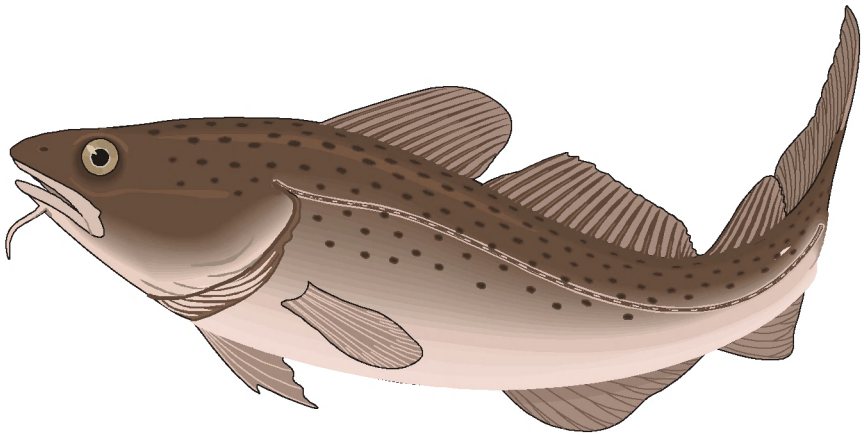
Fish Response to Toxic Environments

Chris Kennedy
Don MacKinlay



International Congress on the Biology of Fish
Towson University, Baltimore MD July 26-30, 1998

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SYMPOSIUM PROCEEDINGS

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PREFACE

Reasonable evidence exists to demonstrate that alteration of aquatic ecosystems is increasing, and is having a deleterious effect on aquatic resources such as fish. We have a vital interest in evaluating and understanding the effects of various environmental stressors on fish in a timely fashion, as current thought perceives the risks to be great. Fish can be affected by stressors through direct effects and by indirect effects on the systems which support them. This symposium proposes to bring together experts in piscine toxicology to highlight and communicate recent research in areas important to increasing our understanding of the impacts of environmental alterations on various aspects of fish biology

The examples of toxic environments discussed in this symposium included those contaminated with anthropogenic and natural sources of xenobiotics such as heavy metals, hydrocarbons, and pesticides, as well as environments of altered physical and chemical characteristics including temperature and oxygen. Several papers discuss some of the more new and novel methodologies in examining and assessing toxic response in fish to environmental insult. The approaches taken in these papers spans several levels of biological organization from the molecular, biochemical, physiological, population to the ecosystem level, to gain a more comprehensive understanding of the relationship between fish and their environments.

Symposium Organizers:

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CONGRESS ACKNOWLEDGEMENTS

This Symposium is part of the International Congress on the Biology of Fishes, whose main sponsors were Fisheries and Oceans Canada (DFO), and Towson University. The main organizers of the Congress, on behalf of the Physiology and Fish Culture Sections of the American Fisheries Society, were Don MacKinlay of DFO (overall chair, program and proceedings), Karin Howard (registration and accommodations) and Jay Nelson of Towson University (local arrangements). I would like to extend a sincere 'thank you' to the many contributors who took the time to prepare a written submission for these proceedings. Your efforts are very much appreciated.

Don MacKinlay
Congress Chair

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CELLULAR FUNCTION AND ENERGETIC DOWNREGULATION IN CULTURED RAINBOW TROUT GILL CELLS EXPOSED TO COPPER *IN VITRO*

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Although normally associated with respiratory gas exchange, the fish gill is multifunctional organ involved in osmoregulation and ion balance as well as acting as a barrier between the internal and external environments. Whilst chloride cells are particularly vulnerable to waterborne Cu^{2+}, which inactivates Na/K - ATPase, the functioning of the most abundant cell type in the gill lamellae of fresh water fish, namely pavement cells (95 % of total cell number), also known as the respiratory cells, is not well-known. Recently developed techniques have allowed these cells to be grown on permeable filters (Wood and Pärt, 1997), where they adopt a clear apical / basolateral orientation. Media, from the apical compartment, can then be substituted for water thus mimicing the gill epithelium *in vitro*. Sodium flux, which has been demonstrated in these cultures, and protein synthesis are both processes which account for significant proportions of the cellular energy budget and are also likely to be involved in the osmoregulatory / barrier properties of the gill cells. The aim of the present study was therefore to investigate the effect of waterborne copper on these aspects of gill cell physiology, within the context of the cellular energy budget.

