Behavior, Physiology
and Toxicology
Interactions in Fish

SYMPOSIUM PROCEEDINGS

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PREFACE

There is an obvious and increasing trend toward multidisciplinary approaches to understanding toxicological research, incorporating the many fields of biology to begin to understand more fully the impact of anthropogenic contamination of the aquatic ecosystem. The large numbers of presentations and the wide variety of topics that they covered in this symposium, highlight the growing enthusiasm for integrating different fields of research and the importance of this approach in aquatic toxicology.

This symposium aimed to draw together internationally recognised speakers whose research focuses on the interactions between behaviour, physiology and toxicology in fish. Presentations covered many areas where behaviour and physiology interlink and the implications that these interactions have for aquatic toxicology. Some of the questions that were addressed within this symposium included:

• Do contaminants affect predator/prey interactions in aquatic ecosystems and are there implications for ecosystem stability?
• Is fish behaviour a suitable tool for monitoring the presence of aquatic pollutants?
• Are early life stages of fish affected by the presence of metals in the environment?
• How does hypoxia affect fish reproduction?
• What pollutants affect swimming behaviour?

We would like to thank the sponsors of this symposium, ICA, ILZRO, NiPERA, Kodak Brazil and Elsevier and we are extremely grateful to everyone who participated in this symposium for presenting and sharing their results and for submitting a written piece of work to these symposium proceedings. We are sure they will serve as a valuable research tool for those people continuing to integrate behaviour and physiology in aquatic toxicology.

Symposium Organizers:

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CONGRESS ACKNOWLEDGEMENTS

This volume is part of the Proceedings of the 6th International Congress on the Biology of Fish, held in Manaus, Brazil in August, 2004. Ten years have passed since the first meeting in this series was held in Vancouver, BC, Canada. Subsequent meetings were in San Francisco, California; Baltimore, Maryland; Aberdeen, Scotland; and again in Vancouver, Canada. From those meetings, colleagues from over 30 countries have contributed more than 2,500 papers to the Proceedings of over 80 Congress Symposia, all available for free viewing on the internet.

We would like to extend our sincere thanks to the many people who helped us organize the facilities and program for this 6th Congress.

The local arrangements team worked very hard to make this Congress a success. The leaders of those efforts were Vera Almeida Val, Adriana Chippari-Gomes, Nivia Pires Lopes and Maria de Nazare Paula Silva (Local Arrangements); Marcelo Perlingeiro (Executive Secretary) and Maria Angelica Laredo (Fund Raising). The enormous contribution of time and effort that was required has led to an unforgettable experience for the participants, thanks to the imagination, determination and dedication of this team.

Many sponsors helped ensure the success of the meeting through both monetary and in-kind contributions, including: Fundação Djalma Batista, Honda, Merse, Cometais, Turkys Aquarium, Banco da Amazônia, Banco do Brasil, FUCAPI, SEBRAE/AM, IDAM/SEPROR, FAPEAM, SECT-AM, SUFRAMA, PETROBRAS, CAPES, FINEP, CNPq, the Physiology Section of the American Fisheries Society, UFAM - Federal University of Amazonas, and INPA - National Institute for Research in the Amazon.

Travel arrangements were ably handled by Atlantic Corporate Travel (special thanks to Maria Espinosa) and Orcal Planet, and the venue for the meeting was the spectacular Tropical Hotel Conference Center in Manaus.

The Student Travel Award Committee of the Physiology Section of the American Fisheries Society, led by Michael Redding, evaluated 65 applications from 15 countries and awarded 40 Travel Grants, after an ambitious and trying fund-raising effort. Special thanks must go the US Department of Agriculture, the US Geological Survey, US National Science Foundation and the World
Fisheries Congress for providing funds. In addition, the American Fisheries Society contributed books to be used as prizes for the best student papers.

The editorial team compiled the short abstracts into an abstract book and formatted and compiled the papers for the Symposium Proceedings. Thanks to Karin Howard, Christie MacKinlay, Anne Martin, Callan MacKinlay and Marcelo Perlingeiro.

In particular, we would like to extend a sincere ‘thank you’ to the organizers of the individual scientific Symposia and their many contributors who took the time to prepare a written submission for these proceedings. Their efforts are very much appreciated. We hope that their participation will result in new insights, new collaborations and new lines of research, leading to new papers to be presented at the 2006 Congress in St. John’s, Newfoundland.

Congress Chairs:

Adalberto Luis Val  Don MacKinlay
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CRUDE OIL, COPPER AND FISH OF THE AMAZON

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Introduction

No other group of fish has thrived spectacular environmental changes as fish of the Amazon, that include extreme changes in dissolved oxygen, ion-poor acidic waters, high temperatures, and seasonal river level oscillation. To copy with these conditions, fish of the Amazon evolved a suite of behavioral, physiological and biochemical responses exhibited by healthy individuals to maintain their organic homeostasis, despite the environmental conditions. These are so-called stress responses to environmental stressors. Under certain environmental circumstances, mostly of anthropogenic origin, there is an over stimulation of these coping responses beyond the capacity of the animal to resume its normal life history what lead to severe ecological disturbances. The activation of the stress response can be a biomarker reflecting combined effects of physiological constraints and changes of habitat quality, natural or caused by men, on the individual animal and on the population as a whole. The increasingly exposure of fish of the Amazon to acute and chronic environmental changes caused by men is a cause of concern, despite the enormous plasticity of this group of fish. The present paper analyzes the effects of two noxious anthropogenic stressors, crude oil and copper, on fish of the Amazon.
Petroleum in the Amazon

Petroleum mining is expanding in the northern Latin America. Large reserves of petroleum and gas are being exploited near the city of Coari, along the Urucu River, a tributary of the Amazon River. Oil barges transport the oil, about 20,000 barrels per day, to Manaus located 700 kilometers down the river, to be refined. Although the Environmental Authorities enforces the observation of strict safety protocols, there are always risks of oil spills both at the wellhead and during the transportation. The pipe system used to move the oil to the barges and from the barges to the refinery also is a cause of concern. In fact, minor accidents have occurred in the Amazon. Another branch of petroleum industry in the Amazon that soon will assume large importance from environmental perspective is the gas-pipe that will connect the mining site to the consuming centers, i.e., Coari and Manaus, and Coari and city of Porto Velho.

Mining and crude oil transport potentially affect the fish of the Amazon in two major ways: the effect of crude oil itself and the effect of physical environmental disturbances caused by machines and barges traffic. After any oil spillage occurs a number of simultaneous processes – collectively known as weathering – that result in physical and chemical modifications of the original compounds present in the crude oil. Weathering of crude oil includes spreading, evaporation, dispersion, emulsification, biodegradation, dissolution, oxidation and sedimentation. The primary and secondary crude oil compounds affect aquatic organisms and those interacting with polluted sites both directly, via physical and toxicological effects, or indirectly, via habitat modifications, including changes in food availability, changes in competition rates, and changes in predation rates, among others (reviewed by Val et al., 2003). Physical environmental disturbances directly affect fish at the ecological level by rupturing fish assemblages and by causing fragmentation and loose of genetic variability in fish populations.

Fish exposed to crude oil experience two sets of challenges. The crude oil on the top of the water column reduces oxygen diffusion and shades the water column, limiting the photosynthesis, what further causes a reduction of dissolved oxygen. As many fish of the Amazon breathe air or depend on the water-air interface to uptake oxygen, an oil spillage is a deadly environmental situation. Tambaqui exposed to crude oil and normoxia, for example, exhibits no major behavioral changes; i.e., it is almost impossible to estimate lethal level for this fish within 96 hours under this condition. Similar situation happens for facultative breathers
and contrasts with that observed for obligatory air-breathing fishes. However, if hypoxia and crude oil are set simultaneously, tambaqui can face only a thin oil slick on the top of the water column, i.e., an oil slick of 1mm. This high sensitivity in hypoxia relates to the specific behavioral adaptation of this fish to hypoxia that is its ability to expand the lower lips to skim water surface when exposed to low oxygen, what increases the amount of crude oil taken in. In the case of the obligatory air-breathing fish, the situation is even drastic, as these fish must surface at regular time intervals to breathe. To protect itself, pirarucu, Arapaima gigas, reduces breathing intervals what results in an increasing oxygen debt and in a reduced locomotion (Figure 1, see an accompanying abstract on this volume), an unsustainable situation if the condition persists for long time.

![Oxygen Debt (%)](chart1.png)

Figure 1. Relationship between oxygen debt caused by crude oil exposure and locomotion of Arapaima gigas.

The crude oil taken in elicits adjustments to reduce its adverse organic effects, in particular the toxicity caused by water-soluble compounds. Changes in gene expression precede these adjustments to unavoidable stressful situations. Currently, two sets of effects of crude oil on fish of the Amazon are under analysis: a) at the molecular level, differential gene expression and expression of CYP1A genes, and b) at physiological and biochemical levels. Differential display reverse transcriptase polymerase chain reaction (dd RT-PCR), a technique used to analyze differentially expressed genes, revealed extensive effect of crude oil exposure on tambaqui. This fish exhibits gene differentially expressed in all analyzed tissues after oil exposure even at very low levels of exposure. The gene “multi-drug resistance associated protein 5”, differentially expressed in the skeletal muscle of tambaqui exposed to crude oil, is the first gene identified so far in this fish species, making this gene a potential biomarker.
for crude oil contamination. However, a clear picture depends on further analysis of the expression of this gene in animals exposed to various environmental stressors. CYP1A subfamily is an important biomarker of exposure to environmental pollutants; its products catalyze the oxidation of planar substrates, such as petroleum compounds. In tambaqui exposed to crude oil for 24 hours, EROD activity increased up to 60 pmol mg\(^{-1}\) min\(^{-1}\) compared to animals from unpolluted sites that had an activity for this enzyme varying between 1.76 and 22 pmol mg\(^{-1}\) min\(^{-1}\). Peculiarly, in specimens of tambaqui acclimated to humic acid and further exposed to crude oil for 24 h, EROD activity doubled relative to crude oil exposure alone (Matsuo et al., 2004). These aspects all deserve further studies for clarification.

Fish exposed to crude oil experience a significant respiratory distress, in many cases associated with changes at other biological level, and significant changes in detoxification mechanisms already above mentioned. In tambaqui, gas transfer is impaired in animals exposed to crude oil because of its behavior – when facing environmental hypoxia, this fish expands the lower lips and skim water surface that is richer in oxygen. Therefore, the animal exposed to crude oil activates all adaptive mechanisms directed towards increasing oxygen uptake, including a significant reduction of intraerythrocytic levels of modulators of Hb-O\(_2\) affinity. In conclusion, fish respond oxygen depletion caused by crude oil pollution using the adaptive mechanisms selected during the evolution to respond to regular environmental hypoxia what makes the animals even more vulnerable to this new extreme anthropogenic challenge.

**Copper in the Amazon**

Copper is a trace element essential for all living organisms. However, excess of copper interfere with a number of organic functions, such as respiration, protein synthesis, ion regulation and reproduction, what requires strong capabilities of the organisms to manage the cellular copper levels. These capabilities display high relationship with the evolutionary history and the historical metal background levels in the habitat of the studied animal. The reduced number of samples from pristine areas of the Amazon so far analyzed indicates low background levels of copper, relatively to the world average. In general, the copper levels follow the general pattern of metal concentrations in white and black waters of the Amazon, i.e., Rio Solimões has higher levels of dissolved metals contrasting with the solute deficient waters of the Rio Negro. The average background level of copper in the Rio Solimões is 2.4±0.6 µg/l, while analyzed samples from Rio Negro had values ranging between 0.03 and 1.0 µg/l.
However, in several spots in the Amazon, the levels of copper are much higher as a consequence of anthropogenic activity, as is the case of some areas around Carajás mining site and in the “Igarapé do 40”, a stream running across the industrial area of Manaus. In addition, oil mining is a potential source of copper pollution in the Amazon as the production water from the Urucu Petroleum Mining Plant contains as much as 240 $\mu$g of copper per liter.

Metals from mining sites and from industries may drain into nearby aquatic ecosystems; once there they can be transported considerable distances downstream. Indeed, several physical processes can give rise to attenuation of metals available to contaminate aquatic animals: advection, dilution dispersion, and sedimentation. However, in the case of the fish of the Amazon, even very low levels can be lethal because of their evolution under an environment roughly free of metals, as copper. In addition, the enormous diversity of fish living in the Amazon allows no generalization; it may be possible that fish from different places or even from different phylogenetic groups present different sensitivities to dissolved metals. Once exposed to metals fish experiences three distinct phases that are species-specific: a) an initial shock-phase; b) a recovery phase; and c) acclimation phase.

Preliminary data on two fish species of the Amazon confirm this difference in sensitivity: the CL$_{50}$ for *Colossoma macropomum* is 700 $\mu$g of copper per liter, while the CL$_{50}$ for *Hoplosternum littorale* is a mere 100 $\mu$g/l, both measured in pH 7, 28°C, after 96 hours. Exposure of *Colossoma* to copper (1/2CL$_{50}$) caused severe liver disturbances, in particular when exposed simultaneously to copper and lead, and an increase in erythrocytic nuclear abnormalities (ENA). Thus, it seems clear that we need to investigate CL$_{50}$ for additional fish species as well as the physiological effects of copper on wide variety of fish of the Amazon before we can have a clear picture.

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References


INTRODUCTION

Cytochrome P450 is a superfamily of heme-thiolate enzymes involved in the biotransformation of endogenous and exogenous compounds, such as hormones and xenobiotics. The CYP1A form is widely used as a biomarker of exposure to organic pollutants. CYP1A induction involves the activation of a specific receptor, the aryl hydrocarbon. Metabolism of petroleum hydrocarbons by CYP1A results in the biotransformation of the parental compound into more toxic forms. These intermediary metabolites are highly reactive and are known to be carcinogenic because they bind to DNA, modifying its structure and function. Analysis of CYP1A induction may therefore indicate the susceptibility of organisms to the toxic effects of petroleum hydrocarbons.

In the Amazon basin, petroleum extraction began in 1988 at Coari (Brazil). Despite care in handling and transport, the risk of an oil spill is always a concern. Petroleum hydrocarbons, especially polycyclic aromatic hydrocarbons, are extremely toxic to fish. High concentrations of dissolved organic matter (DOM) in the water can alter the fate of these pollutants.
We assessed CYP1A inducibility in tambaqui (Colossoma macropomum) acutely exposed to petroleum hydrocarbons and to DOM. Little is known about the effects of DOM itself on CYP1A induction, so we further tested the catalytic activity of CYP1A (EROD) over a range of DOM concentrations and sources.

Materials & Methods

**CYP1A induction by petroleum and DOM**
Tambaqui obtained from a fish farm were acutely exposed to one of the following treatments: a) control, b) petro (2.8% crude oil); c) DOM (22 mgC/l), and d) DOM+petro. CYP1A induction was evaluated by CYP1A protein concentration (Western blots), EROD activity (fluorimetry), and immunohistochemistry (IHC). Liver was sampled for microsomal isolation. Gills and a small sample of liver were fixed for IHC.

**Influence of source and concentration of DOM**
Tambaqui were acclimated for 10 days to either a natural (NOM) or a commercial (AHA) source of DOM at 20, 40, and 80 mgC/l. Hepatic EROD activity was used as an indicator of CYP1A activity.

Statistical analyses used either Student’s *t*-tests or ANOVA, followed by Dunnett’s multiple comparison tests (*α*=0.05).

Results & Discussion

**CYP1A induction by petroleum and DOM**
The results revealed for the first time that DOM induces CYP1A and also exacerbates the induction when combined with petroleum hydrocarbons. CYP1A protein concentration (Fig. 1a) and EROD activity (Fig. 1b) were closely correlated in tambaqui exposed to the treatments. IHC revealed that CYP1A induction in the gills was predominantly linked to pillar cells, whereas in the liver it was mostly in hepatocytes. CYP1A induction was highest in the treatment combining both DOM and petroleum hydrocarbons.

The mechanism of CYP1A induction in fish exposed to petroleum hydrocarbons alone is well known, but the interpretation of CYP1A induction by DOM or by the combination of DOM and petroleum hydrocarbons is more complicated. Because CYP1A induction by DOM had never before been reported, we do not know what metabolites are formed when DOM is metabolized by CYP1A. We
suspect that the intermediary metabolites derived from DOM biotransformation are not toxic, given the great diversity of fish species inhabiting Amazonian waters with a high DOM content (over 1,000 species inhabit the Rio Negro basin where the average DOM content is 35 mgC/l).

Fig. 1. a) Hepatic microsomal CYP1A concentration in tambaqui (N=6 per treatment), b) EROD activity in tambaqui following the same treatments. (*) indicates a significant difference relative to control.
CYP1A induction in the ‘DOM+petro’ treatment is unclear. Although high levels of CYP1A induced by petroleum hydrocarbons suggest high levels of toxic intermediates formed in phase I, the same may not be applicable when DOM is present in solution. Interactions between DOM and petroleum hydrocarbons result in decreased toxicity of the pollutant (Haitzer et al., 1998), but it has also been shown that DOM may increase the solubility of this pollutant, thus resulting in enhanced bioavailability to the organisms (Boehm & Quinn, 1973). Whether DOM decreases or amplifies the toxicity of petroleum hydrocarbons to fish cannot be evaluated by CYP1A analysis.

**Influence of source and concentration of DOM**

Increased levels of EROD activity were closely correlated with increased concentrations of DOM in the water, suggesting a concentration-dependent relationship (Fig. 2).

![Fig. 2. EROD activity in tambaqui (N=6 per treatment) acclimated to either a natural (NOM) or a commercial (AHA) source of DOM at concentrations of 20, 40, and 80 mgC/l. (*) indicates a significant difference relative to control.](image-url)
The degree of induction suggests that increased amounts of DOM (‘inducer’) were involved in increased activation of the Ah receptor. AHA overestimated the influence of DOM on EROD activity relative to NOM, when tested at the same concentration. Although commercial DOM is useful for qualitatively assessing the biological effects of DOM, natural DOM sources result in more accurate extrapolations.

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References


DIFFERENTIAL GENE EXPRESSION
ON TAMBAQUI, COLOSSOMA MACROPOMUM CUvier, 1818
EXPOSED TO CRUDE OIL.


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Introduction

Large reserves of natural gas and petroleum are currently being extracted from the Urucu River Basin, municipality of Coari (Amazon - Brazil), by Petrobras, the Brazilian Petroleum Company. This exploration involves considerable risk due to the dynamics aspects and complexity of the Amazon Basin. Changes in gene expression are known to precede the manifestation of morphological alterations. We can use differential gene expression for early detection of exposure to a contaminant. Differential display (Liang and Pardee, 1992) is a technique useful in the identification of genes that are differentially regulated. Initially, mRNA is reverse transcribed into cDNA using ‘anchored-poli-dT’ primers that anneal to the poly (A+) tail. From the resulting pool of cDNAs, random gene fragments are amplified using ‘arbitrary-randon (AR)’ primers. When these fragments are electrophoresed, patterns of expression can be compared by noting the bands that are present or absent between the treatment groups. We used a differential display RT-PCR (DD-RT-PCR) protocol to
identify genes whose mRNA levels were altered in response to acute exposure of Colossoma macropomum, to crude oil.

Material and Methods

C. macropomum (10 cm) were purchased from “Amazon Fish Farm”, in Rio Petro da Eva, 107 Km from Manaus. Fish were transported to the laboratory (LEEM) at the National Institute for Research in the Amazon (INPA) and were held in 500 L circular tanks with continuous mild aeration ensuring that water was air saturated. Fish were fed to satiation with commercial pellets for at least two weeks prior to experiments. After the acclimation period fifty fishes were randomly assigned to 100 L circular tank containing 100mL/L of Urucu crude oil for 3 h at 30°C. Immediately after capture, the surviving fish (45) were killed with a sharp blow to their head followed by severing the spinal cord (according to animal care association recommendations). The tissues (liver, brain gills and muscle) were removed, placed in tubes and total RNA was extracted using TRIZOL®-Reagent (Invitrogen™ Life Technologies) according to the manufacturer’s instructions. The samples were stored at minus 80°C. Total RNA was diluted in a reaction tube containing RT_PCR beads. For 20µl final volume: 4µl of dH2O, 1µl of 5x RT buffer, 2µl of dNTP (25µM), 5µl of total RNA (3µg diluted with DEPC-treated H2O), 1µl of oligo-dT (10 pmol) and 1µl of ‘arbitrary-primer-1 (AP-1)’ and incubated at 22°C for 10 min, 37°C for 50 min and 72°C for 10 min and stored at 4°C. The cDNA results was re-amplified using: 4µl of dH2O, 1µl of 10x PCR buffer, 2µl dNTP(25µM), 1µl of (AP-1) primer, 1µl of (AP-2) primer, 5U Taq polymerase and 10 µl of RT-product of cDNA. PCR was made as follows: 94°C for 30”, 50°C for 30’’ and 72°C for 1 min for 35 cycles.

Following PCR amplification, the reaction products were run at 100volts on a 8% polyacrylamide gel. After the complete run, the gels were treated with 0.5µg/ml ethidium bromide TAE 1X. Bands were visually inspected in order to determine whether there was differential expression. Differentially Expressed bands were excised, eluted and the cDNA was re-amplified using PCR. The PCR products were cloned into the pGEM-T vector (Promega), transformed into competent E. coli cells (DH5-alpha-F’ strain). The plasmid was sequenced in an automated sequencer. The nucleotide sequences obtained were compared with known sequences by searching the GenBank and EMBL databases with BLAST algorithms and bioinformatics tools.
Table 1 Primer sequences

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence (5’ → 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oligo-dT</td>
<td>TGC CAA GCT TTT TTT TTT TGC</td>
</tr>
<tr>
<td>AP-1</td>
<td>GTA AAA TCG G</td>
</tr>
<tr>
<td>AP-2</td>
<td>CTC AGA TAG CC</td>
</tr>
</tbody>
</table>

Results and Discussion

At least one band was identified and showed a small identity with Platichthys flesus partial mRNA for multidrug resistance associated protein 5 (mrp5* gene), which expresses only in white muscle after crude oil exposure.
Within-sample variation revealed by DDRT-PCR. Tissue samples were obtained from Colossoma macropomum. One independent RNA isolation and reverse transcription was performed for each sample (Labeled RTa, RTb). Two ddPCRs using primer set were performed for each reverse transcribed RNA isolate, resulting in a total of four ddPCRs per individual. Arrows indicate bands that differ among tissues.

Conclusions

It has been demonstrated that even at very low exposure concentration there is a differential gene expression in all tissues studied showing that differential display-RT-PCR technique is powerful technique that can be employed to discover novel genes expressed in organisms for which there is limited genomic information. Consequently, the genes identified in differential gene analyses may be used as tools in environmental monitoring in habitats with chances of crude oil spills. The genes discovered in this study will be the new target for diagnostic techniques for rapid crude oil exposure detection in fishes.

The activation of a multidrug resistance associated protein 5 in muscle contaminated with crude oil reveals the possible use of this gene as a molecular biological marker for fish crude oil contamination.

Additional investigations are undergoing in our laboratory to better define the use of this gene as a molecular marker in ecotoxicological studies.

Acknowledgements

This work was supported by National Institute for Research in the Amazon (INPA), Federal University of Amazonas, FINEP/PETROBRAS (Project PIATAM) and The National Research Council of Brazil (CNPq), KLV, SSO and LKHC are the recipients of fellowships from CNPq/Brasil. ALV and VMFAV are research fellows from CNPq/Brasil.

References


UNIDIRECTIONAL Na⁺ FLUX AND CYP1A IMMUNOHISTOCHEMISTRY IN *Hyphessobrycon erythrostigma*

EXPOSED TO A SURFACTANT AND TO CRUDE OIL

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EXTENDED ABSTRACT – DO NOT CITE

Introduction

Surfactants are organic compounds widely used as dispersants in the mitigation of oil spills. The presence of both hydrophobic and hydrophilic sites in the structure of surfactants results in ‘pseudomicellar’ arrangements that play a role in the sorption of organic pollutants. Although regularly used to clean up oil spills, surfactants have been reported to be toxic to fish (Tovell *et al.*, 1975). Sodium dodecyl sulfate (SDS), one of the most common surfactant agents, has been shown to induce changes in fish gill morphology (Rosety-Rodríguez *et al.*, 2002). Although these changes certainly affect ionoregulatory function, little information is available on the mechanisms of surfactant toxicity. The ionoregulatory processes in fish gills can be disturbed by changes in water
chemistry, which can be assessed by measuring unidirectional ion fluxes. Gills are considered a primary target for uptake of waterborne pollutants and, as such, their physiological function is sensitive to chemicals in the water.

Cytochromes P450 are a diverse group of enzymes involved in the metabolism of both endogenous and exogenous compounds. CYP1A is one of the P450 enzymes often used in environmental monitoring because its high inducibility by pollutants, such as petroleum hydrocarbons, make it an exquisitely sensitive biomarker for exposure to these chemicals. Although the liver is the major site of CYP1A induction for detoxification in vertebrates, induction in extrahepatic tissues of fish such as the gills has also been reported (e.g., Miller et al., 1989; Bainy et al., 1999).

We assessed ionoregulatory disturbances on Na\(^+\) transport and evaluated gill CYP1A immunohistochemistry in an Amazonian fish, *Hyphessobrycon erythrostigma*, upon acute exposure to a surfactant and to petroleum hydrocarbons.

**Materials & Methods**

*Hyphessobrycon erythrostigma* were acclimated to laboratory conditions and exposed to the following treatments: a) control, b) surfactant (SDS, 10 mg/l), c) petroleum (crude oil, 2%), and d) surfactant + petroleum. Na\(^+\) fluxes were based on the disappearance of the isotope \(^{22}\text{Na}\) from the water into the fish (N=8 per treatment) over 6 h. At the end of the flux measurements, four fish from each treatment were sacrificed to sample gills, and the remaining animals were sampled after 24 h of exposure. Gill samples were fixed in buffered formalin for CYP1A analysis. CYP1A immunohistochemistry (IHC) was performed using mouse antibody 1-12-3 (primary antibody; positive slides). Slides were scored based on the intensity of staining in the tissues following the scale: 0-no staining, 1-very mild, 2-mild, 3-moderate, 4-strong, 5-very strong. Results are shown as mean±SEM.

**Results and Discussion**

*Unidirectional Na\(^+\) flux*

Flux measurements revealed that exposure to the surfactant (SDS) and to crude oil caused significant inhibition of branchial Na\(^+\) influx in *H. erythrostigma* (Fig. 1). Fish were unable to restore Na\(^+\) uptake rates to control values. Exposure to
SDS resulted in a 48% inhibition in Na\(^+\) uptake, similar to that found in individuals exposed to crude oil. Flux measurements in *H. erythrostigma* did not exhibit higher ionoregulatory disturbance resulting from the chemically dispersed crude oil in the surfactant + petroleum treatment (36% inhibition on Na\(^+\) uptake, only slightly less than the separate treatments). Surfactants may increase the toxicity of petroleum hydrocarbons to fish by increasing the solubility of the toxic fraction (Ramachandran *et al.*, in press). Despite the inability of the fish to up-regulate Na\(^+\) influx upon exposure to either SDS or crude oil, the ion disturbance was relatively mild. This is because *H. erythrostigma* was able to control Na\(^+\) efflux by adjusting gill permeability quickly, thus preventing an even higher net Na\(^+\) loss (Fig. 1).

![Fig. 1. Unidirectional Na\(^+\) fluxes in *Hyphessobrycon erythrostigma* under various treatments. (*) indicates significant difference relative to control at each time interval (α<0.05).](image)

**CYP1A immunohistochemistry**

CYP1A induction is a common response in fish exposed to petroleum hydrocarbons, and it is known to occur through the activation of the aryl hydrocarbon receptor (AhR). Surfactants generally lack this ability to activate the receptor, but we found that SDS induced CYP1A expression in *H. erythrostigma* (Fig. 2). Induction of CYP1A in epithelial and pavement cells in the gills was very mild after 6 h of exposure to all treatments compared to the control. Induction was only mild after 24 h of acute exposure, as indicated by
the intensity of the staining in the cells (Fig. 2). The gills are a primary route of petroleum hydrocarbon uptake in fish (Miller et al., 1989), so induction of CYP1A is likely to occur in this tissue. CYP1A induction by SDS was unexpected and possible contamination of the surfactant by even small amounts of CYP1A inducers cannot be ruled out. SDS may increase the solubility/permeability of gill membranes through hydrophobic interactions, but this does not lead inherently to activation of the AhR resulting in CYP1A expression.

Fig. 2. CYP1A immunohistochemical detection in the gills of Hyphessobrycon erythrostigma as indicated by the intensity of cell staining (relative scale, from 0-no staining to 5-very strong staining). (*) indicates significant difference relative to control ($\alpha<0.05$).
Acknowledgements

This work was supported by INPA and CNPq/CTPetro grants to A.L. Val, WHOI and EPA grants to J.J. Stegeman, and by a Mary Sears Travel Grant to A.Y.O. Matsuo. IBAMA and the Ministry of Agriculture in Brazil approved the export of samples for analysis. Import permits were granted by the USDA to WHOI. *Hyphessobrycon erythrostigma* were donated by Turky’s Aquarium (Manaus, Brazil).

References


INTRODUCTION

The currently low concentrations of metals in natural aquatic ecosystems tend to increase as a result of increased urbanization, expansion of industrial activity and exploration of natural resources. Copper and lead are potentially dangerous metals and have their background concentrations increased over the recent years. They have been incorporated and accumulated in the biota.

Copper is needed in organic processes while lead doesn’t have a known biological function. High concentrations of these metals cause severe physiological and biochemical disturbances in fish and other animals. The aim of this study was to analyze the effect of copper and lead on Colossoma macropomum.

MATERIAL AND METHODS

Juveniles of C. macropomum were obtained from commercial fish supplier and held in the Laboratory of Ecophysiology and Molecular Evolution (LEEM - INPA) for at least two weeks prior to experimentation.
Experimental protocol

Cu$^{2+}$ and Pb$^{2+}$ toxicities were analyzed in groups of ten individuals (44.58±0.96g), in triplicates, transferred to 60L tanks and exposed to four experimental conditions for 96 hours: a) control; b) 0.368mg.L$^{-1}$ of Cu$^{2+}$; c) 14.25mg.L$^{-1}$ of Pb$^{2+}$; and d) 0.184mg.L$^{-1}$ of Cu$^{2+}$ + 7.17mg.L$^{-1}$ of Pb$^{2+}$. These concentrations represent 50% of LC$_{50}$ (b and c) and 25% of LC$_{50}$ of both metals (d). In another set of experiments, bioconcentration was analyzed in specimens (6.19±0.39g) exposed individually in experimental units of 1L to 50% of LC$_{50}$ of Cu$^{2+}$ or 50% of LC$_{50}$ of Pb$^{2+}$ for 96 h. Control animals were maintained over the same period of time in well water. All experiments were carried out in semi-static systems with 10% renovation of test-solution every 24h. The copper was tested as CuSO$_4$.5H$_2$O and lead as Pb(NO$_3$)$_2$.

Blood sample

Blood samples were collected from the caudal vein. Hematocrit (Hct), hemoglobin concentration ([Hb]), red blood cell counts (RBC), corpuscular constants (MCV, MCH and MCHC) and methemoglobin were estimated by classic methods as described elsewhere (see Benesch et al., 1973; Brow, 1976).

Plasma analyzes

Sodium (Na$^+$) and potassium (K$^+$) levels were measured by atomic absorption spectroscopy (AANALYST 800-PerkinElmer). Chloride levels (Cl$^-$) were measured according to the thiocyanate method. The alanin aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (PA) and cholinesterase (Che) activities were measured according to the manufacturer’s recommendations for each set of assays (Doles Inc).

Metal accumulation in the tissues

Kidney, liver, skeletal muscle and gill tissues were washed, weighted and digested in nitric acid 10% for 24h in 80°C. Metal concentration was measured by a graphite furnace atomic absorption spectroscopy.

Data analysis

Data are presented as mean±SEM. Differences among mean values were determined using one-way ANOVA.
Results and discussion

The effects of sublethal exposure to Cu and Pb on the respiratory capacity, ion regulation and health of *C. macropomum* are shown in Table 1. Basic blood index are affected by Pb$^{+2}$ and by the mixture of Cu$^{+2}$ and Pb$^{+2}$, suggesting that lead potentially causes anemia in tambaqui. According to Hernberg (1976) this metal causes anemia by the inhibition of the hemoglobin synthesis, but this seems unlikely in the present experiment as the animals were exposed to these metals for only 96 hours.

The alterations of ion concentrations in the plasma suggest that exposure to Cu and Pb cause serious damage to ion regulation. In the case of Cu$^{+2}$, the depletion of Na$^+$ and K$^+$ may have resulted from Na$^+$ influx inhibition, increased in the permeability of the apical membrane and Na$^+$-K$^+$-ATPase enzyme inhibition, as depicted by Laurén and McDonald (1984) studying rainbow trout. The individuals exposed to Pb$^{+2}$ displayed an increase in the Na$^+$ and Cl$^-$, probably due to Na$^+$-K$^+$-ATPase enzyme inhibition.

Exposure to Cu$^{2+}$ and Pb$^{2+}$ resulted in liver disturbance, with increased plasma levels of ALT, AST e PA, what suggests that the accumulation of these metals causes liver damage driving the release of those enzymes into the blood circulation. Similar situation has been described for other fish species (Karan *et al.*, 1998).

Cholinesterases are serine hydrolases with two isozymes: cholinesterase, secreted by the liver cells into the plasma, and acetylcholinesterase, the true cholinesterase, found in the nervous tissues, red cells, and muscle. While the former is inhibited in fish exposed to metals, the later is increased, at least in fish exposed to cadmium. In the present experiment, plasma pseudo-cholinesterase was measured and increased levels were found in animals exposed to Pb$^{2+}$, suggesting there should be an interaction of lead and hydrolysis of acetylcholine that requires increased levels of the enzyme.

Tissues levels of Cu and Pb are presented in Table 2. There was an increase in the Cu$^{2+}$ in the gills and liver, in decreasing order, gills>liver. Lead appeared only in the gills of tambaqui, remaining below the detecting limits in all other analyzed tissues.
Table 1. Hematological indexes, ion and enzyme activities of *C. macropomum* exposed to sublethal concentrations of copper and lead for 96h. (*) represents significant difference compared to control (P<0.05).

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>50% LC50 of Cu(^{2+})</th>
<th>50% LC50 of Pb(^{2+})</th>
<th>25% LC50 of Cu(^{2+}) + Pb(^{2+})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hct (%)</td>
<td>25.8±0.67</td>
<td>25.15±0.68</td>
<td>19.83±0.47 *</td>
<td>22.03±0.70 *</td>
</tr>
<tr>
<td>[Hb] (g.dL(^{-1}))</td>
<td>5.60±0.26</td>
<td>5.85±0.19</td>
<td>5.11±0.18</td>
<td>4.80±0.18 *</td>
</tr>
<tr>
<td>RBC (10(^6).mm(^{-3}))</td>
<td>1.38±0.06</td>
<td>1.19±0.04</td>
<td>1.03±0.05 *</td>
<td>1.06±0.06 *</td>
</tr>
<tr>
<td>MCV (µm(^3))</td>
<td>195.27±9.27</td>
<td>216.04±6.64</td>
<td>202.91±7.89</td>
<td>225.45±12.4 *</td>
</tr>
<tr>
<td>MCH (pg.cell(^{-1}))</td>
<td>42.15±2.46</td>
<td>50.01±1.48</td>
<td>52.07±2.36 *</td>
<td>48.12±2.29</td>
</tr>
<tr>
<td>MCHC (%)</td>
<td>21.58±0.78</td>
<td>23.34±0.53</td>
<td>25.76±0.70 *</td>
<td>22.28±1.01</td>
</tr>
<tr>
<td>Meta-Hb (%)</td>
<td>17.78±1.23</td>
<td>13.98±1.30</td>
<td>10.57±1.59 *</td>
<td>13.51±2.23</td>
</tr>
<tr>
<td>[Na(^+)] (mEq.L(^{-1}))</td>
<td>143.04±1.64</td>
<td>130.87±1.82 *</td>
<td>164.56±3.71</td>
<td>144.97±2.57</td>
</tr>
<tr>
<td>[K(^+)] (mEq.L(^{-1}))</td>
<td>7.51±0.16</td>
<td>4.32±0.31 *</td>
<td>7.89±0.37</td>
<td>10.83±0.46 *</td>
</tr>
<tr>
<td>[Cl(^-)] (mEq.L(^{-1}))</td>
<td>95.14±2.23</td>
<td>100.66±3.07</td>
<td>108.97±2.48</td>
<td>116.47±2.59</td>
</tr>
<tr>
<td>ALT (FR.mL(^{-1}))</td>
<td>25.14±2.86</td>
<td>71.80±11.63 *</td>
<td>93.71±4.50 *</td>
<td>53.92±6.42 *</td>
</tr>
<tr>
<td>AST (FR.mL(^{-1}))</td>
<td>75.76±5.16</td>
<td>97.40±7.33</td>
<td>94.86±13.07</td>
<td>157.13±5.54 *</td>
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<tr>
<td>PA (BL.mL(^{-1}))</td>
<td>2.56±0.23</td>
<td>5.14±0.86</td>
<td>11.21±1.78 *</td>
<td>14.09±1.73 *</td>
</tr>
<tr>
<td>Che (U.L(^{-1}))</td>
<td>0.09±0.02</td>
<td>0.07±0.02</td>
<td>0.43±0.04 *</td>
<td>0.65±0.07 *</td>
</tr>
</tbody>
</table>
Table 2. Bioconcentration of copper and lead in kidney, liver, muscle and gills of *C. macropomum* exposed to sublethal concentrations for 96h. The bioconcentration is expressed as mg g⁻¹ wet weight. (*) represents significant difference compared to control (P<0.05).

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>50% LC₅₀</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cu</strong>⁺²</td>
<td>Kidney</td>
<td>7.50±1.02</td>
</tr>
<tr>
<td></td>
<td>Liver</td>
<td>25.22±2.13</td>
</tr>
<tr>
<td></td>
<td>Muscle</td>
<td>0.15±0.05</td>
</tr>
<tr>
<td><strong>Gills</strong></td>
<td></td>
<td>0.08±0.04</td>
</tr>
<tr>
<td><strong>Pb</strong>⁺²</td>
<td>Kidney</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td></td>
<td>Liver</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td></td>
<td>Muscle</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td><strong>Gills</strong></td>
<td></td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

Concluding, *C. macropomum* exposed to Cu⁺² and Pb⁺² experiences severe physiological and biochemical disturbances. Except by ALT, all other analyzed enzymes are increased in animals exposed to Pb⁺².


References


Acknowledgments

The present work was supported by INPA (grant # 1-3140), CNPq/CTPetro (grant # 463654/2000-5), FINEP-BIOTOX (grant # 65000.0070.00) and CNPq (grant # 130010/2001-4)
HISTOLOGICAL ALTERATIONS
IN THE GILLS OF TRACHINOTUS CAROLINUS (CARANGIDAE)

EXPOSED TO NAPHTHALENE

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Abstract

Histopathological investigations have been proved to be a sensitive tool to detect effects of chemical compounds within target organs of fish in laboratory experiments. In this study we wished to find out whether exposure to naphthalene for twelve days has an effect on the gill tissue of Trachinotus carolinus. This study focused on gills because they are the main target for many aquatic pollutants in general, and one of the most seriously affected organ due to direct contact with the aquatic environment. Five types of anomalies in the gills of the fishes were observed. The lesions were typically epitheliocapillary separation, proliferation of epithelial cells and consequent fusion of lamella, aneurysm, hemorrhages and the presence of parasites. The results of these studies clearly indicate that naphthalene has diverse effects on the gill tissues of the fishes. The number of lesions caused by naphthalene is related to its concentration. The naphthalene also makes the fishes more susceptible to diseases and parasites.

Keywords: environmental pollution, fish disease, histopathology, naphthalene.
Introduction

Naphthalene is one of the prominent diaromatic fractions of crude and refined oils and it is a polycyclic aromatic hydrocarbon (PAH). Generally PAHs are less sensitive to photooxidation and therefore are more persistent in water (Rand and Petrocelli, 1985). PAHs are rapidly accumulated by aquatic organisms reaching levels higher than those in the ambient medium and affecting normal function of the aquatic life (Kulkarni and Masurekar, 1984). Hence, naphthalene was selected as a toxicant in the present investigation.

In field studies fish can be used as a monitoring tool for the quality of the aquatic environment. Histopathological studies have been conducted to help establish causal relationships between contaminant exposure and various biological responses. Histopathological investigations have also been proved to be a sensitive tool to detect direct effects of chemical compounds within target organs of fish in laboratory experiments (Schwaiger et al., 1992, 1996). This study focused on gills because they are the main target for many aquatic pollutants in general, and one of the most seriously affected organ due to direct contact with the aquatic environment (Mishra et al., 1985).

The aim of this study was to find out whether exposure of twelve days to naphthalene has an effect on the gill tissue of *Trachinotus carolinus*.

Materials and methods

A. Fish

For the investigations, 33 juveniles of *Trachinotus carolinus* weighting 1.88-2.37 g and measuring 40–75 mm were used for the histological evaluations.

B. Experimental design

The animals were kept in 40 L aquariums with 35 %o saltwater during twelve days. One group of fishes was kept in clean water and another group in ethanol (0.30 ppm) - both were used as controls. Since the pollutant has to be dissolved in ethanol, as it does not dilute in water, it was necessary to keep this control aquarium with ethanol to observe if this compound had any effect on the fish gills. Two other groups of fishes were kept in 0.15 and 0.30 ppm of naphthalene. Therefore three groups of fishes had the same amount of ethanol except the
group in clean water. During the experiments, the water was aerated and it was exchanged every twelve hours to avoid the excessive evaporation of the naphthalene. The temperature was maintained at 24°C ± 1.
**Histological methods**

To examine the effects of naphthalene on the gills of fishes, the first and second gill arch, from both sides of the fishes, were removed and fixed in Dietrich’s solution for 24 hours. They were dehydrated in increasing concentrations of ethanol and included in historesin Leica®. Sections were cut at 4 µm and stained with hematoxylin and eosin (H&E).

**C. Morphological analysis**

Quantitative differences between the anomalies in all groups of fishes were evaluated. The number of each lesion observed (hyperplastic interlamellar occlusion, aneurysms, epitheliocapillary separation (ECS), hemorrhage or infestation of parasites) were registered and tested for its statistical importance.

**D. Statistical analysis**

One-way analysis of variance (ANOVA) was used to test the parameters evaluated. Differences among fishes exposed to clean water, ethanol and the two concentrations of naphthalene were tested by the Tukey’s multiple range test (p<0.05).

**Results**

As in typical teleost gills, the gill arch of *T. Carolinus* is composed by gill filaments and secondary lamellae (Fig. 1A). The secondary lamellae are characterized by blood spaces, which are lined by pillar cells. The respiratory epithelium itself consists of two thin layers of epithelial cells (Fig. 1A).
Histopathological findings in gills of experimental fish could be distinguished from the slight tissue lesions in control individuals. There were observed five types of anomalies in the gills of the fishes. The lesions were typically epitheliocapillary separation (ECS) (Fig. 1B, 2A), proliferation of epithelial cells and consequent fusion of lamella (Fig. 1D), aneurysm (Fig. 1C), hemorrhages (Fig. 2B, 2C) and presence of parasites (Fig. 2D).
The average number of lesions were plotted on the following graphs, significant difference (p<0.05) to the control are marked with an asterisk:

Figure 2. Longitudinal sections of gill lamellae. A. ECS (880X). B. and C. Gill exhibiting subepithelial hemorrhage (Hm) (1660 and 880X). D. Parasite infestation (P) (820X).
Figure 3. Graphics showing anomalies’ average observed in T. carolinus. a. fishes in clean water; b. fishes in 0.30 ppm ethanol water; c. fishes in 0.15 ppm naphthalene; d. fishes in 0.30 ppm naphthalene.
The histopathological alterations were most prominent in individuals exposed to naphthalene. To a minor degree, these findings could also be observed in a few control fish. Differences were not significant between the clean water and ethanol groups for all the parameters evaluated.

