range test.
statistically higher than the 0 and low exposures (Table 3). For this reason, the data for the low-water confinement stress for the two highest doses were combined. Within each dose, the length of low-water confinement stress did not alter the pattern of mortality (Table 4).

4. Discussion

4.1. Physiological responses to confinement stress

The physiological changes of cortisol, glucose and chloride are typical of changes found in channel catfish exposed to a variety of stressors and have been extensively documented (Tomasso et al., 1981a,b; Davis et al., 1984). The response to stress is often characterized as being composed of a primary and a secondary phase (Wedemeyer and McLeay, 1981). The primary phase is considered to be the neural, neuroendocrine hormonal phase represented by plasma cortisol and the secondary phase is the physiological consequence of the primary responses represented by plasma glucose and chloride. Cortisol has several actions including increasing the susceptibility of fish to pathogens. Plasma cortisol increase was most dramatic after 2 h of low-water confinement and had decreased markedly after 6 h. This may be due to the inability of the fish to continue secreting the hormone or an adjustment of the fish to the somewhat mild, non-life threatening intensity of the stressor. Increase plasma glucose can be due to many hyperglycemic hormones, including noradrenaline, adrenaline and cortisol, and represents increased fight or flight activation. Since glucose concentration remained at the same level after both 2 and 6 h of confinement, if there was some physiological adjustment it was not a complete adjustment. Plasma electrolytes decrease in many fish species due to stress in freshwater; however, channel catfish are very resistant to loss of electrolytes (Davis et al., 1993), which may contribute to the ability of this species to tolerate harsh conditions. Low-water confinement was apparently not stressful enough to induce a dramatic change in electrolyte homeostasis.

4.2. Interaction of the stress response with the immune system

Channel catfish are thought to have both an innate (non-specific) and an acquired (specific) immunity against ich (Dickerson and Clark, 1996). Channel catfish which survive

<table>
<thead>
<tr>
<th>CCV exposure (pfu)</th>
<th>n</th>
<th>Hours of low water confinement prior to exposure</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>0</td>
<td>3</td>
<td>6.8 ± 4.4</td>
</tr>
<tr>
<td>$3.3 \times 10^4$</td>
<td>3</td>
<td>1.9 ± 1.9</td>
</tr>
<tr>
<td>$3.3 \times 10^5$ and $3.3 \times 10^6$ combined</td>
<td>6</td>
<td>64.5 ± 13.1</td>
</tr>
</tbody>
</table>

Virus exposure units are in plaque forming units (pfu). There was no statistical difference due to low-water stress in any of the exposure groups.
an initial exposure to ich become resistant to subsequent challenge, and sera from immune channel catfish cause agglutination of *I. multifiliis* theronts in vitro, which suggests an immune system protection (Clark et al., 1987). Infection of fish by ich stimulates immobilizing antibodies in the plasma and mucous of channel catfish (Xu, 1995). Exposure to ciliate protozoans is thought to result in the development of immunity and requires time, and is likely unrelated to any protection available to combat the first exposure.