Primary cultures were prepared from rainbow trout gills and, after an initial 5 days culture in conventional tissue culture flasks at 20°C, these cells were seeded onto permeable, 0.9 cm^2 tissue culture inserts held in 24 well tissue culture plates. Following the formation of an intact epithelial cell layer, Na - free water containing aqueous copper (25 or 751M), or Na - free water only, was added to the apical

surface, whilst L15 based culture media was maintained in the basolateral compartment and the cultures incubated for a further 18 hours. Cellular respiration was recorded by introducing an intact filter, plus the attached cells, into a calibrated Rank System 10 oxygen electrode chamber, containing L15 culture media, and continually monitoring the decline in PO_2 (Smith and Houlihan, 1995). The same cultures were then immersed in L15 containing 1.0 mM phenylalanine labelled with ^{3}H-phenylalanine at 1.0TCi ml^{-1} and protein synthesis rates calculated from the resulting specific radioactivity the free (unbound) and protein bound phenylalanine (Smith and Houlihan, 1995). In homologous filter cultures Na flux was measured by following the cellular uptake, and appearance in the apical compartment, of ^{22}Na added to the basolateral compartment. To investigate the energetics of these processes, these measurements were repeated with cultures treated (in the basolateral culture media) with specific inhibitors of protein synthesis (cycloheximide) and Na/K - ATPase (oubain). Energetic costs, were then calculated from the appropriate inhibitor induced changes and the corresponding decline in oxygen consumption.

Twenty five 1M Cu caused a reduction in oxygen consumption (with 75 1M having no further effect) whereas protein synthesis rates and intracellular sodium concentrations were all maintained at copper free levels (Fig.1.). The cost of protein synthesis was also unaffected by copper but a reduction in the cost of maintaining intracellular Na was observed in copper polluted cells (Fig.2.). The specificity of each inhibitor was confirmed and, from the differential reductions caused to oxygen consumption rates, protein synthesis was shown to account for approximately half of the energy used by Na/K - ATPase. Thus the ability to selectively downregulate ATP dependent Na flux results in the most significant energy saving, thereby providing the mechanism by which gill epithelial cells are able to tolerate a reduced respiratory function caused by sub lethal copper exposure. Furthermore, these data also suggest that maintenance of intracellular Na is achieved by channel arrest; the same mechanism which maintains plasma membrane potential during anoxic suppression and aestivation (Flanigan *et al*, 1993).

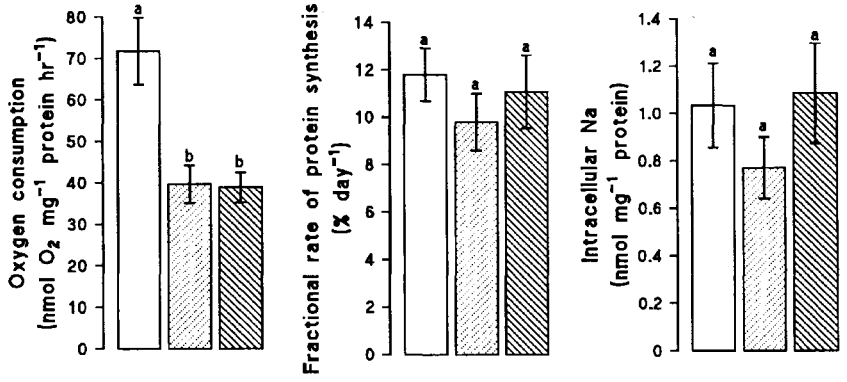

Figure 1. The effect of 251M (/) and 751M (\) Cu on oxygen consumption, protein synthesis and intracellular Na concentration of rainbow trout gill cells *in vitro*.