The mean assessment values based on semiquantitative evaluation of the frequency of lamellar fusion and lamellae hemorrhage showed significant difference between groups exposed to naphthalene, and relative to both control groups. So, the frequency of the lesion was proportional to the amount of pollutant tested.

The results for ECS did not show distinct differences between the fish exposed to the two concentrations of naphthalene but showed significant difference relative to the control groups. The presence of ECS in fishes exposed to naphthalene was extremely high, affecting almost 100% of the gill epithelium.

The number of aneurysms in fishes were not significant different. The maximum average number were 2.43 in 0.30 ppm naphthalene group and the minimum mean value were 0.67 in the control group in clean water.

The parasite infestation tended to increase proportional to the naphthalene concentration. Nevertheless, difference was significant only at 0.30 ppm.

Discussion

The fact that some types of histopathological lesions could also be detected in controls should be considered. The background knowledge concerning the control condition is essential in interpreting biomarker responses in fish because for many of them, control values are used as reference. As the alterations in both control groups did not present significant differences, we suppose that the ethanol concentration is too low to affect the gills of the fishes. So, the main reason for the lesions and effects presented in the tissues is due to the naphthalene exposure.

These lesions consisted primarily of local proliferation of primary and secondary lamellae epithelial cells, occasionally resulting in fusion of adjacent secondary
lamellae (Figure 1D). Another evident effect of the pollutant was the increasing in epitheliocapillary separation - almost affecting 100% of the tissue – as it was in the increasing number of hemorrhages as the concentration of pollutant increased.

Since the number of aneurysms was too low we can only observe a slight tendency of increasing in its number per fish as the naphthalene concentration increases (Fig. 3). This lesion could not represent an important parameter to elucidate the effects in the gills of these fishes.

It is clearly evident that the high number of parasites was related to the higher concentration of naphthalene. So, as for the number of aneurysms in the fishes, there is a tendency in increasing the number of parasites as increases the concentration of naphthalene.

We could observe a large variety of parameters. The mean number of lesions caused by and related to the concentration of naphthalene is evident. The results of these studies clearly indicate that naphthalene makes the fishes more susceptible to diseases and parasites and it affects directly the gills tissues, which are in extremely contact with the water.

In conclusion, our studies of alterations in gills of T. carolinus exposed to naphthalene are continuing. Our next approach is to observe alterations in fishes exposed to another concentrations of the pollutant and different times of exposure.

Acknowledgements

Thanks to FAPESP for the financial support and Instituto Oceanográfico da Universidade de São Paulo for the facilities offered. Special thanks to Arthur Rocha for helping with statistical analysis and graphs discussions.

References


Introduction

Currently, leakage of oil transport pipelines and storage tanks are contributors to hydrocarbon pollution in Iguacu and Tibagi Basin, both located in Paraná State (southern Brazil). Recently (March 2002) a small stream in Tibagi Basin received 80 thousand liters of Diesel Oil as a result of leakage from storage tanks. However, little research has been done on the impact of petroleum hydrocarbons on freshwater tropical ecosystems and aquatic biota. Besides, not many neotropical fish species have been employed in toxicity tests. *Prochilodus lineatus* is native to south/southeast Brazil and is found in Tibagi Basin. This species has been used previously in laboratory studies and has been shown to be a suitable organism for monitoring the effects of xenobiotics (Mazon & Fernandes, 1999; Mazon et al., 2001; Martinez & Souza, 2001; Martinez et al, in press).

In this study, a suite of biological parameters was used to evaluate acute and chronic induced effects/responses in *P. lineatus* exposed to diesel water soluble fraction (DWSF). The following parameters were examined: biotransformation enzyme (liver GST), antioxidant enzyme (liver catalase), hematological parameter (Hb content), metabolic parameters (blood glucose and proteins),
ionic parameters (blood Na\(^+\), Cl\(^-\) and osmolality) and histological parameter (liver histopathology). These assays were designed to detect sublethal biochemical, physiological, and morphological changes in freshwater fish exposed to DWSF. The utility of these methods lies in their ability to provide an early warning of diesel effects before community and ecosystem responses can be detected.

**Material and Methods**

Static toxicity tests were carried out to evaluate DWSF effects to juveniles of *P. lineatus* (9.14±1.32 g, mean ± SD, n=48). To obtain the DWSF one part diesel oil was added to 4 parts water in a glass container. The mixture was then exposed to sunlight for four days, simulating a diesel spill in tropical conditions. After that the upper insoluble phase was discarded and the remaining water phase was collected to prepare the adequate experimental concentration, i.e., 10%. Fish were divided in eight groups (6 fish each), four groups were exposed to DWSF while other four groups were exposed only to clean water, without diesel (control group). Experiments were performed in an 100L glass aquarium with continuously aerated well water. Water temperature, dissolved oxygen, pH and conductivity were continuously monitored. One experimental group plus one control group were terminally sampled at one of the following intervals: 24, 48, 96 h (acute exposure) and 15 days (chronic exposure). Blood samples were taken from the caudal vein by means of heparinezed plastic syringes and subsequently fish were killed by cervical section and liver were immediately removed. Liver was stored frozen at – 80°C.

A small amount of blood was used for hemoglobin determination by the cyanmethemoglobin method. Blood samples were then centrifuged (5 min, 12,000 g) and plasma samples were stored frozen (-20°C) until the moment for chemical analyses. Plasma sodium concentration was measured by flame photometry. Plasma osmolality was measured by freezing point depression. Plasma chloride and glucose concentrations in blood plasma were determined by spectrophotometric enzymatic method using commercial Kit. Total proteins in blood plasma and liver supernatant were measured according to Lowry et al. (1951).

Fish livers were homogenized and centrifuged (14,000 g for 20 min at 4°C). Supernatants were used for glutathione S-transferase (GST) and catalase measurements according to the following spectrophotometric procedures: GST was measured at 340 nm using 1-chloro-2,4-dinitrobenzene as substrate; catalase
at 240 nm by the decay of hydrogen peroxide levels. Liver samples were fixed in Bouin’s, dehydrated in ethanol and embedded in paraplast. Tissue was sectioned (5 µm) and stained with hematoxylin-eosin. Slides were examined under light microscope.

For each parameter analysed differences between control group and DWSF group, for each exposure period, were tested for significance by Student-t test. Means were considered significantly different where P < 0.05.

**Results and Discussion**

All fish exposed to DWSF survived the 15 days exposure and showed some functional changes (Table 1). Glucose concentrations for fish acutely exposed to DWSF were significant higher than those measured for control fish, indicating a stress-induced mobilization of energy reserves. An elevation of blood glucose is generally described as part of the fish stress response, in terms of carbohydrate metabolism. The impaired ability to keep increased blood glucose after 15 days of exposure to DWSF may reflect the depletion of glycogen reserves or a reduced capacity to respond to stress after a longer exposure to DWSF.

*P. lineatus* exposed to DWSF maintained good osmotic balance throughout the 96h study period once plasma osmolality, sodium and chloride concentrations were stable. However, after 15 days of exposure, plasma osmolality was increased significantly. Clearly more work is required to understand the significance of this rise in view of stable monovalent ions.

Liver GST activity was significantly increased only after 15 days exposure to DWSF. This result indicates that GST is induced by compounds present in DWSF. This induction reflects a response to the chemical, resulting in a conjugation process, where diesel compounds or its metabolites are combined with endogenous molecules to form conjugates that could be easier excreted.
Table 1. Catalase and glutathione S-transferase (GST) liver activity, plasma glucose and total proteins, plasma ions (Na\(^+\) and Cl\(^-\)) and osmolality and blood hemoglobin (Hb) of *Prochilodus lineatus* exposed to DWSF and clean water (control) for different time periods.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Group</th>
<th>Experimental Periods</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>24h</td>
</tr>
<tr>
<td>Catalase</td>
<td>Control</td>
<td>38.4±2.8</td>
</tr>
<tr>
<td></td>
<td>DWSF</td>
<td>40.6±3.9</td>
</tr>
<tr>
<td>GST</td>
<td>Control</td>
<td>86.3±14.1</td>
</tr>
<tr>
<td></td>
<td>DWSF</td>
<td>80.0±10.2</td>
</tr>
<tr>
<td>Glucose</td>
<td>Control</td>
<td>34.9±5.8</td>
</tr>
<tr>
<td></td>
<td>DWSF</td>
<td>55.6±6.7*</td>
</tr>
<tr>
<td>Proteins</td>
<td>Control</td>
<td>32.9±7.1</td>
</tr>
<tr>
<td></td>
<td>DWSF</td>
<td>29.9±5.1</td>
</tr>
<tr>
<td>Na(^+)</td>
<td>Control</td>
<td>159.6±5.9</td>
</tr>
<tr>
<td></td>
<td>DWSF</td>
<td>156.8±13.7</td>
</tr>
<tr>
<td>Cl(^-)</td>
<td>Control</td>
<td>108.2±5.4</td>
</tr>
<tr>
<td></td>
<td>DWSF</td>
<td>102.±6.1</td>
</tr>
<tr>
<td>Osmol</td>
<td>Control</td>
<td>284±7.3</td>
</tr>
<tr>
<td></td>
<td>DWSF</td>
<td>262±8.1</td>
</tr>
<tr>
<td>Hb</td>
<td>Control</td>
<td>5.7±0.2</td>
</tr>
<tr>
<td></td>
<td>DWSF</td>
<td>4.9±0.3</td>
</tr>
</tbody>
</table>

All data are expressed as mean ± SD. *different from control in the same period (P<0.05).

No significant differences were observed between control and DWSF exposed fish among the suite of remaining assays: catalase liver activity, blood proteins and hemoglobin content (Table 1).

DWSF exposure did not greatly affect liver histology and no specific lesion was observed. However, it was noted a nuclear enlargement in hepatocytes of fish exposed to diesel. This alteration in the volume of the nucleus can be regarded
as a sign of increased metabolic activity, as documented here by the increased GST activity.

Conclusions

Acute exposure of *P. lineatus* to DWSF caused significant physiological stress, resulting in elevated blood glucose levels. In the longer term (15 days) DWSF induced GST liver activity. Additional investigations are under way in our laboratory to better define DWSF effects to *Prochilodus lineatus* and their suitability as biomarkers in ecotoxicological studies.

References


Acknowledgements

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TOXIC ACTION OF TWO BIODEGRADABLE DETERGENTS
ON THE GILL EPITHELIUM
OF THE SWORDTAIL (Xiphophorus xelleri)

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EXTENDED ABSTRACT – DO NOT CITE

Introduction

The degradation of the environment by toxicants is not a local problem anymore, restricted to small areas, and today it assumes global proportions. The widespread use of synthetic detergents is responsible for a considerable increase of pollution in water bodies. The anionic surfactants are the most used in the world, and the presence of both hydrophobic and hydrophilic sites in the structure of this surfactants makes those molecules present some important properties such as: the tendency to concentrate at surface, the reduction of surface tension in the solution, and the formation of aggregates (micelles) (Abel, 1974), those characteristics play important roles in the manufacturing of many cleaning products, personal care products, and cosmetics. In Brazil, the use of surfactants began in the 50’s, and in the year of 1986 the estimated production of synthetic detergents was 450.000tons (Malagrinho & Rocha, 1987). In the last years the use of synthetic detergents, in Brazil and in other countries, had increasing and the increment of its consumption was one of the mains responsible by pollution of the freshwaters. The presence of surfactants was confirmed in water bodies of the state of São Paulo, by means of the reaction with methylene blue, and the concentrations reached levels of up to 6,71mg/l in some sites of the River Pinheiros after it passes by the city of São Paulo (Cetesb, 1977) Surfactants are recognized as acutely toxic to fish in concentrations
between 0.4 and 40mg/l, and the gill damage is the most obvious acute toxic effect of the surfactants (Abel, 1974). In this way, the aim of this work was to evaluate the toxic effect of two biodegradable surfactants, Sodium Dodecyl Sulafate (SDS) and Linear Alkylbenzene Sulphonate (LAS), on the morphology of the gill epithelium of the fish species *Xiphophorus helleri*.

### Materials & Methods

*Xiphophorus helleri* (0.6-2.4g and 3.0-6.5cm of standard length) were obtained from State Aquarium of Itaquera, São Paulo-SP, and transported for the ponds of the Mackenzie Presbyterian University-UPM. The animals were acclimatized for about two weeks in a tank of 2,000 liters, and the male individuals were kept separate from the females. The fish were placed in glass chambers with capacity of 10 liters and exposed to the detergents at a concentration of 0.1 mg/l for 96 hours in static tests, normoxic conditions, and pH ranging between 5.5 to 6.0. Experiments were conducted in duplicates, and all tests consisted of two treatments (SDS and LAS), besides the control group, which were further separated by gender (males and females). Each group contained 10 individuals. Fish were sacrificed with benzocaine (1ml/l), and the gills were removed, fixed in Bouin’s solution, dehydrated in concentrations of alcohol, cleared with xilol and embedded in paraffin for microtome sectioning (5 µm). The sections were stained with Hematoxylin-Eosin and Mallory for optical microscopy examination.

### Results and Discussion

The results of the toxicity tests revealed a larger mortality of the animals exposed to SDS, even without presenting statistical differences among the treatments, in spite of that substance being considered less toxic than LAS. Morphological analyses of the gills revealed that animals exposed to LAS and SDS presented a thicker epithelium compared to control fish, with the greatest alterations found in animals exposed to the SDS (Fig. 1). The action of LAS on the gill epithelium follows the damage pattern found in short-term exposure, with lamellae epithelium thicker than normal and hyperplasia between the secondary lamellae, suggesting an edema. SDS induced hyperplasia, resulting in an almost total occlusion of the spaces between the secondary lamellae, giving the filament a compact appearance. Large spacings inside the lamellae were also observed, with invasion of leucocytes and disarrange of the pavement cells. In agreement with Rosety-Rodriguez *et al* (2002), hyperplasia and fusion of the secondary lamellae diminishes the amount of available gill surface area.
and offers a protective function against the injury caused for substances dissolved in the water to the animals. Despite the lower toxicity of LAS, the extent of damage induced by SDS in the gill epithelium was larger than those induced by LAS at the concentration of 0.1 mg/l. The morphological changes observed in the gill epithelium of the swordtails exposed to the LAS and SDS suggest the beginning of an inflammatory response of the tissue from the irritation induced by the detergents. This type of response is considered a physiological adjustment commonly found in animals exposed to a wide variety of stressful conditions, and those alterations in the morphology of gill epithelium may result in functional disorder of this organ with great impact on gas exchange and ionic status (Goss et al., 1998).

References


Fig. 1. (A) Light micrograph showing gill epithelium of control fish (x1000). (B) Light micrograph of gill epithelium from a fish sacrificed after exposure to 0.1 mg/l LAS (x1000). Note that the lamellae epithelium is thicker than normal. (C) Light micrograph of gill epithelium from a fish sacrificed after exposure to 0.1 mg/l SDS (x1000). Note the almost total occlusion of the spaces between the secondary lamellae. All micrographs were stained with Hematoxilin-Eosin.
2,3,7,8-TCDD EFFECTS ON VISUAL STRUCTURE
AND FUNCTION IN SWIM-UP RAINBOW TROUT

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EXTENDED ABSTRACT ONLY - DO NOT CITE

Complete article in press in Environmental Science and Technology

Introduction

The study of effects of chemical contaminants on the visual system provides an
interesting paradigm to evaluate links between contaminant effects from the
suborganismal to the individual level of biological organization.

We applied a suite of biochemical, histological, and behavioral endpoints related
to visual structure and function and foraging behavior to evaluate effects of
2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) on swim-up rainbow trout. These
studies were conducted to provide quantitative linkages between biochemical,
tissue, organ and individual level responses of rainbow trout to TCDD.
Material and methods

Fertilized eggs from Eagle Lake strain rainbow trout (Oncorhynchus mykiss) were nano-injected with 2,3,7,8-TCDD standard solutions. Exposure treatments included controls (CT), doses of 38 pg TCDD.egg-gram-1 (T1), 113 pg TCDD.egg-gram-1 (T2) and 300 pg TCDD.egg-gram-1 (T3). Another treatment group called T3-edema (T3-ed) involved fish injected with 300 pg TCDD that developed yolk-sac edema but survived until the swim-up stage. All fish tested in the present study were evaluated at the swim-up stage, ranging between 28 days post-hatch (dph) and 32 dph.

First, we tested the hypotheses that dose-response relationship exists between TCDD exposure and the endpoints of effect related to visual function (acuity, motion detection and low light sensitivity). The testing system addressed optomotor and optokinetic responses, which are unconditioned optical and motor reactions to a series of alternating vertical black and white stripes on the inside of a cylinder that is rotated around a fish. Visual function was evaluated based on these responses using an apparatus and operational procedures described in detail in Carvalho et al. (2002). Second, we evaluated dose-related effects on prey capture rate. Third, we histologically examined key tissues associated with vision. Brain, retina and the eye choroid vasculature were evaluated by immunohistochemistry for dose-related induction of the drug-metabolizing CYP1A protein. Linear densities of retinal photoreceptors (rods and cones) and retinal ganglion cells (RGCs) were quantified.

Data were tested for treatment differences using either one-way ANOVA or Kruskall-Wallis non parametric one-way ANOVA.

Results and Discussion

Rod or cone densities at swim-up did not change in any of the TCDD treatment groups when compared to control fish (Table 1). However, a statistically significant deficiency in the photopic acuity angle and photopic FFT of fish from T2, T3 and T3-ed treatment groups were detected (Table 1). Additionally, deficiencies in behavioral scotopic thresholds were detected at T2, T3 and T3-ed (Table 1). Therefore, in spite of the lack of differences in cone or rod density between TCDD exposed and control fish, generalized dose-dependent visual/motor function impairment was detected in all behavioral visual
parameters evaluated. Visual function is dependent not only on photoreceptor density, but also on the degree of convergence between these photoreceptors and upstream neurons such as RGCs (Browman et al., 1990). RGCs are critical as they are responsible for the synaptic link from the retina to the optic tectum in the brain (Djamgoz & Yamada, 1990). Our results indicated reductions in ganglion cell densities at T2, T3 and T3-ed (Table 1), which are in agreement with the generalized visual function deficits observed. This increase in photoreceptor to ganglion cell convergence could have caused a deficit in visual information transfer in TCDD exposed groups that could be involved in the etiology of the visual neuro-motor deficiencies observed in this study.

Table 1. Summary of results of endpoints measured in swim-up rainbow trout exposed to 2,3,7,8-TCDD through egg-injection

<table>
<thead>
<tr>
<th>Dose</th>
<th>Rod or cone density (cells.100µm⁻¹)</th>
<th>RGC density (cells.100µm⁻¹)</th>
<th>Acuity angle (degrees)</th>
<th>Flicker fusion threshold (cycles.s⁻¹)</th>
<th>Scotopic threshold (log illuminance)</th>
<th>Prey capture rates</th>
<th>Captured Ceriodaphnia</th>
</tr>
</thead>
<tbody>
<tr>
<td>CT</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>T1</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>T2</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>p&lt;0.05</td>
<td>p&lt;0.05</td>
<td>p&lt;0.05</td>
<td>NS</td>
</tr>
<tr>
<td>T3</td>
<td>NS</td>
<td>p&lt;0.05</td>
<td>p&lt;0.05</td>
<td>p&lt;0.05</td>
<td>p&lt;0.05</td>
<td>p&lt;0.05</td>
<td>p&lt;0.05</td>
</tr>
<tr>
<td>T3-edema</td>
<td>p&lt;0.05</td>
<td>p&lt;0.05</td>
<td>p&lt;0.05</td>
<td>p&lt;0.05</td>
<td>p&lt;0.05</td>
<td>p&lt;0.05</td>
<td>p&lt;0.05</td>
</tr>
</tbody>
</table>

RGC: retinal ganglion cell  
NS: no significant difference (p>0.05)  
p<0.05: statistically significant difference when compared to controls

Our results on retinal histology indicate statistically significant decreases in ganglion cell density in T2, T3 and T3-ed, treatment groups where we also detected significant increases in CYP1A induction in vascular endothelial cells of the brain and in the choroid vasculature that perfuse the eye (Figure 1). These results indicate direct effects of TCDD on AHR mediated gene transcription in these tissues and a possible connection to a mechanism leading to ganglion cell death (Cantrell et al., 1998).
Figure 1. Immunohistochemical CYP1A staining index of pseudobranch, choroids capillary and brain vasculature in swim-up rainbow trout injected with 2,3,7,8-TCDD at the egg stage. *: statistically significant difference from controls (Dunn’s test, p<0.05)

Prey capture rates decreased in T3 and T3-ed treatment groups (Table 1), results that could be partially explained by the deficits in visual acuity angle and photopic FFT.

Conclusions

We detected a dose-dependent decrease in densities of retinal ganglion cells (RGC), key retinal neurons that link the eye with the brain. These changes resulted in corresponding deficits in visual/motor function including reductions in visual acuity, and in scotopic and photopic thresholds due to TCDD. Dose-dependent increases in immunohistochemical detection of CYP1A protein in the vasculature of the brain and eye choroid was proportional with decreased ganglion cell densities in the retina. Prey capture rate decreased after TCDD exposure. Collectively, these results show that TCDD causes biochemical and structural changes in the eye and brain of rainbow trout that are associated with behavioral deficits leading to decreased individual fitness.
Acknowledgements

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References


METHYL PARATHION EFFECT IN MATRINXÃ (*Brycon cephalus*)

MUSCLE AND BRAIN ACETYLCHOLINESTERASE ACTIVITY

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Abstract

The farming of the freshwater fish is emerging in Brazil and many species from the wild are promising. The teleost matrixã (*Brycon cephalus*) holds several characteristics, which make it a promising species for commerce. The use of pesticides in aquatic environments is frequent in Brazil, and methyl parathion is very common in aquaculture. We have determined the activity of white muscle and brain acetyl cholinesterase of matrixa exposed to low concentrations of environmental methyl parathion for 24 hours. There was 64.3% and 69.3% of acetyl cholinesterase inhibition in muscle and brain, respectively. These activities were not recovered after 96 hours from exposure. We concluded that acetyl cholinesterase from those tissues was inhibited by small amounts of methyl parathion, and the main effect was observed in the brain.
Introduction

Pesticides are among the most hazardous chemicals to men and ambient. In fact, the lack of information about risks of intoxication causes the improper and excessive use of them in different combinations, mainly in the Southeast of Brazil where the highest selling rates are observed (MEYER et al., 1999).

Insecticides input take place to protect agricultural crop against damaging caused by insects. However, these chemicals may achieve other ecological compartments as lakes and rivers through rains and wind, affecting many other organisms away from the first target. Only 0.1 % reaches the specific target (Aguiar, 2002; Rand & Petrocelli, 1984).

The injuries of insecticides to aquatic environment are clear, and fish are able to bioaccumulate due to the direct exposure to chemicals and ingestion of contaminated preys and food (Rand & Petrocelli, 1984). Methyl parathion is an organophosphorous largely used in Brazil to avoid agriculture losses due to fruit flies, bugs, and other insect attacks. In addition, methyl parathion has been often used in our fish farms to prevent fingerlings losses due to predation by Odonata nymphs and Chironomidae (Figueiredo & Senhorini, 1990).

Matrinxa is a freshwater teleost fish widely reared in Brazil (CASTAGNOLLI, 1992), directly exposed to pesticides in both natural and farming conditions. Acetyl cholinesterase (AChE) is a biomarker extremely used in aquatic ecotoxicology studies (KIRBY et al., 2000), and AChE is fairly sensitive to low environmental organophosphorous concentrations. Methyl parathion inhibits AChE activity in fish, resulting in a neuron system blockage. In this case, the muscular system may keep moving without control until paralysis, convulsion and death (AGUIAR, 2002).

The goal of this work was to determine the methyl parathion effect on AChE activity in muscle and brain of matrinxa as a way to evaluate the methyl parathion risks to consumers and environment.

Material and methods

Fish were purchased from a commercial fish farm and stocked in a 2 m³ at 25°C during 1 month. Water quality parameters were daily monitored for growth of matrinxa. Fish were fed a commercial feed of 35 % of crude protein once a day.
Before the trials, 48 fish (W = 49.5 ± 1.7 g) were equally distributed in 6 tanks (90 liters each) with constant water flow, aeration and heating at 25 °C. In the course of 24 h of experimental exposure, 4 of these tanks received methyl parathion in the commercial form of Folidol-600® at the final concentration of 2 ppm, and the other 2 tanks did not receive any chemical (control groups).

After 24 h of exposure, brains and white muscles samples were collected. Samples were stocked in freezer (-20 °C) for posterior AChE assays. Matrinxa was exposed to methyl parathion concentration of 2 ppm for 24 h, because previous studies of AGUIAR (2002) established these parameters as suitable for AChE inhibition.

The water flow was reestablished in the other 3 tanks for recovery. Fish brains and white muscles samples were collected 24, 48 and 96 h after being reestablished the water flow (recovery). Control groups were sampled after the experimental exposure (0 h of recovery) and the experimental recovery (96 h).

Folidol was analyzed in the water samples at λ = 275 nm in Beckman DU® 520 spectrophotometer (AGUIAR, 2002).

Total protein was determined according to LOWRY et al., (1951) to express the specific AChE activity. AChE activity was determined according to ELLMAN (1961). This method is conducted through the acetylcholine hydrolysis to acetic acid and tiocholine by action of the AChE. All AChE values were express as mmol/min/mg protein.

ANOVA and TUKEY-KRAMER (P<0.05) were used as statistic tests.

Results

AChE of controls groups was constant (7.6 x 10^{-4} ± 7.35 x 10^{-5} mmol/min/mg of protein), which enabled us to observe a clear 64.3 % inhibition of muscle AChE due to methyl parathion exposure.

Specific AChE activity significantly (P<0.05) decreased in matrinxa white muscle and brain, after the 24 h of experimental exposure to methyl parathion. Nevertheless, AChE values kept low even after recovery of 96 h (Figure 1). Fish were alive, but only 31 % of AChE initial activity was observed. Brain AChE activities were also constant in the control groups (4.8 x 10^{-3} ± 1.1 x 10^{-3}
mmol/min/mg of protein). Methyl parathion exposure resulted in 69.7% AChE activity decrease (Figure 1), which kept low even after 96 h of recovery.

![Graph showing AChE activity](image)

**Fig. 1.** Brain and muscle AChE activity specific of matrixxa exposed to methyl parathion and recovery. * means brain and muscle averages are statistically different from the controls.

### Discussion

Pesticides as organophosphorous and carbamates are the main insecticides in the agrochemical market, which yearly circulates more than 2.5 billion dollars. One hundred different organophosphorous are reported in the United States, where 200 million acres are sprayed every year (HOFFMAN *et al*., 1995). In spite of Brazil present unique ecological systems, methyl parathion is extremely used in their fields. The large use of pesticides in Brazil rank it as the five most world consumers (EXTOXNET, 1999).

Methyl parathion is an organophosphorous insecticide classified by the US environmental protection agency (EPA) as restricted use pesticide (RUP). Only authorized staff can manipulate methyl parathion that seems to be not
bioconcentrated and persist in environments (EXTOXNET, 1999; HOWARD, 1989), for plants and animals can quickly metabolize it.

Decrease of AChE activity by methyl parathion intoxication has been reported in different animals and fish as matrinxa (AGUIAR, 2002; RAO & RAO, 1984; CHAMBERS et al, 1996; BENKE & MURPHY, 1974). However, most of the AChE studies are done in fish brains, because the most evident effects are observed in nervous tissues (). Therefore, the brain of matrinxa showed the highest AChE inhibition, as expected by RAO and RAO (1984) who compared the AChE inhibition in different tissues of Nile tilapia exposed to 1/3 of methyl parathion LC 50 for 48 h. Afterwards, they observed that brain had the highest inhibition levels followed by muscle, gills and liver. The AChE inhibition is fairly related to the tissue enervation level. Hence, we can say that the highest AChE concentration the highest inhibition susceptibility.

Matrinxa is a very consumed fish in Brazil and other countries of Latin America (CASTAGNOLLI, 1992). BOONE and CHAMBERS (1996) reported this fish as are more resistant to methyl parathion than mice and other mammalian, which have lower AChE stocks. In addition of the higher tolerance of fish to methyl parathion, matrinxa is kept alive after exposure to methyl parathion in our water ponds directly contaminated with 0.25 to 3 ppm (FIGUEIREDO & SENHORINI, 1990). In such circumstances, methyl parathion may be definitely hazardous to people if contaminated fish are consumed or if people are directly exposed to methyl parathion and contaminated water.

Fish or other animal intoxication by the methyl parathion is characterized by biotransformation into a molecule that is fairly similar to acetylcholine. This molecule binds to AChE, blocking the breakdown of acetylcholine by AChE. Acetyl choline is the primary neurotransmitter in the sensory and neuromuscular systems of fish, and acetylcholine levels are regulated by the AChE, which degrades the acetylcholine into the active products: choline and acid acetic. These are reabsorbed and used as raw materials for the continued acetylcholine production that results in a build up of acetylcholine. This also causes a continuous and excessive stimulation of the nerve/muscle fibers (KIRBY, 2000).

Other authors have observed that fish exposed to methyl parathion did not recover AChE activity after long periods of time (WALLACE & HERZBERG, 1998; CARR et al., 1995; BENKE & MURPHY, 1974). Fish have also AChE recovery period much longer than mammalian (WALLACE & HERZBERG,
Matrinxa did not recover AChE values even 96 h after the reestablishment of the control conditions. WALLACE e HERZBERG (1998) showed the missing brain AChE reactivation capacity in rainbow trout after exposure to methyl parathion, and channel catfish Ictalurus punctatus had no AChE recovery even 10 days after the return of the control conditions (CARR et al., 1995). Pumpkinseed sunfish exposed to parathion and methyl parathion had no AChE recovery even 4 weeks after the reestablishment of the control conditions (BENKE & MURPHY, 1974). Our results corroborate the proposal on the phosphorilation of AChE as a mechanism of increasing the enzyme resistance to hydrolysis (aging). Spontaneous AChE reactivation and aging are the main reason of the no AChE reestablishment.

In conclusion, our results show the hazardous effect methyl parathion in the Neotropical fish matrinxa and points to the needs of other approaches, as histological and cytological studies, of methyl parathion in this fish. Hence, it will be possible to conclude easier upon additional risks to environment and people exposed to methyl parathion, in fisheries or other water dependent farming activities.

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Sites

www.ace.orst.edu/info/externet/ghindex.html Informações toxicológicas e caracterização de pesticidas
Behavior is a sensitive response of animals to contaminants. Behavioral responses may be due to underlying changes in biochemistry of neurotransmitters or other neurotoxicological or endocrine responses. Changes in important behaviors such as prey capture ability may also demonstrate links to ecological effects, such as population or community changes. We have found that killifish, or mummichogs, Fundulus heteroclitus, from a contaminated area in northern New Jersey, Piles Creek (PC) have reduced condition, growth, and longevity, compared with fish from less polluted sites. This may be due to abnormal behavior. PC fish have a lower level of spontaneous activity, and a reduced rate of prey capture. Laboratory studies demonstrated them to be poor predators on grass shrimp, Palaemonetes pugio, compared to fish from the reference site in southern New Jersey, Tuckerton (TK) (Smith and Weis, 1997). Analysis of videotapes showed that the poor prey capture of the PC fish was primarily due to fewer attempts to capture prey, rather than to poor coordination. PC fish generally did not persist and frequently gave up after chasing a shrimp for a brief period of time. When TK fish were maintained in an aquarium with...
PC sediments and water and fed PC grass shrimp for a month, their prey capture ability declined to that of PC fish (Figure 1) and their level of brain mercury increased to that of the PC fish. When PC fish were maintained in the laboratory in clean water for two months, there was only a slight improvement in their prey capture ability, and their brain mercury levels did not decrease (Smith and Weis, 1997). Analysis of field-collected mummichogs’ gut contents indicated that PC fish were eating primarily detritus and much less live food, including less Palaemonetes, than TK fish (Smith and Weis, 1997). This can be considered a field validation of the prey capture experiments performed in the laboratory, and can partially explain the poor growth seen in this population, since detritus is not nutritious for mummichogs. PC fish, being generally "slow," were also more vulnerable to predation by blue crabs (Callinecestes sapidus) than TK fish (Smith and Weis, 1997). Increased vulnerability to predation can contribute to the previously noted observation that PC mummichogs do not live as long as fish from the reference sites.

Figure 1. Prey capture by fish from Tuckerton TK, from Piles Creek PK, Tuckerton fish maintained in PC conditions TK(PC), and Piles Creek fish maintained in clean water, PC(d).
The poor prey capture ability and reduced general activity level of PC fish were correlated with lowered brain levels of the neurotransmitter 5-HT, or serotonin (Smith et al., 1995). While this was only a correlation, further work involving experimental manipulation of serotonin levels indicated that the levels of brain serotonin were indeed directly related to the activity level of the fish. Recently hatched larval mummichogs from PC, which were not behaviorally impaired, had levels of serotonin that were comparable to those of TK larvae, although they did have higher levels of dopamine and its metabolites (Zhou et al., 1999a).

The sluggish behavior in the PC fish prompted an investigation of the thyroid gland, an endocrine gland that is often associated with general activity levels in vertebrates. Endocrine disruption is an issue that is receiving much attention in environmental toxicology, because it is a very sensitive indicator of contaminant effects. PC fish had irregularly shaped and greatly expanded thyroid follicle size compared to TK fish (Zhou et al., 1999b), which can be interpreted as a type of goiter (Fig. 2). In addition to abnormal thyroid histology, PC fish had significantly elevated T4 (thyroxine) levels, and a trend of reduced levels of T3 (tri-iodothyronine), which did not reach statistical significance. The thyroid abnormality in PC fish was found to develop when they are larvae about 15 mm long. Altering the thyroid hormone levels experimentally by dosing the water with T3 or phenylthiourea (PTU, a thyroid hormone antagonist) altered both spontaneous activity and prey capture rates in juvenile fish from both populations (Carletta et al., 2002).

Grass shrimp, Palaemonetes pugio, a major prey species, are not impaired behaviorally at the polluted site, but instead are larger in size and also more numerous than at the reference site, while their mummichog predators are less numerous and smaller (Bass et al. 2001). The larger body size and larger population size of the shrimp is probably due to reduced predation by mummichogs. In this way, behavioral changes can demonstrate linkages of contaminant effects from the biochemical and cellular levels to the organism, population, and community levels.

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Acknowledgments

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Figure 2. Representative thyroid tissue from TK on left and PC on right. Compare sizes of thyroid follicles (t) with the branchial afferent artery (ba).
Prey capture ability was evaluated as a behavioral biomarker of contamination by examining feeding behavior of larval and adult mummichogs (*Fundulus heteroclitus*) from 13 sites on the mid-Atlantic coast, USA (Weis et al, 2001, 2003). Parameters included analysis of metals in sediments and in mummichog liver and brain, gut contents of field-collected adults, and prey strikes/captures in the lab (three adults with 10 grass shrimp (*Palaemonetes pugio*) for 30' in an 80 L tank; individual 3 and 8 d post-hatch larvae in a watch glass with 5 brine shrimp (*Artemia salina*) nauplii. In addition to our data, we utilized existing sediment data on organic contaminants, collected at or near these sites by other investigators, such as the U.S.E.P.A. Environmental Monitoring and Assessment Program (EMAP) (Nelson et al.1996).

*Adults:*

Prey capture ability of adults (Figure 1) was related to sediment and tissue contaminant levels and with previous genetic analyses. The levels of contaminants at a site were highly correlated with each other, confounding the...
impacts of individual contaminants. The number of grass shrimp captured was highest in three of the cleanest sites. Sites with the lowest capture rates were generally more contaminated. The number of captures at all sites was highly variable, with both high and low efficiencies in highly contaminated populations. A significant relationship exists between the Mdh-A(a) allele (Weis et al, 1999) and captures, with higher captures in the southern populations. Gut content analysis of field-collected fish had grass shrimp as the largest proportion of the diet at sites whose fish had the highest laboratory capture rates. Thus, prey capture is ecologically relevant since it corresponds to diet in the field. Behavioral differences related to overall contaminant levels rather than to specific toxicants.

Figure 1. Average number of strikes and captures (± standard error of mean) of grass shrimp in 30 minutes by adult mummichogs of the different populations. The sites are: BB = Bullhead Bay, NY; BC = Berry’s Creek NJ; EH = East Hampton NY; FC = Foundry Cove NY; NB = Newark Bay NJ; ND = New Bedford Harbor, MA; PC = Piles Creek NJ; SC = Scuffletown Creek, VA; SH = Sandy Hook NJ; TK = Tuckerton NJ; UB = Union Beach NJ; VL = Vince Lombardi Rest Stop NJ; WI = West Island MA. R = reference site; I = intermediate, and H = heavy contamination sites.
As part of the EMAP program, some bioindicators were evaluated to see how well they correlated with estuarine conditions (Summers et al. 1997). They focused at both ends of the spectrum, i.e., at reference sites and at heavily impacted sites, and found that certain of these indicators (pathologies, splenic macrophage aggregates, and vertebral abnormalities) showed promise. If we remove our intermediate sites (see Figure 1), and include only reference and heavily impacted sites, more significant associations would be seen. Using only those sites, the correlations of captures increase dramatically with sediment Hg (p = 0.01), sediment Cu (p < 0.0001), sediment Pb (p < 0.0001), sediment Zn (p = 0.0002), and sediment PCB (p < 0.0001).

Larvae:

We were able to obtain fertilized eggs for providing larvae from fish at 10 of the same sites. We evaluated larval prey capture as a behavioral biomarker of contamination by examining feeding behavior of larval mummichogs on brine shrimp (Artemia salina) nauplii and related this prey capture ability to sediment contaminant levels. Since the levels of contaminants at a site were highly correlated with each other, the impact of individual contaminants was confounded. The number of captures of brine shrimp by mummichog larvae from all sites was highly variable, but significant negative correlations of prey capture by 8 d post-hatch larvae (but not 3 d) were seen with Hg (p < 0.001), Pb (p = 0.0055), Zn (p < 0.001), Cd (p < 0.001), and PCBs (p = 0.032). As observed with adults, polyaromatic hydrocarbons (PAHs) did not appear to impair prey capture ability. The only site in which prey capture rates of 8-day old larvae were severely affected was the most highly contaminated Superfund site, Berry’s Creek, NJ (Figure 2). This implies that larval prey capture is not as sensitive a behavioral biomarker for contamination as adult behavior.

Prey capture as a behavioral biomarker is ecologically relevant and corresponds well to fish diet in the field. However, it does not appear to be especially sensitive due to the great variability in behavior among the fish from each site. The behavioral differences in our study seemed to be best related to overall contaminant levels rather than to a specific toxicant, and the development of tolerance in some highly contaminated populations can obscure results.
Figure 2. Captures of *Artemia* nauplii in five minutes by mummichog larvae from 10 study sites.

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Startle reflexes in larval fish provide a simple model to correlate Hg-induced neuronal dysfunction with behavior. Zebrafish embryos were exposed to 0-50 ppb Hg$^{2+}$ for 0-24 hr post fertilization. Embryonic Hg$^{2+}$ uptake was dose-dependant. Larvae (7 day-old) were observed with high-speed videography for response to a single tap. While no significant difference existed for latency of response, larvae from eggs exposed to 50 ppb Hg$^{2+}$ separated into two distinct groups: 1) a delayed response that was twice as long as either the control or 25 ppb Hg$^{2+}$ groups and 2) no significant difference with the control group. Total reaction time showed a significant, asymptotic, dose-dependent response. There were no significant differences between exposure regimes for maximum velocity normalized for body length. Thus, differential effects of developmental Hg exposure on components of the startle reflex arc may exist.

Introduction

Developmental exposure to Hg$^{2+}$ and organomercurials is a major health concern (Clarkson, 1998) due to inducing neuron dysfunction. It is often expressed as altered behavioral outcomes directed by the central (CNS) and peripheral (PNS) nervous systems (Zhou et al., 2001; Zhou and Weis, 1998; Ososkov and Weis, 1996). While adults are exposed directly to Hg compounds,
the primary route of embryonic exposure is through maternal transfer. This is particularly true for populations that rely on marine species in their diet, e.g., Native Americans, Inuits, and oceanic island populations (Power et al., 2002; Grandjean et al., 1997; Delling et al., 1995; Myers et al., 1995). Hg-induced CNS effects are observed in sensory neurons, neural centers involved in processing and coordinating information, and brainstem evoked potentials (Urban et al., 1996). Adult Hg-induced neurobehavioral effects include loss of hand-eye coordination, increased tiredness and confusion, and deficits in vision and hearing (Gobba, 2000; Letz et al., 2000). Early neurodevelopmental stages are more sensitive than adults to Hg$^{2+}$ exposure (Rice et al., 2000). Such exposures cause age-specific hyperactivity in fishes (Eaton et al., 1977) and reversible dysfunction of lateral line sensory neurons, i.e., fish tended to collide more often when in dense groups vs. control groups (Ososkov and Weis, 1996). This study focused on startle reflex responses in zebrafish (*Danio rerio*) larvae, after embryonic exposures to Hg$^{2+}$.

**Methods**

**Exposure Regime:** Fertilized eggs (~500) were evenly divided between 3 sublethal exposure regimes (0, 25, 50 ppb Hg$^{2+}$) for 0-24 hours post fertilization. Eggs then removed and either placed in 0 ppb Hg$^{2+}$ until 7 days post hatch for behavioral testing or used immediately for embryo Hg analysis.

**Embryo Hg Analysis:** Eggs (N = 50) were weighed, placed in Teflon™ microvials (3.0 mL), and acid digested (2.0 mL of 4 parts concentrated HNO$_3$: 1 part digestion solution–5% HNO$_3$, 0.1% HCl, 500 ppb Au in ultrapure Milli-Q™ water) in a microwave oven (MARS 5, CEM Corp., Matthews, NC). Gold added to the digestion solution to scavenge Hg that might adsorb to the vessel wall. Digestions occurred under a temperature-controlled program (25-130°C over 10 min, held at 130°C for 10 min, cooled to 80°C). Samples decanted into 20-mL autosampler vials and brought to a final volume of 10.0 mL with 8.0-mL digestion solution. Hg analyzed with a MicroMass Platform ICP-MS (Manchester, UK) equipped with a CETAC ASX 500 autosampler (Waters Corp.) under MassLynx NT software control for Hg measurements. Hg calibration standards prepared from a 10 ?g/mL (in 5% HNO$_3$) Hg standard (Certiprep, NJ). Solvent system solution blank was 5% HNO$_3$, 0.1% HCl and 500 ppb Au in ultrapure water (18 MegOhm). Acids used were ICP grade (Optima, Fisher Scientific). All analyses measured in SIR Mode (Single Ion Recording) for 60 seconds. Labware immersed in 30 mM EDTA overnight and
rinsed in glass-distilled water to remove all metal contaminants.

Behavioral Tests: D. rerio larvae (7-day-old; N = 8/trial) were placed into a 3 cm-diameter, 2 mm deep chamber lit by fiber optics (Solarc Lighting Technologies; New York). Fish acclimated for 5 min before tested. Adjacent to the chamber was a metal pin that a plastic hammer, attached to a spring, hit to create a vibration. A control box regulated stimulus intensity. Behavior recorded using a Photron PCI 500 CCD camera (Motion Engineering Co., Indianapolis, IN) at 500 Hz and analyzed for latency of response, maximum velocity normalized for body length \( V_{\text{max}} \) after single stimulus applied, and total reaction time (WINanalyze software, Mikromak, Germany).