Innate, or non-specific defense mechanisms remain the same no matter what parasite is encountered and are present before exposure. Evidence for innate protective mechanisms comes from several sources. Many laboratories have reported significant variations in susceptibility among naïve fish within and between host species (Pickering and Christie, 1980; Maule and McCallum, 1982). Innate protective mechanisms can include passive physical barriers, such as skin epithelium and mucous secretion, as well as cellular components, which are capable of phagocytosis or release of chemicals toxic to the parasite. Non-specific cytotoxic cells (NCC) have been implicated in the protection of channel catfish against ich. These cells are thought to be the fish equivalent of mammalian natural killer (NK) cells which lyse mammalian tumor cells (Graves et al., 1985). Leukocytes migrate to the sites of ich trophonts; however, because there is no evidence for active cell adherence or cell-mediated damage to the parasite, any protection to the fish by leukocytes has been questioned (Cross, 1994). Cross and Mathews (1993) found that neutrophils migrated to infection sites of naïve and immune carp early in infection (1–2 days), and other polymorphonuclear cells migrated about 2 days later. However, it is not clear whether these cells were associated with immune or inflammatory responses. Innate mechanisms of protection are very important since fish must depend on this mechanism to combat the first parasite exposure. Cortisol release during stress is known to suppress differentiation and growth of monocytic and granulopoietic colonies in mammals and to suppress the secretion of elastase, collagenase, non-specific neutral proteases, endogenous pyrogens and prosta-glandins from mammalian mature macrophages (Werb, 1978). Further, corticosteroid treatment of immunized carp increased their susceptibility to infection by ich even though serum antibody levels remained high and suggested that the steroid-depleted immune effector cells are involved in protection (Houghton and Mathews, 1990). Net confinement has been shown to reduce apoptosis in channel catfish; however, in vitro cortisol did not duplicate the stress effect on apoptosis (Alford et al., 1994). Short-term (2 h) crowding stress depressed complement and phagocytic activities in gilthead seabream, *Sparus aurata* (Ortuno et al., 2001). The data presented here suggest that low-water confinement stress, either directly by depressing phagocytosis or by suppressing cell secretions, is likely responsible for increasing the susceptibility of channel catfish to infection by ich.

### 4.3. Interaction of stress with viral infections

Less is known about defense mechanisms to channel catfish virus (CCV) than to either bacterial or parasite infections. Natural infection by CCV is thought to be by oral or gill routes and the disease is usually fatal with 95% mortality of exposed fish within 3–8 days (Stingley and Gray, 2000). Most tissues are infected and show necrotic lesions. Fish which survive infection may become asymptomatic carriers and express antiviral antibodies (Plumb, 1973; Amend and McDowell, 1984). Gray et al. (1999) reported that 140 days
after infection, PCR analysis detected CCV DNA in the blood, brain, intestines, kidney, liver and peripheral blood leukocytes of infected fish, which indicates a latent infection. Latent CCV has also been detected in channel catfish broodstock; CCV detection was much more effective when the fish were immunosuppressed with dexamethasone, a synthetic immunosuppressive steroid similar to cortisol, the native interrenal steroid of channel catfish (Bowser et al., 1985). Immunosuppressed fish from which CCV virus was isolated had no detectable CCV antibody. Our data did not demonstrate an effect on CCV-induced mortality due to confinement stress in naïve fingerling channel catfish. These results suggest that any immunosuppressive effect induced by the stress is not affected by components of the stress response, at least in fish exposed to CCV for the first time. It is possible that stress does not have an effect on mortality or the methods were not sensitive enough to detect stress-related differences.

Stress has been shown to increase susceptibility of naïve channel catfish to infection by the bacteria Edwardsiella ictaluri following both immersion and injection challenges, although the immersion route was associated with a high degree of variability (Wise et al., 1993; Ciembor et al., 1995). Channel catfish stressed with low oxygen and high ammonia concentrations are reported to have higher total bacterial counts of Aeromonas hydrophila (Walters and Plumb, 1980). The bacteria were isolated from a small number of controls, but the controls had no associated pathology, which raises the question of whether the fish had been exposed to A. hydrophila prior to the experimental challenge.

Many factors likely influence the susceptibility of fish to pathogens, including the route of entrance, the type of pathogen and whether the exposure is made to naïve fish or fish with previous exposure which did not result in a disease outbreak. In the present study, confinement stress increased susceptibility of channel catfish to I. multifiliis but not to CCV. These fish had not been previously exposed to the pathogens and the results with I. multifiliis likely represent innate protective systems which were suppressed by the confinement stress. Stress-enhanced ich pathology was similar to previous work with bacteria and the lack of stress-induced increase in susceptibility to CCV may be because virus protection must be acquired during the exposure.

Acknowledgements

The authors thank Jan Franks and Deborah Davis for excellent technical support and Mary Anna Davis for editorial support. Drew Mitchell, Brian Small and David Wise provided valuable comments on the manuscript. Mention of a trade name, proprietary product or specific equipment in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

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