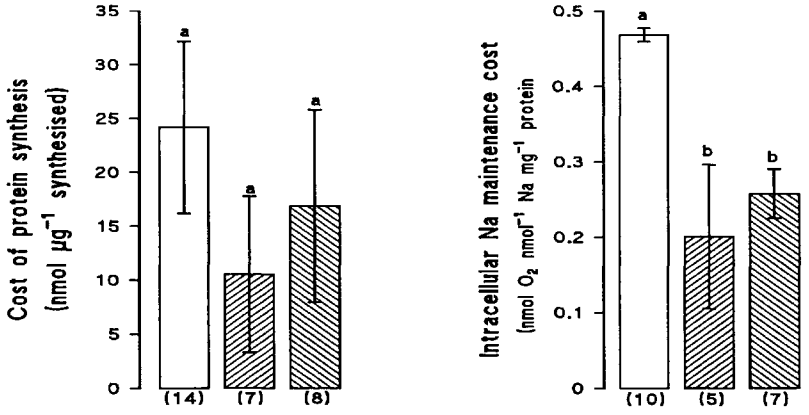

Figure 2. Costs of protein synthesis and the maintainance of intracellular Na in rainbow trout gill cells exposed to 251M (/) and 751 (\) Cu *in vitro*.

Acknowledgment

This study was supported by The European Commission DG XII, "Environmental and Climatic Change".

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USE OF CRITICAL THERMAL METHODOLOGY AS A BIOASSAY FOR FISHES

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The temperature tolerance zone represents the thermal arena within which individuals of a species can operate. Consequently, quantifying and determining the effects of environmental factors on the limits of this arena are important. In the Critical Thermal Methodology (CTM), a laboratory method to quantify temperature tolerance, a random sample of fish is subjected to a constant, linear change in temperature until a predefined sublethal, but near lethal endpoint is reached. The endpoint, either the CTminimum or CTmaximum, is the pre-death thermal point at which locomotory movements become disorganized and a fish loses its ability to escape from conditions that may lead to its death. The endpoints in this bioassay, estimates of lower and upper temperature tolerance limits, are unambiguous, biologically defensible and important attributes of an individual. The CTM is rapid, easy, requires few fish and approximates natural conditions better than static temperature tolerance methods.

We have employed the CTM to (1) estimate the upper and/or lower temperature tolerance of >15 fish species, (2) determine the effects of cycling temperature, oxygen concentration and reproductive stress on temperature tolerance, (3)

measure rates of heat gain and loss during tolerance acclimation, (4) distinguish among ecotypic field populations and (5) bioassay the effects of toxicants on temperature tolerance of fishes.

Via the CTM we have bioassayed the effects of seven inorganic and organic chemicals on the temperature tolerances of fishes (Table 1). In two cases, no adverse effects were observed. Mean CTmaxima of red shiners exposed to 1 and 30 mg L^{-1} of the aquatic herbicide endothal are not different than controls. One mg L^{-1} is within, and 30 mg L^{-1} is six times the recommended endothal application. Similarly acetone (a carrier solvent) at 0.76µg L^{-1} did not effect either CTmaxima or CTminima of larval fathead minnows. Results with cadmium differed interspecifically. Three species were exposed to 96-h LC_5, LC_{10} and LC_{20} concentrations, and tested after 1, 5 and 10 days of cadmium exposure. Although mean CTmaxima of green sunfish were not effected, cadmium caused significant decreases in the mean CTmaxima of both red shiners and fathead minnows.

Table 1. Influence of various chemicals on CTmax and/or CTmin of fishes.