Statistical Analyses: All variables evaluated for normality of data and variance using Kolomogorov-Smirnov tests followed by one-way ANOVA and Tukey’s post hoc tests. Level of significance was \( \alpha = 0.05 \). Correlations determined with Pearson’s Correlation Coefficient: two-tailed test for increases and decreases in total reaction time; one-tailed test for latency of response with controls assumed to be fastest possible time.

Results

Embryo Hg Analysis: Hg\(^{2+}\) uptake (ppb Hg\(^{2+}\) /egg) was dose-dependant (Figure 1; ANOVA, P < 0.001): 0 ppb Hg\(^{2+}\) - 1.23±0.16; 25 ppb Hg\(^{2+}\) - 5.46±0.41; 50 ppb Hg\(^{2+}\) - 7.57±0.82; 100 ppb Hg\(^{2+}\) - 49.99±4.93.

Behavioral Tests: There was no significant correlation (Pearson’s Correlation Coefficient) between Hg\(^{2+}\) exposure concentration and \( V_{\text{max}} \) or latency of response or total reaction time, or latency of response and total reaction time. There were significant correlations between Hg exposure concentration and total reaction time (\( R = 0.633; P < 0.001 \), two-tailed test) and Hg exposure concentration and latency of response (\( R = 0.371, P < 0.05 \), one-tailed test).

There was no dose-dependent increase in the mean latency to respond (Figure 1; ANOVA, P > 0.05), although an upward pattern was observed.

However due to the large standard error of the mean at the 50 ppb Hg\(^{2+}\) exposure level, these data were divided: one set of larvae did not respond significantly
differently from controls, and a second set displayed significantly longer latency of responses (Figure 2; ANOVA, P < 0.05).

Figure 1: Effect of developmental Hg$^{2+}$ exposure (0-24 hpf) on latency to respond (msec) after application of a single vibrational stimulus to 7-day-old zebrafish larvae (N = 8).
Figure 2: Effect of developmental Hg\(^{2+}\) exposure (0-24 hpf) on the latency to respond (msec) after application of a single vibrational stimulus to 7-day-old zebrafish larvae (N = 8). Larvae from the highest exposure regime separated into two groups (listed as 50 and 50.1 ppb Hg\(^{2+}\)) based on response time.

A significant, non-linear, dose-dependant relationship occurred for total reaction time (Figure 3; ANOVA, P < 0.005), reaching an asymptote at approximately 25 ppb Hg\(^{2+}\) (122.8 ± 24.5 [control] vs. 206.1 ± 11.6 [25 ppb Hg\(^{2+}\)] vs. 239.4 ± 25.7 [50 ppb Hg\(^{2+}\)] msec).

There was no dose-dependant relationship between exposure regime and \(V_{max}\) normalized for body length under the exposure regimes of this study.
Conclusion

Differential sensitivity of sensory, descending, and neuromuscular neurons to embryonic Hg\textsuperscript{2+} exposure may exist. 1) A lack of altered latency of response (Figure 1) suggests lateral line neurons may not be affected during very early embryonic exposures. Conversely, nerve impulses sent from the Mauthner cell to the appropriate trunk muscles facilitate rapid escape responses. Data (not shown) indicate that V\textsubscript{max} is not altered by developmental Hg\textsuperscript{2+} exposure, suggesting that effects on the neuromuscular junction or specific interneurons may not occur during the first 24 hours after fertilization. 2) Zebrafish possess a dual system of neural control of reflex movements that induce fish movements in a sequential manner after a directional stimulus is applied (Gahtan et al., 2002). Initial reflex movements (latency of response) are controlled by circumferential descending interneurons. Continued swimming is controlled by

Figure 3: Effect of developmental Hg\textsuperscript{2+} exposure (0-24 hpf) on total reaction time (msec) after application of a single vibrational stimulus to 7-day-old zebrafish larvae (N = 8).
the multipolar commissural descending interneurons. Prolonged responses to a single stimulus after early embryonic exposure (Figure 3) suggest Hg-induced dysfunction may occur in these multipolar commissural descending neurons.

There appears to be a Hg-induced, nonlinear, asymptotic relationship for total reaction time (Figure 3). There was no significant difference between 25 and 50 ppb Hg\(^{2+}\), suggesting that this plateau occurs at exposure levels below the LC\(_{50}\) for zebrafish eggs (between 100-200 ppb Hg\(^{2+}\) [personal observation]).

Individuals within a population show a greater range of responses under stress than under control conditions. This was evident at 50 ppb Hg\(^{2+}\) (Figure 1) with data falling into two distinct groups. When these were analyzed separately, one group was significantly different than the control and all other treatments (Figure 2), suggesting potential genetic influences in behavioral outcomes.

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TOLERANCE AND WITHDRAWAL IN GOLDFISH
EXPOSED TO ETHANOL

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EXTENDED ABSTRACT ONLY: DO NOT CITE

Animals typically respond to chronic drug or toxin exposure by developing tolerance. Continued exposure often leads to a dependent state, which is revealed by the withdrawal symptoms which occur when the drug or toxin is removed. Tolerance and withdrawal are usually studied in mammals, although it is likely that similar processes occur in fish as well. In this study we investigate these processes in the goldfish, Carassius auratus. We utilize ethanol as the drug and selected temperature (Tsel) as the response.

Tolerance is studied in one of three administration regimes. Acute tolerance effects occur within one exposure, rapid tolerance is measured as reduced response to a second dose about 8 - 24 hours after the first, and chronic tolerance obtains following many exposures over a longer period of time [4]. Acute tolerance is the least understood, since in mammals assessing the time course for its development is difficult because the standard protocol involves a single
injection. Responses are compared, at the same concentration, for the rising and falling concentration of drug in the body. However, drug levels are constantly in flux and change at different rates in the various compartments of the body. Thus, while it is straightforward to demonstrate the phenomenon of acute tolerance, estimating the rate at which it develops can require many groups of animals and complicated protocols. The development of acute tolerance can be estimated by procedures involving continuous monitoring and arterial infusion, but this procedure is not applicable in all situations. In this experiment we take advantage of the thin gill epithelium of fish which permits rapid entry of many waterborne chemicals into the blood. When goldfish are placed in water containing ethanol, steady state brain concentrations that are directly related to the concentration of ethanol in the water are soon attained [3]. This provides a method for evaluating response changes under constant blood ethanol levels.

To study the development of acute tolerance, we monitored Tsel of goldfish (Carassius auratus) for 9 h while they were exposed to one of three doses of ethanol (v/v). After initial exposure, Tsel was: Control: 24.1 ±0.07 °C; 0.4% ethanol: 21.9 ±0.09 °C; 0.8% ethanol: 21.3 ±0.05 °C; 1.1% ethanol: 18.4 ±0.10 °C. The difference between control and experimental Tsel decreased by the following amounts for the final 1.5 h in the gradient: 0.4% ethanol: 2.60 ±0.12 °C; 0.8% ethanol: 1.58 ±0.09 °C; 1.1% ethanol: 4.08 ±0.12 °C. At all 3 doses, tolerance proceeded in a stepwise manner rather than continuously.

In most cases, responses seen during withdrawal are of opposite sign to the initial effects of drug or toxin administration. Thus, in the case of ethanol (a sedative), withdrawal signs reflect stimulation and are thought to represent a rebound response, i.e., an overcompensation for the organism’s adjustment to the presence of ethanol. In humans, withdrawal signs include anxiety, tremor, insomnia, and tachycardia. There is disagreement about the presence of a rebound effect on the regulated body temperature during withdrawal.

We studied the effects of withdrawal from chronic ethanol exposure on the regulated temperature by maintaining goldfish in a 0.8 % ethanol solution for three days and subsequently measuring changes in Tsel after the goldfish were transferred to an ethanol free temperature gradient for 36 h. The process of withdrawal appeared to have no effect on Tsel, since values for experimental and control groups were similar for the entire 36 h period in the gradient. Activity during withdrawal appeared slightly lower for withdrawing animals (as compared to controls) for the first 16 h of withdrawal.
The most important result of this study was the delineation of a continuous function representing the development of acute tolerance to the effects of ethanol on the regulated body temperature. Particularly noteworthy was the discontinuous nature of the function, with all three doses eliciting a relatively similar pattern. At about 160 min and 300 min there was a rapid increase in the rate of tolerance development, while at other times tolerance developed at a much slower rate. There were no obvious alterations in activity during these periods of rapid tolerance development.

The initial decrease in Tsel in the current experiments confirmed the regulated decrease in body temperature that is caused by ethanol. While it is still generally held that the major effects of ethanol on body temperature derive from influences on peripheral blood flow and a derangement of the central nervous system thermoregulatory areas, previous experiments on mice and goldfish, as well as the current work, indicate that low and moderate blood ethanol concentrations decrease the regulated body temperature.

Responses that occur when ethanol is withdrawn from a dependent organism are normally opposite to the initial reaction. This is postulated to occur because the affected systems have adapted to function in the presence of ethanol and thus rebound in the opposite direction when the ethanol is no longer present. Body temperature often changes during withdrawal, and the direction of change can vary: humans typically are hyperthermic, while small rodents are often hypothermic. While it is clear that the regulated body temperature is decreased by ethanol [2], there is evidence that a rebound increase in the regulated body temperature does not occur during withdrawal [1]. The observed changes in the body temperature of mammals have been postulated to be due to a combination of a rebound basal hypermetabolism and a blunted metabolic scope which compromises the ability of the organism to respond to large metabolic demands. Thus, when mice were allowed to select their thermal environment, withdrawing individuals chose cooler temperatures than controls but maintained their body temperature at the same level [1]. A similar result was obtained in the current study: Goldfish removed from a 3 day exposure to ethanol exhibited a Tsel that was nearly identical to that of control fish. Thus, in goldfish as well as mice, the regulated body temperature is not altered during the period following continuous ethanol exposure.

In conclusion, we have shown that tolerance develops to the acute hypothermic effect of ethanol on Tsel and that this tolerance develops in a step-wise manner. Following a long-term residence in ethanol, removal from this environment does
not alter Tsel. The lack of change in the regulated body temperature during withdrawal corroborates a similar finding in mice.

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References


THE VELVET HAMMER: USING SWIM PERFORMANCE AS A 
SUBLETHAL INDICATOR OF EXPOSURE TO TOXIC COMPOUNDS.

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EXTENDED ABSTRACT ONLY - DO NOT CITE

In fish toxicity studies, death is often used as the ultimate bioindicator of effect. However, as the investigation of toxicity has become more sophisticated, sublethal, whole-organism indicators of effect are becoming more frequently used, and of greater importance. Indeed, sublethal exposures are velvet hammers causing noticeable effects in whole animal performance or behavior without striking a lethal blow. The purpose of this paper is to illustrate how a specific whole-organism performance (critical swimming speed, \( U_{\text{crit}} \)) has been used to focus studies regarding individual variation in the copper tolerance of adult fathead minnows (\textit{Pimephales promelas}).

Genetics

Individual variation in the metal tolerance of fish is, in part, genetic. For example, in fish exposed to lethal concentrations of a variety of metals, allozymic variation of a few polymorphic enzyme loci is often strongly correlated with their time to death. Attempts (e.g. Tatara et al. 2001) to identify a functional association between copper tolerance and the identified allozymes have not been successful. Therefore, the most parsimonious explanation for the observed correlations is genetically linkage between the allozymes and other, currently unidentified but functionally relevant, proteins.

Sublethal indicators of copper tolerance are of more value to physiologists, as the relative copper tolerance of the individual can be evaluated without the animal dying. These sublethal indicators of copper tolerance also have a genetic basis. For example, in fathead minnows, individual variation in the percent
reduction in $U_{\text{crit}}$ following a sublethal exposure to copper is significantly correlated with variation in the same enzyme loci that were found to correlate with copper tolerance in time-to-death studies (Kolok et al. 2004).

**Physiology**

*Branchial Na$^+\text{-}K^+$ ATPase activity.* The physiological mechanism by which copper impairs the performance of fish is well understood. Copper is known to impair Na$^+$ ionoregulation (Lauren and McDonald 1987a), specifically by impairing the activity of chloride cell Na$^+\text{-}K^+$ ATPase pumps (Lauren and McDonald 1987b). Our findings (Kolok et al. 2002) are consistent with this; those of Lauren and McDonald in that we found that copper exposure reduced gill Na$^+\text{-}K^+$ ATPase activity in fathead minnows by 47%. However, when individuals with different copper tolerances were compared there was no significant correlation between gill Na$^+\text{-}K^+$ ATPase activity and relative copper tolerance. Rather, significant correlations were found between individual variation in copper tolerance and whole body Na$^+$ (Kolok et al. 2002). While we have yet to identify the mechanistic basis underlying the observed differences in whole body Na$^+$, we have ruled out the most parsimonious explanation, that being chronic differences in branchial Na$^+\text{-}K^+$ ATPase activity.

*Whole body copper.*

When chronically exposed to copper, fish accumulate it in a dose-dependent manner (Marr et al. 1996). Generally speaking, it is axiomatic that fish with the greatest whole body copper concentrations are the ones that will experience the most dramatic adverse effects. Among individual fathead minnows however, the relationship between whole body copper concentration and relative copper tolerance is surprising. The most copper tolerant individuals are those that have the greatest whole body copper concentrations, (Kolok et al. 2002) suggesting that these individuals are accumulating copper in a non-toxic form.

*Genetic inheritance of physiological traits.*

If the physiological traits discussed above are important for copper tolerant minnows, and if copper tolerance is genetic, then the copper tolerant parents
may pass these traits on to their adult offspring. This is indeed the case (Figure 1). Adult offspring of copper tolerant parents are better able to maintain whole body Na\(^+\) when exposed to a sublethal dose of copper than are fish produced by randomly paired parents (Figure 1A). Furthermore, adult offspring of copper tolerant parents accumulate significantly greater concentrations of Cu than do fish produced by copper susceptible parents (Figure 1B).

![Graph](image_url)

Figure 1. The mean (± standard error) whole body Na\(^+\) (A) and Cu (B) concentrations for adult fathead minnows from three different breeding lines exposed to 150 \(\mu\)g Cu/L for eight days. Different numbers denote significant differences in the whole body ions among the three groups. The dashed lines represent the mean whole-body Na\(^+\) or whole body Cu concentration in unexposed minnows.
Conclusions

A great deal of information is already known regarding the genetics and physiology of metal tolerance in fish. Previous research, however, has avoided the issue of individual variation in the metal tolerance of conspecific individuals. Sublethal indicators of effect, such as percent reduction in $U_{crit}$, provide physiologists with a velvet hammer, a tool that can be used to uncover differences in tolerance among individuals while keeping those individuals alive. By using sublethal indicators of effect, physiologists can test hypotheses that would be otherwise not be testable.

Acknowledgements

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References


COPPER AFFECTS SWIMMING PERFORMANCE IN TROUT:

THE PROBLEM IS AMMONIA

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Toxic metals and ammonia accumulation

Exposure to toxic metals has repeatedly been shown to affect ammonia
excretion in fish. Acutely lethal levels of metals such as copper caused obvious
structural damage to the gill epithelia and a breakdown of ionoregulation,
oxygen transport and acid-base balance in freshwater rainbow trout (Wilson &
Taylor 1993a). Plasma total ammonia accumulated to ~40 times baseline levels,
peaking at 2 mM shortly before death. Exposure of rainbow trout to equivalent
levels of copper in saline waters was not lethal, caused no obvious gill damage
and had little effect on ionoregulation or respiratory gas exchange (Wilson &
Taylor 1993b). However, plasma total ammonia levels were elevated in these
fish, peaking at 200 µmol l-1 in seawater.

Exposure of brown trout to sublethal levels of copper caused significant
disruption of the functional integrity of the gill epithelia, as revealed by electron
microscopy (M.S.I. Mujallid, 1996). In water at pH 5, with a calcium
concentration of 50 µmol l-1 the levels of copper that were just below the mean
lethal concentration (see Beaumont et al, 1995) were 30 µg l-1 (0.47 µmol l-1) at
5oC and 5 µg l-1 (0.08 µmol l-1) at 15oC. At these levels there were marked
changes in the ultrastructure of the gill lamellae, compared to gills from control
fish held in water at pH 7 without copper. At 5oC there was some hyperplasia of
epithelial cells plus fusion between neighbouring lamellae. The harmonic mean
diffusion distance increased fourfold. There was ultrastructural evidence of the
necrosis of chloride cells and their numbers decreased significantly. The
numbers of mucocytes increased sixfold. At 15oC there were only minor
changes in the ultrastructure of gill lamellae, with some vacuolation and curling
of the tips of lamellae. Nevertheless, the harmonic mean diffusion distance doubled, there was a significant decrease in chloride cell numbers and mucocyte numbers increased fourfold.

Chronic exposure of brown trout to sublethal levels of copper (e.g. 0.08 µmol-1 of copper at 10°C) in soft water at pH 5.0 caused a pronounced increase in plasma ammonia concentration, up to 1 mmol l-1 at 96h (Beaumont et al. 1995a,b; 2000a). Chronic exposure to low pH alone exerted a similar effect (Day and Butler, 1996). This accumulation of ammonia was accompanied by slight reductions in plasma Na+ and Cl- concentrations, while blood oxygen and carbon dioxide levels were relatively unaffected. So the significant ultrastructural damage caused by exposure to sublethal levels of copper was without effect on respiratory gas exchange, had only minimal effects on ionoregulation, but was associated with a marked reduction in the ability of the fish to excrete ammonia.

How do heavy metals such as copper cause ammonia to accumulate?

One potential mechanism leading to the accumulation of ammonia could be a large increase in the rate of endogenous production. For most aquatic, ectothermic animals ammonia is the primary waste product of protein catabolism, with its rates of production varying with protein intake and breakdown. Beaumont et al. (1995a; 2003) did find that ammonia production increased following exposure to copper, partly in response to elevated levels of cortisol. This increased rate of ammonia production was not, however, considered sufficient to account for the high plasma and tissue levels in trout exposed to copper and, when cortisol release was blocked using metyrapone, ammonia continued to accumulate in the plasma (Beaumont et al., 2003). When infused with large loads of ammonia, trout exposed to copper and low pH exhibited a reduced ability to excrete it (Beaumont et al., 2003). This indicates that the ammonia accumulation is derived in large part from an impaired excretory process. The presence of a low external pH in these experiments should have favoured “ammonia trapping” at the gill boundary layer, with the consequent maintenance of a NH3 gradient favouring diffusive excretion (Wright et al., 1989). Accordingly, some form of active branchial transport of NH4+ is likely to have been disrupted.

Perfused, isolated gill preparations were used to investigate rates of branchial ammonia excretion in brown trout, exposed for 96 h to copper and acid or to copper-free water at neutral pH (Shingles, 2000). The activities of Na+/K+...
ATPase and H+ ATPase were also investigated to identify any impact of copper and acid on these enzymes.

Am
When perfused with saline containing 600 µmol l⁻¹ total ammonia, gills from fish exposed to copper and acid extracted 25% less ammonia from the saline than gills from control fish.

The mean activity of H+ ATPase in control gills was almost double that of its activity in the gills exposed to copper and acid (P = 0.025; Figure 2). However, the Na+/K+ ATPase activity was almost identical in gills of both control and copper-exposed fish (Figure 2).

The results indicate that copper and acid have two measurable effects on the gills. Firstly, at the highest concentration of ammonia in the perfusate, ammonia extraction by gill cells was 25 µmol kg⁻¹ h⁻¹ lower than that of control gills. This figure is similar to the rate of ammonia accumulation in the plasma of copper and acid exposed whole animals (8-16 µmol kg⁻¹ h⁻¹, Shingles, 2002). Thus the present study suggests that a reduction in the extraction capability of copper and acid exposed gills is sufficient to account for the accumulation of ammonia in whole animals (Shingles, 2002). Secondly, this reduced extraction of ammonia by gill cells was associated with a reduction in H+ ATPase activity.

Thus, the ability of an individual fish to tolerate sublethal copper and acid pollution is likely to result from a combination of the magnitude of increased ammonia production, the degree of the loss of H+ ATPase activity, the number of available apical NHE sites and the ability of the gills to maintain the stability of ion channels.
Ammonia impairs exercise performance in trout.

The first evidence that ammonia impaired the exercise performance of fish was provided by Beaumont et al. (1995a,b). These authors exposed brown trout to sublethal concentrations of copper (0.08 or 0.47 µmol l⁻¹) in soft acidic water (pH 5.1) at water temperatures of either 5°, 10° or 15°C, and found that their sustained aerobic exercise performance was significantly impaired by comparison with that of trout in neutral (pH 7) water with no added copper. A negative linear relationship between Ucrit and plasma ammonia concentrations was revealed whereby plasma ammonia accounted for almost 70% of the variation in Ucrit (Beaumont et al., 1995). This led to the suggestion that it was the accumulation of ammonia in the tissues that caused the decline in exercise performance (Beaumont et al., 1995b; 2000a).

Shingles et al. (2001) exposed rainbow trout for 24 h to a hard water (280 mmol l⁻¹ as CaCO₃, pH 8.4) containing a sub-lethal concentration (290 µmol l⁻¹) of ammonium chloride yielding a water NH₃ concentration of 20 µmol l⁻¹. This ammonia concentration was equivalent to 50% of the 96h LC50 for juvenile rainbow trout in that water at the prevailing temperature (14°C) and elicited arterial plasma ammonia concentrations that were similar to those which impaired swimming performance in brown trout exposed to copper (Beaumont et al., 1995a,b; 2000a). When the data for plasma ammonia concentration was plotted against Ucrit for these rainbow trout a negative linear relationship very similar to that described for brown trout by Beaumont et al. (1995b; 2000a) was revealed.

How does ammonia impair exercise performance?

Beaumont et al. (1995b; 2000a; 2003) were unable to find evidence that the impaired performance of brown trout exposed to copper in soft acidic water was linked to metabolic or physiological disruptions such as problems with oxygen utilisation or cardiovascular function. Similarly, the impairment of swimming performance in brown trout exposed to high concentrations of ammonia appears to be independent of tissue oxygen supply (Shingles et al. 2004). That is, in control trout, exposure to mild hypoxia (a water O2 partial pressure of 11 kPa, equivalent to 54% of atmospheric O2 saturation) caused a 45% decline in Ucrit, as a consequence of a limit to aerobic scope (Jones, 1971; Bushnell et al., 1984). However, exposure to hypoxia did not cause the same proportional decline in performance in trout exposed to either 100 µmol l⁻¹ or 200 µmol l⁻¹ NH₄Cl. Indeed, at 200 µmol l⁻¹ NH₄Cl, hypoxia made no further contribution to the
impaired performance of the trout. These results indicate that the impaired exercise performance of hyperammonemic trout is due to a direct effect of ammonia (Beaumont et al. 1995b; 2000a; 2003; Shingles et al. 2004). In teleosts, ammonia is produced by protein catabolism but also by deamination of adenylates in working muscle (Mommsen and Hochachka, 1988). There are, therefore, a number of potential ways by which it might exert an influence upon muscle and CNS function and fatigue (Beaumont et al., 2000a; Shingles et al., 2001; 2004; Wicks et al., 2002).

Accumulation of NH4+ can have disruptive effects upon both anaerobic and aerobic metabolism, because of its regulatory role in a number of metabolic pathways (Katunuma et al., 1966; Sugden and Newsholme, 1975; Lai and Cooper, 1991). Ammonia exposure causes depletion of glycogen stores, NADH and adenylates in fish tissues (Arillo et al, 1981a,b). Beaumont et al. (2000a) reported that ammonia accumulation in brown trout was associated with high lactate levels in red muscle at rest, and depletion of white muscle glycogen and phosphocreatine. Shingles et al. (2004) also found that ammonia accumulation caused elevated levels of lactate in white muscle, heart and brain of brown trout at rest. Thus, ammonia may impair performance by influencing the metabolic status of brain, heart and locomotory muscle. Nonetheless, no simple link between such effects and performance has yet been described (Beaumont et al., 2000a; Shingles et al., 2004), although they may contribute to the reduced swimming efficiency (i.e. the energetic cost of swimming at a given speed) observed in hyperammonemic rainbow trout and brown trout (Shingles et al., 2001; 2004).

One of the major toxic effects of ammonia is that it can substitute for potassium at vertebrate muscle and nerve membranes, thereby compromising their function (Raabe and Lin, 1985; Cooper and Plum, 1987). Beaumont et al. (1995; 2000a) hypothesised that impairment of swimming performance in hyperammonemic trout might arise, at least in part, as a consequence of depolarisation of muscle membrane potential (EM), due to the replacement of K+ with NH4+. Indeed, in fish with elevated plasma ammonia concentrations, application of the Nernst equation to the prevailing distributions of ammonia between extracellular and intracellular compartments consistently predicts a significant depolarisation of white muscle (Beaumont et al., 1995a; 2000a; Shingles et al., 2001;2004; Wicks et al., 2002). These predictions are based on the assumption that membranes have a relatively low (Alex - not "high"??) membrane permeability for ionic NH4+ relative to gaseous NH3. Beaumont et al. (2000b) exposed brown trout to sub-lethal copper in soft acid water and measured membrane potential in white
muscle myocytes directly, to reveal that ammonia accumulation did indeed cause a measurable decrease in the EM of white muscle that was very similar to that predicted from the distribution of ammonia between intra- and extra-cellular compartments.

Both the measured and predicted decrease in white muscle EM (Beaumont et al., 1995b; 2000a; Shingles et al., 2001; 2004) would be sufficient to cause a complete loss of electrical excitability in that tissue (Jenerick, 1956). White muscle is recruited to achieve the highest swimming speeds in fish, in rainbow trout this occurs at swimming speeds above 70% of Ucrit (Taylor et al. 1996; Burgetz et al. 1998). Day and Butler (1996) used electromyographic recordings to demonstrate that brown trout exposed to acidic water (pH 4) showed little or no continuous white muscle recruitment during swimming, and attributed this to an accumulation of plasma ammonia. White muscle relies upon substrate-level phosphorylation, primarily glycolysis, for ATP generation, and exercise to fatigue typically leads to a pronounced increase in white muscle lactate levels in salmonids, as a consequence of the increased glycolytic activity (Moyes and West 1995). Shingles et al. (2004) measured a pronounced increase in lactate in the white muscle of control brown trout at fatigue, but no such increase in trout exposed to ammonia. Shingles et al. (2004) also found that control trout showed a significant increase in white muscle ammonia content at fatigue, which is known to be derived endogenously in working muscle from the deamination of adenosine 5’-monophosphate in the purine nucleotide cycle (Mommsen and Hochachka, 1988; Weicker et al., 1990). The ammonia content of white muscle was elevated in hyperammonemic trout at rest, but did not show any further significant increase at fatigue (Shingles et al. 2004). Thus, there is good evidence that electrophysiological impairment to white muscle, resulting in it not being recruited into the swimming effort, may be the primary mechanism underlying the effects of ammonia accumulation on the swimming performance of trout.

It is worth noting, however, that exposure to ammonia was also associated with a partial depolarisation of EM in the brain of brown trout (Shingles et al., 2004). Although the validity of using the Nernst equation to predict white muscle EM was confirmed by Beaumont et al. (2000b), its application to the brain predicted an extreme depolarisation (Shingles et al. 2004) that requires confirmation in future studies. Nonetheless, depolarisation of neural tissue may have contributed to the reduced swimming efficiency if it impaired the co-ordination of swimming movements, by affecting central and/or peripheral nervous function, and transmission at the neuromuscular junction. There is also
evidence that ammonia flux from working muscle into the plasma is responsible for causing central fatigue in mammals, namely an unwillingness to generate and maintain an adequate CNS drive to working muscle (Davis and Bailey, 1997), and the acutely toxic effects of ammonia in the brain are a consequence of membrane depolarisation (Raabe and Lin, 1985; Cooper and Plum, 1987). Thus, depolarising effects of ammonia upon nervous function may also contribute to the decline in the exercise performance of fish.

In conclusion, it seems clear that the processes of ammonia excretion over fish gills remain incompletely understood, that these processes are vulnerable to inhibition by low levels of toxic metal ions and that the resulting accumulation of ammonia in the fish reduces rates of sustainable swimming. This effect apparently arises from depolarisation of white muscle due to accumulation of NH4+ across the cell membranes of myocytes and possibly a similar effect on the nervous system, leading to a lack of recruitment of white muscle and reduced co-ordination of the swimming effort. As swimming is central to the life of fish, enabling them to maintain station or migrate within their watery environment, to find mates, catch prey or avoid predators, then any impairment of this function will be deleterious to their continued survival. This must be an important factor limiting fish populations in polluted urban rivers and an improved understanding of the combined effects of ammonia and metal ion pollution on fish performance must surely educate our future development of standards for improvement of conditions in our rivers.

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Figure 1. The mean (± S.E.M.) rates of ammonia (a) extracted from saline and (b) excreted into water when isolated gill arches from fish exposed to either 0.08 µmol l⁻¹ copper at pH 5 for 96 h (open blocks), or exposed to copper free water at neutral pH (solid blocks), are perfused with saline containing either 0, 200 or 600 µmol l⁻¹ total ammonia for 1 h. * denotes a significant difference from control values, P<0.05.
Figure 2. The mean (± S.E.M.) activities of two branchial enzymes from fish exposed either to copper free water at neutral pH for 96 h (‘control’ solid blocks), or to 0.08 μmol l-1 copper at pH 5 for 96 h (‘copper and acid’ open blocks). * denotes a significant difference from control values, P<0.05.
Figure 3. Linear relationships between plasma ammonia concentration and Ucrit in brown trout (Salmo trutta) and rainbow trout (Oncorhynchus mykiss). The blue symbols are data for individual brown trout (Salmo trutta) in which plasma ammonia accumulated following exposure to sub-lethal concentrations of copper in soft acidic water, replotted from Beaumont et al. (1995a). The blue line describes a least squares linear regression equation whereby $U_{crit} = -0.0020 \times \text{[ammonia]} + 2.089$ ($R^2 = 0.670$, $n = 30$). The large black symbols are data for brown trout exposed to three water concentrations of ammonia, replotted from Shingles et al. (2004). Plasma ammonia and $U_{crit}$ were measured on separate groups of fish ($n = 6$ or $7$), and the black line describes a least squares linear regression equation whereby mean $U_{crit} = -0.0018 \times \text{mean[ammonia]} + 2.347$ ($R^2 = 0.903$, $n = 3$). The red symbols are data for individual rainbow trout (Oncorhynchus mykiss) exposed to elevated water ammonia, plotted from data reported in
Shingles et al. (2001). The red line describes a least squares linear regression equation whereby $U_{crit} = -0.0024 \times [\text{ammonia}] + 2.677$ (R$^2 = 0.590$, n = 12).
Ammonia is one of the primordial toxicants with which organisms had to cope. In modern vertebrates, ammonia is a potent neurotoxin, but the mechanisms of its effects are not well understood. In the course of our research on mechanisms and rationales for urea production and excretion in the gulf toadfish, *Opsanus beta* (see Wood et al., 2003, for review), we discovered that this species is rather ammonia tolerant, with 96h LC50 values in the range of 10 mM total ammonia concentration at normal seawater pH (Wang and Walsh, 2000). Recent research has also demonstrated that these high level of ammonia tolerances extend to larval and juvenile stages of the gulf toadfish, and that urea production and excretion are fully functional in all life stages of this species (Barimo et al., 2004; Barimo and Walsh, unpublished). We believe that this species can serve as a model vertebrate with which to try to understand the mechanisms of ammonia toxicity and tolerance, and this presentation will show recent data on this topic. First we will present evidence from in vivo MRI studies demonstrating that, contrary to the situation in mammals, ammonia intoxication does not result in brain swelling, but rather in brain (and plasma) dehydration. These results imply that the mechanisms of ammonia’s action in the toadfish likely also include systemic osmoregulatory effects. We will also present data from field observations on the microhabitat ammonia and urea concentrations of wild toadfish. These data are the first to demonstrate that toadfish in fact are
substantially ureotelic in nature, and may offer an explanation for why toadfish express ureotely in all life stages. Lastly, potential behavioral implications of the pulsatile nature of urea excretion in this species will be discussed.

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MECHANISM OF AMMONIA TOLERANCE
IN THE GULF TOADFISH

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EXTENDED ABSTRACT ONLY – DO NOT CITE

Introduction

The gulf toadfish, *Opsanus beta*, is more tolerant to high ammonia concentration in the blood than most fish or mammals. The 96hLC50 for most fish expressed as un-ionized ammonia is below 200 µmol/L whereas for gulf toadfish it is 600 µmol/L (equivalent to 10 mM total ammonia) (Wang and Walsh, 2000). In patients, cerebral edema (one of the symptoms of hyperammonemia) is observed with concentrations of total ammonia in the plasma being 600 µmol/L. Hyperammonemic events in mammals can induce brain swelling (Brusilow, 2002). It is believed that an enzyme, glutamine synthetase, located in the astrocyte cells of the brain converts excess ammonia into glutamine. It is hypothesized that water enters brain tissue to relieve the osmotic imbalance created by the accumulation of glutamine leading to brain swelling. Studies in rodents using the drug methionine sulfoximine, an irreversible blocker of the enzyme glutamine synthetase, partly prevent the accumulation of glutamine and alleviate brain swelling during hyperammonemia (Takahashi et al., 1991).

Aim

To understand the mechanism by which *Opsanus beta* can tolerate hyperammonemia in the context of the brain glutamine hypothesis, we used magnetic resonance imaging technique (MRI) to monitor changes in water
content in the brain. The MRI technique gives information about the movement of water between the intracellular and extracellular milieu of cells (apparent diffusion coefficient) and about the movement of water in and out of the whole brain tissue (T2 parameter) (Van der Linden et al., 2001).

Methods

We exposed toadfish to two types of ammonia treatments in seawater: a chronic exposure to a sublethal concentration of ammonium chloride 3.5 mM (1/3 the 96hLC50 value) for 16 and 40 hours and an acute exposure to 10, 20, and 30 mM ammonium chloride consecutively for one hour in each concentration. The values obtained for the apparent diffusion coefficient and the T2 parameter in the hyperammonemic state were compared to values obtained in control situations. For the chronic exposure, plasma and tissue samples (brain, liver, gills and muscle) were collected at 16 and 40 hours to monitor the levels of glutamine, glutamate, ammonia and urea in the fish.

Results

The results from the MRI analysis show no significant changes in the apparent diffusion coefficient for the chronic and acute exposure suggesting that water molecules didn’t shift from the extracellular to the intracellular compartment of the cell. However, a significant decrease in T2 parameter for the chronic exposure suggests a loss of water to the whole brain. This result was corroborated by a significant increase in plasma osmolality for fish exposed for 16 and 40 hours suggesting whole body dehydration. Measurements of blood pH and plasma bicarbonate concentration show no disturbance in the acid-base status of the fish. There was no significant change in the T2 parameter during the acute exposure.

Conclusion

Toadfish and rodents seem to have a different response to hyperammonemia in the context of brain water. While hyperammonemonic rodents tend to gain water to the brain leading to brain edema, toadfish lose water from the brain and display whole body dehydration without an impairment of the acid-base regulatory mechanism located at the gill surface. Whole brain glutamine concentration in
the toadfish is significantly raised during high ammonia exposure (Wang and Walsh, 2000). However, this increase is not as pronounced as for rats. Comparing two studies (Brusilow, 2002 and Wang and Walsh, 2000) where rats and toadfish were made hyperammonemic by raising the concentration of ammonia in the plasma to about 12 times its original concentration, rat brain glutamine concentration raised by 3.5 times its control value while toadfish brain glutamine concentration raised by only 1.2 times its control value. It is possible that toadfish could survive the toxic effect of ammonia to the brain by preventing brain glutamine to accumulate to a certain threshold value.

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References


Social hierarchies develop among groups of stream dwelling salmonid fish and add stability to fish populations. Physiological consequences have been associated with social status, especially in laboratory based experiments. In particular, subordinate fish often display elevated plasma cortisol concentrations, reduced growth rates and decreased resistance to disease (Øverli et al., 1999). Subordinance is also commonly associated with changes in brain serotonin synthesis and metabolism (Winberg et al., 1991). The social behaviour of fish is, like many other behaviours, susceptible to disturbance from aquatic contaminants.

Initial studies investigating the effects of trace metal exposure on agonistic encounters in juvenile rainbow trout demonstrated that of the five metals, copper, nickel, cadmium, lead and zinc, cadmium had the most pronounced effects on behaviour (Figure 1; Sloman et al., 2003).
In a following suite of studies the effect of the neurotoxic metal cadmium (Cd), on social interaction between pairs and among groups of fish was investigated and alterations in physiological parameters such as brain monoamines were considered.

To examine the behaviour of fish in the presence of cadmium, rainbow trout were confined in either pairs of fish in small tanks, or groups of ten fish in stream tanks designed to simulate the natural environment of the fish. These social groups could then be exposed to waterborne cadmium before social interactions were observed.
interaction to investigate the effect of Cd on the formation of hierarchies, or once dominance was established to consider the effects of Cd on the maintenance of social structures. Tissue Cd was measured by acid digestion followed by graphite furnace atomic absorption spectrophotometry. Brain monoamines were measured by HPLC. Tissue distribution of Cd was analysed by whole body autoradiography following exposure to 109Cd.

Pre-exposure to 3 µg l-1 Cd for 24 h, followed by a 24 h depuration period in clean water significantly affected the ability of exposed rainbow trout to become dominant over non-exposed rainbow trout. This effect persisted when the depuration period was increased to 2 and then 3 days, but was eliminated if the fish were allowed 5 days to recover in clean water before social encounters. Whole body autoradiography demonstrated that Cd accumulated in the olfactory rosette following a 24 h exposure period, and only after 5 days depuration in clean water was this Cd accumulation significantly reduced. It is therefore possible that Cd alters the determination of social status between pairs of fish, in part, by olfactory disruption.

In a subsequent experiment, pairs of rainbow trout were exposed to 4 µg l-1 or 7 µg l-1 Cd for 48 h, following establishment of dominance hierarchies. In this instance, no effect of Cd on behaviour was seen; dominant fish remained dominant during exposure and no obvious changes in social behaviour were detected. Significant Cd accumulation occurred in the brain following exposure to 7 µg l-1 for 48 h (Table 1).

Table 1: Brain Cd concentrations of rainbow trout exposed to waterborne Cd.

<table>
<thead>
<tr>
<th>Exposure</th>
<th>Control</th>
<th>4 µg l-1 Cd</th>
<th>7 µg l-1 Cd</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain Cd Concentration (µg g-1)</td>
<td>4.17 ± 0.77</td>
<td>3.04 ± 0.26</td>
<td>13.09 ± 0.90*</td>
</tr>
</tbody>
</table>

As expected, control subordinate fish had higher levels of circulating plasma cortisol compared with dominant fish and higher 5-hydroxyindoleacetic acid/serotonin (5-HIAA/5-HT) ratios in the telencephalon. However, the effect of social status on 5-HIAA/5-HT was abolished following exposure to Cd, and
indeed there was a trend towards higher serotonergic activity in dominant fish in the 7 μg l-1 treatment. Dominant fish exposed to 7 μg l-1 Cd also had higher 5-HIAA/5-HT ratios in the hypothalamus. A positive correlation existed between plasma cortisol and 5-HIAA/5-HT ratios in the control fish but was lost in the Cd exposed fish, suggesting a disruption of the link between monoaminergic activity and the HPI axis.

In conclusion, Cd has obvious effects on the social behaviour of rainbow trout. However, these effects may not be seen during all aspects of social behaviour, as an effect was seen here only during establishment of hierarchies. Maintenance of hierarchies was not affected. Behavioural effects may be mediated in part by olfactory disruption. Cd also has the potential to interfere with physiological correlates associated with social status. Physiological differences that exist between control animals may be eliminated in exposed fish, even in the absence of behavioural effects.

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SOCIAL HIERARCHY AND GENDER-RELATED BEHAVIOURAL PHYSIOLOGY: RESPONSE TO ENDOCRINE DISRUPTORS IN FRESHWATER FISH

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The position or rank of a fish within a social hierarchy can influence its behaviour and physiology. In view of the clear correlation of physiological variables and social rank in salmonids fish it has further been hypothesised that there may be rank-related differences in the sensitivity to environmental toxicants. As a dramatic example to demonstrate this principle, copper accumulation rate in the gills of rainbow trout was shown to be ~20-fold higher in subordinate fish than in dominant fish (Sloman et al., 2002). This may reflect the elevated metabolic rate of subordinates leading to greater 'functional' exposure of the uptake surface to the toxicant, coupled with higher uptake rates of Na⁺ which shares a similar pathway to copper uptake (Sloman et al., 2002). If metabolic rate is an important factor, then this explanation would presumably apply to many if not all contaminants that are absorbed across the gills. Thus one would predict similar rank-related differences in the uptake of, and subsequent sensitivity to, other toxicants.
The first objective of the proposed study was to test this hypothesis using nonylphenol as an example organic toxicant, which is a known endocrine disrupting chemical (EDC). Once nonylphenol is absorbed into the bloodstream, it acts upon oestrogen receptors in the liver of male fish, stimulating the production of vitellogenin which is normally only seen at significant levels in sexually maturing females (Tyler et al., 1998). Thus plasma concentrations of vitellogenin were used as a surrogate for estimating the effective uptake of the EDC nonylphenol.

To allow individual recognition, mixed sex, diploid juvenile rainbow trout were anaesthetised (0.1 mg l$^{-1}$ MS222) tagged with PIT tags. Blood samples were taken at the same time to quantify baseline vitellogenin levels (using a monoclonal antibody ELISA) and based on this the fish were separated according to gender (plasma vitellogenin levels typically <15 ng/ml in male, and > 200 ng/ml in female juvenile trout; Tyler et al., 2002).

The male juvenile rainbow trout were then divided into size-matched pairs, each pair being held in a separate flow-through freshwater tank at 15°C. Pairs were then exposed to either 5 or 50 µg/L of nonylphenol for 1 week. Control fish were exposed to the solvent carrier alone (<0.02% ethanol). During the exposure fish were recorded on video to determine their social rank (dominant or subordinate) according to aggression scores and body colour (Hoglund et al., 2000). After this exposure, fish were anaesthetised and blood sampled to determine plasma vitellogenin, and returned to clean freshwater. Three weeks later, fish from all 3 treatments were transferred to a single tank of ~50% seawater to test their ability to osmoregulate when challenged with a hyper-osmotic environment. After 18 hours in this high salinity all fish were anaesthetised and blood sampled to assess plasma osmolality and chloride.

Following exposure to nonylphenol plasma vitellogenin was elevated in a dose dependent manner (controls = <200 ng ml$^{-1}$; Low Dose Nonylphenol = 200-2500 ng ml$^{-1}$; High Dose Nonylphenol = 3-37 x 10$^6$ ng ml$^{-1}$). Within pairs, subordination was associated with higher plasma vitellogenin in 6 out of 8 pairs across the two doses of nonylphenol. However, despite this trend there was no significant difference between dominant and subordinate fish with respect to the plasma vitellogenin concentration. It is suggested that further data is required before concluding whether or not social subordination is associated with elevated uptake and effect of nonylphenol.

When fish were transferred to 50% seawater, 3 weeks after their treatments had ceased, the fish which had been exposed to the 2 doses of nonylphenol had
significantly higher plasma osmolality and chloride when compared with the solvent control group (Table 1).
Table 1 - Plasma osmolality and chloride (means ± SEM) in rainbow trout challenged with 50% seawater for 18 hours, 3 weeks after a 1 week exposure to either 0.02% ethanol (solvent control), 5 or 50 µg l⁻¹ nonylphenol (NP). Asterisks represent significantly different from the solvent control value (P<0.05, one-way ANOVA).

<table>
<thead>
<tr>
<th></th>
<th>Osmolality (mOsm kg⁻¹)</th>
<th>Chloride (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solvent Control</td>
<td>328 ± 7</td>
<td>140 ± 6</td>
</tr>
<tr>
<td>Low Dose NP</td>
<td>357 ± 7*</td>
<td>156 ± 3*</td>
</tr>
<tr>
<td>High Dose NP</td>
<td>352 ± 7*</td>
<td>149 ± 5*</td>
</tr>
</tbody>
</table>

Madsen et al. (1997) has previously shown that injection of nonylphenol can inhibit the smolting process in Atlantic salmon. However, Moore et al. (2003) showed no effect of 30-day exposure to nonylphenol on osmoregulatory performance after 24 h seawater challenge (although they also found no induction of vitellogenin). This therefore represents the first evidence that acute exposure to nonylphenol can cause long-lasting impairment of the ability to osmoregulate when challenged with a high salinity environment. This could have important consequences for the successful seaward migration of salmonids derived from EDC-contaminated rivers.

Acknowledgements

AG was supported by a BBSRC MSc studentship during this work.

References


MACROPHAGE SUPEROXIDE PRODUCTION BY RAINBOW TROUT EXPOSED TO A SINGLE PULSE OF WATERBORNE NONYLPHENOL: POSSIBLE ECOLOGICAL IMPLICATIONS AND POTENTIAL AS A BIOMARKER OF EXPOSURE TO AN ENDOCRINE DISRUPTING COMPOUND.

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EXTENDED ABSTRACT ONLY: DO NOT CITE

To date the toxicology of oestrogen mimics, or Endocrine Disrupting Compounds (EDC’s), such as nonylphenol, has been largely restricted to measuring the induction of vitellogenin (e.g. Harris et al, 2001). The aim of the present study was to investigate the action of nonylphenol exposure on superoxide production in head kidney macrophages (i.e. an essential aspect of non-specific immunity) and therefore expand the study of an important EDC into immunotoxicology.

Six groups, each of 4 weighed, measured and individually tagged (Passive Integrated Transponder; PIT. Biomark, USA), fish were introduced into 100 l aquaria. The water flow rate in each was adjusted to 2.0 l min⁻¹. The fish were
fed once daily, via a fixed location feeding tube, with a commercial diet equivalent to 1.5% the total fish biomass. At weekly intervals the fishes were anaesthetized and individual body mass and fork lengths were recorded. After 3 weeks, 2 tanks (selected at random) were given a single dose of 15 mg nonylphenol dissolved in 100 ml ethanol: i.e. an immediate concentration of 150 µg l⁻¹. Another 2 tanks (again selected at random) were treated with 100 ml ethanol only whilst the remaining 2 tanks were left untreated. The water flow was not altered thus allowing the nonylphenol (and ethanol) to flux out. Water samples were taken at intervals after the nonylphenol addition and nonylphenol concentration was determined by LC-MS. Waterborne nonylphenol concentrations were found to exponentially decline according to the following model (Fig 1).

Figure 1. The logarithmic decline in nonylphenol concentration following the addition of a single dose of 15 mg 100 l aquarium (i.e. generating an initial concentration of 150 µg l⁻¹) supplied with a 2.0 l min⁻¹ water flow-through. The decline in waterborne nonylphenol concentration may be described as follows: \( \log_{10} Y = 3.52 -1.31 \log_{10} X \ (r = -0.934, \ n = 16) \), where \( Y = \)
nonylphenol concentration (µg l⁻¹) and X = elapsed time since nonylphenol addition (min).

Two weeks after the nonylphenol pulse (or a comparable time period on non-exposed fish) a final measurement of weight and fork length was made. Anterior kidney macrophage cell cultures were then prepared from each fish and superoxide production measured by Nitro-Blue Tetrazolium (NBT) reduction in both non-activated and Phorbol Myristate Acetate (PMA) activated macrophages (Secombes, 1990).

Irrespective of fish treatment superoxide production was found to be independent of growth rate for non-stimulated and PMA-stimulated macrophages (data not shown). Similarly, superoxide production was also independent of body mass, in non-stimulated and PMA-stimulated macrophages in non-exposed (r = 0.067 and 0.052, n = 8, respectively) and ethanol exposed fish (r = 0.079 and 0.058, n = 8, respectively) In contrast there was a direct relationship between body mass and superoxide production in nonylphenol exposed fish (Fig 2).
Figure 2. The induction of a linear relationship between rainbow trout head kidney macrophage superoxide production and total body mass two weeks after exposure to a pulse of waterborne nonylphenol (refer to Fig 1).

This study raises 2 issues; the possible ecological consequences of nonylphenol acting to sensitize non-specific immunity on a size specific basis and if the induction of this body mass / superoxide production relationship can be used as a biomarker of nonylphenol exposure.

The first of these is intriguing and suggests that, if rainbow trout are challenged by a pathogen after having been exposed to a pulse of nonylphenol, larger fish may have the potential to mount a more effective immunological defence. As this study is the first to suggest such an effect there is little in the way of existing data with which to support or disprove this result. However additional regression analysis of data presented by Alcorn et al (2003) and by Lin and Shiau (2003) also indicates there is no direct relationship, between body weight and NBT stimulation index in chinook salmon (Oncorhynchus tshawytscha), or between
weight gain (i.e. growth) and superoxide production in grouper *Epinephelus malabaricus*, under non-polluted conditions: \( r = -0.352, n = 11 \) and \( r = -0.203, n = 4 \), respectively. In addition, the fact that the present study employed two independent groups of 4 fish for each treatment does suggest the relationship between head kidney macrophage superoxide production and body mass, found after the nonylphenol pulse exposure, is less likely to be the result of an experimental artefact than if a single group of 8 were used.

As a possible biomarker to EDC exposure the induction of this relationship offers two significant advantages over vitellogenin induction (i.e. the most widely used biomarker), the first being sensitivity. Vitellogenin induction in rainbow trout requires a longer exposure time or higher concentration; e.g. 8.3 or 85.6 \( \mu g \text{ l}^{-1} \) for 6 – 18 weeks (Harris et al, 2001) or 150 \( \mu g \text{ l}^{-1} \) for 9 days (Pedersen et al, 1999). Secondly vitellogenin is assayed immediately following nonylphenol treatment. The practical application, in terms of environmental monitoring, is therefore restricted to such situations where fish can be sampled immediately following exposure. In contrast the data presented here offer the potential of being able to detect prior nonylphenol exposure in rainbow trout; e.g. after an encounter with an accidental exposure which has been subsequently diluted or in the case of a migratory fish swimming through a discharge plume and into cleaner water.

In conclusion, nonylphenol does appear to impact on rainbow trout superoxide production. However these data also clearly indicate the need for similar studies to both validate the findings presented here and extend to this subject to other potential EDC’s.

References


**Acknowledgements**

The authors express their grateful thanks to Robert Loos, Inland and Marine Waters Unit, JRC, Italy, for assistance with the waterborne nonylphenol measurements presented in this study.
THE BIOTIC LIGAND MODEL: PREDICTING METAL TOXICITY TO FISH IN THE REAL WORLD

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EXTENDED ABSTRACT ONLY – DO NOT CITE

Introduction

Historically, ambient water quality criteria (AWQC) for metals have been based simply on total metal levels in the water, or more recently on dissolved metal levels. This occurred despite a wealth of physiological and toxicological research dating back to early in the previous century which demonstrated that the bioavailability and toxicity of metals to aquatic organisms is dependent on site-specific water chemistry factors such as hardness, salinity, specific ion levels, pH, alkalinity, and dissolved organic matter (DOM, earlier referred to as dissolved organic carbon, DOC). The Biotic Ligand Model (BLM) is a relatively new computational approach for the generation of AWQC that incorporates these influences in a quantitative manner, thereby replacing insensitive and inefficient “blanket” regulations. The BLM procedure has been provisionally accepted by the U.S.EPA for the generation of acute freshwater AWQC for some metals (copper, silver), and is under various stages of review by other regulatory agencies (EU, Canada, Australia/New Zealand, Chile). BLMs for many other metals are currently under development, and the ultimate goal is to generate chronic and estuarine-marine AWQC as well.

The BLM approach integrates basic principles in physiology, aquatic geochemistry, and toxicology, using the known water chemistry and the experimentally determined binding constants of key “toxic” sites (physiological processes) on the receptor surface (biotic ligand) of the organism to predict the metal load causing a defined toxic endpoint (e.g., 96-h mortality). The historical framework of the BLM has been comprehensively reviewed in a treatise by Paquin et al. (2002), which served as the introduction to a compendium of
BLM-related papers occupying a whole journal issue to which the reader is referred (*Comparative Biochemistry and Physiology* 133C, 2002). Niyogi and Wood (2003) have detailed the conceptual basis of the BLM, and a number of organism-related issues (acclimation to different water hardness and sublethal waterborne metal levels, dietary ion and metal levels) which affect the predictions of the BLM. McGeer et al. (2000) provided an example of a physiologically-based BLM for the prediction of the acute toxicity of silver to freshwater rainbow trout.

**Theory**

The BLM assumes that the free cationic forms of metals are the source of acute toxicity, that these do their toxic damage by binding on or in the gill surface, and that the extent of short-term gill metal binding (i.e., before pathology develops at 3 h or 24 h) is directly predictive of longer term mortality (e.g., at 96 h). The greater the binding affinity of a metal for the gill (i.e., the higher the log K value), the greater the toxicity (Fig. 1).

![Figure 1. Relationship between 96-h LC50 and log K values for rainbow trout in Lake Ontario water](image-url)
Based on fish gill research, these “toxic” sites appear to fall into three categories: active Na\(^+\) uptake sites targeted by silver and copper, resulting in hyponatremia and hypochloremia; active Ca\(^{2+}\) uptake sites targeted by zinc, cadmium, lead, and cobalt, resulting in hypocalcemia; and allergic reaction sites targeted by nickel, resulting in gill edema and associated inhibition of respiratory gas exchange (Fig. 2).

![Figure 2. Model of toxic action of metals at three different sites in the freshwater fish gill, with associated log K values for protective cations. Possible complexing anions are also shown.](image)

A variety of water chemistry factors can protect against metal binding to these sites either by cationic competition (e.g., Ca\(^{2+}\), Mg\(^{2+}\), Na\(^+\), H\(^+\)), or by anionic complexation (e.g., OH\(^-\), HCO\(_3^-\), CO\(_3^{2-}\), Cl\(^-\), thiosulfate, chromium reducible...
sulfide, and most importantly DOM) of the cationic metal, thereby preventing it binding to the toxic sites. In addition, these naturally occurring cations and can interact among themselves. Based on the concentrations of all moieties present, the geochemical stability constants governing all relevant reactions, including those with the toxic sites on the biotic ligand itself, and the experimentally determined relationship between short term metal binding and longer term toxicity, the BLM predicts the ultimate outcome for any given metal level in a particular water quality. Therefore, the BLM incorporates competition, complexation, and concentration (the “three Cs”) into a geochemical modelling framework that includes the organism itself. Advantages of the BLM are economy and speed, the minimization of animal testing, and the ability to generate AWQC which are site-specific (i.e., cognizant of receiving water chemistry).

References


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THE EFFECTS OF HYPOXIA ON REPRODUCTION IN FISH

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Introduction

There has been a large increase in aquatic hypoxia over the past decades in rivers and coastal regions of oceans around the world where there are large human populations, for example the Gulf of Mexico, the Black Sea, and the Danube (National Research Council, 1999). Animals have not had time to adapt to these changes, which have gained in strength recently. Very little has been published on the relationship between hypoxia and reproduction in fish. However, in the past decade, hypoxia in coastal marine waters has been associated with a major change in fish species composition, with a reduction in the total biomass. One possible explanation of this phenomenon is the impairment of gonad development and failure in spawning, fertilization, hatching and survival in fish associated with aquatic hypoxia.

Effects of hypoxia in the aquatic ecosystem

Prolonged aquatic hypoxia causes changes in species composition, some organisms leave and other more sensitive species die. What is left are those species that are more tolerant of hypoxia. The overall effect is a reduction in species diversity and a marked reduction in biomass (Diaz and Rosenberg, 1995).
Physiological responses to hypoxia in fish

At the individual level, animals exposed to hypoxic conditions attempt to maintain oxygen delivery to the tissues in the face of reduced levels in the environment. Fish increase gill ventilation and gill diffusing capacity to enhance the transport of oxygen across the gills into the blood. Heart rate is reduced but stroke volume is increased and the changing pattern of blood flow through the gills increases gill diffusing capacity for oxygen (Randall, 1982). Decreased red blood cell phosphate levels result in an increase in hemoglobin oxygen affinity and this also facilitates oxygen uptake at the gills (Val and de Almeida-Val 1995). Blood erythrocyte levels are increased initially due to release from the spleen and then subsequently due to erythropoiesis in response to the hormone, erythropoietin (EPO), produced by the kidney (Kakuta and Randall, unpublished observations). Anaerobic metabolism increases during hypoxia (Randall, 1982; van den Thillart and van Waarde 1985). There is an up-regulation of anaerobic enzymes, increased glucose transport and utilization of liver glycogen. The magnitude of the glycogen stores are an important determinant of hypoxic survival. There is a down-regulation of energy expenditure coupled to the up-regulation of anaerobic metabolic pathways. Fish respond to aquatic hypoxia by initially increasing activity in an attempt to leave the area, escaping from hypoxic areas, which are often patchy in nature. Fish decrease swimming activity during prolonged hypoxia and tend to move to colder waters (Schurmann and Steffensen, 1994). This move to colder temperatures reduces energy metabolism and is associated with an increase in the oxygen content of water. Fish exhibit reduced food intake during hypoxia (Zhuo et al. 2001) and this also decreases energy expenditure. Zebrafish (Danio rerio) stopped feeding about six hours after the onset of hypoxia. Protein synthesis was reduced by 40% during anoxia in carp (Smith et al. 1996; 1999), with the liver showing a much larger reduction (85%) than the muscle (40%). Protein synthesis in the brain was maintained at the normal low rates seen under normoxic conditions.

Hypoxia inhibits reproduction

Wu et al., (2003) reported on the affects of hypoxia on reproduction of the common carp (Cyprinus carpio). Gonad development was reduced when fish were exposed to hypoxia for 8 weeks. There was a significant reduction in the number of spermatocytes and spermatids, lowered incidents of mitosis, decreased lobular diameter of testes and reduced sperm motility in male carp. In female carp, oocytes from hypoxic fish remained in the early stages of the
developmental process, whereas normoxic ones had oocytes that were near completion of the developmental process. Successful spawning females were 71.4% in the normoxic group, significantly higher than the hypoxic group (8.3%). There was a rapid decrease in the percentages of fertilization success (99.4% in normoxia and 55.5% in hypoxia); hatching (98.8% in normoxia and 17.2% in hypoxia); and survival of larvae (93.7% in normoxia and 46.4% in hypoxia).

Hypoxia has been shown to reduce egg production and food intake in carp and zebrafish. In addition starvation and β-naphthoflavone (βNF) reduce egg production in zebrafish. Thus the inhibition of reproduction during hypoxia could be a direct effect of hypoxia or an indirect effect caused by reduced food intake; most likely both play a role. Zebrafish produce eggs every day, but hypoxia (0.5 – 0.8 mgO₂/L), starvation, and β-NF (1.0 mg/L) reduced egg production (Figure 1). There were increased numbers of undeveloped eggs, but no effect was observed on hatching rates when the eggs are allowed to develop under normoxic conditions.

![Figure 1](image1.png)

Figure 1 Both hypoxia (0.5-0.8 mgO₂/L) and β-NF (1 mg/L) inhibit egg production in zebrafish

There were significant decreases in serum testosterone and estradiol levels in carp exposed to hypoxia (Wu et al., 2003). The size of the gonads was related to
steroid levels in the blood. What is clear is that hypoxia results in reduced egg production, reduced sperm motility and reduced larval survival and this could account for the demise of a number of fish species seen in areas subjected to hypoxia. Possible mechanisms of inhibition of steroid metabolism will be discussed.

References


Acknowledgements

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INTRODUCTION

The Amazonian fish fauna is one of the most diversified and richest freshwater ichthyofauna in the world, with approximately 3000 species described. The fishes of the Amazon basin have developed a marked degree of physiological plasticity that allows them to adapt to a highly variable natural environment (Val & Almeida-Val, 1995). The Amazonian fish fauna consists mainly of the lungfish, Lepidosiren paradoxa, the osteoglossids like Osteoglossum and the giant freshwater fish, Arapaima gigas, numerous cyprinodontiform killifishes, characoids, siluroids, gymnnotoids and an array of cichlids.

Cichlid fishes have undergone a spectacular adaptive radiation in the Neotropical region. Adaptive changes in response to the varying ecological conditions, sexual and social selection, in addition to behavioural attributes, parental care and competition act as creative forces contributing to the outstandingly splendid speciation of cichlids (Lowe-McConnell, 1999; Chellappa, 2000). Brazil has many cichlids with marked specialisation for living under special environmental conditions. Mention could be made of Cichla monoculus, Crenicichla lacustris; C. lenticulata; Geophagus brasiiliensis; Cichlasoma facetum; Symphysodon discus; Pterophyllum scalare, and Astronotus ocellatus. The cichlids S. discus and P. scalare could be considered...
as typical representatives of the Brazilian freshwater ornamental fish (Chellappa et al., 1999).

The fish fauna of the semi-arid northeastern Brazil consisted mainly of the native piranhas, *Serrasalmus* sp., pirambeba, *Serrasalmus rhombeus*, and the swamp eel, *Synbranchus marmoratus*, which were not commercially important (Câmara et al., 2002). Amazonian fishes of commercial importance, such as *Cichla monoculus*, *Astronotus ocellatus*, *Colossoma macropomum*, *Piaractus brachypomus* and *Plagioscion squamosissimus* have been introduced in the lakes and reservoirs of the semi-arid north-eastern Brazil, besides the exotic tilapias, *Tilapia rendalli* and *Oreochromis niloticus*. Of the introduced species, the nonmigratory fishes, especially the cichlids have become well adapted to the semi-arid conditions, and effectively contribute to fisheries production. As such, an understanding of the reproductive plasticity of the cichlids introduced to the semi-arid region of Brazil is considered important.

**Materials and Methods**

The three natural lakes (Lake Urubu, Lake Bonfim and Lake Extremoz) and the three man-made reservoirs (Assu, Campo Grande and Marechal Dutra), located in northeastern Brazil, are all utilized for the distribution of potable water and for fishery purposes. The seasons are muted in this region and are related to variations in the rainfall pattern. The climate is tropical semi-arid, with large water deficit, high temperatures and high evaporation rate outstripping the yearly precipitation. The Lake Urubu is situated in Nizia Floresta, and is an oligotrophic water body, with high phytoplankton diversity and productivity (Chellappa et al., 1995). Lake Bonfim is small, shallow and its water level had reduced by 30% in recent years, due to the construction of aqueducts, which draw water to distribute to the drought-ridden regions. The water loss in this lake eliminated the marginal aquatic vegetation and periphytic algae, thus reducing considerably the overall primary productivity (Chellappa et al., 2002). Lake Extremoz is situated in the urban industrial complex, is shallow, oligo-mesotrophic and with predominance of cyanophycean populations (Chellappa et al., 1996; Araújo et al., 2000).

Assu Reservoir has a water holding capacity of $2400 \times 10^6$ m$^3$, and supplies potable water for habitants spread over 25 cities and 97 rural communities in the semi-arid region. This impounded water is the largest in the State of Rio Grande do Norte and was built by damming the River Piranha. This reservoir is eutrophic with predominance of cyanophycean phytoplankton (Costa et al.,
1998). The Campo Grande Reservoir was constructed by impounding the River Potengi and has a maximum stocking capacity of 34,000,000 m$^3$ of water. Marechal Dutra Reservoir was built by damming the River Acauã, belonging to Piranhas–Açu hydrographic basin. It has a storage capacity of 40,000,000 m$^3$, with a drainage area of 2400 Km² (Costa, 2000).

Principally occurring cichlid species, such as, *Cichla monoculus*, *Astronotus ocellatus* and *Cichla temensis* were collected on monthly basis from all the six study sites, with the help of local fishermen, from 2001 to 2003. Fish were captured using gill nets of different mesh sizes.

**Results and Discussion**

The seasonality of the six freshwater ecosystems is defined by the short spell of intense rainfall (March to July) coupled with an extended dry period during the rest of the year (August to February). These aquatic ecosystems receive consistently high insolation and constant periods of daylight. In their native Amazonian basin, the cichlids inhabit both river channels and forest streams. The adaptability of *C. monoculus*, *C. temensis*, and *A. ocellatus* for growth, development and reproduction in their new introduced environments, is due to their marked degree of physiological plasticity, which allows them to adapt to a highly variable natural environment. Seasonal water fluctuations in the Amazon basin are commonly in the order of 4 m with concomitant changes in electrical conductivity of 50 to 300 $\mu$Scm$^{-1}$ (Sioli, 1984; Val & Almeida-Val, 1995). By contrast, the lakes and reservoirs in the semi-arid northeast of Brazil, such as the Campo Grande Reservoir, have characteristically high electrical conductivity (800$\mu$Scm$^{-1}$). During the dry season the impounded waters were found to have high nutrient levels and alkaline pH. The cichlid fish communities successfully occupy the lakes and reservoirs and contribute to about 60% of fishery production of northeastern Brazil.

Reproductive plasticity is generally more conservative in comparison with the other vital activities and result from selection pressures on a species to produce the maximum number of young that survive to maturity under prevailing environmental conditions, thereby maximizing fitness (Potts & Wootton 1984). The cichlid fishes of the semi-arid region clearly show phenotypic plasticity in allocation of resources to growth or reproduction according to the environmental conditions and increases reproductive effort with the onset of adverse conditions. Cichlids also present a high reproductive output coupled with repeated breeding (Iteroparity), which provides for immediate population increase and
effective exploitation of environmental resources. In all the lakes and reservoirs of the semi-arid region, adult cichlids presenting different gonadal maturity stages, were captured throughout the study period. Thus they tend to spawn during rainy as well as dry seasons, indicating that water level changes have no significant effect on the reproductive process of these fish.

Sexual maturation occurs early in cichlids and once sexual maturity is attained the females are able to complete successive breeding cycles producing broods within 2 to 3 weak intervals. Male reproductive colouration is strikingly manifested during the reproductive phase. Changes in eye colour of males indicate their importance for dominance and territory establishment. Whereas, the adults of \textit{C. monoculus}, show considerable sexual dimorphism, and the onset of reproductive activity in the male is characteristically marked with the appearance of a post-occipital cephalic hump with lipid stores, yellowish-orange in colour, which gradually becomes darker and disappears after the breeding season (Chellappa et al., 2003). Accumulation of lipid stores in the cephalic hump and their mobilization during the reproductive phase is considered as an important strategy of this species.

Competition for territory and mates are well marked in cichlids. The bigger sized male cichlids are more aggressive in defending territories and gain priority in courting females. Defence of the spawning territory by males is important in ensuring egg survival, and forms a basis for female preference of mate choice. Breeding in cichlids occurs year-round and they produce batches of mature oocytes at frequent intervals. The number of eggs produced by a female cichlid over a breeding season depends on her batch fecundity and the number of times she spawns. Batch fecundity increases with the body size. In \textit{C. monoculus} the fecundity ranges from 3000 to 4000, whereas in \textit{P. scalare} the fecundity ranges from 50 to 300 per batch, and they produce a second batch of eggs in two to three weeks (Chellappa et al., 1999). The capacity to tolerate dry seasons, high degree of parental care, ranges from substrate cleaning, nest building, guarding eggs and the hatchlings, enhances survivorship of the offspring and reproductive success. Reproductive success of the cichlids is facilitated by the presence of many mature females and males throughout the year, with a capacity to complete successive breeding cycles in short intervals.
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References


THE EFFECT OF SILVER ON GROWTH AND IONOREGULATORIAL DEVELOPMENT IN EARLY LIFE STAGES OF RAINBOW TROUT

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EXTENDED ABSTRACT ONLY - DO NOT CITE

Introduction

Silver is one of the most toxic metals to aquatic organisms when present in its ionic form (Ag"). In juvenile and adult trout and fathead minnows, the mechanism of acute silver toxicity is through direct impairment of gill Na⁺,K⁺ ATPase activity and inhibition of gill carbonic anhydrase activity. This impairment disrupts ion regulation, resulting in a reduction in plasma Na⁺ and Cl⁻ levels leading to secondary effects ultimately causing circulatory collapse and death (Wood et al., 1996).

During acute exposure to silver, natural ligands such as dissolved organic matter (DOM), water Cl⁻ and water hardness offer a great degree of protection against silver toxicity (Bury et al., 1999). Few studies have investigated the protective effects of these ligands during chronic silver exposure, which is of particular concern in early life stages which tend to be the most sensitive to contaminants in general.
The objective of this presentation is to summarize the protective effect of various water ligands (DOM, water Cl$^-$ and water hardness) on chronic silver exposure (from fertilization to swim-up) in early life stages of rainbow trout. The ultimate goal is to use this data to generate a Biotic Ligand Model to predict chronic silver toxicity.

**Materials and Methods**

Freshly fertilized rainbow trout (*Oncorhynchus mykiss*) eggs were obtained from Rainbow Springs trout farm (Thamesford, Ontario) and maintained in darkened chambers in flowing, dechlorinated Hamilton tap water at a constant temperature of 11-12 °C. To investigate the protective effects of varying water composition during chronic silver exposure, embryos and larvae were exposed to sublethal levels of silver in a flow through set up (from 3 h following fertilization to swim-up) in water of varying composition. Total silver concentrations were 0, 0.1, 1.0 and 10 µg/l total silver (as AgNO$_3$) in the presence of varying levels of dissolved organic carbon (3 and 12 mg C/l), water Cl$^-$ (30, 300 and 3000 µM) or water hardness (2, 150 and 400 mg/L CaCO$_3$). Embryos and larvae were continuously monitored for growth, mortality, time to hatch, and time to swim-up. Every 5-8 days, embryos or larvae were collected for measurement of silver accumulation as well as indicators of ion regulatory status including Na$^+$ uptake, whole body Na$^+$ and Cl$^-$ content, as well as whole body Na$^+$,K$^+$ ATPase activity.

**Results and Discussion**

While no significant effects were observed at the lowest silver concentration (0.1 µg/L total silver), continuous exposure to 1.0 µg/L total silver from fertilization to swim-up at low water water Cl$^-$ (30 µM), low DOC (3 mg C/L) and low water hardness (2 mg/L CaCO$_3$) resulted in a pronounced reduction in whole body Na$^+$,K$^+$ ATPase activity, unidirectional Na$^+$ uptake (J$_{in}$ Na$^+$) and whole body Na$^+$ and Cl$^-$ levels with development. Furthermore, there was a reduction in extractable protein and wet weight relative to controls. Thus, the mechanism of chronic silver toxicity (Brauner and Wood, 2002) appears to be similar to that observed during acute silver exposure in juvenile and adult fish, specifically an ionoregulatory disturbance (Wood et al., 1996). Higher water Cl$^-$ levels (300 and 3000 µM Cl$^-$), dissolved organic carbon (DOC, 12 mg carbon/L) and water hardness (150 and 400 mg/L CaCO$_3$) offered some degree of protection against the silver induced ionoregulatory disturbance. In general, these factors appear to be less protective during chronic than during acute silver exposure.
exposure. In some cases, mortality and larval Na\(^+\) concentration, I\(_{\text{Na}}\), Na\(^+\), and Na\(^+\)K\(^+\) ATPase activity all appear to be correlated with silver body burden and calculated water Ag\(^+\) during chronic silver exposure. These results are promising in terms of development of a Biotic Ligand Model for prediction of chronic silver toxicity; however, a physiologically based model may be more appropriate because Na\(^+\)K\(^+\)-ATPase activity may be a better endpoint for prediction of toxicity rather than gill or body silver accumulation.

**References**


**Acknowledgements**

This research was supported by Kodak Canada and NSERC CRD Program.
The effect of elevated dietary calcium (as CaCO3) on the responses of juvenile rainbow trout (Oncorhynchus mykiss) to dietary and acute waterborne Cd exposure were examined. Endpoints measured included whole body uptake and internal distribution of both newly accumulated and total Cd and Ca2+.

**Methodology**

Fish were fed with four diets 20 mg Ca2+/g food (control), 50 mg Ca2+/g food, 300 µg Cd/g food, and 300 µg Cd/g food + 50 mg Ca2+/g food. After 15 or 30 days of exposure to experimental diets, ten fish from each group were collected, weighed and transferred to 450 mL flux chambers containing dechlorinated Hamilton tap water (moderately hard water) for each measurement of uptake rates at the gills (whole body uptake rate) and internal distribution (tissue specific accumulation) of the newly accumulated metal. For each diet, the following flux (including internal distribution) measurements were made: Ca2+ influx rate, Ca2+ influx rate in the presence of acute exposure to 50 µg/L Cd (as CdCl2), and Cd influx rate during acute exposure to 50 µg/L Cd. The flux chambers contained an isotope solution (10 µCi/L 45Ca++ or 2 µCi/L 109Cd, from New England Nuclear, Boston, MA) and CdCl2 (to Cd-exposed fish).
Controls for each diet treatment were submitted to the same treatment but without the addition of Cd to the water.

After 3 h, fish were anesthetized, their blood collected and then sacrificed, and gills, kidney, liver, and the remaining carcass were collected. Tissues were then partitioned for radioactivity analysis (for newly accumulated metal concentrations). Thereafter, gills, kidney, carcass, liver, and bone were digested in 3-5 volumes of 1N HNO₃ for 24-48 h at 60°C. These tissues and water samples were analyzed using flame (AAS; Ca²⁺) or graphite furnace (GFAAS; Cd) atomic absorption spectrophotometry (Varian AA-1275 fitted with a GTA-95 graphite tube atomizer). Comparisons among different diets, times of exposure to these diets and acute Cd exposure (for Ca²⁺ fluxes) were made by multivariate analysis of variance and Tukey test.

Results

Mortality was low (2-5%) through the 30 days of experiment and there were no significant differences among treatments. There were also no significant differences in specific growth rates among the treatments. All treatment diets significantly reduced rainbow trout whole body waterborne Ca²⁺ uptake after 15 days of feeding (P < 0.05). Dietary Cd exposure resulted in decreased whole body Ca²⁺ uptake. Increase of dietary Ca²⁺ did not prevent the inhibitory effect of dietary Cd on Ca²⁺ uptake, and also did not allow the recovery of this uptake after 30 days. Treatments diets also did not alter the inhibition of whole body Ca²⁺ uptake and newly accumulated Ca²⁺ caused by acute exposure to waterborne Cd. Dietary Cd supplementation for 15 days led to a significantly higher whole body waterborne Cd uptake (P < 0.05), but after 30 days it decreased and was not significantly different from that in fish fed with control diet (Figure 1).
Figure 1 – Cadmium whole body influx rates of rainbow trout exposed to diets with different Ca 2+ and Cd concentrations. Means ± 1 SEM (N = 8–9). Means with different letters in the same time of feeding are significantly different (P < 0.05) as determined by two-way ANOVA and by the Tukey test. * significantly different from group exposed to the same diet at 15 days (P<0.05)

Dietary Ca2+ supplementation abolished the increase of whole body waterborne Cd uptake and newly accumulated Cd in the gills provoked by the Cd-supplemented diet 15 days after feeding. Moreover, it also kept whole body waterborne Cd uptake significantly lower than did the control diet and newly accumulated Cd in the gills significantly lower than did Cd-supplemented diet
after 30 days (P < 0.05). Fish fed with Cd- and Ca2+-Cd-supplemented diets showed significantly higher total Cd after 15 and 30 days of feeding in all compartments (except plasma, only after 15 days of feeding) than those fed with control and Ca2+-supplemented diets (P < 0.05). However, fish fed with Ca2+-Cd-supplemented diet showed significantly lower total Cd in most compartments after 30 days of feeding than those fed with the Cd-supplemented diet (P < 0.05). After 30 days of feeding, total Cd in the plasma was significantly higher in fish fed with Ca2+-Cd-supplemented diet than in fish fed with all other diets (P < 0.05) (Table 1).

Table 1 – Effect of treatment diets on total Cd in several compartments of rainbow trout. Values in µg/g tissue or µg/mL (plasma). Means ± 1 SEM. N = 9-10 fish

<table>
<thead>
<tr>
<th>Compartment</th>
<th>Diet</th>
<th>Day of exposure</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>15</td>
</tr>
<tr>
<td>Gills</td>
<td>Control</td>
<td>0.34±0.002a</td>
</tr>
<tr>
<td></td>
<td>Cd</td>
<td>1.48±0.100b</td>
</tr>
<tr>
<td></td>
<td>Ca2+</td>
<td>0.31±0.017a</td>
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<td></td>
<td>Ca2+ + Cd</td>
<td>1.34±0.106b</td>
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<tr>
<td>Carcass</td>
<td>Control</td>
<td>0.006±0.0004a</td>
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<tr>
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<td>Cd</td>
<td>7.15±0.803b</td>
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<tr>
<td></td>
<td>Ca2+</td>
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<tr>
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<td>Ca2+ + Cd</td>
<td>1.67±0.144c</td>
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<tr>
<td>Kidney</td>
<td>Control</td>
<td>0.039±0.004a</td>
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<tr>
<td></td>
<td>Cd</td>
<td>3.59±0.354b</td>
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<tr>
<td></td>
<td>Ca2+</td>
<td>0.072±0.008a</td>
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<td>Ca2+ + Cd</td>
<td>2.82±0.386b</td>
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<tr>
<td>Liver</td>
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<td>Ca2+</td>
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<td>Ca2+ + Cd</td>
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<td>Control</td>
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<td>Cd</td>
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<td>Ca2 + Cd</td>
<td>0.218±0.055b</td>
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For each compartment, means with different letters in the same columns are significantly different (P < 0.05) as determined by two-way ANOVA and by the Tukey test. * significantly different from group exposed to the same diet at 15 days (P<0.05)

Conclusion

Dietary Cd exposure led to an accumulation of this metal in the tissues, affected waterborne Cd uptake and internalization, and for some days reduced waterborne Ca$^{2+}$ uptake and internalization in some tissues. Dietary Ca$^{2+}$ supplementation did not change the inhibitory effect of dietary Cd on waterborne Ca$^{2+}$ uptake, but strongly reduced Cd uptake in most tissues. It is therefore possible that dietary Ca is protective against both waterborne and dietary Cd toxicity.

Acknowledgement

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CADMIUM TRANSPORT IN ISOLATED GILL CELL POPULATIONS
OF FRESHWATER RAINBOW TROUT

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EXTENDED ABSTRACT ONLY – DO NOT CITE

Cadmium disrupts calcium regulation in freshwater fish through its actions on high-affinity Ca$^{2+}$ channels and Ca$^{2+}$-ATPase transporters in the gill epithelium (Wong and Wong, 2000). These proteins are presumably localized in the mitochondria-rich (MR) chloride cells of the freshwater fish gill. It is generally thought that Cd is transported across MR chloride cells, or more specifically across a specific population of MR cells on the filamental epithelium. Unfortunately, standard experimental techniques are unable to definitively localize the site of Cd transport in the gill epithelium.

Recently, a number of novel cell separation methodologies have been reported to isolate different gill cell populations in an attempt to characterize the relative roles of each cell type in ion transport. Galvez et al. (2002) have used a combination of density centrifugation, lectin histochemistry and magnetic bead separation techniques to purify at least four specific cell types from the gill epithelium. In brief, discontinuous Percoll gradients are used to fractionate mucocytes, pavement cells and mitochondria-rich cells based on differences in density. Second, differential staining of MR cells with the protein peanut lectin agglutinin is used to identify two distinct subtypes of MR cells (Galvez et al., 2002; Goss et al., 2002). Finally, a magnetic bead separation technique is used to separate these cell populations into two highly enriched MR subtypes. One of the MR cell subtypes possesses features characteristic of chloride cells (PNA$^+$ MR), while the other cell population exhibits ultrastructural features similar to those of pavement cells (PVC) (PNA$^-$ MR). Moreover, these MR cell subtypes appear to have differing roles in ion and acid-base regulation (Galvez et al., 2002).
2001; Reid et al., 2003). PNA− MR cells are implicated in acid-inducible Na⁺ transport mediated by Na⁺ channels sensitive to phenamil and bafilomycin inhibition. Alternatively, PNA⁺ MR cells are thought to be the site of apical Cl−/HCO₃⁻ exchange and unidirectional Ca²⁺ transport. Based on this model, Cd uptake in the freshwater fish gill is hypothesized to occur largely through PNA⁺ MR cells. Current studies are investigating the cellular localization and mechanistic basis of Cd transport in isolated cells of the gill epithelium of rainbow trout.

In initial experiments, adult rainbow trout were exposed to 10 µg/L Cd (as Cd(NO₃)₂) for one hour, followed by a 30-minute depuration period to remove any superficially-bound metal. Exposures were performed in Hamilton dechlorinated tap water (in mM: Na⁺ ≈ 0.6; Cl⁻ ≈ 0.7; Ca²⁺ ≈ 1.0 and pH 8.0). Afterwards, gills were excised from fish and fractionated into mucocytes, pavement cells, PNA− MR cells and PNA⁺ MR cells, using the protocol described above. Results show that Cd accumulation in PNA⁺ MR cells is approximately 4 to 10-fold higher than that measured in other gill cell types, including the PNA− MR cells (Fig. 1). In comparison, PV cells and PNA− MR cells accumulate almost equal amounts of Cd following a 1-h exposure to 10 µg/L Cd.

Experiments were also performed to characterize the kinetics of Cd transport in isolated gill cells using an in vitro approach. In brief, enriched gill cell populations were first obtained from control fish. Cells were incubated with

![Figure 1. Cadmium-109 accumulation in isolated mucocytes, pavement cells (PVC), total mitochondria-rich (MR) cells, PNA- MR cells and PNA+ MR cells after 1-hour exposure to 10 µg/L Cd (as CdNO₃).](image-url)
109Cd at concentrations ranging from 1 to 16 µg/L for 60 s in either phosphate-buffered saline (PBS) (Fig. 2) or Cl− free PBS (Fig. 3). Cd concentrations and time of exposure were chosen based on preliminary range-finder experiments (data not shown). PNA+ MR cells showed the highest level of Cd accumulation of all isolated cell fractions when cells were fluxed in standard PBS. Similar to the in vivo Cd fluxes, PNA+ MR cells accumulated approximately 4-fold more Cd than did PNA− MR cells. Surprisingly, Cd uptake in PV cells reached unexpectedly high levels, tending to increase almost linearly with Cd concentration (Fig. 2).

Figure 2. Cadmium-109 accumulation in isolated pavement cells (PVC), PNA− MR cells and PNA+ MR cells of the freshwater rainbow trout gill. Gill cells were fluxed with Cd in phosphate-buffered saline (PBS).

In vitro Cd exposures were also performed in Cl− free PBS to investigate whether Cd as Cd-chloride complexes was accumulating in cells by passive diffusion. Removal of Cl− from the flux medium reduced Cd accumulation in all gill cell types, however Cd uptake was most affected in PNA+ MR cells (a reduction of approximately 50 % from levels measured in PBS). Current studies are attempting to elucidate the mechanisms of Cl− dependence on Cd transport.

The gill cell isolation technique of Galvez et al. (2002) allows us, for the first time, to assess the cellular localization of Cd transport in the freshwater fish gill. In conclusion, we provide strong evidence that Cd is preferentially accumulated
in one specific subtype of mitochondria-rich cell of the gill epithelium, termed the PNA\(^+\) MR cell. Furthermore, environmental stressors that increase the relative abundance of PNA\(^+\) MR cells in the gill epithelium are expected to influence the transport of Cd at the freshwater fish gill.

**References**


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RESPONSE OF THE NUCLEOLAR ACTIVITY IN THE GILL AND LIVER OF THE NEOTROPICAL SILVERSIDE, *Odontesthes bonariensis* EXPOSED TO SUBLETHAL WATERBORNE CONCENTRATION OF TWO HEAVY METALS - Cd(II) AND Cr(VI) - WITH DIFFERENT MECHANISMS OF ACTION.

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EXTENDED ABSTRACT ONLY - DO NOT CITE

Introduction

The nucleolus is a subcellular domain within the cell nucleus involved in ribosome biogenesis and other several functions that play key roles in cell growth, proliferation and aging (Scheer and Hock, 1999). Morphology of the nucleolus reflects the nucleolar activity (Mêlêse and Xue, 1995). Some factors related with nucleolar functions modulation (Leary and Huang, 2001) are potential targets for toxic metals as Cd (Hartwing et al, 2002) and Cr (Hamilton et al, 1998).
Cd and Cr are heavy metals with environmental relevance in aquatic systems and fish are susceptible to suffering exposure to sublethal concentration of these elements. During waterborne exposures, gill represents the main route of uptake for both metals and liver and kidney the main detoxifying organs. Despite these similarities, the mechanisms of toxic actions described for Cd (Beyersmann and Hechtenberg, 1997) and Cr (Costa, 1997) are different as it is the lethal toxicity on the selected fish species (Carriquiriborde and Ronco, 2002).

In spite of the biological role of the nucleolus, the environmental relevance of the Cd and Cr, and the mechanistic linkage between the toxic action of these metals and nucleolar functions, no previous studies were identified on this subject. The aim of the present study was to evaluate, by means of available methods, the response of the nucleolar activity in the gill and liver of the neotropical silverside *Odontesthes bonariensis* exposed to sublethal waterborne concentrations of Cd(II) and Cr(VI).

**Materials and methods**

Two experiments were performed using juveniles of *O. bonariensis* (Cuvier and Valenciennes, 1835, Pisces Atherinidae). Six fish per treatment were exposed in dechlorinate tap water (hardness, 215 mgCaCO$_3$/l) to sublethal concentrations of CdCl$_2$ and K$_2$Cr$_2$O$_7$ ranging from 1 to 10 $\mu$gCd$^{2+}$/l and 100 to 1000 $\mu$gCr$^{6+}$/l respectively, from 0.5 to 384h. Gill and liver of each fish were dissected and fixed in methanol-acetic acid (3:1). Air-dried slides were prepared from nuclear suspension and then stained with AgNO$_3$ according to Howell and Black (1980). Ten pictures per slide, containing a mean number of 6 nuclei, were acquired by a CCD-Iris color video camera on a light microscope using the 100X objective. The number of nucleoli per cell was counted directly from each image and volume of each nucleolus was calculated using image analysis software. Nucleolar activity was assessed by the mean nuclear volume (MNV) and mean nucleoli number (MNN) per nucleus. Effects between treatments were assessed by Factorial-ANOVA and post-hoc analysis was performed by LSD test.
Figure 1. Response of nucleolar parameter MNV (A, B, E, F) and MNN (C, D, G, H) in the gill (A, B, C, D) and liver (E, F, G, H) of O. bonariensis exposed to sublethal concentrations of Cd\(^{2+}\). A, C, E, G experiment 1; B, D, F, H experiment 2. LSD: significant (* p<0.05) or highly significant (** p<0.01) differences.
Results

In the gill, Cd\(^{2+}\) significantly reduced MNV in a dose response fashion from 6 h of exposure (Panel A and B, Figure 1) and then values seemed to reach a new state of equilibrium depending on the Cd\(^{2+}\) concentration. These new values in fish exposed to 1, 5 and 10?gCd\(^{2+}\)/l were respectively 23, 28 and 33% lower than those in the control fish. No effect of Cd\(^{2+}\) was observed on MNN (Panel C and D, Figure 1). Only transient decrease was observed in MNN at 48h exposure.