Chemical	Species	Effect	Reference
Endothal	Cyprinella lutrensis	CTmax uneffected at two exposure concentrations	Takle et al. 1983
Selenium	Pimephales promelas	CTmax (C) = 35.20 - 0.0016 (mg Se L^{-1})	Watenpaugh & Beitinger 1985
Nitrite	Ictalurus punctatus	CTmax (C) = 38.12 - 1.44 (mg NO_2 L^{-1})	Watenpaugh et al. 1985
Cadmium	Lepomis cyanellus	CTmax not effected by concentrations as high as 5.17 mg L^{-1} for 10 days	Carrier & Beitinger 1985a
Cadmium	Cyprinella lutrensis	CTmax decreased by concentrations as low as LC_5 and exposure times of 1-10 days	Carrier & Beitinger 1985b
Cadmium	Pimephales promelas	CTmax decreased by concentrations as low as LC_{10} and exposure times of 1-10 days	Carrier & Beitinger 1985b
Acetone	Pimephales promelas	Neither CTmax nor CTmin were effected at 0.76 ug L^{-1}	Heath et al. 1994
Cyfluthrin	Pimephales promelas	Tolerance zone decreased by 30%	Heath et al. 1994
Copper	Pimephales promelas	Inverse relationship between CTmax and copper at three acclimation temperatures	Richards & Beitinger 1995

Exposure-effect relationships between CTmaxima and sublethal concentrations of nitrite and selenate-selenium occurred in channel catfish and fathead minnows, respectively. Mean CTmaximum of fatheads at the highest selenate concentration was nearly 6 C below that of controls. The zone of temperature tolerance of larval fathead minnows exposed for 24-h to cyfluthrin (synthetic pyrethroid) was decreased by 30%. Cyfluthrin concentrations causing significant effects were as low as 170 parts-per-trillion. Finally, acute sublethal exposure to copper significantly decreased CTmaxima of fathead minnows at three of the four acclimation temperatures tested.

In summary, the endpoint of a CTM test, i.e., ecological death, is more obvious, dramatic and easier to interpret than other types of bioassays in which a behavioral or physiological process is used as a biomarker. The standard deviations of all 17 control groups were less than 4% of their respective CTmaximum mean, making it easy to detect statistically significant changes in mean CTmaximum, which may or may not be ecologically important. Temperature tolerance was adversely effected in significant "dose - response" relationships at concentrations less than those directly responsible for death in several of the chemicals studied. Also, some chemicals, e.g., selenate, nitrite, cadmium and copper, not only decreased mean CTmaximum but also increased variation at higher concentrations. The results of these studies confirm that the CTM is a sensitive, simple, rapid and inexpensive indirect method to bioassay the potential effects of water-borne chemical on fishes.

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BOTH CONTAMINANTS AND HABITAT LIMIT NEOSHO MADTOM *(NOTURUS PLACIDUS)* NUMBERS IN THE SPRING RIVER, A MIDWESTERN WARMWATER STREAM EFFECTED BY RUNOFF FROM HISTORIC ZINC AND LEAD MINING

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Many studies have implicated water chemistry along with physical habitat factors as important to understanding the distribution and abundance of stream fishes (e.g., Maret et al., 1997). Other studies have implicated mining contaminants as factors limiting stream fish populations (e.g., McCormick et al., 1994). Yet, refereed scientific literature contain no studies that simultaneously address effects of both habitat quality and environmental contamination on stream fish communities.

The Federally-listed threatened Neosho madtom (*Noturus placidus*) is a small ictalurid (generally < 75 mm total length) found in unconsolidated pebble and gravel with moderate to slow flow, and moderate depths (Moss, 1983). Currently, Neosho madtoms are found in mainstems of the Neosho, Cottonwood, and Spring rivers in Kansas, Missouri, and Oklahoma. Spring River and its tributaries drain remnants of the Tri-State Mining District where lead (Pb)-zinc (Zn) mining occurred (Barks, 1977). Spring River and its tributaries have elevated levels of cadmium (Cd), Pb, and Zn from abandoned mines and weathering of tailings piles (Barks, 1977). Neosho madtom population densities are much greater in the Neosho River system than in the Spring River (Wilkinson et al., 1996). The Spring River reach where the great majority of Spring River Neosho madtoms are found is upstream of the primary

sources of mining-derived pollution in the Spring River (Barks, 1977). The primary objective of this paper is an integrated evaluation of natural and anthropogenic factors that may be limiting populations and densities of riffle-dwelling benthic fishes in the Spring River with emphasis on the Federally-listed threatened Neosho madtom.