Figure 2. Response of nucleolar parameter MNV (A, C, D) and MNN (B, E, F) in the gill (A, B) and liver (C, D, E, F) of *O. bonariensis* exposed to sublethal concentrations of Cr\(^{6+}\). C, E experiment 1; A, B, D, F experiment 2. LSD significant (* p<0.05) or highly significant (** p<0.01) differences.
Liver MNV was also significantly affected by cadmium concentration in both experiments (Panel E and F, Figure 1). However in this tissue, MNV was increased (28 and 41% in experiment 2) in fish exposed to 1 and 5\(\mu\)gCd\(^{2+}\)/l. On the other hand, MNV in fish exposed to 10\(\mu\)gCd\(^{2+}\)/l remains similar to that in control fish or shows a tendency to decrease. MNN was not affected by cadmium exposure during the first experiment (Panel G, Figure 1) but it was during the second experiment that it showed a significant increase in the MNN of fish exposed to 1\(\mu\)gCd\(^{2+}\)/l (Panel H, Figure 1).

Gill MNV was significantly decreased from 6h of exposure in response to waterborne Cr\(^{6+}\) concentrations equal or greater than 500\(\mu\)gCr\(^{6+}\)/l (Panel A, Figure 2). MNV seemed to reach a new state of equilibrium depending on the concentration of Cr\(^{6+}\) with values 28, 33 and 41\% lower than those in control fish for individuals exposed to 100, 500 and 1000\(\mu\)gCr\(^{6+}\)/l. No effect was observed on the MNN (Panel B, Figure 2), which only showed a transient non significant reduction after 48h exposure.

Liver MNV significantly decreased from 1h of exposure to 100\(\mu\)gCr\(^{6+}\)/l during the first experiment but only after 192h of exposure to 500\(\mu\)gCr\(^{6+}\)/l in the second one (Panel C and D respectively, Figure 2). MNN was not affected by chromium in the liver at any Cr\(^{6+}\) concentration or time of exposure (Panel E and F, Figure 2), showing only a marginal decreases during the second experiment.

**Conclusions**

- The studied nucleolar parameters were affected by Cd and Cr, responding in a different way depending on the metal and the tissue.
- MNV was the most sensitive parameter indicating that both metals mainly induce quantitative changes in the level of the nucleolar activity more than qualitative modifications in the pre-established nucleolar genetic program.
- Observed differences in the sensitivity of the response of the nucleolar parameters to Cd and Cr agree with the different levels of toxicity and bioaccumulation rates observed of these metals and the particular mechanism of action described for each one (see introduction).
Further studies on the genomic and proteomic of the nucleolus would be useful to elucidate the underling mechanism involved in the observed nucleolar responses.

Acknowledgments

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References


FUNCTIONAL ANALYSIS OF THE HEMOLISATE
OF MATRINCHÃ
IN THE PRESENCE OF CADMIUM

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EXTENDED ABSTRACT ONLY: DO NOT CITE

Abstract
The exposition of fish to sublethal concentrations of cadmium can harm gas exchange between the external environment and tissues. In this study we analysed the effect of cadmium on oxygen affinity of the hemolysate of matrinchã (Brycon cephalus) at different pHs by tonometry, according Riggs & Wolbach (1956). The results suggest that Hb-O2 affinity do not differ at low cadmium concentrations in the Bis-Tris and Tris-HCl pH 7.0 buffer. However, it is possible that cadmium can indirectly modify the Hb-O2 affinity by complexing with ATP, making this phosphate unavailable to bind to hemoglobin.

Introduction
The hemoglobin structure is designed to transport oxygen from the environment to tissues. Hb-O2 affinity can be modulated by protons, temperature, chloride and organic phosphates like ATP, although there are hemoglobins insensitive to these parameters. Phosphates and chloride, for example, bind to specific sites of
the Hb molecule and can stabilize its desoxy structure, decreasing the Hb-O2 affinity. Cadmium is a transition metal that causes great damages to the ichthiofauna. In fish specifically, cadmium enters the branchial chloride cells via apical Ca2+ channels. Once in the blood, cadmium induces hematological changes, namely changes of antioxidant enzymes activities, levels of lipid peroxidation, blood cell numbers, hematocrit and hemoglobin levels. In vitro studies show that cadmium affects methemoglobin reductase activity. There are direct evidences that cadmium enters human red blood cells, binding intracellularly to hemoglobin (Rabenstein et al. 1983). The aim of the present paper is to analyze the effect of cadmium on the Hb-O2 affinity of matrinchã, supposing that cadmium also enters fish red blood cells of this fish species.

Material and Methods

Specimens of matrinchã (Brycon cephalus) were obtained from a fish farm near Manaus, Brazil. Blood was collected from the caudal blood vessels using heparinized syringe. The erythrocytes were washed three times by centrifugation against a large excess of saline buffer. Haemolysis was achieved by the addition of 50mM Tris-HCl buffer, pH 8.5, 1:1 volume. The haemolysate was centrifuged and the debris were removed. The stripped hemoglobin (Hbt stripped) was prepared by gel filtration in Sephacryl S-100 column (1.5x40cm) balanced with 50mM Tris-HCl buffer, pH 8.5. Hemoglobin (Hb) multiplicity was checked by non-denaturating PAGE 7%. Functional aspects were analysed by tonometry according Riggs & Wolbach (1956). Hbt in presence of 1mM of cadmium was analysed at pHs between 6.0 and 6.5 (50mM Bis-Tris buffer) and the effect of different cadmium levels was analysed at pH 7.0 (50mM Bis-Tris and 50mM Tris-HCl buffer). Detection of Cd2+ after tonometry was made by digestion of samples with HNO3 and metal concentration determined by furnace atomic absorption spectrophotometry.

Results and Discussion

Two Hb fractions, one more anodic Hb-II and one less anodic Hb-I, were detected on PAGE 7%. Hbt of matrinchã presented a Bohr index (ΔH+=Δlogp50/ΔpH) of –1.46 between pH 6 and pH 6.5. In the presence of cadmium, ΔH+ exhibits a value of –1.06 in the same pH range (Fig. 1). Above pH 6.3 a small decrease of Hb-O2 affinity was detected, which may result from the formation of insoluble Cd-buffer or Cd-Hb complexes, under this specific
Cd2+ concentration. Likewise, they probably occur in the presence of ATP and Cd2+ presence (ΔH+= 0.5) because of the ATP-Cd complex (Toledo-Maciel et al., 1998).

![Graph of Hb Bohr effect](image)

**Figure 1.** Hb Bohr effect of Brycon cephalus in the presence of CdNO3 and between pH 6.0 and 6.5, 50mM Bis-Tris buffer.

There are no major effects of different Cd2+ levels on Hb-O2 affinity checked at pH 7.0, prepared with Bis-Tris and Tris-HCl buffer; logp50 were, respectively, 0.48 (SD= 0.04 n=19) and 0.5 (SD=0.04 n=10) (Fig. 2). In relation to stripped Hb at pH 7.0 (logp50 = 0.42±0.04, n=4), those values are higher but the causal factor is unclear.
In the erythrocyte, Cd2+ can bind to other proteins (Garty et al., 1981), such as metallothionein (Tanaka et al. 1986), immobilizing it and thus substantially reducing its effect on ATP-Cd complex.

**Conclusion**

The results suggest that Hb-O2 affinity do not differ at low cadmium concentrations in the Bis-Tris and Tris-HCl pH 7.0 buffer. However, it is possible that cadmium can indirectly modify the Hb-O2 affinity by complexing with ATP, making this phosphate unavailable to bind to hemoglobin.
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OXIDATIVE STRESS ON THE CARDIAC MUSCLE
OF THE LUSITANIAN TOADFISH:
CADMIUM AND VANADIUM IN VIVO EFFECTS

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Introduction

Several biological studies associate cadmium and vanadium with the ability to produce reactive oxygen species (ROS), resulting in lipid peroxidation and antioxidant enzymes alterations, leading to oxidative stress. Further, these metals have been associated mainly with hepato and renal toxicity. Recent studies have focused on the involvement of toxic metals in cardiac oxidative damage and suggested that this organ shows a great vulnerability to metal intoxication (Aureliano et al., 2002; Borges et al., 2003). However, the comparative in vivo oxidative effects of these metals in the cardiac tissue are still unclear. Therefore, the present study aims to analyse and compare the oxidative stress responses induced by an acute exposure (1 and 7 days) to a sub-lethal concentration (5 mM) of a cadmium and two vanadium solutions containing different oligovanadates (metavanadate and decavanadate), on the cardiac muscle of the marine teleost Halobatrachus didactylus (Lusitanian toadfish).
Material and Methods

*H. didactylus* individuals (n=40; 358±127g) were collected in Ria Formosa lagoon (Portuguese south coast) and divided into four groups: Placebo, injected intravenously (i.v.) with 0.9% NaCl; Cd, injected i.v. with a 5 mM cadmium chloride solution; Meta, injected i.v. with a metavanadate solution containing 5 mM of total vanadium and Deca, injected i.v. with decavanadate containing 5 mM of total vanadium. Metal solutions were prepared in 0.9% NaCl. Metavanadate and decavanadate solutions were prepared from ammonium metavanadate, as described elsewhere (Aureliano and Madeira, 1994). All solutions were administrated in a dosage of 1mL/Kg of body mass.

Subgroups of 5 individuals of each group were sacrificed 1 and 7 days after injection with anaesthetic overdosage of 2-phenoxiyethanol; blood was collected and the heart was immediately removed and homogenised in adequate buffers. Mitochondrial and cytosolic fractions of *H. didactylus* heart tissue were obtained as described by Aureliano *et al.* (2002). Oxidative stress was determined in cardiac subcellular fractions evaluating: 1) antioxidant enzymes activities – catalase (CAT), superoxide dismutase (SOD), total glutathione peroxidase (GPx\text{total}) and selenium-dependent peroxidase (Se-GPx), estimated by UV/VIS spectroscopy; 2) lipid peroxidation, analysed by thiobarbituric acid test and by cis-parinaric acid assay (fluorimetry) and 3) metal subcellular distribution, on heart tissue and blood (plasma and red blood cells), measured by atomic absorption spectroscopy. Additionally, ROS production (DCF assay) was estimated in vitro by titration with different concentrations of meta and decavanadate, in cardiac mitochondrial fraction obtained from non treated individuals.

The Mann-Whitney test was applied to test differences between placebo and contaminated groups, for all the analysed parameters. The significant level used was \( p <0.05. \)

Results and Discussion

The results obtained in this study showed that, although both metals significantly affected antioxidant enzymes activities and reactive oxygen species production, they have different targets in the cells and affect differently the enzymes studied.

*Antioxidant enzymes activities and lipid peroxidation*
In the mitochondrial fraction, only the decavanadate induced significant variations ($p < 0.05$) of the antioxidant enzymes activities: both CAT and SOD increased immediately 24h after injections (+100% and +119%, respectively), although CAT showed a more pronounced effect after 7 days (+147%) (Fig. 1). Further, in the cytosolic fraction, only SOD was affected by vanadium. Metavanadate induced a significantly decrease ($p < 0.05$) of SOD activity upon 1 day of exposure, while 7 days after exposure, both vanadate solutions decreased SOD activity (-52%). On the other hand, cadmium was the only metal with significant effects ($p < 0.05$) on both GPx$_{\text{total}}$ and Se-GPx activities and also induced increases on cytosolic CAT activity (Fig. 1). Lipid peroxidation levels in heart tissue remained unchanged upon cadmium or vanadate exposure (data not shown).

The described results are somewhat different from those obtained for the same species but injected intraperitoneally, with the same vanadium solutions (Aureliano et al., 2002). According to that report, both vanadate solutions induced a decreased in both subcellular fractions of all antioxidant enzymes activities – CAT (except mitochondrial CAT activity), SOD and GPx. Additionally, a significant increase of lipid peroxidation products (+82%) in $H.\ didactylus$ heart, was observed upon intraperitoneal decavanadate intoxication. These differences indicate that the metal administration protocol is determinant for the results obtained and it should be taking into account when interpreting and comparing results from in vivo metal exposure.
Figure 1. Antioxidant enzymes activity of *Halobatrachus didactylus* heart: A) mitochondrial catalase (CAT); B) cytosolic CAT; C) mitochondrial superoxide dismutase (SOD); D) cytosolic SOD; E) total glutathione peroxidase and F) selenium dependent glutathione peroxidase. Reported values are means±S.D. (N=5). *significantly different from control (p<0.05).

*Metal subcellular distribution*
Cadmium and vanadium were primarily accumulated in the blood (preferentially in plasma), where vanadium accumulation (5.1±2.0 and 6.6±2.8 µg V/g dry tissue for Meta and Deca groups, respectively) was 6-fold higher than cadmium (1.8±5.7 µg V/g dry tissue). In the heart, vanadium accumulated 2-fold more than cadmium; decavanadate, was mainly accumulated in cardiac mitochondria (79±23 and 57±2 ng V/g dry tissue after 1 and 7 days exposure, respectively). This result is in agreement with the increase of antioxidant enzymatic activities observed in mitochondrial CAT and SOD activities.

**Reactive Oxygen Species (ROS) production**

*In vitro* ROS production in cardiac mitochondria was increased by both vanadate solutions, although metavanadate showed a stronger effect (Fig. 2).

![Figure 2. In vitro metavanadate (?) and decavanadate (?) effects on reactive oxygen species (ROS) production in *Halobatrachus didactylus* cardiac mitochondrial fraction.](image)

**Conclusions**

Cadmium and vanadium seem to have different toxicological patterns in the heart of the toadfish since the different metal solutions injected affected
differently the antioxidant enzymes studied and metal accumulation. This study point out that vanadate oligomers induce in vivo and in vitro prooxidant effects in the toadfish heart.

Acknowledgements

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References


RENAL FUNCTION IN RAINBOW TROUT
ACUTELY AND CHRONICALLY
EXPOSED TO WATERBORNE NI

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In the rainbow trout, acute and chronic waterborne Ni exposure leads to marked renal Ni accumulation (Calamari et al., 1982; Pane et al., 2003; Pane et al., 2004). The effects of extensive Ni loading on renal function following either acute or chronic Ni exposure, however, have not been well studied.

The investigation of renal function during acute, high-level Ni exposure is particularly relevant given that acute Ni exposure causes a respiratory acidosis in the rainbow trout (Pane et al., 2003), and given that the kidney is an important extrabranchial organ of acid-base regulation. In addition to characterizing the impact of Ni exposure on basic renal function, we sought to determine the extent of renal compensation of Ni-induced respiratory acidosis.

Additionally, in the rainbow trout, chronic Ni exposure limits maximal aerobic swimming capacity (Pane et al., 2004). While a substantial portion of this limitation is attributable to reduced branchial diffusing capacity, it has been suggested that additional limitation of exercise performance may stem from extrabranchial organ damage. Because renal water and electrolyte handling is important in swimming performance (Wood and Randall, 1973), and because chronic Ni exposure leads to high levels of kidney Ni, we also evaluated renal function following chronic Ni exposure.
The present study used in vivo techniques to investigate renal function during acute and chronic waterborne Ni exposure. Adult rainbow trout (200 – 400 g) were divided into three groups. A control group (C) received clean, moderately hard (140 mg L\(^{-1}\) as CaCO\(_3\)) Lake Ontario tap water for 35 days (the acclimation phase), followed by clean water for 96 h during acute experiments. Unacclimated fish (U) received clean water during the acclimation phase, but were challenged acutely by 96 h exposure to 10 mg Ni L\(^{-1}\) (~30% of the 96 h LC50). Acclimated fish (A) were exposed to 400 \(\mu\)g Ni L\(^{-1}\) (a completely sublethal concentration) for 35 days, followed by 96 h exposure to 10 mg Ni L\(^{-1}\). Over the 96 h acute challenge period, blood and urine were sampled repetitively via surgically implanted indwelling arterial and urinary catheters, respectively.

Over 96 h of acute Ni exposure, glomerular filtration rate (GFR) fell sharply in both acclimated and unacclimated fish (~66%) with no change in control rates (Figure 1). Chronic Ni acclimation had only a minor effect on acute, Ni-induced reductions in GFR, delaying the onset of significantly reduced GFR in

![Figure 1](image-url)

Figure 1. Glomerular filtration rate (GFR) measured during acute Ni challenge. A, U, and C, are chronically acclimated - acutely challenged, chronically unacclimated - acutely challenged, and control, respectively. Values are means ± 1 S.E.M. (n = 4 – 8). Asterisk denotes significantly different (P < 0.05) from simultaneous control mean by means of a one-way ANOVA and 2-sided Dunnett’s post hoc test.
acclimated fish by 36 h. By 78 h, GFR values in both acclimated and unacclimated fish reached the theoretical minimum value in that they were equivalent to (and not significantly different from) urine filtration rates (UFR). Unchanged urine flow rates (UFR) in all three of the groups (data not shown), suggests concomitant reductions in water reabsorption to keep UFR constant in fish subjected to acute Ni challenge.

Urine pH fell sharply in unacclimated fish and was significantly lower than control pH from 48 h onward (Figure 2a). The 96 h urine pH value of unacclimated fish was approximately 1 unit lower than the simultaneous control value. Interestingly, urine pH was well conserved in acclimated fish. The pH drop in unacclimated fish was consistent with a significant increase in the 96 h urinary excretion rate of titratable acid equivalents for unacclimated fish as compared to the rate for acclimated fish (5.06 mol H+ kg⁻¹ h⁻¹ vs. -0.55 mol H+ kg⁻¹ h⁻¹). Acid-buffering capacity in the urine of unacclimated fish was augmented by increased appearance of urine inorganic phosphate (Figure 2b).

Renal ion handling was ion-specific, as demonstrated by the differential handling of Ca²⁺ and Mg²⁺. After 96 h of acute Ni challenge, the urinary excretion rate of Ca²⁺ was higher in unacclimated fish than in acclimated fish, yet both of these rates were lower than control rates (1.47 mol kg⁻¹ h⁻¹ acclimated, vs. 2.27 mol kg⁻¹ h⁻¹ unacclimated, vs. 3.24 mol kg⁻¹ h⁻¹ control). These results suggest that urine Ca²⁺ handling is not greatly perturbed by Ni exposure, and are consistent with the lack of significant effect of acute Ni challenge on plasma Ca²⁺ concentration measured in either a previous study (Pane et al., 2003), or the present study (data not shown).

Conversely, renal Mg²⁺ handling appeared to be more sensitive to acute Ni challenge. Similarly to Ca²⁺, urinary excretion rates of Mg²⁺ were higher in unacclimated fish than in acclimated fish, again suggestive of a protective effect of chronic acclimation. However, urinary Mg²⁺ excretion rates in these two groups of acutely challenged fish were higher than control rates (1.72 mol kg⁻¹ h⁻¹ acclimated, vs. 3.00 mol kg⁻¹ h⁻¹ unacclimated, vs. 0.76 mol kg⁻¹ h⁻¹ control). These results, combined with a significantly decreased plasma Mg²⁺ concentration in unacclimated fish at 96 h (0.59 ± 0.05 mM unacclimated vs. 0.78 ± 0.02 mM control) strongly suggest that increased urinary Ni in unacclimated fish (1985.5 ± 549.7 μg L⁻¹ vs. 7.9 ± 4.1 μg L⁻¹ control) directly interferes with tubular Mg²⁺ reabsorption in the rainbow trout kidney.
Figure 2. Renal acid-base parameters measured during acute Ni challenge. All symbols are as described in Figure 1. Values are means ± 1 S.E.M. (n = 4 – 8). A) Urine pH. B) Urine inorganic phosphate concentration.

Analysis of renal function during acute Ni exposure confirmed that the trout kidney was actively involved in compensation of a Ni-induced respiratory acidosis via increased net acid excretion in conjunction with increased urine buffering capacity achieved by increased urinary phosphate excretion.
Comparison of this acid excretory response in unacclimated fish during acute Ni challenge with the lack of acid excretion (and well-conserved urine pH and inorganic phosphate concentration) in chronically acclimated fish suggests that chronic, sublethal Ni exposure allowed trout to better resist acid-base disturbances elicited by acute Ni exposure.

Additionally, as both the urinary excretion rate and plasma concentration of Mg\textsuperscript{2+} were better conserved in chronically Ni-acclimated fish as compared to unacclimated fish, sublethal Ni exposure protected against acute Mg\textsuperscript{2+} disturbance induced by acute Ni exposure. The potential mechanisms of this protective effect of chronic Ni acclimation are currently being investigated via ion transport studies using brush border membrane vesicles isolated from kidneys of chronically acclimated and unacclimated rainbow trout.

References


Acknowledgements

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COPPER UPTAKE ACROSS FRESHWATER FISH GILLS –

CU(I) OR CU(II)?

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EXTENDED ABSTRACT ONLY – DO NOT CITE

Introduction

Copper is an essential element involved in numerous physiological processes but is also a known, potent toxicant especially to aquatic animals. The gill epithelium is the primary target for acute water-borne copper toxicity in fish (Grosell et al., 2002) and is also involved directly in maintaining copper homeostasis. The gill exhibits regulated copper uptake depending on copper status; deficiency is associated with increased uptake while reduced uptake is seen when copper is present in excess (Grosell et al., 1997; Kamunde et al., 2002). As an epithelium involved in vertebrate copper homeostasis, the fish gill offers a unique model for studies of copper transport mechanisms because copper chemistry can be easily manipulated in freshwater media. We have previously exploited this feature to describe a novel, sodium-sensitive copper uptake pathway in the freshwater rainbow trout, *Oncorhynchus mykiss* (Grosell and Wood, 2002). The purpose of the present study was first to quantify the importance (if any) of the sodium-sensitive copper uptake pathway in another model teleost, the zebrafish, *Danio rerio*. Second, realizing that the copper uptake mechanisms which have been characterized at the molecular level in vertebrates (e.g. Ctr type copper transporters, Cu ATPases) utilizes Cu(I) rather
than Cu(II) (Lee et al., 2002) we investigated the influence of Cu redox state on branchial copper uptake. We hypothesized that reducing Cu(II), the dominant ionic form of copper in oxygenated waters, to Cu(I) would enhance copper uptake.

Materials and Methods

Adult zebrafish were acclimated to ion-poor soft water for > 3 weeks before experimentation and were not fed for 48 hours prior to copper uptake studies. Copper uptake studies using $^{64}$Cu were performed in an artificial freshwater medium consisting of deionized water supplemented with 4 µM CaSO$_4$. This medium was chosen on one hand to ensure low background levels of copper and sodium and on the other hand to ensure sufficient calcium concentrations to maintain tight junction integrity. Two hour flux periods were employed to measure copper uptake rates, as determined by $^{64}$Cu accumulation, at a range of sodium concentrations and in the presence and absence of dithiotreitol (DTT), a reducing agent.

Results and Discussion

Fig. 1 illustrates copper uptake saturation kinetics in zebrafish at different sodium concentrations. A high-affinity, carrier mediated copper uptake across zebrafish gills is evident from the low affinity constant ($K_m = 11.8$ nM). Branchial copper uptake may occur even in non-contaminated environments since the affinity of the copper uptake system
coincides with background copper concentrations typical of most freshwaters. Although a sodium sensitive component is evident in zebrafish at copper concentrations above 100 nM this pathway seems less prevalent than in rainbow trout. Furthermore, copper uptake at concentrations above 100 nM exhibits an IC50 of ~700 µM sodium in zebrafish (data not shown) which is much higher than the IC50 of ~100 µM seen in rainbow trout (Grosell and Wood, 2002).

Contrary to expectations, addition of the reducing agent dithiothreitol (DTT) lowered copper uptake substantially (Fig. 2). This strongly suggests that Cu(II) is the predominant substrate for branchial copper uptake in zebrafish. It should be noted, however, that apparently Cu(I) continues to be taken up via a parallel high affinity, saturatable carrier system. Also worth noticing is the markedly increased copper uptake when ambient copper as Cu(II) is elevated above 100 nM. This increase occurs at concentrations approaching toxic levels. These observations may suggest that a divalent metal transporter (DMT) is involved in copper uptake across fish gills and metal competition studies have offered some support for this hypothesis (data not shown).

![Fig. 2. Copper uptake by zebrafish in the presence and absence of the reducing agent DTT, illustrating both Cu(I) and a Cu(II) uptake systems. N=10.](image)

\[
\begin{align*}
\text{Cu(II)} & : r^2=0.99 \\
V_{\text{max}} &= 43.2 \pm 0.7 \text{ pmol g}^{-1} \text{ h}^{-1} \quad K_m = 31.5 \pm 1.1 \text{ nM} \\
\text{Cu(I)} & : r^2=0.94 \\
V_{\text{max}} &= 20.2 \pm 1.1 \text{ pmol g}^{-1} \text{ h}^{-1} \quad K_m = 51.3 \pm 7.6 \text{ nM}
\end{align*}
\]

Conclusions

Perhaps the most important conclusion to be drawn is that species-specific differences in copper uptake mechanisms are pronounced among freshwater
fishes. In addition, it is clear that several uptake pathways operate in parallel and that their relative importance may depend on the ambient copper concentration. These uptake pathways include, but may not be limited to, a sodium-sensitive pathway (perhaps through a sodium-channel), divalent copper uptake pathway (likely DMT) and finally a monovalent copper uptake pathway which could be similar to Ctr-type copper transporters.

Acknowledgements

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Reference List


Copper is an essential element for all the organisms. However, copper can be extremely toxic, depending on its concentration in water. The toxicity of copper depends on a variety of factors including the chemical and physical characteristics of the water and the biology of the species (Fernandes and Mazon, 2003). The assimilation of toxic compounds by the aquatic organism is through the water or food intake. The assimilation of these compounds from water by freshwater fish occur mainly through the surface of the gills, the
primary target organ of all pollutants, due to their great surface area, reduced diffusion distance between the water and the blood and large water volume flowing over the gills during the respiratory cycle.

Extended biochemical and physiological alterations induced by copper on the gill cells cause morphological changes in the gill structure (Cerqueira and Fernandes, 2002) and, consequently, gas transfer, ion and acid-base disturbances. Such disturbances are usually characterized by an initial “shock” phase in which the disturbance develops quickly and a long-term recovery phase where the disturbance gradually diminish. Recovery implies in a partial restoration of the gill transport function, establishing a new physiological steady state, as a result of specific biochemical, physiological and/or morphological adaptations of the organ.

Morphological changes of gills have been intensively studied after exposure to pollutants however, no studies have applied the stereological principles in the gills to evaluate the changes caused by copper exposure in these organs. The stereological methods avoid the bias of data and make possible that all parts of organ can be sampled. The aim of this study was to evaluate the changes in the gills the freshwater fish, the curimbatá Prochilodus scrofa, exposed to copper (96h) and during subsequent recovery in water free of copper, applying stereological methods.

Juveniles Prochilodus scrofa supplied by the Hydrobiology and Aquaculture Station, Furnas Power Plant, Furnas, MG, Brazil were kept in tanks with a continuous flow of aerated (100% O₂ saturation) water (water constitution: pH 7.3 ± 0.2; alkalinity 23.7 ± 1.9 mg.L⁻¹ as CaCO₃, conductivity 8.3 ± 0.3 µS and hardness 24.5 ± 0.2 mg.L⁻¹ as CaCO₃) at 25°C for 30 days prior to the experiments.

After acclimation to laboratory conditions, the fish were randomly divided in controls groups, kept in water free of copper, and exposed to copper (29 µgCuL⁻¹, CL50-96h, pH 7.0). After copper exposure fish from both groups were randomly sampled. The gills were fixed in 2.5% glutaraldehyde buffered with 0.1M phosphate buffer pH 7.4. The remaining fish were transferred to tanks with water without copper, continuous aerated water flow for recovery. Fish (n=5) were sampled at 1, 2, 7, 15 and 30 days after transference and the gills were removed and fixed as above.
The changes in the gill tissue were done applying the stereological methods (Howard and Reed, 1998). Sampling and the embedding procedures of gills were designed in order to combine the Cavalieri principle for determining the reference volume with the vertical sectioning method for measuring surface area and the gill area and pathologies were analyzed using an Olympus BX light microscope with video camera coupled to a computer possessing a specialized image analyzer for stereology C.A.S.T. - Grid System (Olympus Danmark A/S). ANOVA was used to determine the significance of the data and the Tuckey test with 95% confidence limit was applied to compare the means whenever the data were significant.

The histopathology found in the gills are shown in Table 1 and, in general, they occupied up to 25% of gill of surviving fish. On the 30th day of recovery in water free of copper they were not significant different from the controls although the histopathology still slightly higher. The water-blood barrier distance was significant differences (p>0,05) between control and 96h of exposure to copper. The results showed that the gills of the surviving fish from acute copper exposure are functional making possible fish recovery.

Table 1. Histopathology of the gills of *P. scrofa* after 96h copper exposure during the recovery period.

<table>
<thead>
<tr>
<th>Histopathology</th>
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<tbody>
<tr>
<td>Hipertrophy and hyperplasia of mucous cells</td>
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<tr>
<td>Hipertrophy and hyperplasia of chloride cells</td>
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<tr>
<td>Hipertrophy and hyperplasia of lamellar epithelium</td>
</tr>
<tr>
<td>Hipertrophy and hyperplasia of filament epithelium</td>
</tr>
<tr>
<td>Incomplete fusion of several lamellae</td>
</tr>
<tr>
<td>Cell degeneration</td>
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</tbody>
</table>

Acknowledgements

FAPESP and CNPq for financial support. Hydrobiology and Aquaculture Station, Furnas Power Plant, Furnas, MG for providing the fish. E.T. Gravi is grateful to CNPq for the award of scholarship.
References


It is known from ecotoxicological studies that freshwater fish, even with the same life history, show a species specific resistance against pollutants. However, the mechanisms underlying these differences are not well understood. We examined effects of sublethal waterborne copper exposure on three freshwater fish: rainbow trout, *Oncorhynchus mykiss*, common carp, *Cyprinus carpio*, and gibel carp, *Carassius auratus gibelio*. Besides disturbance of ion regulation, one of the primary actions of copper is disruption of gas exchange causing impairment of oxygen uptake. Therefore, species were selected because of their differences in hypoxia tolerance. Indeed, the hypoxia sensitive rainbow trout was most sensitive to copper (96h LC₅₀: 3.3 µM), while copper tolerance of the hypoxia tolerant common carp was 3 times higher (96h LC₅₀: 10.4 µM) and of
the anoxia resistant gibel carp almost 7 times higher \(96h \text{ LC}_{50}: 22.0 \, \mu\text{M}\) (De Boeck et al., 2004). To unravel the physiological mechanisms that bring about these differences, we examined a broad range of physiological parameters, including effects on whole organism respiration and blood gases, anaerobic metabolism, swimming capacity, ion regulation, protein synthesis, induction of metal binding proteins, tissue damage etc. The differences in copper tolerance did not relate to the hypoxia sensitivity per se, although rainbow trout clearly differed from the cyprinids by showing hyperventilation, high blood oxygen levels and increased energy consumption, compared to a transient reduction in blood oxygen levels accompanied by a temporary peak in blood carbon dioxide levels suggesting hypoventilation in the cyprinids. Both carp species switched to anaerobic metabolism when this occurred. Disturbance of ion regulation, measured as plasma sodium and chloride was similar for all species. Gill damage showed different patterns in all species, showing more severe damage in carps. Copper accumulation rates differed between species, and whereas common carp mainly showed accumulation in the liver, gibel carp showed a peak accumulation in plasma and kidney followed by recovery to control levels or lower suggesting excretion through the kidney. Also, only gibel carp seemed capable to respond to copper exposure with the synthesis of metal binding protein in a dose dependent way in all tissues, protecting cells from heavy metal damage (De Boeck et al. 2003). In common carp, and even more in rainbow trout, dose response curves of metallothionein were limited.

The aim of the presented experiment is threefold. First, clarify the differences in uptake, accumulation and excretion of copper between the three species. Using the \(^{67}\text{Cu}\) isotope, we assessed uptake, internal distribution and excretion of the ‘newly accumulated’ copper over a period of three weeks. Adding a short-term experiment with \(^{64}\text{Cu}\) isotopes at regular time intervals, allowed taking into account possible effects of acclimation on copper uptake rates. Secondly, \(^{22}\text{Na}\) uptake was tracked in these short-term experiments in order to take a closer look at the disturbance, and possibly acclimation, of ion regulation. Indeed, it was stated by Grosell and co-workers (2002) that sodium turnover rate determines sensitivity to acute copper and silver exposure in freshwater animals. Although the disturbance of absolute plasma sodium concentrations was similar in all species, sodium turnover rates might differ. Possibly the difference in sodium turnover explains the difference in copper sensitivity of our fish. Besides \(^{22}\text{Na}\) uptake, the activity of the most important ion-transporting enzyme, namely \(\text{Na}^+/\text{K}^+\)-ATPase activity was measured. Copper is known to block the activity of this enzyme, and differences in this event again could help explain differences in sensitivity. Finally, plasma and muscle ammonia and ammonia excretion rates
were assessed since increased ammonia production, in combination with impaired ammonia excretion is thought to be the cause of the reduced swimming capacity under copper exposure (Beaumont et al, 1995).

References


COPPER TOXICITY AND METALLOTHIONEIN INDUCTION IN FISH: EFFECT OF WATER TEMPERATURE AND PH

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Abstract

Copper accumulation and metallothionein (MT) concentration were determined in the liver of Prochilodus scrofa exposed to the respective 96h-LC50 at 20 and 30°C and pH 4.5 and 8.0. Copper accumulation in the liver of copper exposed fish was greater at lower temperature and pH 8.0. MT concentration in the liver was higher in fish exposed to copper at 30°C. Copper accumulation is related to copper toxicity and the water pH is a determinant factor in this process. MT concentration in the liver seems to depend on water temperature rather than copper concentration in water.
Introduction

Ecotoxicologists have used the expression of metallothionein (MT) as a biomarker for heavy metal contamination due to its rapid increasing concentration by induction with ions such as cadmium, mercury, zinc, and copper; although, MT may be also induced by a great variety of pathophysiological factors, including restriction of food intake, bacterial infection, and exposure to physical and inflammatory stress (Kägi, 1993; Muto et al., 1999).

Copper is an essential element that serves as a cofactor in a number of enzyme systems for most living organisms but, at high concentration, copper become a toxic pollutant. Copper is used today a chemotherapeutic agent in aquaculture however, the increased level of copper in aquatic environments coming from industrial or agricultural wastes. The physical and chemical characteristics of water play an important role in copper toxicity for aquatic animals. Copper speciation is directly affected by water pH, and the free cupric ion concentration is higher in water with low pH, while copper hydroxide complexes prevails in water with high pH (Payle et al., 1992; Tao et al., 2001).

Water temperature acts as a regulator of the physiological processes of poikilothermal animals, such as the fish. All life processes of living organisms are physicochemical in nature therefore, temperature affect all these processes. Although the aquatic environments are in general thermally stable, seasonal variations in temperature characterize the subtropical regions. High temperatures tend the speed up the physiological processes and, as the temperature changes, the rate of various processes must be balanced and coordinated, and the organisms must compensate or minimize the changes in its body (Reynolds and Casterlin, 1980).

Therefore, changes in water temperature, as occurs in the Southeast Brazil that range from 15-20°C during the winter and 28-35°C during the summer and in pH due to episodic ecologically accidents, as most Brazilian freshwaters is ion-poor and soft having low buffering capacity, may affect profoundly copper toxicity and also the MT response in fish living in such environment. Carvalho et al. (2004) demonstrated high copper accumulation in the liver of Prochilodus scrofa with concomitant expression of MT in the liver. To evaluate the effect of temperature on copper toxicity in fish, copper accumulation and the MT
induction in the liver of *Prochilodus scrofa*, we exposed fish to copper in water with 4.5 and 8.0 pH at 30°C. *P. scrofa* is highly sensitive to copper (Mazon and Fernandes, 1999) and copper toxicity is strongly influenced by water pH (Takasusuki et al., 2004; Carvalho, 2003) however, this species also show fast physiological recovery from copper effects and long-term gill histological recovery after transference to water free of copper (Cerqueira and Fernandes, 2002a,b). Furthermore, this species has a high tolerance for changes in temperature (Barrionuevo and Fernandes, 1995). The thermal range for the temperature acclimation of *P. scrofa* goes from 15 to 35°C (Barrionuevo and Fernandes, 1995, 1998; Fernandes et al., 1995), and displays a thermal tolerance zone, determined by the critical minimum and maximum temperatures, equivalent to 1046°C for small fish (Barrionuevo and Fernandes, 1995).

**Materials and Methods**

Juvenile *P. scrofa* (body mass 18.5 ± 3 g) were holding tanks at 25 ± 1°C with a continuous flow of aerated dechlorinated tap water (water composition: pH = 7.3 ± 0.2; alkalinity = 23.7 ± 1.9 mg L⁻¹ as CaCO₃; hardness = 24.5 ± 0.2 mg L⁻¹ as CaCO₃) for 30 days, after which the water temperature was lowered or increased by 1°C on alternate days until it reached 20 or 30°C which were the average lower and higher temperature of *P. scrofa* natural habitat. The fish were kept at this temperature for a minimum of 30 days prior to the experiments. The experiments were done in static-systems. Control groups were maintained in aquariums with copper-free water and the pH was adjusted to 4.5 and 8.0. Copper exposed groups were maintained in aquariums, which received a concentration of copper (CuSO₄.5H₂O) equal the nominal 96h-LC₅₀ of copper for *P. scrofa*, in water with pH 4.5 (88 ± 0.8 µgCu L⁻¹) and pH 8.0 (14 ± 0.2 µgCu L⁻¹) at 30°C (Carvalho, 2003). The water temperature in each aquarium was kept stable at 30°C. All experiments were done in duplicate. Copper concentration in water was measured using Atomic Absorption Spectrophotometry.

After 96h the controls and the surviving fish from groups exposed to copper were killed and the liver was dissected. A sample of liver was taken to determine the total copper, which was done using Atomic Absorption Spectrophotometry. Cytosolic proteins were isolated from the remaining liver tissue to determine MT and total protein concentrations. MT was determined in the supernatant from the centrifuged homogenate of liver, following the method described by Ahmad et al. (2000) with slight modifications. Purified protein fractions contained MT were obtained using a Sephadex G75 column and DEAE-Sepharose column.
following by polyacrylamide gel electrophoresis (SDS-PAGE) as described by Laemmli (1970). The protein concentration in the liver tissue was determined by Lowry’s method (Lowry et al., 1951).

The significance of the data was determined using the ANOVA analysis of variance and the Tukey test with a 95% confidence limit was applied to compare mean values whenever the data was significant.

Results and Discussion

The study reveals important aspects regarding the effect of environmental water pH on copper toxicity and MT induction in fish. The percentage of surviving fish from groups exposed to copper (96h-LC50) in water at pH 4.5 and 8.0 was 50 and 46% at 30 ºC, respectively. No fish died from the control group. As expected, the results found by Carvalho et al. (2004) in the same species exposed to the respective 96h-LC50 copper concentration in water with pH 4.5 (98 ± 0.8 µgCu L⁻¹) and 8.0 (16 ± 0.5 µgCu L⁻¹) at 20°C was also close to 50% and confirms the influence of water pH on copper toxicity evidencing no effect of temperature in copper toxicity as reported by Carvalho (2003).

The Cu²⁺ form of copper, considered the more toxic form of copper species, is bioavailable in waters with pH lower than 6.0 (Tao et al., 2001) and, paradoxically, copper toxicity was lower in water with pH 4.5. In this case, the H⁺, which concentration is high at low pH, and Cu²⁺ competition for Ca²⁺ sites in the gill membrane may be the main reason for lower copper toxicity in low water pH (Meador, 1991). In waters with high pH, the reduced concentration of H⁺ and low levels of Ca²⁺ as found in the Brazilian freshwaters may favor copper binding to gill surface membrane, increasing copper uptake and, hence, its toxicity.
Figure 1. Copper concentration in the liver of *P. scrofa* exposed to copper (96h-LC$_{50}$) in water with pH 4.5 and 8.0 at 20°C (data from Carvalho et al., 2004) 25°C (Takasusuki et al., 2004) and 30°C (present results). Values from the control groups (0 µgCuL$^{-1}$; open bars) and from groups exposed to 96h-LC$_{50}$ copper concentration in water with pH 4.5 and 8.0 (black bars). The bars indicate the mean values ± SEM; (*) indicates a significant difference from the respective control group ($p < 0.05$); and (#) indicates a significant difference in relation to copper – pH 4.5 and pH 8.0; and (+) indicates a significant difference in relation to temperature ($p < 0.05$).

Copper accumulation in the liver of fish exposed to copper was significantly higher ($p < 0.05$) than in the controls at the end of the 96 h of copper exposure fish and higher ($p < 0.05$) at pH 8.0. Similar results was found by Carvalho et al. (2004) in fish acclimated at 20°C. Figure 1 shows a comparison between copper concentration in liver tissue of fish copper exposed groups at 20°C (Carvalho et al., 2004), 25°C (Takasusuki et al., 2004) and 30°C (present results).

The MT-mass protein identified in the liver of *P. scrofa* was lower than 15 kDa as revealed by SDS-PAGE electrophoresis. Among the numerous factors stimulating MT biosynthesis, the increased of MT concentration in aquatic animals has been associated with increased levels of metals in the aquatic environment and with the length of exposure time (Olsvik et al., 2000). However, some authors have emphasized that MT production depends, at least up to a
certain critical concentration (Roméo et al., 1997), on the effective increase in metal inside the animal, implying a response to intracellular heavy metal content (Mayer et al., 2003).

The effect of temperature seems to be evident in the MT concentration. The mean liver MT concentrations from fish exposed to copper in water with pH 4.5 and pH 8.0, are shown in the table I. MT concentration in the liver of fish exposed to copper at 20°C were not difference on both water pH (Carvalho et al., 2004). Similar results were found in the MT concentration in the liver of fish at 30°C ($p>0.05$).

Table I. MT concentration in the liver of $P. scrofa$ from groups exposed to 96h-LC$_{50}$ copper concentration in water with pH 4.5 and 8.0 at 20 and 30°C. Values are means ± SEM.

<table>
<thead>
<tr>
<th>Water Temperature ($^\circ$C)</th>
<th>Metallothionein concentration (µg g$^{-1}$ liver wet mass)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pH 4.5</td>
<td>pH 8.0</td>
</tr>
<tr>
<td>20</td>
<td>126.8 ± 21.5</td>
<td>167.4 ± 25.6</td>
</tr>
<tr>
<td>30</td>
<td>710 ± 70*</td>
<td>1010 ± 60*</td>
</tr>
</tbody>
</table>

(*) indicates a significant difference in relation to copper – pH 4.5 and pH 8.0 at 20 and 30°C ($p<0.05$).

The diffusion rate and all biological processes are higher at high temperatures. The lower copper accumulation and higher MT concentrations in the liver of fish exposed to copper at 30°C in both water pHs may be due to the higher activity of metabolic processes related to copper detoxification including the excretion rate and suggests an imbalance between copper uptake and detoxification/excretion rate in neotropical fish at low temperature, seeming to favor higher copper accumulation, since the 96-LC50 of copper in water at pH 4.5 and 8.0 was the same at water temperatures of 20°C and 30°C (Carvalho, 2003). It could be explained by an extremely slow elimination of copper in low temperature.

In conclusion, this study provides evidence that copper accumulation is related to copper toxicity and the pH of water is a determinant factor in this process. MT
concentration in the liver seems to depend on water temperature rather than copper concentration in water.

Acknowledgements

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accumulation of copper in the tropical freshwater fish, Prochilodus scrofa

changes induced by copper exposure in the South American tropical

Hematological and physiological changes induced by short-term exposure


NO INHIBITION OF Na⁺ INFLUX IN TAMBAQUI EXPOSED TO HIGH COPPER CONCENTRATIONS IN EXTREMELY SOFT WATER

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EXTENDED ABSTRACT – DO NOT CITE

Introduction

Copper occurs in the aquatic environment as a result of the natural geochemistry of the water and through anthropogenic action. Though essential for metabolic processes, copper can be acutely toxic to fish. This toxicity varies based on the physicochemistry of the water, the chemical speciation of the metal, and the differential tolerance of various species. Water hardness is known to affect copper toxicity, with toxicity generally higher in organisms inhabiting soft water. In the Amazon basin, natural waters are mostly soft, with calcium (Ca²⁺) concentrations between 10 and 100 µmol/l. Contamination of Amazonian waters by copper is mostly attributed to industrial activity, with concentrations over 1,000 µg/l reported at some sites (Sampaio, 2000). Such high concentrations of copper may severely affect the fish fauna as a result of the soft nature of Amazonian waters. We assessed the ionoregulatory disturbance on Na⁺ balance in tambaqui (Colossoma macropomum), a species native to the Amazon basin, exposed to copper in extremely soft water. For comparison, we also tested the effects of copper exposure in rainbow trout (Oncorhynchus mykiss) acclimated to soft water.
Materials & Methods

Tambaqui and rainbow trout were obtained from fish farms in Brazil and Canada, respectively. Tambaqui were acclimated in soft water (Ca\(^{2+}\)=11, Na\(^+\)=34, K\(^+\)=15, Cl\(^-\)=27, Mg\(^{2+}\)=0.8 – all in \(\mu\)mol/l; pH 6.5; dissolved organic matter (DOM)=0.9 mgC/l; 28°C). Rainbow trout were acclimated gradually from hard to soft water (Ca\(^{2+}\)=100, Na\(^+\)=70 \(\mu\)mol/l (other ions not measured); pH 7.2; DOM=3.0 mgC/l; 12°C). Unidirectional Na\(^+\) fluxes were based on the disappearance of \(^{22}\)Na from the water over time, indicating incorporation by the fish. Tambaqui were exposed to copper at concentrations up to 400 \(\mu\)g/l, whereas trout were exposed to copper at concentrations to 300 \(\mu\)g/l. Water samples were taken to determine Na\(^+\) concentration (flame atomic absorption spectrophotometry), \(^{22}\)Na counts (beta or gamma counting), and total copper concentration (graphite furnace absorption atomic spectrophotometry). Calculations were based on Wood (1992). Statistical analysis used ANOVA, followed by Dunnett’s multiple comparison test.

Results & Discussion

Tambaqui showed an impressive tolerance to copper toxicity when tested in soft water, as indicated by a lack of inhibition on Na\(^+\) influx, even at 400 \(\mu\)g/l of copper (Fig. 1). Inhibition of Na\(^+\) influx is a typical response to copper toxicity in fish (Laurén & McDonald, 1985). Trout had 50-66% inhibition in Na\(^+\) influx relative to control, upon exposure to 70 and 300 \(\mu\)g/l of copper (Fig. 2).

In tambaqui, however, exposure to increased copper concentrations was associated with increased Na\(^+\) efflux, although diffusive losses were controlled over time (Fig. 1). The resulting net flux was still higher relative to the control, but not as high as that of trout, which were not able to control the diffusive losses at the highest copper concentration tested (Fig. 2). The pattern of copper toxicity in tambaqui therefore differs from that of trout, affecting only the paracellular pathway (as indicated by increased Na\(^+\) loss at increased copper concentrations). The explanation for a lack of inhibition in Na\(^+\) influx in tambaqui is unclear. The presence of high affinity Na\(^+\) channels in tambaqui would explain in part the unaffected influx rates upon exposure to copper. But if true, copper accumulation on the gills would be expected to be relatively low upon exposure to the metal. Preliminary gill binding assays in tambaqui revealed, however, an accumulation of high copper concentration at the gills resulting from waterborne exposure (Matsuo & Val, unpubl.).
Fig. 1. Unidirectional Na\(^+\) fluxes (mean±SEM; N=10 per treatment) in tambaqui exposed to copper. (*) indicates significant difference relative to control at each given time interval.

Fig. 2. Unidirectional Na\(^+\) fluxes (mean±SEM; N=7 per treatment) in rainbow trout exposed to copper. (*) indicates significant difference relative to control at each given time interval.
That copper toxicity and the effects of low pH appear to share a common pathway is worthy of discussion. The copper 96h-LC50 value documented for tambaqui (735 µg/l copper; Oliveira, 2003) is exceptionally high compared to that for trout acclimated to soft water (14-80 µg/l copper). Moreover, the tambaqui is known to be extremely tolerant of low pH (withstanding pH levels around 3.5; Wood et al., 1998), whereas rainbow trout cannot tolerate pH levels below 4.5. The extremely high tolerance of tambaqui to both H+ and copper toxicity, both of which target Na+ transport, suggests an unusual Na+ uptake system that differs from the mechanisms currently known in fish. This would also explain the lack of disruption in Na+ influx upon exposure to copper.

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HEMATOLOGICAL RESPONSE
OF FRESHWATER FISH COLOSSOMA MACROPOMUM
EXPOSED TO SUBLETHAL COPPER CONCENTRATION

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Abstract

The hematological response of freshwater fish *Colossoma macropomum* exposed to subletal copper concentration was evaluated during 7 and 15 days. After exposition, the fish were depurated during 21 days. Hemoglobin (Hb), hematocrit (Ht), red blood cells (RBC), Mean Corpuscular Hemoglobin (MCH), Mean Corpuscular Volumen (MCV), and Mean Corpuscular Hemoglobin Concentration (MCHC) were determined. A significative decrease was observed in the Hb, Ht, MCV and MHC without changes on the MCHC; the RBC showed significant increase. After copper depuration, Hb, Ht, RBC, MCH and MCV increase in depurated fish, to reach a range similar to control organisms. The copper induced microcytic anemia in *C. macropomum*, although the effect is not permanent.

Key words: anemia, *Colossoma*, copper

Introduction

Copper exposure causes a series of cellular and physiological changes in fish, such as reduction of lymphocytes circulating and B-cell depressed, elevation of
neutrophils, monocytes and thrombocytes (Dick and Dixon, 2000), acute oedema and epithelial lifting in the gill, cell proliferation and necrotic and apoptotic chloride cell (Dang et al., 2000), inhibition of the branchial Na+ K+ ATPasa (Li et al., 1998), proliferation of mitochondria in enterocytes (Kamunde et al., 2001).

Hematological studies in fishes have assumed greater significance because this parameters were to be used as an effective and sensitive index to monitor physiological and pathological changes induced by natural or anthropometric factors, for example, bacterial or fungi infections or pollution of water resources. There are numerous reports on the short-term effects of Cu exposure on fish hematology (Dick and Dixon, 2000; Mazon, 2002;) but there are only a few papers that explore chronic copper effects and physiology mechanisms of fish depuration using hematological parameters as sensitive index to monitor the depuration capacity.

The freshwater fish *Colossoma macropomum* Cuvier 1818 (Osteischthyes: Characidae) is a living in Amazonan rivers. In Venezuela it is found in Orinoco and Apure rivers; actually, *C. macropomum* is been employing in pisciculture because its great potential as monoculture adaptable species.

In the work, we were studied the hematological changes induce to copper in the blood of *C. macropomum* after exposure to subletal copper concentration in water and again, we evaluate hematological changes after copper depuration fish; in order to know about possible adaptative to environmental copper and homeostatic status of the fish.

**Material and Methods**

*Animals*

Juvenile *C. macropomum*, weighing between 31.43 ± 48.41g and size between 12.47 ± 14.31 cm were obtained from Instituto Nacional de Investigaciones Agrícolas (INIA), Delta Amacuro state, Venezuela. Following their transfer to Ecophysiology Laboratory, IOV, Universidad de Oriente, Cumaná, Venezuela. The fish were maintained at 28 ±2 °C in tanks (1000L) with continuously aerated and flowing dechlorinated tap water at least 15 days prior to the experiments. Fish were fed ad libitum with balanced fish food for this species provided by INIA.
Experiment protocol

Previously, we calculate LC50 of copper for this species, (LC50 96 hours: 8 mg Cu L\(^{-1}\)). Groups of 12 fish were exposed for 7 and 15 days to sublethal doses of 0.2 mg Cu L\(^{-1}\), using a static test system. Each aquarium was continuously aerated and the same physical and chemical characteristics of the water as those in laboratory acclimation were maintained. The copper agent was CuCl2.2H2O and the concentration in the water was measured using an atomic absorption spectrophotometer. Control fish were maintained under the same conditions in water devoid of copper detectable. All days during the assay, the fish were fed ab libitum previous change of metal and water. The food rest were cleaning before to add the metal. After 15 day copper exposition, the hematological parameters were determined in the fish and then, theirs were depuration for its colocation in aquarius with copper free water during 21 days. After this time, the blood samples were, again, analyzed.

Blood analysis

The blood was obtained from the caudal vein into heparinized plastic tubes. Analyses of hematological parameters were conducted immediately. Ht was determined by spinning the blood sample contained in heparinized capillay tubes in a microhematocrit centrifuge. The RBC count was carried out in a modified Neubahuer chamber and the Hb was determined by the cyanomethahemoglobin method. The blood indexes, mean corpuscular volumen (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) were then calculated using the blood measurements above.

Statistical analysis

The analysis of variance (ANOVA) was used to determined the significance of the date and the SNK test with 95% confidence limited was applied to compared the means whenever the date were significant.

Results

The freshwater fish *C. macropomum* exposed to 0.2 mg L\(^{-1}\) Cu showed a significant decreased in Hb, Ht and indexes: MCV, MHC without changes in
the HMCH (ANOVA: 13.07 p<0.001; 10.76 p>0.001: 3.08 p<0.05; 4.05 p<0.01, respectively; Figure 1).
The erythrocyte counts (RBC) showed a significant increased (ANOVA: 4.77 p< 0.01) in exposed to copper fish. The depuration for 21 days allowed a recovery of parameters Hb, Ht, VCM and HCM (Figure 2A, B, C and D). A significant decrease in the RBC was observed in copper depurated organism (ANOVA: 16.06 p< 0.001; Figure 2E).

Discussion

Chronic sublethal effects of copper tend to result in physiological changes an altered function in a range of body systems in fish (Handy, 2003). The sublethal copper exposure during 15 days and at 2 ppm Cu concentration of C. macropomum induce anemia for decrease of Hb, Hct and MCV and MHC. A microcytic-hypocromic anemia is observed nby copper in C. macropomum suggesting that the metal affect the haematopoyetic system and biochemical pathway of hem formation. This response has been showed in other fish exposed to short term or long time to copper (Mazon, 2002).

During copper exposure, the RBC in C. macropomum showed a tendency to increase. This response was maintained after copper depuration fish, indicating that the copper depuration triggered proliferation of RBC; also, these results suggest that C. macropomum have a good depuration capacity. The RBC increased indicates a compensatory response of this species to increase the oxygen demand induced by anemia (Handy et al 1999).
Figure 1. Hematological parameters in the freshwater fish C. macropomum exposed to copper. A. Hemoglobin (Hb), B. Corpuscular Mean Hemoglobin (CMH), C. Hematocrit (Ht), D. Mean Corpuscular Volumen (MCV), E. Red blood cells (RBC). Horizontal line, vertical rectangle and vertical line denote mean, standard deviation and range respectively. Sample size given number above range. 0: before Cu exposed, 7a: Cu-exposed 7 days, 15a: Cu-exposed 15 days, 7 control: control 7 days, 15 control: control 15 days. * 0.01<p<0.05; **p<0.01; ***p<0.001.
Figure 2. Hematological parameters in the freshwater fish *C. macropomum* exposed to copper. A. Hemoglobin (Hb), B. Corpuscular Mean Hemoglobin (CMH), C. Hematocrit (Ht), D. Mean Corpuscular Volume (MCV), E. Red blood cells (RBC). Horizontal line, vertical rectangle and vertical line denote mean, standard deviation and range respectively. Sample size given number above range. E: Cu-exposed 15 days (0.2 ppm) and D: Depured during 21 days. * 0.01<p<0.05; **p<0.01; ***p<0.001.
We observed change in the behavior of the fish during the copper exposure, such as, slow swimming speed and slow appetite; may be, this behaviour are produced because the fish want to preserve metabolic scope for aerobic metabolism. When fish were copper depuration, their recovery the rapid swimming response and appetite confirmed the observation made above.

The change produce for copper exposition were a physiological and metabolic adjustment the fish during metal exposure and, maybe the copper effect on this parameters are reversible if the organisms are depurated above 21 days.

Conclusion

The copper induces hypochromic microcytic anemia in *C. macropomum*. The Copper effect on the hematological parameters of *C. macropomum* evaluated and during the period used in this study is reversible.

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REASONS OF EXOGENOUS STEROIDS ENHANCE Cu²⁺-RESISTANCE IN TILAPIA LARVAE (Oreochromis mossambicus)

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EXTENDED ABSTRACT ONLY - DO NOT CITE

The toxic effects of sub-lethal concentrations of Cu²⁺ on larvae have also been reported. Whole body Na⁺ and K⁺ contents of larvae significantly decreased following Cu²⁺ exposure up to 96 h with Ca²⁺ contents statistically significantly decreased for larval exposure times exceeding 72 h (Wu et al., 2003). Besides, the Na⁺/K⁺-dependent adenosine triphosphatase (Na⁺-K⁺ ATPase) specific activity was found to be very sensitive in fish exposed to waterborne Cu²⁺ (Li et al. 1998; de Boeck et al., 2001). Exogenous cortisol has been demonstrated to induce a 14-65% increase gill Na⁺-K⁺-ATPase activity in tilapia, and has been found to significantly increase gill Na⁺-K⁺-ATPase activity in SW-adaptive fish (Mancera et al., 2002). Therefore, it may be expected that the role of increased usefulness to increase Na⁺-K⁺-ATPase activity may enhance resistance of copper challenge in larvae through administration of exogenous steroids. The present study was to approach the effects on copper resistance of exogenous steroids and the relationship between steroids, Na⁺-K⁺ ATPase and ionic homeostasis after challenge with copper toxicity in tilapia larvae (Oreochromis mossambicus).

Methods and Experiment Design

Tilapia larvae were reared with artificial feed containing 0 (control), 50 (low dose), 125 (middle dose) and 250 mg / kg (high dose) cortisol or progesterone
for 10 days. On the day of feeding end, tilapia was collected and whole body Na⁺-K⁺-ATPase activation, cortisol levels and aldosterone was measured, or the larvae was exposed to 1 mg/L Cu²⁺ for 72 hr and the mortality was examined. In addition, per alive larva was for one sample and 5-8 samples were measured whole body of K⁺, Na⁺ and Ca²⁺ contents.

Cortisol and aldosterone extracted from whole body of tilapia larvae with ether, and the final combined extract was reconstituted with 0.6 ml EIA assay buffer (PBSG) for ELISA (enzyme-linked immunosorbent assay) of cortisol or aldosterone. Whole body of K⁺, Na⁺ and Ca²⁺ contents were measured by atomic absorption spectrophotometer.

**Results and Discussion**

The results indicate both cortisol and progesterone significantly increased Na⁺-K⁺-ATPase, while only progesterone was able to increase the survival rates and whole body Na⁺, K⁺, and Ca²⁺ contents are retained for a prolonged period in larvae fish after Cu²⁺. These effects of progesterone were clearer than cortisol. On the other hand, endogenous cortisol significantly increased after 10 days of exogenous administration of cortisol. While larvae aldosterone level increased, the cortisol content was not enhanced after 10 days of oral progesterone treatment. We suggest that in addition to progesterone involvement in copper resistant mechanisms in tilapia larvae by ion retention, and aldosterone effectively enhances this mechanism.
Fig. 1. Changes in mortality of tilapia larvae after Cu$^{2+}$ exposure (1 mg/L for 72 h). Data (n=3) were compared with one way ANOVA using Dennett’s test analysis. * Indicate $p < 0.05$.

Fig 2. The Na$^+$-K$^+$-ATPase activation was compared. In tilapia larva after 10 days of treatment with steroids hormone (n = 35) Different superscripts indicate a significant difference among treatments ($p < 0.05$, ANOVA analysis with Tukey’s comparisons).
References


BIOMARKERS AS TOOLS
TO CHARACTERIZE THE HEALTH STATUS
OF FISH COLLECTED ALONG AN URBAN STREAM

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EXTENDED ABSTRACT ONLY – DO NOT CITE

Introduction

Literature has documented the difficulty of predicting a toxicological hazard for the aquatic community of a given area using only data on water and sediment chemistry. Several studies have shown that investigating biomarkers in benthic fish of a given area can provide evidence of contaminant exposure, toxicant bioavailability and toxicological effects (Viganò et al., 2001). In the present work a battery of potential biomarkers has been evaluated in a native fish species collected along an urban stream which receives contaminants from different sources.

Material and Methods

Fishes were collected along Cambé stream, which is 27km long and crosses the city of Londrina (PR, Brazil), where mixed effluents are discharged into it at several points. Five sampling sites along Cambé stream were chosen: site A, which is located near its headwaters and receives agricultural waste; site B, which receives effluent from a soluble-coffee factory; site C, which receives waste discharged from an agroindustrial cooperative; sites D and E, where two
lakes are formed and receive domestic and industrial effluents. For this study a medium-sized detritivorous benthic fish *Hypostomus ancistroides* (Siluriformes; Loricariidae) was collected, this species is indigenous to Paraná state (southern Brazil). Immediately after being caught fish were sampled. Blood was taken from the caudal vein into a heparinized syringe. Subsequently gills, liver and kidney were taken from each fish for histological and biochemical analysis. An amount of blood was used for hemoglobin determination by the cyanomethemoglobin method, comet assay (single-cell gel electrophoresis) as described by Singh *et al.* (1988) and for micronucleus test (MN), by the method recommended by Hooftman & De Raat (1982).

For each fish, images of 100 randomly-selected nuclei were assigned visually to comet classes 0, 1, 2 and 3, according to the length of the stained tail and each animal received a score of DNA damage calculated by multiplying the number of cells in each class by the class index. Hence, the damage index for the group of 100 cells ranged from 0 (all undamaged) to 300 (all with maximum damage). For the presence of micronucleus, 3000 erythrocytes per animal were analyzed. After blood centrifugation, plasma sodium concentration was measured by flame photometry, plasma chloride, glucose and total lipids concentrations were determined by spectrophotometric enzymatic method using commercial Kit. Protein content in blood plasma and liver were estimated by the method of Lowry *et al.* (1951). Fish livers were homogenized and centrifuged (14,000 g for 20 min at 4°C) and supernatants were used for glutathione S-transferase (GST) measurement at 340 nm using 1-chloro-2,4-dinitrobenzene as substrate. Gills, kidney and liver samples were fixed in Bouin’s, dehydrated in ethanol and embedded in paraplast.

Tissue was sectioned (5 μm) and stained with hematoxylin-eosin. Histopathological alterations were classified according to two criteria: 1) semi quantitatively by ranking the severity of tissue lesions (Schwaiger *et al.*, 1997). On the basis of these data mean assessment value (MAV) of lesions was calculated for gills, kidney and liver for each caging site; 2) based on the scope for repair of the lesions (Poleksc & Mitovic-Tutundžic, 1994). With this method it was possible to compare the degree of tissue change (DTC) in fishes from different sites. For each parameter differences between sites were tested using a one-way ANOVA (when assumptions of data normality and equal variance were confirmed) or a Kruskal-Wallis test (nonparametric one-way ANOVA). Student-Newman-Keuls (parametric) or Dunn’s (non parametric) multiple range test were used if the ANOVA test was significant (P < 0.05).
Results and Discussion

The most meaningful results found with *H. ancistroides* collected at different sites of an urban stream are shown in Fig. 1 and Fig. 2.

Plasma glucose concentrations were higher in fish from sites C and D (Fig.1); such hyperglycemia may be attributed to an increased energy demand associated with chemical induced stress. This result is in agreement with the increased lipids level in fish from site C. Thus, metabolic parameters indicate a higher contaminant load in sites C and D. The average scores in the comet assay for fish from all sites were high, suggesting that *H. ancistroides* were under environmental stress and suffering damage in their genetic material. Thus, all sites appeared to be suffering the genotoxic effects of pollutants. However, there was a rise in the severity of damage to genetic material as the distance of the sites from the headwater of the stream increased.

Among the studied sites, no site was found at which histopathological alterations were completely absent (Fig.2). This might be regarded as evidence of environmental stressors along Cambé stream, since its headwaters, which can alter gill, liver and kidney structural integrity. The results of the degree of tissue change (DTC) indicated that kidney is the most affected organ, presenting more severe changes, which probably affect tissue function.
Fig. 1. Results found with *Hypostomus ancistroides* collected at five sampling sites of Cambé stream. Graphs show the mean ± SE. * indicates difference from site indicated by the letter (P < 0.05).
Fig. 2. Mean Assessment Value (MAV) and Degree of Tissue Change (DTC) calculated for gill, liver and kidney of *Hypostomus ancistroides* collected at five sampling sites of Cambé stream. Graphs show the mean ± SE. * indicates difference from site indicated by the letter (P < 0.05).

**Conclusions**

The results, based on several biomarkers, strongly suggested that the health of the *H. ancistroides* collected along the stream is impaired probably due to chronic exposure to pollutants.
References


Acknowledgements

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LIVER HISTOPATHOLOGY OF FERAL FRESHWATER FISH
POPULATIONS COLLECTED ALONG A CONTAMINATED STREAM

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EXTENDED ABSTRACT ONLY – DO NOT CITE

Introduction

Aquatic animal health status has been shown to be a useful indicator of water quality. A variety of factors of the aquatic medium, including environmental pollutants, can induce organic lesions on fish, which involve mainly the gills, the kidney and the liver. The liver, as the major organ of metabolism, comes into close contact with xenobiotics absorbed from the environment, and liver lesions are often associated with aquatic pollution. The primary objective of this study was to describe liver histopathological alterations in two freshwater fish species collected over one year in five sites along an urban stream which receives contaminants from different sources. In addition, histopathological responses were quantified using semi quantitative procedures to compare the frequency and severity of lesions in fish collected in different sites along the stream.
Material and Methods

Fishes were collected along Cambé stream, which is 27 km long and crosses the city of Londrina (PR, Brazil), where mixed effluents are discharged into it at several points. Five sampling sites along Cambé stream were chosen: site A, located near its headwaters and receives agricultural waste; site B, which receives effluent from a soluble-coffee factory; site C, which receives waste discharged from a tannery and an agroindustrial cooperative; sites D and E, where two lakes are formed and receive domestic and industrial effluents. Two fish species were collected for this study *Astyanax altiparanae* (Characiformes; Characidae) and *Hypostomus ancistroides* (Siluriformes; Loricariidae), both species are indigenous to Paraná state (southern Brazil). The former is a small omnivorous fish that lives in the pelagic zone, whereas *H. ancistroides* is a mediumsized detritivorous benthic fish. *H. ancistroides* was caught at all sites, while *A. altiparanae* was found only at sites C, D and E, as this species is rarely seen in the shallower and narrower parts of the Cambé stream, such as points A and B. Immediately after being caught fish were dissected in the field and liver was rapidly removed. Liver samples were fixed in Bouin’s fluid, routinely prepared, embedded in paraplast and 5 µm sections were stained with hematoxylin-eosin (HE) and PAS, and analyzed under an Olympus light microscope. Histopathological alterations were classified semi quantitatively according to two criteria: 1) by ranking the frequency of tissue lesions (Schwaiger et al., 1997). On the basis of these data mean assessment value (MAV) of lesions was calculated for each site; 2) based on the scope for repair of the lesions (Poleksic and Mitrovic-Tutundzic, 1994). With this method it was possible to compare the degree of tissue change (DTC) in fishes from different sites and to correlate the intensity of pollution with the intensity of changes found. Differences among sites were tested with a one-way ANOVA followed by the Student-Newman-Keuls (SNK) multiple range test. Means were considered different where P < 0.05.

Results

The hepatic lesions observed in both fish species collected along Cambé stream consisted primarily of nuclear and cellular degeneration (Fig. 1A), nuclei with irregular shape (Fig. 1B), hepatocyte cytoplasmic vacuolization (Fig. 1B), vacuolar degeneration of nuclei (Fig. 1A and 1C), pyknotic nuclei
(Fig. 1C), nuclear hypertrophy (Fig. 1D), bile stagnation (Fig. 1E), presence of melano-macrophages (Fig. 1F).

Figure 1. Hepatic histology of fish collected along Cambé stream (HE, bar = 10 µm). (A) and (B) *Hypostomus ancistroides* from site C; (C) *Hypostomus ancistroides* from site D; (D), (E) and (F) *Astyanax altiparanae* from site E. DC, cellular degeneration; DF, nuclei with irregular shape; DN, nuclear degeneration; EB, bile stagnation; HN, nuclear hypertrophy; MM, melanomacrophage; PC, pyknotic nuclei; S, sinusoid; VC, hepatocyte cytoplasmic vacuolization; VN, vacuolar degeneration of nuclei.

The frequency of liver lesions observed for both fish species did not vary among sampling sites, as evidenced by statistical evaluation of MAV. However, the severity of liver lesions were found to be more pronounced in
*H. ancistroides* from sites A, C and E and in *A. ancistroides* from sites D and E, as indicated by DTC values (Fig. 2).

**Figure 2.** Mean assessment value (MAV) and degree of tissue change (DTC) of liver of *Hypostomus ancistroides* and *Astyanax bimaculatus* collected in different sites along Cambé stream. * different from sites without asterisk (P < 0.05).
Conclusions

Among the studied sites, no site was found at which histopathologic alterations were completely absent. This might be regarded as evidence of environmental stressors along Cambé stream, since its headwaters, which can alter liver structural integrity.

Based on the results of the quantitative analyses of liver lesions (DTC) it was possible to discriminate different pollution degrees along Cambé stream. Liver damage was more severe in *H. ancistroides* from sites A, C and E and in *A. bimaculatus* from sites D and E.

References


Acknowledgements

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POPULATION STRUCTURE ANALYSIS
OF TWO SPECIES OF ASTYANAX (PISCES, CHARACIDAE)
FROM A CONTAMINATED URBAN STREAM

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EXTENDED ABSTRACT ONLY – DO NOT CITE

Introduction

In the last years, the biochemical and molecular approaches have deeply investigated the correlation between genetic structure of natural populations and the levels of environmental stress (Bickham et al., 2000). Studies developed with populations of fishes and other aquatic organisms have shown a close correlation between genetic polymorphism and ecological response to habitat challenge (Cimmaruta et al., 2003) and changes in genotype frequencies in populations living in contaminated streams (Foré et al., 1995). In Londrina city, Northern Paraná State (Brazil), Cambé stream is a very disturbed ecosystem, where mixed effluents are discharged into it at several points. However, until this moment, there is no study about the genetic structure of native fish populations living in this urban stream. In South America rivers, species of fish belonging to Astyanax genus, commonly known as lambari, are important components of the food web, with a significant participation in the diet of larger fish species. In this study, RAPD markers were used to analyze genetic variability and structure of populations of two species of Astyanax (A. scabripinnis and A. altiparanae), collected along Cambé stream.
Material and Methods

Fishes were collected in six sampling sites along Cambé stream: site N, located just below its headwaters; site A, which receives agricultural waste; site B, which receives effluent from a soluble-coffee factory; site C, which receives waste discharged from a tannery and an agroindustrial cooperative; sites D and E, where two lakes are formed and receive domestic effluents, site E also receives the waste from a battery factory. A total of 37 specimens of *A. scabripinnis* were sampled in four sites: N (n=7), A (n=12), B (n=11) and C (n=7). *A. altiparanae* was collected only in sites: C (n=13), D (n=16) and E (n=16). Immediately after being caught fish were dissected in the field and muscle samples were rapidly removed. DNA was extracted from the fish muscle following the procedure described by Almeida *et al.* (2001). The RAPD profiles were generated from total genomic DNA as described by Williams *et al.* (1990). Final reaction volumes were 15 µL and contained approximately 15-25 ng of template DNA. RAPD marker profiles were determined by direct comparison of the amplified DNA electrophoresis profiles, and the obtained data were analyzed as binary variables (band presence or absence). Only RAPD bands that could be unequivocally scored were counted in the analysis. Each band was considered an allele of a locus. The following parameters were examined by the TFPGA 1.3 software: a) estimation of genetic variability from the proportion of polymorphic loci (P), using the 95% criterion; b) unbiased average heterozygosities (He); and, d) estimation of gene frequency divergence among populations by the theta (θ), performing 1000 iterations in order to find an approximate 95% confidence interval. Genetic similarity dendrograms among the different groups of individuals analyzed were constructed applying Jaccard (J) coefficient and the UPGMA grouping method, using NTSYS-PC package.

Results and Discussion

Ten primers were used in RAPD analysis of *A. scabripinnis*, which produced 159 loci, among which 129 (81.3%) were polymorphic. Estimates of genetic variability (P) for each subpopulation of this species were: 64.8% (N), 63.5% (A), 64.2% (B) and 63.5% (C) revealing values very similar among the four subpopulations. Values of average heterozygosity (He) estimated for each subpopulation of this species were also very similar: 0.2433 (site N), 0.2271 (A), 0.2518 (B) and 0.2296 (C). For *A. altiparanae*, the 9 primers used in RAPD
analysis produced 157 bands, among which 142 (90.44%) were polymorphic (95% criterion). The estimates of $\bar{P}$ were higher than observed for A. *scabripinnis*: 72.6% (C), 77.7% (D) and 82.2% (E). Also, for A. *altiparanae* the estimation of $\bar{H}$ for the three subpopulations were alike: 0.2521 (C), 0.2751 (D) and 0.2626 (E). The levels of heterozygosity found in subpopulations of both species of *lambari* were higher than the range estimated by allozymes for subpopulations of other species of teleosts present in disturbed streams (Foré *et al.*, 1995a,b; Cimmurata *et al.*, 2003). However, this divergence can be resultant of intrinsic differences of both molecular markers, since RAPD technique frequently produces dozens of loci to be screened because each primer typically produces multiple bands (Bickham *et al.*, 2000), while allozymes products are less numerous. Despite the anthropogenic interference in the Cambé stream, both estimates of genetic variability found for two species of *lambari* suggest that the subpopulations from different sites are being able to maintain satisfactory levels of genetic variability. Allele frequencies were significantly different among subpopulations of *A. scabripinnis* from four sites (Table 1; $\Theta_p$), indicating some genetic structuring in these subpopulations. According to Wright (1978) values of $F_{st}$ ranging from 0.05 to 0.15 are indicative of moderate population genetic structuring, while values from 0.15 to 0.25, indicate high genetic structuring.
Table 1. Theta$_p$ test and number of migrants per generation ($Nm$) of *A. scabripinnis* (above) and *A. altiparanae* species (below) sampled from different sites across Cambé stream.

<table>
<thead>
<tr>
<th>Subpopulations</th>
<th>Theta$_p$</th>
<th>Jackknife</th>
<th>$\chi^2$</th>
<th>Nm</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A. scabripinnis</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N-A</td>
<td>0.1737$^*$</td>
<td>0.1744(0.0338)</td>
<td>6.60</td>
<td>1.18</td>
</tr>
<tr>
<td>N-B</td>
<td>0.1452$^*$</td>
<td>0.1457(0.0292)</td>
<td>6.68</td>
<td>1.47</td>
</tr>
<tr>
<td>N-C</td>
<td>0.1566$^*$</td>
<td>0.1570(0.0256)</td>
<td>5.95</td>
<td>1.34</td>
</tr>
<tr>
<td>A-B</td>
<td>0.1367$^*$</td>
<td>0.1372(0.0287)</td>
<td>4.92</td>
<td>1.57</td>
</tr>
<tr>
<td>A-C</td>
<td>0.2075$^*$</td>
<td>0.2082(0.0325)</td>
<td>5.81</td>
<td>0.95</td>
</tr>
<tr>
<td>B-C</td>
<td>0.1459$^*$</td>
<td>0.1461(0.0204)</td>
<td>5.25</td>
<td>1.46</td>
</tr>
<tr>
<td><strong>A. altiparanae</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-D</td>
<td>0.0735</td>
<td>0.0735(0.0144)</td>
<td>4.26</td>
<td>3.15</td>
</tr>
<tr>
<td>C-E</td>
<td>0.0721</td>
<td>0.0791(0.0157)</td>
<td>4.18</td>
<td>3.21</td>
</tr>
</tbody>
</table>

250
Thus, on the basis of the theta_p test, which corresponds to Fst estimates, the present results suggest a moderate differentiation between the subpopulations: N–B, A-B and B-C. Simultaneously, subpopulations from sites N-A, N-C and A-C showed a high genetic differentiation (Table 1). Similarly, A. altiparanae subpopulations (C-D, C-E and D-E) showed a moderate differentiation among them (Table 1). The estimations of gene flow (Nm) were higher than those found for A. scabripinnis. Likewise to A. scabripinnis, the results indicate that despite the values of Nm > 1, genetic differentiation among subpopulations of A. altiparanae is occurring throughout Cambé stream. Therefore, the higher values of theta_p between subpopulations of A. altiparanae from sites C and D, reflects the lowest value of Nm between these subpopulations. Genetic differentiation among populations is expected when gene flow is low (Nm < 1). However, the estimates of gene flow based on models that assume that populations are in equilibrium, grossly overestimate the true number of migrants under these conditions (Widmer and Schmid-Hempel, 1999), and consequently, the gene flow is not necessarily equivalent to Nm. Therefore, for both lambari species, the values of Nm obtained could be overestimated and gene flow might not be occurring between subpopulations from different sites.

It has been suggested that population genetic structure of natural organisms can be used as bioindicators of water quality and health of aquatic populations in contaminated environments (Foré et al., 1995a). Recent works concerning to Astyanax responses to environmental stressors have shown that they are relatively resistant to disturbed waters (Winkaler et al., 2001; Martinez and Cólus, 2002). The results suggest a higher tolerance of A. altiparanae to contaminated waters than A. scabripinnis populations, which exhibited a higher trend in undergoing genetic differentiation along the stream. The differences between both species could be attributed to species-specific genetic responses to anthropogenic stress (Foré et al., 1995a). Wright’s distance model and empirical data (cf. Foré et al., 1995a,b) indicate that populations from adjacent sites should be more similar genetically because of the greater probability of gene flow. However, in disturbed streams the water quality could alter the distribution of alleles in fish populations (Foré et al., 1995a,b). Therefore, the high genetic differentiation between subpopulations N-A could be consequence of the
polluted conditions of the Cambé stream (Yabe and Oliveira, 1998; Winkaler et al., 2001; Martinez and Côlus, 2002).

Conclusions

Despite the satisfactory levels of genetic variability found for the *A. scabripinnis* and *A. altiparanae* from Cambé stream, the process of genetic differentiation showed by both species could be reflecting some limited capability of these species to migrate along the stream.

References


Acknowledgements

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HEALTH STATUS OF CATHOROPS SPIXII (ARIIDAE) LIVING IN AN IMPACTED AREA IN PARANAGUÁ BAY, PR, BRASIL

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Abstract

Paranagua Harbor in Parana, Brazil, receives industrial and municipal effluents. In order to characterize the influence of these different anthropogenic sources in the environment health, this study was performed evaluating the species Cathorops spixii. Ten adult fishes were sampled from an impacted and from a reference area in August, 2003. Somatic indices and histopathological aspects of liver and gills were evaluated. Fishes from impacted area showed necrosis lesions in liver, hyperplasia of secondary lamellae in gills, and an increase on hepatossomatic index. This results suggest that C. spixii living in this impacted area present impaired health and could be used as a biomonitoring species.

Introduction

Paranagua Harbour as other harbour areas is exposed to several contaminant sources as industrial and urban wastes, oil spills and agricultural effluents. Due to the diversity of sources, a mixture of contaminants as heavy metals, polycyclic
aromatic hydrocarbons and pesticides are frequently detected in these areas, causing several adverse effects on fish health. Once harbour areas are located in coastal and estuarine regions, the concern about this kind of ecosystem health is increasing, reinforcing the significance of biomonitoring such areas. The main purpose of this study is to evaluate the health status of *C. spixii* through histopathology analysis of gills and liver as well as through somatic indices.

**Material and Methods**

Ten adult fishes were sampled from an impacted and ten from a reference area in Paranagua Bay, Paraná, Brasil. Total length, total weight, liver and gonads weight were measured, gills and liver were collected for light microscopy analysis. These organs were fixed in ALFAC (80% ethanol, 10% formol and 5% acetic acid) for 16 hours, dehydrated in alcohol and included in Paraplast. The 5 μm sections were stained with HE (hematoxylin and eosin).

Hepatossomatic index (liver weight/total weight x 100), gonadossomatic index (gonad weight/total weight x 100) and condition factor (total weight/length²), b represents the angular coefficient of weight-length relationship.

**Statistics**

All data were expressed as mean ± standard deviation. Statistical significance between somatic indices data was determined by t-test and between histopathologic alterations by Mann-Whitney test. A statistical significance of p<0.05 was adopted.

**Results**

Fusion of secondary lamellae (Fig. 1B), that is a typical acute effect of pollutants, was observed, but did not present significant difference. Similar situation was observed for the presence of secondary lamellae hyperplasia (Fig. 1C).

The liver of *C. spixii* seems to be organised like most other teleosts (Fig. 1D). In the contaminated fish the most evident alteration observed was the presence of necrotic areas (Fig.1E). In such areas is observed a large liver necrosis where the absence of cells is a characteristic of this kind of lesion. The incidence of necrosis in the current studied species was higher if compared with the reference animals.
A tendency of increase in HSI was observed in the contaminated fish if compared with reference animals, but the data did not differ significantly (Fig. 2A). Condition factor (Fig. 2B) and gonadosomatic index (Fig. 2C) also did not present significant differences.
Figure 2. C. spixii somatic indices.
Discussion

Gill alterations described in the present work were frequently described in situations of aquatic pollution stress. Despite the presence of gill alterations, their occurrence were insufficient to evaluate the impact of contaminants on *C. spixii*.

Liver necrosis, was the most relevant damage caused by the contaminated water on *C. spixii* collected at Paranaguá Bay. This kind of alteration is related by other authors in conditions of environment contamination (Spiers et al. 1996, Valdez Domingos, 2001).

Somatic indices of *C. spixii* were not significantly altered by the contaminant exposure in the current study; similar results were observed by Felder et al. (1998) in fish exposed to paper industry effluents.

Despite the mild alterations noticed in this work, effluents treatment and severe water policies are recommended, besides the investigation of another species in this area.

Acknowledgements

We would like to thank Dr. Henry Louis Spach and Dr. Paulo Lana and their staff for field sampling and logistic resources (CEM-UFPR). This research work was supported by RECOS – Instituto do Milênio. Valdez Domingos acknowledges CAPES for the award of a scholarship.

References


Valdez Domingos, F. X. Utilização de biomarcadores imunológicos e morfológicos em *Fundulus heteroclitus* (Teleostei, Ciprinodontidae) na
Abstract

Neotropical fishes have been increasingly exposed to xenobiotics, in nature and in farms, but little is known about the biochemistry of their intoxication. Glutathione S-transferases (GST) conjugate xenobiotics with reduced glutathione (GSH) increasing their hydrosolubility and thus favoring their excretion. Specific substrates were used to standardize optima conditions for assays of cytosolic GST found in liver of pacu (Piaractus mesopotamicus). Two GST-mu were identified, a homodimer with molecular mass of 23.8 kDa per subunit, and a heterodimer with two polypeptides of 21.3 kDa and 23.8 kDa. The GST-pi found in liver of pacu is a homodimer with molecular mass of 23.8 kDa per subunit.

Introduction

Aquatic animals are exposed constantly to pollutants like pesticides, polycyclic aromatic hydrocarbons and polychlorinated biphenyls. Unfortunately, the lipophilicity of these xenobiotics is an obstacle to their elimination from the body because lipophilic compounds can be readily reabsorbed. Consequently, the elimination of xenobiotics depends on the increasing of their solubility in water through biotransformation. The glutathione S-transferases (GST) are isoenzymes of the biotransformation system. Cytosolic GST isoenzymes are classified into five classes designated alpha (α), mu (µ), pi (π), theta (θ) and
sigma (σ). Their function is to catalyze the combination of several xenobiotics with reduced glutathione (GSH), producing more polar GSH-xenobiotic conjugates, which are more hydrophilic and thus more easily excreted. Glutathione-xenobiotics conjugates may be excreted in bile and urine.

Despite the fact that Neotropical fishes have been increasingly exposed to xenobiotics, both in the environment and in fish culture, very little is known about the biochemistry of the enzymes that promote their biotransformation. In the present study, specific substrates were used to standardize optimal conditions for assaying the activity of three classes of GST. In addition, we purified pi and mu GST isoenzymes responsible for the enzymatic activities we found in pacu liver.

**Methods**

Sexually mature males and females specimens of fish were used. Cascudo (*Hypostomus punctatus* Valenciennes, 1840, Loricariidae) weighed approximately 230 g and measured between 25 and 28 cm. Pacu (*Piaractus mesopotamicus* Holmberg, 1887, Characidae) measured around 25 cm and weighed approximately 350 g. Matrinxãs (*Brycon cephalus* Günther, 1869, Characidae) measured around 33 cm and weighed approximately 400 g. Dourados (*Salminus brasiliensis* Cuvier, 1816, Characidae) measured around 50 cm and weighed approximately 1,400 g.

Total GST activity, and alpha, mu, and pi GST-isoenzymes were assayed in liver cytosolic aliquots. We employed 1,2-dichloro-4-nitrobenzene as substrate to determine the activity of GST of the mu class, ethacrynic acid to assay the activity of class pi GST, and cumene hydroperoxide to assay the activity of class alpha GST. The non-specific substrate 1-chloro-2,4-dinitrobenzene was used to assay total GST activity. The fishes were arranged into two groups with 12 animals. The fishes from the first group received an intracelomal injection of benzo(a)pireno (15 mg per kg body weight) suspended in corn oil (assay group). The other group received only corn oil (control group). Six fishes from each group were sacrificed 7 days after the injection and the other sacrificed 14 days after injection.

Isoenzymes of mu and pi classes from liver of pacu were purified from liver cytosol through three steps: affinity chromatography using an S-hexylglutathione-agarose resin; ionic exchange chromatography using a
resource™ Q (Pharmacia Biotech) and reversed phase high-pressure liquid chromatography using a C4 Supelcosil™ LC-304 (Supelco Park).

Results and Discussion

The predominant GST isoenzyme activity in liver of pacu and matrinxã was determined to pertain to the alpha class. As seen in Table 1, when we compared alpha-GST activities found in pacu with those activities reported for other fish species, pacu’s liver alpha-GST activity was 14 times higher than matrinxã’s (*Brycon cephalus*), 125 times higher than gudgeon’s (*Gobio gobio*) and 830 times higher than sea trout’s (*Salmo trutta*). GST isoenzymes of mu and pi classes are predominant in liver of cascudo (Table 1). The mu class GST activity in pacu liver was half the one found in cascudo (*Hypostomus punctatus*) liver, 1.5 times the activity found in matrinxã liver, three times the liver activity found in dourado (*Salminus brasiliensis*), and 20 times the activity reported for channel catfish (*Ictalurus punctatus*) liver. The pi class GST activity of pacu was six times lower than that from cascudo and it was similar to those from other species (Table 1).

Two GST of the mu class were identified in liver of pacu, a homodimer with molecular mass of 23.8 kDa per subunit, and a heterodimer with two polypeptides of 21.3 kDa and 23.8 kDa molecular masses. The class pi GST purified from liver of pacu is a homodimer with molecular mass of 23.8 kDa per subunit.
Table 1. Activities of hepatic GST isoenzymes from liver of some different vertebrate species (µmol min⁻¹ mg⁻¹ protein).