Our basic approach was to quantitatively characterize the riffle environment of and aquatic communities found with the Neosho madtom in the summer during daylight in the Neosho River system, where no mining has occurred, for use as a baseline to which the mining-affected Spring River could be compared. We used an empirical model based on physical habitat, water chemistry, and nutrient measurements from the Neosho River system in 1991 to predict species distribution of the Neosho, Cottonwood, and Spring rivers in 1994. Our comparison of 1994 measurements collected from the Neosho River and Spring River systems and of 1994 predicted and observed Neosho madtom distributions allowed us to assess the extent to which basic environmental quality and/or metals contamination limited Neosho madtom distribution in the Spring River. Along with Neosho madtom densities, our measurements included: 1) aquatic community; 2) physical habitat; 3) water chemistry; 4) nutrients; and 5) metals in surface and pore waters, invertebrates, or both.

We analyzed the data at the level of site averages to assess differences between the Neosho-Cottonwood River and the Spring River systems and among sites within the Spring River with and without Neosho madtoms. We calculated site densities of Neosho madtoms and, as a group, the riffle-dwelling fishes that could be considered benthic fish competitors to Neosho madtoms by dividing the total number of Neosho madtoms or benthic fish competitors collected at a site by the total area sampled with the kick seine. Determination of the list of benthic fish competitors was based on spatial orientations and feeding habitats of each species as described by Plieger (1975). The statistical methods used included analysis of variance, correlation analysis, multivariate analysis of variance, principal components analysis, and discriminant analysis.

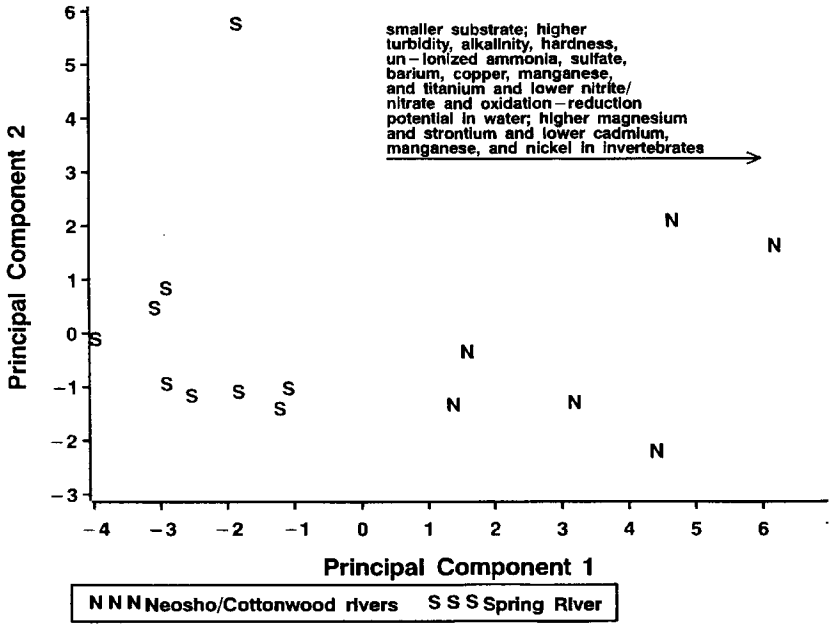

Figure 1:
Higher Neosho madtom densities, smaller substrate, and lower concentrations of Cd and Pb in benthic invertebrates in the Neosho-Cottonwood River system than in the Spring River system suggest that differences in Neosho madtom densities are due both to differences in habitat and contaminants.