<table>
<thead>
<tr>
<th>VERTEBRATES</th>
<th>MU</th>
<th>PI</th>
<th>ALPHA</th>
</tr>
</thead>
<tbody>
<tr>
<td>cascudo</td>
<td>0.297</td>
<td>0.245</td>
<td>–</td>
</tr>
<tr>
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<td>–</td>
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<tr>
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<td>0.037</td>
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</tr>
<tr>
<td>b trout</td>
<td>–</td>
<td>0.048</td>
<td>0.015</td>
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<tr>
<td>c salmon</td>
<td>–</td>
<td>0.070</td>
<td>–</td>
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<tr>
<td>d gudgeon</td>
<td>–</td>
<td>–</td>
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<td>0.029</td>
<td>0.372</td>
</tr>
<tr>
<td>e mouse (female)</td>
<td>0.05</td>
<td>0.032</td>
<td>0.598</td>
</tr>
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</table>

a Gallagher et al., 1996  
b Egaas et al., 1999  
c Nóvoa-Valiñas et al., 2002  
d Almar et al., 1998  
e Egaas et al., 1995a
Figure 1. Activity levels of GST isoenzymes from pacu liver 7 and 14 days after an intracelomal injection of 15 mg benzo(a)pyrene /kg body weight.

Class mu GST activity augmented significantly after the injection of pacu with benzo(a)pyrene (Figure 1).

References


Cytotoxicity of *Stryphodendrum adstringens*, *Stryphodendrum poliphylium* and *Eugenia dysenterica* extracts evaluated in the guppies (*Poecilia vivipara*).

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Extended abstract only – do not cite

Abstract

It has been suggested to use the extracts of *S. adstringens*, *S. poliphylium* and *Eugenia dysenterica*, to control the vector snails of schistosomiasis. In this work were used the guppies to evaluate the effects of these plants extracts (20-60 ppm) in the aquatic environment. The fishes showed increased lipids droplets in the hepatocytes, damage of junctional complex of intestine epithelia, swollen of blood vessels in the gills and death within 2h of exposition. The bioactive substances containing in these plants are highly toxic with acute effect to the aquatic organisms and their releasing into to the water should be carefully controlled.

Background

“True-Barbatimão” (*Stryphodendrum adstringens-SA*), “Barbatimão” (*Stryphodendrum poliphylium-SP*) and “cagaita” (*Eugenia dysenterica-ED*) are native plants widely distribute in the Goias woodsy pasture. These local people use these plants as edible fruits with anti-inflammatory or abortifacient agents (Silva et al, 2003) and more recently, was reported as potential molluscicides (Bezerra et al., 2002). Although generally harmless to humans, some snails, most notably of the genera *Biomphalaria*, *Bullinus* or *Oncomelania*, are directly implicated in the transmission of schistosomiasis (bilharzia), the endemic
disease affecting more than 200 million people. Therefore, great effort has been made to find an efficient and cheaper way to control either the parasite and the vector snails. The use of plants with molluscicidal properties could be a simple, inexpensive and accessible technology for focal control of the snail vector (Marston and Hottestmann, 1985). In the present work, it was aimed to characterize the main active compound of bulk extracts of SA, SP and ED and then evaluate the toxicity of these plants extracts in the aquatic animals. The dosage and time-dependant effects were evaluated with guppies (Poecilia vivipara).

Material and Methods

Plant extracts
The leaf and bark of ASP, SA and ED growing in the Goias woodsy pasture were collected and after air-dried they percolated with 95% EtOH. After evaporation of the EtOH the bulk extracts were lyophilized. Samples of these extracts were evaluated by high-pressure liquid chromatography (HPLC) in standard procedure to identify the major plant derived chemical compounds.

Experimental procedures
Guppies collected from clean lakes and small rivers are routinely reared in our laboratory. Forty-five specimens of these aclimated fishes were randomly chosen and separated in 9 small tanks supplied with fresh tap water and fed with commercial food for fish. The lyophilized extracts of SA, SP and ED were previously diluted in destilled water and than added into the tank in a final concentration of 20, 40 and 60 ppm for dosage evaluations and 20 ppm for time-effect evaluation. The control groups were kept in a tank without addition of extracts. From each experimental group, the fishes were sacrificed to collect the gills, liver and intestine for historessin or Spurr resin embedding. The 2 µm thick historessin sections were processed for PAS, Alcian blue (AB) pH 0.5 and 2.5 staining. The ultra-thin sections of Spurr-embedded samples were analyzed in transmission electron microscopy.

Results and Discussion

The HPLC analyses showed the tannins (Fig 1) as the main compound of the alcoholic extracts from the three plants used.
The effects of these extracts on guppies are very strong and acute since both the 20ppm extracts of SA and SP killed all the animals within 2 hs of exposition. The toxic effects of ED are not so strong, since the animals survive over than 24h of exposition at 20 ppm, but at 60ppm all the animal dead after 2h. The PAS and AB pH 2.5 histochemistry showed increased reaction in the gill’s mucous cells and intestine’s goblet cells if compared to the control groups, suggesting increased mucous secretion as response against astringent action of tannins.
The ultrastructural evaluation showed lost of cell junctions followed by cell disruption in the epithelia with swollen of gill’s blood vessels (Fig 2a). The hepatocytes lost their glycogen contents and increased the lipid droplets (Fig 2b). These results clearly showed the strong cytotoxic effects on epithelial cells that took contact with toxic compounds, but the effects were not confined at epithelial level, since the hepatocytes were also affected. Therefore, the differences of lethal effect between AS/AP and ED cannot be attributed to the tannin only. The complex compositions of the plant extracts and their combined effects seem to be more or less lethal to fishes used in this experiment. Further and fine evaluation of these bulk extracts should be performed to determine precisely the compositions. However, based on the data of the present experiments, it is not recommendable to use the bulk extracts of these plants to release in the aquatic environment. Actually the 20 ppm concentration of AP, AS and ED extracts could act as good bioactive molluscicida but they are also harmful and lethal to other aquatic organisms.

References


OXIDATIVE STRESS IN THE HEART AND LIVER
OF Sparus aurata (GILTHEAD SEABREAM):
IN VIVO META AND DECAVANADATE EFFECTS

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EXTENDED ABSTRACT ONLY – DO NOT CITE

Introduction

Vanadium is a heavy metal with increased environmental circulation, resulting from various anthropogenic activities. The deleterious effects of vanadium have been associated mainly with nephrotoxicity and hepatotoxicity (Sto hs and Bagchi, 1995). Additionally, previous studies indicated that in vivo oxidative stress induced by vanadium is dependent on the oligovanadates present (Aureliano et al., 2002; Soares et al., 2003). However, the contribution of different vanadate oligomers to the toxicological effects mediated by vanadium stills unclear. Further, these studies also subject that more pronounced effects may happen in a short-term basis. Therefore, the objective of the present study was to compare the in vivo oxidative stress related effects of an acute exposure (1h, 6h and 12h) to a sub-lethal concentration of two vanadate solutions, meta and decavanadate (5 mM), in the heart and liver of the teleost Sparus aurata (gilthead seabream). S. aurata is a species with high commercial interest which has been extensively used in aquaculture. However, although its reproductive and nutritional physiology has been largely studied, toxicological studies on this species are almost absent (Vaglio and Landriscina, 1999).
Material and Methods

This study was performed with *Sparus aurata* individuals (N=120; 400-600 g) obtained from a local fish-farm. After the acclimation period, fish were divided into four groups: Control 1, non injected; Control 2, injected intravenously (i.v.) with 0.9% NaCl (placebo); Meta, injected i.v. with a metavanadate solution containing 5 mM of total vanadium and Deca, injected i.v. with decavanadate containing 5 mM of total vanadium. Metal solutions were diluted into final concentration (5 mM) in 0.9% NaCl. Metavanadate and decavanadate solutions were prepared from ammonium metavanadate, as described by Aureliano et al. (2002). All solutions were administrated in a dosage of 1 mL solution/Kg of body weight.

Subgroups of 10 individuals of each group were sacrificed 1, 6 and 12 hours after injection, with anaesthetic overdosage of 2-phenoxyethanol; blood was collected and the heart and liver were immediately removed. Five pools of two individuals were prepared for each organ and homogenised in adequate buffers to prepare mitochondrial and cytosolic fraction. Mitochondrial and cytosolic superoxide dismutase (SOD) and catalase (CAT) and total glutathione peroxidase (GPx_total) and selenium-dependent peroxidase activities were analysed by UV-VIS spectroscopy. Metal subcellular distribution was determined on blood (plasma and erythrocytes) and cardiac and hepatic tissues (total, mitochondrial and cytosolic), by atomic absorption spectroscopy.

The Mann-Whitney test was applied to test differences between groups, for all the analysed parameters. The significant level used was $p < 0.05$. Control 1 and Control 2 showed no significant differences and for result analysed were considered together as Control.

Results and Discussion

In the present study the intravenous injection of meta and decavanadate clearly resulted in acute oxidative stress in both heart and liver of *Sparus aurata*.

The first significant observation was a stress effect of the Meta solution, which was evident by a marked behavioural alteration (fish became very agitated) on ≈50% of the fish, after recovery from anaesthesia and injection. In fact, 33% of the metavanadate injected fish died within few hours and were not considered for enzymatic analysis.
Further, a placebo effect was observed in cardiac antioxidant enzymes activities. We suggest that this effect was related with the intravenous injection, since in previous studies where the same saline solution was administrated intraperitoneally, there was not a significant placebo effect (Aureliano et al., 2002; Soares et al., 2003).

Both vanadate solutions induced alterations on antioxidant enzymes, although stronger effects were observed for metavanadate. The injection of this solution affected CAT activity in both organs, with a decrease in the activity in cardiac mitochondria and an increase in hepatic cytosol, both 6 hours after injection. Minor effects were observed upon decavanadate intoxication. Lipid peroxidation levels remained unchanged, in heart and liver tissues, upon vanadate exposure. Vanadium was mainly accumulated in the blood.

These data indicate that, for seabream, intravenous injection is a source of oxidative stress, mainly in cardiac tissue. Moreover, this species seems to be highly sensitive to metavanadate intoxication. Quantification of ROS production and pyridine nucleotides will allow us to evaluate S. aurata cardiac and hepatic prooxidant status and antioxidant susceptibility to vanadate.

Acknowledgements

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References


Abstract

We assayed in serum of piauçu (Leporinus macrocephalus Garavello & Britski, 1988), a Brazilian Neotropical fish, a cholinesterase that catalyzes butyrylthiocholine hydrolysis with the highest activity ever described. We managed to purify this cholinesterase for the first time ever from fish serum. It is a dimer with 66 kDa subunits under reducing conditions and it shows isoelectric points in the range between pH 4.7 and pH 5.1. Its N-terminal presented homology with BChE from other sera. The enzyme was activated by high concentrations of butyrylthiocholine and presented high sensitivity to iso-OMPA and low to BW284c51. Serum BChE may avoid poisoning of fish by scavenging pesticides.

Introduction

Cholinesterases (ChE) are hydrolases found in several animals and plant species. There are two cholinesterases in vertebrates, corresponding to distinct genes: acetylcholinesterase (AChE; E.C. 3.1.1.7) and butyrylcholinesterase (BChE; E.C. 3.1.1.8). They are distinguished based on substrate specificity; AChE
hydrolyzes acetylcholine faster than other choline esters, while BChE shows more hydrolyzing activity upon butyrylcholine.

Although the physiological roles of BChE remain to be definitely established, it has been suggested that BChE inhibition can function as a scavenging reaction for anticholinesterase poisons (Wolfe et al., 1987 & Doctor et al., 1993). The interaction between organophosphate and BChE splits the ester linkage in the organophosphate but leaves the phosphate moiety fastened to the active center, producing an inactive stable phosphate-BChE complex. This explains why BChE can stoichiometrically remove organophosphate molecules from blood before they reach their target, the AChE located at cholinergic synapses and myoneural junctions. Therefore, high levels of BChE in an animal might make it resists more to poisoning by organophosphorus pesticides.

Information about molecular characteristics and functions of plasma cholinesterases from fish is scarce. Owing to the increase of organophosphorus pesticides in water of rivers in South America, it is important to establish if different levels of poisoning by organophosphates among Neotropical fish could pertain to scavenger esterases. The work has been done to obtain information about a serum cholinesterase from Leporinus macrocephalus, a freshwater Neotropical fish important to fishery in Brazil. We aimed at establishing the characteristics of its inhibition to know if it had the potential for protecting piauçu from organophosphate poisoning.

Methods

ChE activity was determined according to Ellman et al. (1961) using acetylthiocholine (ASCho) or butyrylthiocholine (BSCho) as substrates. Polyacrylamide gradient gel (4 - 18%) for native electrophoresis was electrophoresed at 100 V for 20h at 4°C. Lanes contained 0.016 U of enzyme activity. The gel was stained for cholinesterase activity (Karnovsky & Roots, 1964) or, in the case of denaturing electrophoresis, with a silver-staining technique (Morrissey, 1981). BChE was purified from piauçu serum using procainamide-Sepharose 4B-affinity column and gel filtration high-pressure liquid chromatography. Each complete purification process started with 10 mL of serum. Isoelectrofocusing gels were carried out using Phast gel (4-6.5 pH range). The samples contained between 5 and 10 µg of purified BChE from piauçu serum and the gels were revealed by the Coomassie blue staining. N-terminal sequences of the purified enzyme were analyzed on a Shimadzu PPSQ-10 Automated Protein Sequencer by Edman degradation.
**Results**

Piauçu serum hydrolyzed BSCho about three times faster than ASCho. We purified the enzyme 1,700 times, with an 80% yield. BChE from piauçu, as well as human BChE, was activated by high concentrations of BSCho and presented high sensitivity to iso-OMPA and low sensitivity to BW284c51 (Table 1). Kinetic studies indicated that typical BChE inhibitors (Table 1) inhibit it. As seen in Figure 1-A, differently from human serum BChE, piauçu serum presented a single BChE molecular form. In the reduced SDS-PAGE (Figure 1-B) this protein had a molecular mass of 66 kDa, but 134 kDa under non-reducing conditions, which showed that piauçu’s BChE is a dimer. Isoelectric focusing analysis showed that the BChE from piauçu serum displayed a continuous smear on the gel, extending from pH 4.7 to 5.1 (Figure 1-C). N-terminal amino acid sequence of BChE of piauçu serum (LEELVVSTNKGKIRG) presented homology with BChE from sera of mammals and hens.

**Table 1. IC50 inhibition values of BChE from *L. macrocephalus* purified serum**

<table>
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<tr>
<th>Cholinesterases inhibitors</th>
<th>IC50 - 30 min (molar)</th>
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<tr>
<td>Iso-OMPA</td>
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</tr>
<tr>
<td>Methyl-paraoxon</td>
<td>$3.9 \times 10^{-10}$</td>
</tr>
<tr>
<td>Fisostigmine</td>
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<tr>
<td>Methyl-parathion</td>
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</tr>
<tr>
<td>Procainamide</td>
<td>$8.4 \times 10^{-4}$</td>
</tr>
<tr>
<td>BW284c51</td>
<td>$1.8 \times 10^{-4}$</td>
</tr>
</tbody>
</table>

**Discussion**

We have found that piauçu (*Leporinus macrocephalus*) has a single and very active cholinesterase in its serum. Piauçu presented the highest level of serum butyrylcholinesterase activity described in a wild organism; at least three times higher than BChE of human serum. The range of isoelectric point values we found for piauçu serum BChE suggests that this Neotropical fish enzyme
presents an amino acid composition similar to that from humans. In addition, the enzyme showed high sensitivity to iso-OMPA, a specific BChE inhibitor, and a contrasting low sensitivity to BW284c51, a specific AChE inhibitor. Moreover, piauçu serum hydrolyzed BSCho more efficiently than ASCho.

Altogether, our findings indicate that piauçu has in fact a dimeric BChE circulating in its plasma. In addition, the enzyme inhibition characteristics support that it has the potential for protecting piauçu from organophosphorus poisoning.

Figure 1. Biochemical characterization of serum BChE from piauçu. A - Polyacrylamide gradient gel (4-18%) native electrophoresis; B - SDS-PAGE (1) reducing conditions and (2) non-reducing conditions; C - Isoelectrofocusing gel (4-6.5 pH range).
References


Acknowledgements

We are grateful to Dr. Oksana Lockridge from the Eppley Institute, University of Nebraska Medical Center, Omaha NE, U.S.A for supplying procainamide-Sepharose 4B affinity gel. We are also grateful to Mr. José Borges Nogueira (owner) and Mr. José Marcelino Lima de Sousa (manager) from Morro Grande farm (Cachoeiras de Macacu – RJ - Brazil) for supplying the piauçus.
TEMPORAL EVOLUTION

OF MERCURY CONTAMINATION IN CICHLA SPP

IN BALBINA HYDROELECTRIC RESERVOIR, AM, BRASIL

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EXTENDED ABSTRACT ONLY - DO NOT CITE

Introduction

Recent studies have been demonstrating that the soils of the Amazonian region possess a great reservoir of natural mercury (Hg) (Silva-Forsberg, 1999; Roulet et al., 1998) serving as primary source of the contamination of the water and of the organisms. In areas with soils enriched with mercury the creation of hydroelectric reservoirs can play an important part in the mercury mobilization for the column of water. Tucunaré (Cichla spp) is a fish predator, very appreciated in the local trade. This work aimed at to investigate the temporary evolution (1992 to 2003) of mercury contamination in Tucunaré in the Hydroelectric Reservoir of Balbina. Determining the relationships among them concentration of total mercury and the size of the fish.

Material and Methods
The dam of Balbina with an area of 2,360 Km$^2$ was closed in 1987 and it is located to 180 kilometers of Manaus - AM, constructed among the rivers Uatumã and Pitinga in the center of the Amazonian region, to supply energy to Manaus.

The fish were collected to amount of the dam in the years of 1992, 1997 and 2003, they were submitted to measures of total and standard length. A sub sample of number muscle of the each fish, was extracted, being this frozen immediately. The analyses of Hg total were made at the Laboratory of Environmental Biology of the Federal University of Pará. The used analytical technique was the one of Pichet (1999), which consists of digestion acid of the samples and analysis for spectrometry of atomic fluorescence. The reliability of the analytical method was tested through analyses of a standard of reference of National Research Council of Canada (TORT-2).

**Results and Discussion**

The bioaccumulation of total Hg, in *Cichla* spp, was quite pronounced in function of the standard length of the fish, with positive correlations in all of the studied years (Figure 1A). The mercury concentrations (in µg/g wet weight) found in the species *Cichla* spp in this work for the year of 1992 varied of 0,1 - 0,22, with average of 0,145 ± 0,069 (n=6). After five years, in 1997 the levels of Hg found in that fish species were of the order of 0,15 - 1,18, with average of 0,654 ± 0,300 (n=16). In 2003, eleven years after the first measures, the concentrations were of 0,155 - 0,568, with a medium concentration of 0,316 ± 0,137 (n=7) (Figure 1B).

Through variance analysis it was possible to observe that the highest concentrations of total Hg in the tucunarés were found in the year of 1997 (F2, 25 = 21,7; p <0,05) differing significantly of the years of 1992 (Tukey, p = 0,000) and 2003 (Tukey, p = 0,001). on the other hand significant difference didn't exist among the years of 1992 and 2003 (Tukey p = 0,237) (Figure 1B).
Figure 1: A) Bioaccumulation of total Hg in Cichla spp, in the different years, in function of the lengths pattern measured in centimeters and B) differences in the medium concentrations of Hg along the studied years (F2,25 = 21.7; p <0.000).

In 1992 no tucuná presented levels of Hg above 0.5 ppm, which is the established for the World Organization of Health. Already in 1997, of the total of sixteen analyzed copies, eleven of them presented values above referred him limit. In 2003, of a total of seven fish only one crossed the limit. There was not significant difference (F2, 26 = 0.33; p = 0.719) as for the standard length of the fish analyzed along the years.

The levels of total Hg of the fish in the dam from 1992 to 97 increased in a factor of 4.5, and for the total period of eleven years (1992 - 2003) the factor
was of 2.2. Though, in the interval from 1997 to 2003 there was a decrease of 0.48 times. In Balbina the mobilization of the mercury in the first years seems to be 3 times more accelerated than in countries of Northern Hemisphere (Bodaly et al., 1984).

The increase of the levels of Hg, in Tucunarés, found in our work immediately after the flooding, and the subsequent decrease of the concentrations after the first decade, it is corroborated in the work of Weisser (2001), which observed an increase in the levels of the residents' of the town of Balbina total Hg from of 1995 up to 1999, starting from then the levels of the element began to return for more basal levels (Figure 2).

![Figure 2: Temporary evolution of the medium concentrations of total Hg (µg/g) in residents' of the town of Balbina hair with feeding of originating from fish the dam (courtesy of Weisser, 2001).](image)

The processes of decomposition of organic material flooded in the reservoirs and the liberation of coming products of this decomposition added to the chemistry of the water of the reservoir promote the metilação of the mercury (Bodaly, 1997).

The creation of hydroelectric reservoirs in the Amazonian can contribute to the mobilization of inert natural mercury in the soils, following by absorption and
biomagnification for the chain aquatic trófica and, consequent contamination of the populations through ingestion of polluted fish.

Acknowledgments

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References


TOXICITY OF NATURAL PRODUCTS IN

*Brachdanio rerio AND Prochilodus lineatus*

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EXTENDED ABSTRACT ONLY – DO NOT CITE

Natural product chemists have devoted many years on the isolation and structural determination of plant constituents. Most of scientific approach is the selection and isolation based on bioassay information, which permits to locate quantitatively, minor, but often very active, compounds.

Several products from plants have been considered as a potential compound to control ants in agriculture procedures. The aquatic environment may receive this compounds and it is important to know how they affect the animal living in such environment.
The main goal of the present study was to determine the toxicity of selected natural products from plants with potential application in the agriculture procedures in two fish species: *Brachidionio rerio* (zebrafish) and *Prochilodus lineatus* (curimbatá).

Acute toxicity tests using selected natural products such as flavone, flavanone, 7-methoxyflavone, catechin, xantiletin, arborinin, ricinine (Figure 1) were carried out in *Brachidionio rerio*, recommended fish for test toxicity, following the American Public Health Association (APHA) to estimate the 96h-LC\textsubscript{50}. Briefly, the experiments with two replications were done in aquariums (static systems) containing 8 fish each, never exceeding 1g fish/L. Fish mortality was counted every 24h and the 96h-LC\textsubscript{50} was estimated using the Trimed Spear-Karber method and the LC\textsubscript{50} programs JSPEAR test (Hamilton et al., 1977) with 95% confidence limit.

![Figure 1. Structure of the bio-assayed compounds.](image)

Ricinine and flavanone were not toxic to *B. rerio* and the flavone was the highest toxic compound. The comparison between the flavonoids activity showed that the presence of double bond in the ring C is essential to the toxicity of them to the *B. rerio*. Flavone was isolated from *Pilocarpus spicatus* (Family: Rutacea) and it is a potential compound to be used in large scale to control leaf-
cutting ants due to efficiency in inhibiting the development of the fungi *Leucoagaricus gongylophorus*, a symbiont of leaf-cutting ant *Atta sexdens rubropilosa*, and indirectly controlling the nest development (Della Lucia and Araujo, 1993).

The estimated 96 h-LC$_{50}$ concentration of flavone (6.21 ± 0.3 mg flavona/L, Figure 2) to *B. rerio* was assayed in a Brazilian fish species, the curimbatá *P. lineatus* and showed similar toxicity. *P. lineatus* exposed to the same flavone concentration during 96h were irreversibly anaesthetized with benzocaine 0.1% and the gills, liver and kidney were taken to evaluate the flavone accumulation. These organs were extracted with dichloromethane during 1 h., the solvent was filtered, evaporated and analyzed by thin layer chromatography.

![Figure 2. Mortality percentages curve of *B. rerio* in response to Flavone concentration in water showing the 96h-LC$_{50}$.](image)

The analyzes showed accumulation of flavone in all these organs. Further studies will be done in order to determine quantitatively this accumulation as well as the metabolism of flavone by these organs.

**Acknowledgements**

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References


BIOCHEMISTRY RESPONSES
OF MATRINXÃ (BRYCON CEPHALUS)
EXPOSED TO ENVIRONMENTAL NITRITE.

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Abstract
Nitrite is usually present in water bodies. It comes from nitrification processes. Environmental increase of nitrite injures several aquatic species, particularly fishes. Nitrite reacts with hemoglobin yielding the non-functional methemoglobin, and many physiological consequences can arise, among them functional anemia and functional hypoxia. Metabolic profile and oxidative enzymes of metabolism were studied in the freshwater teleosts Brycon cephalus (matrinxã) exposed to 0,6 mg/L N\textsubscript{2}O\textsubscript{2} of nitrite for 24 and 96 hours. The metabolic profile and oxidative enzymes suggest that aerobic metabolism prevails. The protein and amino acid catabolism is likely the cause of plasma ammonia increase. Brain and heart were not injured under nitrite exposure.

Introduction
Water pollution and industrial waste usually enhance the nitrite concentrations (Nikinmaa, 1992; Heckman et al., 1997). Many problems are raised due to nitrite concentration increase. It comes from ammonia oxidation and this nitrification process depends on the bacterial activity and oxygen level. High ammonia concentrations can be observed in aquaculture systems as consequence.
of high-density stocks (Hargreaves, 1998; Hagopian & Riley, 1998). Nitrite from such conditions brings many physiological to reared fish.

Environmental nitrite cross the gill barrier through chloride cells (Gaino et al., 1984; Williams & Eddy, 1986), is accumulated in the plasma (Shechter, 1972; Bath & Eddy, 1980), and spreads through the tissues (Arillo et al., 1984). Plasma nitrite concentrations are usually higher than environmental ones (Williams & Eddy, 1986). Within the red blood cells it oxidizes the hemoglobin-Fe$^{2+}$ to Fe$^{3+}$ yielding methemoglobin, which is unable to transport oxygen and resulting in functional anemia (Scarano & Saroglia, 1984). This effect is supposed to lead to tissue hypoxia (Cameron, 1971; Bath & Eddy, 1980; Doblander & Lackner, 1996; Vedel et al., 1998) even in the presence of oxygen (functional hypoxia). Its content varies among fish and depends on few factors as the external nitrite concentration and the time of exposure. Nitrite cause other blood disturbs as decrease of hemoglobin, hematocrit and red cell counting. This phenomenon is consequence of hemolytic anemia (Scarano & Saroglia, 1984). Several osmoregulatory responses as hyponatremia, hypochloremia (Jensen et al., 1990), branchial chloride cells failure (Gaino et al., 1984) and inhibition of chloride uptake (Williams & Eddy, 1986), are observed in freshwater teleosts exposed to nitrite.

Nitrite can also accumulate in tissues as brain, liver and plasma (Margiocco et al., 1983). However, there are a small number of works that try explaining what happen with metabolism during the exposure to nitrite. Some authors imagine that they are similar to the responses to the environmental hypoxia, but few studies explore this assumption. These studies have shown glycogenolysis in liver during nitrite exposure in sea bass Lates calcarifer (Woo & Chiu, 1997) and traíra Hoplias malabaricus (Moraes et al., 1998), similarly to the environmental hypoxia in Hypostomus regani (Moraes et al., 1997). During environmental hypoxia, white muscle accumulates lactate and increases LDH activity, but the studies on nitrite exposure do not present such characteristic.

In the present study metabolic responses in the freshwater Neotropical teleost Brycon cephalus (Günther, 1869) (matrinxã) exposed to environmental nitrite were investigated. This Amazon species is widely reared in South America but is little resistant to environmental nitrite.
Materials and Methods

Juveniles of *B. cephalus* ranging 90 ± 5 g (± S.D.) were obtained from the fish farm Águas Claras, Mocóca, SP, Brazil. The fish were brought to the lab aquaculture facilities, acclimated for 2 months fed on commercial food, natural photoperiod, controlled temperature (25 ± 1°C) and aerated water. The water quality parameters in the experiment were: pO₂, 7.5 mg/L; pH, 6.8 ± 0.2; temperature, 24 ± 1°C; conductivity, 74.3 µS. cm⁻¹; total alkalinity, 37 mg/L as CaCO₃; hardness, 28 mg/L as CaCO₃, ammonia concentration, 0.01 mg/L; chloride concentration, 0.294 mg/L; and nitrite concentration, 0 mg/L.

Experimental design
Twenty-four fish, starved for one day, were equally divided into four dark tanks with 250L of nitrite-free water. The fish rested for 24 hours, and 0.6 mg/L of N-NO₂ (sub-lethal concentrations) were added to each tank except to the control. The nitrite exposure remained for 24 hours. At the same time and conditions other group of tanks were set for 96 hours of exposure. Both experiments, 24 and 96 hours of exposure to nitrite, were maintained in semi-static system. After the exposure to nitrite, the fish were collected, anesthetized with MS 222 and a blood sample was drawn from the caudal vein with an heparinized syringe. Afterward, fish were killed by pinching of the spinal cord.

Blood
The blood samples were centrifuged at 12,000Xg for 3 minutes at room temperature and plasma (100 µl) was deproteinized by the addition of 20% trichloroacetic acid -TCA (900 µl). Protein-free plasma samples were centrifuged at 12,000Xg for 3 minutes at room temperature. The supernatants were used to determine glucose, pyruvate, lactate and ammonia.

Biochemical analysis
After defrosted, the excised organs (100mg) were mechanically disrupted in a Potter Evelhjein homogenator with two 15-second strokes, using 20% TCA (900µl). The free protein extracts were centrifuged at 12,000 x G for three minutes at 5°C and the supernatants were used for biochemical analysis. The total sugars, assumed in this work as glucose, were determined after acid hydrolysis by sulphuric acid, (Dubois et al., 1956) in free protein extracts. The other metabolites were estimated by colorimetric methods; pyruvate by the 2,4-dinitrophenilhydrazine modified from Lu (1939) and lactate by p-phenylphenol (Harrower & Brown, 1972). Ammonia levels were determined by Nessler’s method adapted by Gentzkow (1942). The glycogen analysis was modified from
Bidinotto and collaborators (1997), as follows. After alkaline digestion of 100-200mg of tissue per ml of 6N KOH in a boiling water bath, 100µl of extract were transferred to 3.0ml of ethanol and 250µl of saturated K₂SO₄ was added. The samples were centrifuged at 3000 x G for 3 minutes at room temperature. The supernatant was discarded and the pellet resuspended in distilled water. The carbohydrate content was determined in suitable aliquots (Dubois 1956).

**Enzyme assay**

Tissues for enzyme assays (100mg) were mechanically disrupted in a Potter-Elvehjem homogenator with two 15-second strokes, using Glycerol-Phosphate pH 7.0 (900µl). The extracts were centrifuged at 600 x G for three minutes at 0°C. The supernatants were centrifuged at 6000 x G for eight minutes at 0°C and supernatants were used as enzyme source. LDH, MDH and GDH were assayed (Hochachka et al, 1978), based in the NADH oxidation. The enzyme activity was expressed in units of enzyme per mg of protein. The total protein in supernatants was determined by UV-method. Enzyme activity was expressed in nmol of NADH/min/mg of the protein (mU/mg of the protein).

The chemicals standards were analytical grade, purchased from Sigma Chemical Co. St. Louis, Mo or Merck. MS222 was from Sandoz. All other reagents were of analytical grade.

Tests for significance were applied using the program “Statistica 5.5”. The data were submitted to normality test SHAPIRO-WILK, with 95% C.I, and after that was used parametric ANOVA test, with DUNCAN test for multiple comparison, with 95% C.I.

**Results and Discussion**

During nitrite exposure occurred functional hypoxia and it was expected that responses were similar to environmental hypoxia. Others studies have shown that hematological responses are different to environmental hypoxia (Scarano & Saroglia, 1984). However, hematological parameter in matrinxa did not increase during nitrite exposure. Instead, it was observed decrease and consequent functional and hemolytic anaemia

Metabolic profile of fish under environmental hypoxia is usually the increase of muscle lactate followed by occasional increase of LDH activity and
neoglucogenesis in liver to support glucose to other tissues (Jorgensen & Mustafa, 1980; Hochachka & Somero, 1984; Yu & Woo, 1987; Woo & Chiu, 1997; Moraes et al., 1998; Hochachka & Somero, 2002; Moraes et al., 2002). In this work it was not observed these classics responses during nitrite exposure. In liver (TABLE 1) enzyme activities of the oxidative metabolism was kept constant during the trials. In 24 hours no changes of metabolism were observed and there was no decrease of lactate or LDH in 96 hours of exposure. Consume of glucose and glycogen was also observed. Our work showed the occurrence of oxidative metabolism in liver of matrinxã for nitrite exposure. Matrinxã exposed to nitrite, reaches high levels of methemoglobin, 79%, after 96 hours of nitrite exposure (Avilez, 2002). Our data showed that the oxidative metabolism was kept constant. In *H. regani* submitted to environmental hypoxia (Moraes et al., 1997), *L. calcarifer* exposed to nitrite (Woo & Chiu, 1997), and *H. malabaricus* exposed to environmental hypoxia (Moraes et al., 1995; Moraes et al., 1996) or nitrite exposure (Moraes et al., 1998) it was reported hepatic glycogenolysis. Constant levels of lactate and LDH activity were observed in liver of *L. calcarifer* (Woo & Chiu, 1997) as exposed to nitrite, but the same was not verified in trout (Arillo et al., 1984).

Increase GDH activity in liver of matrinxã plus ammonia increase in white muscle (TABLE 1) and plasma (TABLE 2) after 96 hour of nitrite exposure suggest protein mobilization. This should be used for amino acid production to energetic support of other tissues, for example brain.
Table 1. Metabolite concentration and enzyme activities in liver and white muscle of *matrinxa* exposed to nitrite for 24 and 96 hours.

<table>
<thead>
<tr>
<th>Metabolites and enzymes</th>
<th>0 mg/L 24 h</th>
<th>0.6 mg/L 24 h</th>
<th>0 mg/L 96 h</th>
<th>0.6 mg/L 96 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>713,02±32, 90</td>
<td>557,82±60,50</td>
<td>24,24±1,04</td>
<td>26,46±1,83</td>
</tr>
<tr>
<td>Glycogen</td>
<td>485,25±42, 34</td>
<td>330,60±55,47</td>
<td>14,12±0,75</td>
<td>15,66±0,56</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>0,99±0,03</td>
<td>1,04±0,02</td>
<td>0,62±0,08</td>
<td>0,52±0,039</td>
</tr>
<tr>
<td>Lactate</td>
<td>10,34±0,87</td>
<td>7,2±0,36*</td>
<td>42,21±2,66</td>
<td>43,03±0,91</td>
</tr>
<tr>
<td>Ammonia</td>
<td>56,11±5,21</td>
<td>70,13±8,77</td>
<td>9,86±1,69</td>
<td>8,47±0,66</td>
</tr>
<tr>
<td>GDH</td>
<td>911,11±45, 94</td>
<td>1042,21±87,1</td>
<td>19,62±2,66</td>
<td>22,98±3,83</td>
</tr>
<tr>
<td>MDH</td>
<td>18548,62±1048,08</td>
<td>13860,86±7,08</td>
<td>2092,48±341,72</td>
<td>3417,76±181,13*</td>
</tr>
<tr>
<td>LDH</td>
<td>249,44±13, 01</td>
<td>217,35±12,15</td>
<td>19,33±1,30</td>
<td>20,55±1,68</td>
</tr>
</tbody>
</table>

In liver, metabolites are expressed in µmol/g of tissue and enzyme specific activity in mU/mg of protein. (*) means significant difference against the control for p <0.05. (X±SE)
In white muscle, metabolites are expressed in μmol/g of tissue, enzyme specific activity (GDH and MDH) in mU/mg of protein, and LDH are U/mg. (*) means significant difference against the control for p <0.05. (x ±SE)

White muscle is the more responsive tissue to anaerobic metabolism under environmental hypoxia, however, this did not occur during functional hypoxia. Enzymatic activity did not change during the experiment. And also, lactate was kept constant, with increase of glycogen in 24 hours and glucose in 96 hours. This suggests that anaerobic metabolism did not occur. Lactate is constant in most tissues of *L. calcarifer* exposed to nitrite, and this response is different to environmental hypoxia (Woo and Chiu 1997). Kidney (TABLE 2) also showed this characteristic during the exposure of 24 hours, with increase of glucose, but in 96 hours this metabolic profile changed toward consume of the glucose, as suggests the increase of pyruvate. Compared to *H. malabaricus* under environmental hypoxia as neoglycogenesis was observed in the kidney (Moraes et al., 1995), this profile was different.

In plasma, constant values of lactate of lactate were observed. This fact is not typical of anaerobic metabolism. Constant glucose concentrations show that some tissue provide glucose to other tissue through plasma. *C. carpio* submitted to nitrite did not increase plasma lactate (Jensen, 1990). Cardiac and brain enzymes did not show any change during nitrite exposure. These data were expected because the immediate relevance of that tissue to the organism. Moreover, there are few protections against metabolism injury (Driedzic e Almeida-Val, 1996). Arillo and collaborators (1984) did not observe alterations in brain metabolism of trout during nitrite exposure in spite of nitrite accumulation in the brain.
Table 2. Plasma and kidney metabolites of matrinxã exposed to nitrite for 24 and 96 hours.

<table>
<thead>
<tr>
<th>Time</th>
<th>Metabolites</th>
<th>Plasma</th>
<th>Kidney</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0 mg/L</td>
<td>0.6 mg/L</td>
</tr>
<tr>
<td>24 h</td>
<td>Glucose</td>
<td>10.3±1</td>
<td>33 15.6±1 20 33.5±1 61 25.5±2 00</td>
</tr>
<tr>
<td></td>
<td>Glycogen</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>Pyruvate</td>
<td>0.41±0 0 5 0.91±0 20* 2.05±0 09 1.42±0 13*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Lactate</td>
<td>3.00±0 5 1 4.15±1 23 2.19±0 40 3.07±0 53</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ammonia</td>
<td>10.36±0 52 3.85±0 87* 23.22±3 02 19.33±1 76</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Glucose</td>
<td>7.04±0 3 8.49±0 60 16.90±0 55 15.93±0 82</td>
<td></td>
</tr>
<tr>
<td>96 h</td>
<td>Glycogen</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>Pyruvate</td>
<td>0.28±0 0 7 1.14±0 17* 1.57±0 06 1.24±0 08*</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Lactate</td>
<td>1.29±0 1 5 1.92±0 13 3.47±0 21 4.24±0 36</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ammonia</td>
<td>6.72±0 2 12.25±1 20 36.24±1 33 36.14±2 36</td>
<td></td>
</tr>
</tbody>
</table>

- Metabolites are expressed in µmol/g of tissue or ml of plasma. (*) means significantly different against the control for p <0.05. (x±SE).

Table 3. Enzyme activities in brain and heart of matrinxã exposed to nitrite for 24 and 96 hours.

<table>
<thead>
<tr>
<th>Time</th>
<th>Enzymes</th>
<th>brain</th>
<th>heart</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0 mg/L</td>
<td>0.6 mg/L</td>
</tr>
<tr>
<td>24 h</td>
<td>GDH</td>
<td>86.5±2 98</td>
<td>71.6±3 09</td>
</tr>
<tr>
<td></td>
<td>LDH</td>
<td>2455.8±2 09</td>
<td>2213.6±5</td>
</tr>
<tr>
<td></td>
<td>MDH</td>
<td>10.3±0 55</td>
<td>8.01±0 42</td>
</tr>
</tbody>
</table>
In brain, GDH and LDH specific activities are expressed in mU/mg of protein, and MDH in U/mg of protein. (*) means significantly different against the control, p <0.05. (x ±SE).

In heart, MDH and LDH specific activities are expressed in mU/mg of protein and GDH in U/mg of protein. (*) means significantly different against the control, p <0.05. (x ±SE).

**Conclusion**

This work showed an oxidative preference of metabolism of matrinxã and the use of protein metabolism to hold levels of tissular glucose. The action of nitrite on matrinxã also showed that toxicity of such anion manifest the first problems at hematological and osmoregulatory level. After that, metabolic alterations should occur in liver, but our methodology and / or time of exposure were not enough to worsen it in matrinxã.

**References**


Moraes, G., Chippari, A., Souza, R.H.S. Comparison between metabolic responses in traíra (Hoplias malabaricus) and pacu (Piaractus


COMPARISON BETWEEN THE RESPONSES OF THE TELEOST PACU AND ITS HYBRID TAMBACU

(Piaractus mesopotamicus X Colossoma macropomum)

TO ACUTE SHORT TERM NITRITE EXPOSURE.

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Introduction

Organisms are exposed to many environmental factors. Among them there is pH, temperature, dissolved oxygen, ammonia and ions. The natural environment is very dynamic; however in many systems its parameters are almost constant or vary in a predictable way. That, over the process of evolution, has allowed animals to adapt to their habitats (Hochachka & Somero, 2002). However men’s interference in natural processes has modified the characteristics of several environments. One example of this is the sewage dumping in rivers and lakes. In artificial systems, such as fish farms, these parameters vary greatly, especially in high-density systems. One ion can be of major importance in both natural and artificial systems because of its toxicity. That ion is nitrite.

Nitrite is an ion naturally found in the environment due to the process of nitrification. Although its concentrations are usually low (Eddy & Williams, 1987), several processes can interfere in the balance of the nitrogen cycle. Among these, pollution of aquatic environment is known to cause considerable increase in nitrite concentration (Nikinmaa, 1992). As a matter of fact high levels of nitrite are used as indicators of impacted areas. Nitrite toxicity to fish is due to several processes including the formation of methemoglobin, which is non-functional form hemoglobin, and the cytolysis of hepatocytes and gills’ chloride cells (Arillo et al., 1984). Fish can be especially sensitive to nitrite because it has the ability to compete with the uptake of chloride by the gills’
chloride cells (Bath & Eddy, 1980). Nitrite can actually be found in high concentrations in fish plasma. (Margiocco et al., 1983)

Methemoglobin formation can induce cyanosis and a state similar to the one find in cases of environmental hypoxia (Smith & Russo, 1975). On the other hand the damage caused to gills and liver is altering two of the most important regulation organs in fish and can, therefore, cause serious problems in maintaining homeostasis. Moreover nitrite is known to induce hemolytic anemia (Scarano & Soroglia, 1984), which can aggravate the metabolic hypoxia stress. Pacu (Piaractus mesopotamicus) and its hybrid Tambacu (Piaractus mesopotamicus X Colossoma macropomum) were vastly reared in Brazil. The aim of this work was to evaluate the effects of environmental nitrite (20 ppm of NaNO₂) on the hematology and intermediary metabolism of both fish over the period of 8 hours.

Materials and Methods

The fish used for experimentation had a mean weight of 51.21 ± 16.50 g and were kept in natural photoperiod, constant supply of oxygen, termostatized and filtered water for aclimatation. They were then transferred to two 200 L tanks (N=6 for Tambacu and N=10 for P. mesopotamicus) where they stayed for five days. The conditions on these tanks were equal to those described above. Then 24 hours before the experiment the feeding was suspended. Finally we added NaNO₂ to one of the tanks (the other was kept as negative control) until was reached the desired concentrations. After eight hours of exposition the fish were taken from the tanks and blood, white muscle and liver samples were collected, being the tissue samples frozen in liquid nitrogen for future analysis.

Blood was used for hematocrit (Lima, 1969), hemoglobin (Drabkin, 1948) and methemoglobin (Benesch et al., 1973) determinations. The remaining blood was centrifuged to obtain plasma, in which were determined the contents of glucose (Dubois, 1956), lactate (Harrower & Brown, 1972) and pyruvate (Lu, 1939). In the sampled tissues the same parameters were analyzed in addition to the content of glycogen (Bidinotto et al., 1997). The data obtained were analyzed for normality using the KOLMOGOROV-SMIRNOV test and afterwards the means were compared using the parametric T-Student test. All tests were performed at 5% level of confidence utilizing GRAPHPAD PRISM™ software.
TABLE 1. Comparison between the metabolic intermediates in the tissues of Tambacu and P. mesopotamicus exposed to 20 ppm of nitrite during 8 hours. All values ± S.E expressed in µmols/mg of wet tissue (except L/P Ratio). Significant differences indicated by * (when p=0.05) and ** (when p=0.01)

<table>
<thead>
<tr>
<th>Metabolic Intermediates in P. mesopotamicus</th>
<th>Liver</th>
<th>WM</th>
<th>Plasma</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group</td>
<td>Control</td>
<td>20 ppm</td>
<td>Control</td>
</tr>
<tr>
<td>Glucose</td>
<td>647.0±</td>
<td>496.1±</td>
<td>26.93±</td>
</tr>
<tr>
<td></td>
<td>49.67</td>
<td>13.26**</td>
<td>1.239</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>1.357±</td>
<td>1.402±</td>
<td>0.4536</td>
</tr>
<tr>
<td></td>
<td>0.1002</td>
<td>0.09176</td>
<td>±0.044</td>
</tr>
<tr>
<td>Lactate</td>
<td>7.750±</td>
<td>7.320±</td>
<td>43.02±</td>
</tr>
<tr>
<td></td>
<td>0.4949</td>
<td>0.2208</td>
<td>1.856</td>
</tr>
<tr>
<td>Glycogen</td>
<td>480.7±</td>
<td>544.8±</td>
<td>17.01±</td>
</tr>
<tr>
<td></td>
<td>20.50</td>
<td>15.19*</td>
<td>0.4741</td>
</tr>
<tr>
<td>L/P Ratio</td>
<td>5.932±</td>
<td>5.419±</td>
<td>112.6±</td>
</tr>
<tr>
<td></td>
<td>0.2955</td>
<td>0.3772</td>
<td>20.18</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Metabolic Intermediates in Tambacu</th>
<th>Liver</th>
<th>WM</th>
<th>Plasma</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group</td>
<td>Control</td>
<td>20 ppm</td>
<td>Control</td>
</tr>
<tr>
<td>Glucose</td>
<td>396.7±</td>
<td>484.4±</td>
<td>12.25±</td>
</tr>
<tr>
<td></td>
<td>25.36</td>
<td>25.43*</td>
<td>0.9678</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>1.55±</td>
<td>1.006±</td>
<td>0.9171</td>
</tr>
<tr>
<td></td>
<td>0.1133</td>
<td>0.05349</td>
<td>±</td>
</tr>
<tr>
<td>Lactate</td>
<td>9.96±</td>
<td>6.85±</td>
<td>30.52±</td>
</tr>
<tr>
<td></td>
<td>0.5624</td>
<td>0.3158*</td>
<td>2.024</td>
</tr>
<tr>
<td>Glycogen</td>
<td>329.4±</td>
<td>438.6±</td>
<td>6.606±</td>
</tr>
<tr>
<td></td>
<td>28.04</td>
<td>34.19*</td>
<td>0.5343</td>
</tr>
<tr>
<td>L/P Ratio</td>
<td>6.337±</td>
<td>7.027±</td>
<td>29.42±</td>
</tr>
<tr>
<td></td>
<td>0.3169</td>
<td>0.2374</td>
<td>1.658</td>
</tr>
</tbody>
</table>
TABLE 2. Comparasion between the hematological parameters of Tambacu and P. mesopotamicus exposed to 20 ppm of nitrite during 8 hours. Values ± S.E expressed for Hematocrit and Methemoglobin in %, for hemoglobin in g/dL and for RBC in millions of cells per mm$^3$. Significant differences indicated by * (when p=0.05) and ** (when p=0.01)

<table>
<thead>
<tr>
<th>Fish</th>
<th>Hematological Parameters in P. mesopotamicus</th>
<th>Tambacu</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P. mesopotamicus</td>
<td>Tambacu</td>
</tr>
<tr>
<td>Group</td>
<td>Control 20 ppm</td>
<td>Control 20 ppm</td>
</tr>
<tr>
<td>Hematocrit</td>
<td>29,25± 1,055</td>
<td>36,70± 1,457**</td>
</tr>
<tr>
<td>Hemoglobin</td>
<td>9,42± 0,1847</td>
<td>8,68± 0,2063*</td>
</tr>
<tr>
<td>Methemoglobin</td>
<td>0,0</td>
<td>8,84± 1,372</td>
</tr>
<tr>
<td>RBC</td>
<td>38,40±1,523</td>
<td>29,52± 3,160*</td>
</tr>
</tbody>
</table>

Results and Discussion

The stress imposed to the fish because of the formation of methemoglobin has a major difference when compared to environmental hypoxia. Functional hypoxia results not from lack of oxygen, but rather from the impossibility to carry the available oxygen due to a reduction in the concentrations of functional hemoglobin. The results of the contents of methemoglobin found are low (the highest was around 18%) when compared with the data presented in the literature (Souza et al., 1995; Bath & Eddy, 1980; Schoore et al, 1995; Tucker et al., 1989; Hilmy et al., 1987). On the other hand the levels of methemoglobin in catfish take from 6 to 18 hours to reach its maximum concentrations (Huey et al., 1980). In fact Moraes and collaborators (1998) found similar methemoglobin concentrations in Hoplias malabaricus exposed to 30 ppm of environmental nitrite.

The hematological responses in Tambacu differ in one aspect to those found in cases of environmental hypoxia. Nitrite can cause hemolytic anemia (Scarano & Soroglia, 1984), to support this hypothesis we found hematocrit reduced after exposition to nitrite in Tambacu. P. mesopotamicus does not present a characteristic hematological response to nitrite intoxication and the levels of
methemoglobin are somewhat lower than in tambacu (8%). We actually found an increase in hematocrit, which is a common response to environmental hypoxia. Hemoglobin concentration was significantly reduced but that might be a result of the initial process of elimination of red cells that have methemoglobin. This process cause the hemolytic anemia referred above. On the other hand the number of red cells increased in both Tambacu and P. mesopotamicus. This is also a strategy that fish present when exposed to low dissolved oxygen concentrations (Fievet et al., 1987; Peterson et al., 1987; Moraes et al., 1995; 1996). These hematological responses suggest that at least to some extend these animals are exposed to a lack in oxygen supply.

One of the major strategies to survive hypoxia is through the regulation of the glycolytic pathway (Hochachka & Somero, 2002). In both fish the liver showed a neoglucogenic metabolic pattern. The Tambacu’s liver appear to have a more intense neoglucogenic profile since in this tissue there is a significant reduction in lactate levels and a decrease in the L/P ratio along side a reduction of pyruvate. Also glycogen increased in content in both fish. In P. mesopotamicus glucose levels were reduced. This can be explained in two non-exclusive ways: glucose can be exported to other tissues; especially the brain or it can be converted in glycogen. In Tambacu the glucose levels increased and glycogen levels remained the same. The liver is very likely preparing itself to an attempt to sustain oxidative functionality under hypoxia. Two enzymes play a major role in this process, namely: Pyruvate Kinase (PK) and Glycogen Phosphorylase. Both enzymes are subject to allosteric regulation and under of the control of hormones such as catecholamines (Wright et al., 1989). Other enzyme to play a major role in neoglucogenesis is Lactate Dehydrogenase (LDH) (Hochachka & Somero, 2002). Although in this work there is no direct measure of the activity of this particular enzyme, it can account to the decrease in lactate contents because it is being turned in pyruvate. PK subsequently converts the lactate converted in piruvate to phosphoenolpyruvate, which is used in the neoglucogenic pathway. An increase in Pk could account for the reduction of pyruvate in Tabacu’s liver.

The white muscle is adapted to sustain its activity through the anaerobic consumption of glucose (Hochachka & Somero, 2002). However the glucose levels were found stable in this tissue in Tambacu. The origin of this glucose can be the liver. This organ might be exporting an excess of glucose, produced in neoglucogenesis. To support this idea one can observe a significant increase in glucose in tambacu’s plasma. White muscle glycogen also increased. The glucose used for this has probably the same origin. On the other hand the
tendency of increase of pyruvate contents and the tendency of lactate contents reductions might suggest that the Tambacu’s white muscle has a neoglucogenic profile too. These strategy aims to build up a reserve of metabolic fuel to sustain activity under low oxygen tensions. To achieve this, the white muscle must be able to increase also its glycolytic capacity. In order to accomplish its capacity several strategies are found in vertebrate muscle. Firstly, the amounts of enzyme such as PFK (phosphofructokinase) are higher. Secondly, the system is strongly poised for the glycolytic direction. And thirdly, it is tightly regulated (Hochachka, 1980). Moreover white muscle can sustain much higher levels of lactate then other tissues (Hochachka, 1980). The Tambacu’s muscle presented a strategy that is the same that the one observed by Souza and collaborators (1995) for C. macropomum when exposed to nitrite. The P. mesopotamicus’ white muscle presented a different pattern. The glycogen and glucose contents were smaller in the fish exposed to nitrite. The pyruvate levels were higher. Lactate contents were significantly reduced, which might suggest that white muscle is turning it to pyruvate, whose fate is oxidative metabolism. These results suggest that this tissue in hydrolyzing glycogen and using the resulting glucose in oxidative metabolism. Therefore these findings suggest that in the white muscle of both fish, oxidative metabolism prevails over fermentative metabolism.

The metabolic profile found in P. mesopotamicus’ liver and white muscle is very similar to the one presented when this fish is exposed to severe environmental hypoxia (Moraes et al., 1997a). This together with the hematological response indicates that even low levels of methemoglobin can trigger a metabolic response to a reduction in oxygen availability. Considering that, in general, the first strategy to hypoxia is related to regulation in transportation pigments affinity to O₂ (Hochachka & Somero, 2002), the formation of methemoglobin may reduce the effectiveness of this particular response. Thus, the metabolic adjustments become necessary as a backup strategy. Figure 1 depicts the probable metabolic profile of both fish in response to nitrite exposure.
Figure 1. Suggested metabolic profile of *P. mesopotamicus* (A) and Tambacu (B) exposed to environmental nitrite.

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EFFECTS OF WATER QUALITY ON THE 
BEHAVIOURAL AND ELECTROPHYSIOLOGICAL 
RESPONSES TO ODOUR SIGNALS IN CYPRINID FISH

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In eutrophic and poorly buffered water bodies pH shows diurnally variations, reaching as high as 9.5 at daytime in periods of heavy algal bloom, which can occur in a hot sunny calm day. The assimilation causes a reduction in carbon dioxide and the water becomes more alkaline. Also the water effluence from industry can be extremely alkaline e.g.: by wool scouring, by retorting of oil shale, and by manufacturing of certain chemicals. Such alkaline effluents may have a pH of 12-14 and can be lethal to all types of water life. The exposure of fish to elevated pH causes inability to dispose of metabolic ammonia and often cause acute fish kills. Relatively short time exposure of fish to high pH (pH ~ 9.5) is rarely lethal for many fish species (Jones, 1964) but nevertheless this water can damage outer surfaces like gills, eyes, and skin. Cypriniformes seem less sensitive to high pH than Perciformes but prolonged exposure to sub-lethal high pH levels can cause serious stress. High pH can also affect the sensory epithelium of the fish olfactory system as it is directly exposed to the environment. The olfactory system in fish is very sensitive and mediates essential life functions, like finding food, homing, and predator avoidance induced by alarm substances from conspecifics. Studies have shown that EOG recordings can be used to evaluate the effects of pollutants, e.g., low pH and pesticides (Moore and Warning 1996), and heavy metals (Baatrup et al. 1987, Baatrup and Døving, 1990, Winberg et al. 1992). Water with heavy metals has effect on predatory and pray capture ability (Weis et al., 1999). However,
little is known about the potential sublethal effects of high pH exposure to functions of the olfactory system. The effect of water with high pH on the olfactory sensitivity was studied by recording the electro-olfactogram (EOG) in crucian carp (*Carassius carassius*). The test odorants (molar concentration) were the amino acids, L-alanine ($10^{-5}$) and L-serine ($10^{-5}$) representing food signals; steroid hormone 17α,20β-P ($10^{-11}$) and prostaglandine 15-keto-PGF$_2$α ($10^{-8}$) representing sex hormones/pheromones, and a putative alarm pheromone, hypoxanthine-3(N)-oxide ($10^{-8}$). We exposed the olfactory organs with artificial pond water with pH 7.0, 8.5, 9.0, 9.5. Additionally, we also tested the effect of alkaline extracts of the waste from oil shale industry.

Acute exposure of the olfactory organ to pH 9.0 and 9.5 decreased the olfactory sensitivity to food signals, sex hormones or pheromones, and the putative alarm substance (Fig. 1). The EOG amplitudes at pH 9.5 were between 9.5 and 53 percent of the amplitude recorded at pH 7. Similar reduction of sensitivity of olfactory organ was seen after perfusion of the olfactory rosette with mixture of industrial alkaline leach (pH ~9) of oil shale industry. We consider the high pH of leach to be the main factor causing the reduction of olfactory sensitivity to these stimuli. Secondary importance for reduction of the sensitivity of olfactory epithelium had the toxic substances, primarily heavy metals, polycyclic aromatic hydrocarbons, and phenols, present in leach. Heavy metals and phenols are known to impair the olfactory sensitivity. However, our experiments with water saturated with polycyclic aromatic hydrocarbons (benzo(a)pyrene,
Figure 1. Reduction of olfactory sensitivity by alkaline water. The EOG amplitudes from the olfactory epithelium of crucian carp induced by L-serine (10^{-5} M), 17a,20ß-P (10^{-11} M), 15-keto-PGF_{2a} (10^{-8} M), Hypoxanthine-3(N)-oxide (10^{-8} M) at different pH. benzo(a)antracene, mixture of 16 PAHs) demonstrated no effect to the olfactory sensitivity of crucian carp. In a further series of acute behavioural experiments in fluviarium with crucian carp and roach (*Rutilus rutilus*) it was observed that these cyprinid fish do not avoid water at pH ranging from 9.3 to 10.0. Thus, these cyprinids species do not avoid water with high alkalinity. Nevertheless, the ability to detect essential odour signals is dramatically reduced at high pH.

**Acknowledgements**

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ROLE OF OLFATORY TRACTS MEDIATING
FEEDING BEHAVIOR AND ALARM REACTION
IN THE CATFISH PINTADO, PSEUDOPLATYSTOMA CORUSCANS

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Introduction

Chemical signals released by fishes are among the most important factors mediating intra- and interspecific relationships. Such signals convey information about alarm (Giaquinto & Volpato, 2001), social hierarchy recognition (Giaquinto & Volpato, 1997), and predator-prey assessment (Jordão & Volpato, 2000). The olfactory system of fish responds to amino acids, bile acids, gonadal steroids and prostaglandins, the two last acting as pheromones. In gadids, silurids and cyprinids, the olfactory tracts are long and divided into distinct bundles. Each bundle connects to different areas of the brain and this organization can be an indicative of separate functional properties for each bundle.

The way this information is used in fish which have developed distinct degrees of differentiation of extra olfactory chemicals senses, has yet to be determined.
The Brazilian catfish pintado, *Pseudoplatystoma coruscans*, is a voracious, carnivorous, nocturnal Ostariophysian fish. As a nocturnal species, chemically-mediated behaviors are expected to play a major role in agonistic relationships. In the present study we attempt to investigate the role of olfactory tracts in feeding and alarm reaction behaviors. Also, the decision whether to eat or to flee when simultaneously stimulated with alarm substance skin extract and food was tested in fish with intact and sectioned olfactory tracts.

**Methods**

We investigated the role of olfactory tracts in feeding and alarm reaction behaviors in the decision making in the dilemma ‘to feed or to flee’ exposing the fish, with intact and sectioned olfactory tracts, to alarm substance extract (distilled water as a control) and food. The following groups were set:

- Sham operated, in which the olfactory tracts were exposed during surgery but left intact;
- LOT section group, in which lateral olfactory tract was bilaterally transected;
- MOT section group, in which medial olfactory tract was bilaterally transected;
- TOTAL section group, in which MOT and LOT were bilaterally transected.

The following responses were considered components of feeding behavior: barbell movements, bottom food search, exploring, biting on the bottom and prey catching. Conspecific skin extract induced the following sequential alarm reactions: (1) *dashing* - alarmed fish swam at high velocities in unpredictable trajectories; (2) *freezing* - the fish suddenly stopped ongoing behavior remaining immobile on the bottom of the aquarium.

**Results**

Feeding behavior occurred in sham operated and in fish with olfactory tract section (either LOT, MOT and TOTAL) when distilled water (control) was introduced into the aquarium (Table 1). Latency to feeding was significantly lower in sham-operated fish (2.54s ± 1.02) when compared with olfactory tracts sectioned (LOT=36.56s ± 6.11; MOT=12.17s ± 4.72; TOTAL=12.25s ± 4.56; p<0.05).

Feeding also occurred when fish were exposed to the skin extract, but this was
restricted to fish with olfactory tract section. Alarm reaction, however, did not occur in any fish with sectioned tract (under skin extract: LOT= 0 alarm reaction vs 6 feeding events, Dunn’s test: Q=0.04; MOT= 0 alarm reaction vs 6 feeding events, Dunn’s test: Q=0.02; TOTAL= 0 alarm reaction vs 5 feeding events under skin extract, Dunn’s test: Q=0.03; p < 0.05; Table 1).

Table 1. Number of pintados with bilateral olfactory tract sections or sham operated that presented alarm reaction or feeding when exposed to skin extract and food stimulus.

<table>
<thead>
<tr>
<th></th>
<th>LOT Section</th>
<th>MOT Section</th>
<th>Total Section</th>
<th>Sham</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alarm</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>Feeding</td>
<td>6</td>
<td>6</td>
<td>5</td>
<td>0</td>
</tr>
</tbody>
</table>

Feeding always involved obvious reaction: barbell movements, exploring, biting on the bottom and prey catching.

Sham-operated fish showed the typical alarm reaction and did not eat when stimulated with skin extract (4 alarm reaction vs 0 feeding events under skin extract, Dunn’s test: Q=0.05; p < 0.05, Table 1).

Despite all groups with sectioned olfactory tracts react to food when stimulated with skin extract, the latency to such behavior was different according to the sectioned tract. Fish with LOT and TOTAL section showed a higher latency to react to food (33.48s ± 23.14 and 29.42s ± 11.71, respectively) than fish with MOT section (4.28s ± 1.44) (Figure 1).
Conclusion

The results of the present study indicates that the so called taste subsystems (taste buds and solitary chemosensors) are sufficient to trigger and elicit feeding behavior and that the integrity of the olfactory tract, particularly LOT which has been implicated in feeding (Doving and Selset, 1980), is not necessary to maintain the quantitative extent of food detection. Information through the gustatory via (and its respective sub-systems) seems to be sufficient to elicit feeding behavior, even in the absence of a refined olfactory cue. Integrity of the tracts, although, seems to be required for mediating alarm reaction in *Pseudoplatystoma coruscans*.

The results of our study stress the discussion to caution simplistic interpretations of experiments with suppression of one sensorial channel. A lack of behavior after olfactory suppression cannot be taken as a positive evidence for olfactory versus taste function.
Acknowledgements

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IDENTIFICATION OF CLUB CELLS AND BEHAVIOURAL RESPONSES OF PIAU *Leporinus piau*, TO ALARM SUBSTANCE

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Introduction

In ostariophysan fish, the detection of a conspecific (and some heterospecific) alarm substance may elicit alarm reactions or antipredator behavioral responses (Pfeiffer, 1963, for review). The present study was undertaken to describe and quantitatively characterize the behavioral responses of solitary and in shoals of piau *Leporinus piau* (Ostariophysi, Anastomidae) to the conspecific alarm substance, under laboratory conditions.

Methods

Juvenile unsexed piau (5.5 - 7.0 cm total length) were placed in freshwater glass aquaria (40x22x20 cm, 18 L) at 26 ± 1.5°C, exposed to a 12:12 light-dark photoperiod. The conspecific alarm substance (CAS) was prepared of skin from the both sides of some fish. Small pieces of the skin were preserved in 4% formaldehyde for histological examination of the presence of specialized epidermal cells (Lawrence & Smith, 1989). During the experiments, solitary fish were stimulated with 0.1 ml of different dilutions of CAS (1:100, 1:10, 1:4 or stock solution) or distilled water (DW). Group size of 5 fish were tested with 0.1 ml of stock solution (n=10) or distilled water (n=6).
Results and Discussion

The histological examination of juveniles piau skin epithelium revealed large cells arranged in a single layer in the middle region of the epithelium and containing a single centrally located nucleus. This analysis revealed a type of cell that can be admitted as the club cell (Pfeiffer, 1963).

The responses of individually housed piau to CAS were the initial dashing behavior followed by a longer-lasting period of immobility (biphasic response), slowing its swimming activity and freezing. Biphasic responses decreased at more diluted solutions (Tab. 1). Comparing to the immediate period preceding the stimulus, the animals presented significant reduction in the swimming activity when increasing diluted solutions were introduced: stock solution ($X^2=30.983; P≤0.001; n=8$), 1:4 ($X^2=36.214; P≤0.001; n=7$), 1:10 ($X^2=42.265; P≤0.001; n=9$), 1:100 ($X^2=7.916; P=0.637; n=8$). Statistical differences were denoted among 1:10, 1:4 and stock solutions, when compared to the control group ($P<0.05$; Kruskall-Wallis’ Test). These data suggest that the minimum stimulus dilution required to elicit an alarm response for the animals tested.

Under laboratory conditions, tested solitary fish such as fathead minnows (Lawrence and Smith, 1989; Chivers and Smith, 1993) and matrixxãs (Ide et al., 2003), similar to piau.

Table I. Number and percent (in parentheses) of piau exhibiting behavioral responses after exposition to 0.1 ml of DW or different dilutions of CAS (1:100, 1:10, 1:4 or stock solution).

<table>
<thead>
<tr>
<th>Behavioural response</th>
<th>DW</th>
<th>1:100</th>
<th>1:10</th>
<th>1:4</th>
<th>Stock</th>
</tr>
</thead>
<tbody>
<tr>
<td>+++</td>
<td>1 (12.5%)</td>
<td>7 (87.5%)</td>
<td>6 (66.7%)</td>
<td>4 (57.1%)</td>
<td>3 (37.5%)</td>
</tr>
<tr>
<td>++</td>
<td>0</td>
<td>0</td>
<td>1 (11.1%)</td>
<td>2 (28.6%)</td>
<td>4 (50.0%)</td>
</tr>
<tr>
<td>+</td>
<td>1 (12.5%)</td>
<td>0</td>
<td>2 (22.2%)</td>
<td>1 (14.3%)</td>
<td>1 (12.5%)</td>
</tr>
<tr>
<td>-</td>
<td>6 (75.0%)</td>
<td>1 (12.5%)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

(++) slowing; (+++) biphasic; (+) immobility; (-) no response.

Piau swan in a more cohesive school and decreased their area use, 30 seconds immediately after CAS exposition, when compared to the immediate period preceding the stimulus ($X^2 = 25.889; P = 0.004$) or with DW controls ($T = 70.0$;
P = 0.045). Immediately after exposition to CAS, some alarmed shoals showed an increase in swimming and/or dashing responses. Other shoals became motionless, and 90% shoals settling to the substratum. Grouped fish such as zebra danios (Rehnberg and Smith, 1988) showed reduction in swimming activity, increased shoaling and area avoidance to the conspecific skin extract. The maximum cohesion time in piau was from 2.2 until 66 minutes, with members constantly changing aggregations or maintaining motionless.

Figure 1 – Latency and duration of piau cohesion (n=10) after skin extract exposition.

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FIELD BIOASSAYS WITH COMMON FISHES AND INVERTEBRATE FOOD RESOURCES NEAR CONSTRUCTED AND RECLAIMED WATER MARSHES ON SAN FRANCISCO ESTUARY

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Abstract

Filtration by constructed marshes might help detoxify wastewater and help restore fish populations nearby. In San Francisco Bay, our field bioassays in plastic/mesh enclosures for two-week periods suggested generally high survival of threespine stickleback, *Gasterosteus aculeatus*, and most other common organisms enclosed *in situ* in treated reclaimed water marsh effluent (~5 ppt salinity) compared with similar survival in reference marshes at various higher salinities. Dilution with bay water attracted diverse animals to cages more than in reference sites, possibly due to dense zooplankton from the wastewater marsh. This marsh effluent has shown no other major short-term detriments compared with other marsh water, cautiously supporting filtration by constructed marshes and future use of field bioassays for environmental monitoring. However, each marsh shows risks of mortality for some species under extreme conditions, and fish behavior might attract these species to concentrated food resources and low salinities, even if areas are polluted or would be otherwise detrimental for prolonged exposure.
Introduction

Laboratory bioassays can assess toxicity originating from water chemistry. One means to integrate effects of multiple environmental stressors under relatively natural conditions may be bioassays under field conditions, which may fluctuate day and night, and may add additional combinations of factors that together determine distribution, abundance, mortality, and behavior of aquatic organisms, “Environmental Health,” including fishes and their foods. Confining local animals under prolonged exposure to particular stressors may help determine realistic worst-case responses of animals.

Pollution from wastewater discharge into bays has caused severe damage to local fauna (Filice, 1959; Nichols, 1979; Luoma and Cloern, 1982; Josselyn, 1989) and more economical and environmentally wise alternatives for wastewater disposal are in demand. Biological filtration of sewage pollutants by marsh sediment and plants has received special attention locally as a technique to dispose of wastewater, decreasing pollutants in water (Demgen, 1989; Josselyn, 1989).

The Hayward Constructed Wastewater Marsh Project, in southern San Francisco Bay, receives nearly 20 million liters of secondary treated sewage from municipal and light industrial sources daily. Preliminary chemical analysis of the wastewater and animals throughout marsh filtration over nearly one week of residence time, showed significant net decreases in major metals, yielding low concentrations of manganese, iron, and copper, detectable in most invertebrates (~400-600 ppm) but not in nearby fishes. Absence of fishes up in the wastewater mash is attributed to high ammonia. Kitting et al. (1994) provides more detailed descriptions of these areas.

Our initial ecological surveys on effects of this experimental marsh effluent reported higher species richness and densities of invertebrate and fish populations compared to our similar control sites (Ouverney, 1993; Kitting et al. 1994; Kitting, 1996). It was feasible that fishes found inside this experimental marsh effluent might only tolerate the channel as a brief feeding ground, for its high concentration of invertebrates, and predators might not tolerate remaining permanently, due to the possibly excessively polluted conditions or salinity fluctuations from that effluent. This investigation tested whether common fishes and invertebrates could indeed withstand continuous exposure to direct effluent from a marsh fed by treated sewage.
Methods

During spring (April) and summer (July) of 1992, a 1m$^2$ aluminum cage was placed at an experimental wastewater marsh effluent site (EXP) and at a pair of control sites (CNT), 500 meters south of EXP, in South San Francisco Bay. Mean salinities at EXP reached ~15 ppt (1:1 marsh effluent to bay water) during high tides and ~25 ppt at CNT. Cages had open bottoms, enclosing mud to a depth of 10 cm, with 5-mm mesh secured atop the cages. Approximately eight shrimp, *Palaemon macrodactylus*, and threespine stickleback, *Gasterosteus aculeatus*, were added to cages, then monitored every 2 days for mortality rates.

High survival of animals at mid salinity and intermediate levels of wastewater marsh effluent in cages led to these subsequent field bioassays right at the point of wastewater marsh discharge to San Francisco Bay.

Organisms tested in these field bioassays were collected at locations 500 meters south from the wastewater marsh effluent. Organisms were then measured, and multiple individuals ~5~10 cm in total length were placed immediately in 20-liter plastic buckets at an experimental site (EXP) and control site (CNT) during summer (from July 24 to August 14) and fall (from October 7 to 30) of 1992. Subsequent, less systematic sampling sought to detect any major changes in survival of common aquatic animals at this and an additional reclaimed water marsh, in Martinez, CA, in northern San Francisco Estuary.

Replicates of bioassays at both Hayward sites took place simultaneously. Buckets were prepared by cutting a 20-cm hole in each lid, covered with 1-mm nylon mesh, attached with inert thermoplastic glue. Buckets were rinsed with tap water, deionized water, and then sea water several times, before being attached inside channels to remain submerged even during low tide, about 5 m away from the marsh effluent pipes. Animals inside EXP buckets were exposed daily to a full range of salinities and water quality from 100 % marsh effluent at 5 ppt (as up in the marsh) during low tides, to higher bay water concentrations at 15-20 ppt during high tides (~20 % marsh effluent). Animals in CNT buckets were exposed to higher salinities reflecting that of bay water and an analogous salt marsh, between 25 and 30 ppt.

Numbers of live and dead animals in bioassays were recorded every 2-3 days, over a two-week period. Salinity and temperature were measured during each visit. Despite occasional disappearance of buckets, four to twenty individuals...
for each species (except for a single longjaw mudsucker goby, *Gillichthys mirabilis*, as noted) were tested per season. Low populations of organisms and loss of buckets limited the number of individuals tested (Ouverney, 1993). Hence, some species were tested numerous times during each season and results represent a compilation of data from the various tests. For each test, two replicate buckets were prepared, with equal numbers of animals of the same species inside each bucket. *Palaemon* shrimp had to be tested separately from fishes, mainly gobies, due to predation. Most fishes tested were juveniles, except for threespine stickleback (TL 4 cm) and yellowfin goby (TL 15 cm,) *Acanthogobius flavimanus*, which were available primarily as small adults. Native California horn snails, *Cerithidea californica*, and eastern mud snails, *Ilyanassa obsoleta*, included equal numbers of juvenile and adult sizes, ~1cm and ~3cm long.

After the summer bioassays, snails were not replaced with new individuals in the fall, but instead remained inside both EXP and CNT buckets until the fall bioassays. Hence snails were exposed to conditions in both sites for up to three months, while bioassays for all other animals restarted with new individuals in the fall.

Small food particles could enter the bioassay buckets through the top mesh. In addition, small pieces of wood and roots covered with epifauna were enclosed with organisms in buckets to serve as protection and food resources.

Percentage of live animals over time was plotted for each site during summer and fall of 1992. Because most juveniles and adults of both snail species showed high survival in fall buckets at both sites, they were not plotted for that season.

The first author’s subsequent field bioassays at these and additional marshes, through 2004, used modified plastic minnow traps with mesh and plant debris inside with most of these species, and additional common fishes and invertebrates, year round.
Results and Discussion

Threespine stickleback and *Palaemon* shrimp added to 1m² cages in 15 ppt salinity and at CNT (at ~25 ppt) persisted over one week. The 5-mm mesh size allowed for recruitment of other, smaller, threespine stickleback into the cage during high tides, and these smaller stickleback accumulated over long periods. Results for most invertebrates and fishes after eight days of exposure to ~100 % marsh effluent generally showed close similarities between control (CNT) and experimental (EXP) sites, described below. Statistically, no significant nor nearly significant differences appeared in numbers of surviving individuals at the end of the field bioassays comparing the two major sites, using the two seasons as replication (Mann-Whitney U test, U’>0.5, P>0.32 for each species). Mortality for some species was detectable while others showed no mortality at both sites.

Summer bioassay controls include data only for the first eight days, due to loss of both buckets (Fig. 1A), while all 14 days of bioassays at EXP are shown in Fig. 1B. During the first eight days of summer bioassays in CNT buckets, all animals survived except for 100% mortality of topsmelt, *Atherinops affinis*, and 20 % of threespine stickleback (Fig. 1A). At EXP (Fig. 1B), all animals survived these first eight days of summer bioassays, except 100 % of the topsmelt, 20 % of the stickleback, and 20 % of juvenile eastern mud snails and 10% of horn snails.

At CNT, adults and juveniles of both snail species remained active through the eight days of the experiment. Also, none of the rainwater killifish, *Lucania parva*, died in either CNT (Fig. 1A) or EXP (Fig. 1B) field bioassays. After eight days, those animals that remained in EXP showed no major mortality except for declines in *Palaemon* shrimp and yellowfin goby. During summer, neither of these animals were included in controls, nor plotted in figures, because very few were found at control sites during this season.

In the fall, field bioassays during 14 days yielded results roughly similar to those of the summer. Except for one adult horn snail, all other snails of both species remained alive beyond the summer bioassays and throughout the later 14 days in the fall bioassays. Snails were not included in fall figures (Figure 2A and 2B) to make figures clearer. In addition, a 50 % decline in *Palaemon* shrimp occurred at CNT buckets (Fig. 2A), and the remaining four shrimp survived until the end of the experiment. At EXP, fewer *Palaemon* shrimp died early in the experiment (20 %), but all died after day 10 (Fig. 2B). Among the fishes in fall
bioassays, 100% of threespine stickleback died after day 12 at CNT (Fig. 2A) and after day 6 at EXP (Fig. 2B). All rainwater killifish survived at both sites. Pacific staghorn sculpin died by day 5 at CNT, and by day 3 at EXP. Furthermore, a single longjaw mudsucker goby caught along the Hayward shore was placed in buckets at EXP and survived all 14 days of the test.

Overall, field bioassays showed that animals exposed directly and continuously to maximum concentrations of the Hayward experimental wastewater marsh effluent, at nearly 5 ppt salinity, yielded mortalities similar to those animals exposed to bay water at control sites, at 25 ppt. In almost all cases, animals with high mortalities at CNT site also showed high mortality at EXP. Subsequent, analogous tests at a range of additional, lower-salinity control sites (Kitting, 1996) and Mt. View Sanitary District’s wastewater McNabney Marsh near Martinez, CA, showed that survival of common fishes and invertebrates in these additional control and wastewater marshes is very high, with exceptions (in most marshes) due to periodic warm, calm weather and low oxygen.

Among the four fish species tested in both major (Hayward) sites, Pacific staghorn sculpin, *Leptocottus armatus*, and topsmelt, *A. affinis*, were the ones unlikely to withstand test confinements at either site. On the other hand, rainwater killifish, *L. parva*, showed high survival under these extreme conditions, even subjected to ~100% wastewater marsh effluent, as in maximum exposures used in other assays, e.g. MacKinlay and Buday (2002). High killifish survival suggests that killifish may play an important role in the colonization of a wastewater marsh. Threespine stickleback, *Gasterosteus aculeatus*, appeared to have intermediate tolerance of exposure in small enclosures, and may be a suitably sensitive bioassay species.

Better understanding of fish behaviors may be an important factor to consider in confinement experiments such as these. To some species, such as topsmelt, which requires large areas (Green, 1968), testing survival by confining individuals into 20-liter buckets evidently is not a suitable approach for such species.
Figure 1A and 1B. Summer field bioassays, where each paired enclosure began with 4-6 fish of each species, and 20 snails of each species. Control site is Figure A. Experimental Wastewater Marsh outflow is Figure B. The arrow at 8 days indicates the end of the comparison period (due to loss of control enclosures).
Figure 2A and 2B. Fall field bioassays, where each paired enclosure began with four threespine stickleback, ten rainwater killifish, two staghorn sculpin, one longjaw mudsucker goby, four *Palaemon* shrimp, plus (not shown with 95% survival over 3 months) twenty horn snails and twenty mudsnails.
Control site is Figure A. Experimental Wastewater Marsh outflow is Figure B.

Furthermore, interpretations of bioassay tests on juvenile fishes are difficult due to their high natural mortality rate, up to 99% during first 130 days of life (Bagenal and Braum, 1978), and high sensitivity to most pollutants (Johansen et al., 1985). Therefore, basic evidence for survival in bioassays under hypothetically extreme conditions in the field can be instructive. Field bioassays may integrate multiple field effects on aquatic animals, as ionic stress (Rogers et al., 2003) and dissolved organics (McGeer et al., 2002) can modify metal sensitivity in certain fishes, particularly likely in wastewater marshes. Yet bioassay survival merely shows that an animal tolerates those persistent conditions, while contaminants may be building up in these predators apparently attracted to high food concentrations, despite possible contaminants.

In general, the Hayward treated wastewater effluent does not seem to affect animals very differently than San Francisco Bay water. Hence, biological filtration by marsh soil and plants may indeed be a possible alternative to open-water waste disposal, to pursue. Yet the bay control sites themselves may be detrimental with prolonged exposure, possibly too saline for these estuarine species. Even water from a wastewater marsh, lowering salinities toward historic estuarine levels, may be as suitable as are high-saline conditions in this bay subjected to extreme freshwater diversion. Roughly 60% dilution with bay water showed no mortality in 1m² cages and also appeared to attract diverse animals to be more common than at reference sites, possibly due to dense zooplankton from the wastewater marsh. This marsh effluent has shown no other major short-term detriments compared with other marsh water, cautiously supporting filtration by marshes and future use of field bioassays. However, fish behavior might attract various estuarine species at least periodically, during tidal migrations, to concentrated food resources and low salinities, even if sites are contaminated or otherwise detrimental to some of those species during prolonged exposure to extreme environments.

Major longer-term advantages and disadvantages (Lau-Wong, 1990) of such techniques for constructing marsh habitats remain to be tested (Josselyn, 1989) as experimental marshes themselves continue to be developed while alternative sources for estuarine low salinities tend to be diverted from historic estuaries.
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References


Abstract

The aim of this work was to verify the effects of water soluble fraction (WSF) of diesel oil in histological features of pompanos fishes (*Trachinotus sp.*). The fishes were exposed to two concentrations of diesel WSF (2.5% and 7.5%), during 24 hours and 72 hours. The histological analysis showed increasing damages dose-dependent and time-dependent. The most common alterations were hyperplasia of the lamellar epithelium, cellular lifting, aneurysms and lamellar fusion. These alterations may play the role of organism defense, because these histological changes acts as a barrier that increase the distance between the toxicant and the bloodstream. However, the alterations may be very harmful to the fish community, once they can impair the respiratory or the osmoregulatory systems, and therefore increasing the risk of disease and death of the fishes.

Introduction

When we consider the marine regions, we can observe that the coastal regions are very touched by the human action. Tourism, transportation by a different kind of boats and the degradation of the coastal zones are responsible for the alterations of the marine community.
Different kinds of organic pollutants can be found in the coastal regions, and one has been very important in the last decades, is the hydrocarbons. The contamination by crude oil and by diesel oil are originated by spills or even others sources. Despite the fact that usually we cannot see spills of diesel, little and frequent accidents occur and are very harmful to the coastal zone. The chronic pollution at the ports, bays and places with great ship movements or without a good water circulation are very representatives of this affected environment.

In the composition of the diesel oil, we have a large amount of the toxic and low fractions of the hydrocarbons. These fractions are usually very soluble in seawater and may act on the different organs and tissues of marine animals, especially on the respiratory system of the fishes.

Fishes which are widely distributed in different habits are good indicators of the ambient health. In addition, many species are economically important for fishery communities. The low cost of maintenance and ready acceptance to the captivity make them suitable for the laboratory experiments (Bolis et al., 2001). Several parameters in fish health can be used as a sensitive indicator for the assessment of adverse aquatic pollutants effects. Numerous methods can be used to evaluation the water quality, and morphological, in special the histological, observations of the animal structures and its alterations can provide a good response (Bolis et al., 2001).

Even not being specific the effect of toxic substances on the cellular structure, is detectable at very low concentrations. Histological analysis may be very useful to the experimental or environmental studies (Wester et al., 2002).

The branchial system is very dynamic, and their cellular structure may respond in a short time to the environmental situations because they are in direct contact with the pollutant. The juvenile phase is often very sensitive to the environmental perturbations, and biological alterations at this life stage may produce damages at the population structure. This is very important, especially when we consider the species with economics value. In the Brazilian coast the genus, *Trachinotus* is fished all over the marine coast and have a good market price, so alterations in their population may affect the fisheries.

Therefore, the aim of this study is to verify the effects of the water-soluble fraction (WSF) of diesel oil, in the histology of gills of *Trachinotus sp.*
Material and Methods

The present work was conducted at Ubatuba, São Paulo State – Brazil, in the installations of Oceanographic Institute of São Paulo University. The fishes were collected in the summer, the best period for the catch of juveniles of pompanos. The capture was made by a beach seine, and after that, the fishes were acclimated in 500 l boxes for 7 days, with daily exchanged water. Fishes were allowed to feed once a day with commercial food.

Afterward the fishes were removed to laboratory conditions, with controlled temperature of 21 °C, for 2 days before the experiments. The water-soluble fraction (WSF) of diesel oil was produced by the method described by Anderson et al. (1974), and than diluted at the chosen concentrations (2.5% and 7.5%).

The fishes were transfered to glass aquariums with 8 liters of total volume (marine water and WSF), with aeration. After 24 and 72 hours fishes were sacrificed and had the gill removed. The gills were fixed in McDowell, included in historesin, cutted in sagital sections of ??m thickness and stained with Toluidin Blue and Fucsin. The material was photographed in a photomicroscope.

Results

The gill epithelium consisted of different cell types including pavement, mucous and pillar cells. Histological analysis permitted observe that common damages, like lifting of the epithelium of the secondary lamellae, hyperplasia lamellar, fusion of adjacent secondary lamellae, edema of subepithelial space and tumors. There were not homogeneous gill damage: areas with severe damage were often directly beside healthy areas.

The photograph analysis of the gill material, show us an effect dose-relation, so in general, we can see a greater damage at the highest concentration and exposure time. The area covered by the damage epithelium increase when we compare the two concentrations of 24 hs. of exposure, and also, when the comparisons is made between the same concentration at different times (Fig. 1).
Figure 1 – Gills alterations between the two different concentrations and different times. A) 2.5% of WSF in 24 hs exposition; B) 7.5% of WSF in 24 hs exposition; C) 2.5% of WSF in 72 hs exposition; and D) 7.5% of WSF in 72 hs exposition. (Toluidin & Fuscin, 128 x)

The alterations that we can find in the gills epithelium can be observed below (Fig. 2).
Fig. 2. Histological sections of gills with the most important alterations: A) 2.5% of WSF, 24 hs exposure (560x); B) 7.5% of WSF, 24 hs exposure (560x); C) 7.5% of WSF, 72 hs exposure (960x) and D) 7.5% of WSF, 72 hs exposure (480x). Pl = primary lamellae; Sl = secondary lamellae; m = mucous cell; ec = epithelial cell; pi = pillar cell; an = aneurysm; he = hyperplasia epithelial; el = epithelial lifting; t = tumour; es = epithelial separation; lf = lamellar fusion

Discussion

Analysis of the photos obtained with this preliminary study allow us to detect a gradual increase in the effects of the WSF of diesel oil in the histological structure of the gills of Trachinotus sp. The diesel oil is composed by a great variety of light elements, and their toxicity is reported in a large amount of literature.

Exposure to the polluted water is associated with the increase appearance of gill abnormalities, in most instances the literature conclude that this histological changes are symptomatic of physical or chemical stress and that such changes are not generally diagnostic of particular chemicals or mode of action (van den Heuvel et al., 2000).

Despite this fact, we can observe that the alterations are very close to that verified in others studies. Arellano et al. (1999), working with the effect of cooper in the Senegales Sole, concluded that certain alterations, like lifting, swelling and hyperplasia of the lamellar epithelium could serve as a defense function, because these histological changes could increase the distance across which waterborne irritants must diffuse to reach the bloodstream. Similar conclusion may be found in Karan et al. (1998), with carp exposed to copper sulfate; Poleksic and Karan (1999), with carp exposure at trifluralin; van den Heuvel et al. (2000), with yellow perch exposed to oil sands mining-associated waters and others.

In general, we can observe that the great part of anthropogenic pollutants is responsible by similar alteration in the gill tissue. However, the acute spill and especially the chronic released of diesel oil may be very harmful to the fish community, since those cellular changes in a long time may induce a irreversible
alteration, with damage to the respiratory and osmoregulatory systems. This may improve the risk of diseases and death of the animals.

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References