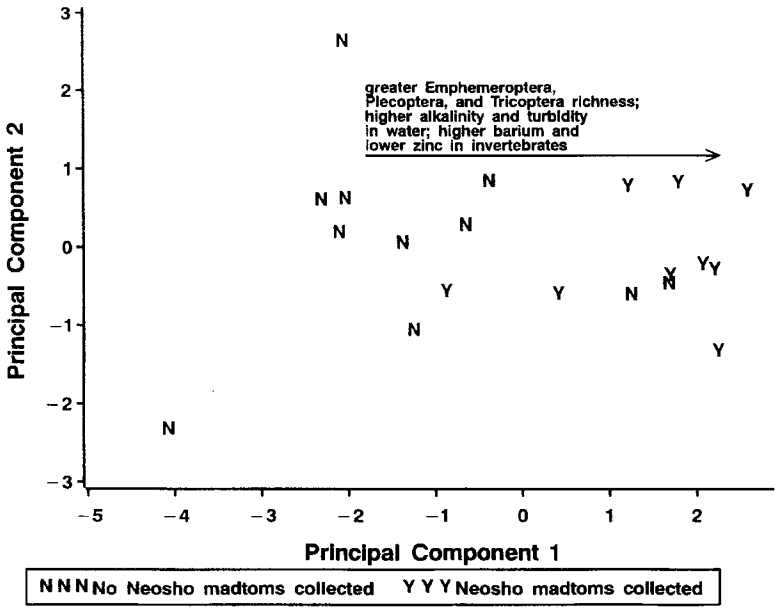

Figure 2:
Hypothesized effects of contaminants on Neosho madtom densities were further supported by higher fish densities and species richness, no substrate differences, higher benthic invertebrate taxa richness, and lower benthic invertebrate concentrations of Cd and Zn at sites with Neosho madtoms than at sites without in the Spring River.

Through this study we have shown that an integrated approach that includes assessment of both natural and anthropogenic factors is necessary to effectively estimate the level to which anthropogenic factors influence fish populations and communities. Our investigation documented that fishes of the Spring River, the Neosho madtom in particular, may be limited not only by the presence of Pb, Zn, and Cd in both water and benthic invertebrate food sources as a result of runoff from historic Pb-Zn mining sites but also by basic differences in water chemistry, nutrients, and available physical habitat. Our results also indicate that competition between Neosho madtoms and other fishes probably does not limit the Spring River Neosho madtom population, but lower food (i.e., benthic invertebrates) abundance at sites without Neosho madtoms, possibly as a result of metals contamination, may be limiting.

Acknowledgments

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ENVIRONMENTAL INFLUENCES ON FISH METABOLIC SCOPE:

BEHAVIOURAL ADAPTATIONS

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Introduction

To analyse the environmental constraints and their consequences on fish energetics and behaviour, one method consists in monitoring the metabolic expenditure of free ranging animals. In spite of the difficulties inherent to this approach, successful attempts have been made using various telemetry techniques. However, although valuable information can be gained from *in situ* evaluations of routine energy expenditure, the full explanatory power allowed by this approach is achieved only if the metabolic scope of the animal is simultaneously taken into consideration. The most important problem facing an animal trying to survive in an heterogenous environment is indeed to attain the power output necessary to live in its selected niche, while operating well below its maximum power rating (Priede, 1977, 1985). As a contribution to the understanding of the environmental influences on metabolism and their potential ecological consequences, we modelled the impact of water temperature and oxygenation on the metabolic scope of 3 marine species, the European sea bass (*Dicentrarchus labrax*), the Atlantic cod (*Gadus morhua*) and the European sole (*Solea solea*). In the sea bass, the results of the experimental and modelling procedures were then used to pre-examine the possibility that fish may behaviourally optimise their aerobic metabolic capacity.

Materials and methods

Respirometry

Fish of both sexes were acclimated to 10, 15, 20 and 25 °C (bass), to 2, 5 and 10° C (cod) and 4, 8, 12, 16 , 20 and 24 °C (sole). Fasted fish (sole) or groups of 3-5 fish (bass and cod) were successively introduced in a respirometer chamber (sole, 4 l; bass and cod, 240 l) supplied with fully aerated 28-30 ‰ sea water. Fish routine metabolic rate (RMR) was measured in normoxic condition and during stepped decreases in ambient oxygen concentration (decrement 10 % down to 15 % air saturation). On occasion, fish were fed and/or chased to exhaustion prior to hypoxia. Standard or resting metabolic rate (SMR) was estimated as the minimal RMR measured during the night where fish tended to remain motionless on the bottom of the respirometer.

Behavioural study

Experiments were carried out in a 35 m^3 indoor tank in which the temperature and oxygenation conditions were accurately controlled. Water temperature and oxygen content were monitored using a custom built computer-controlled water sampler which hourly sampled 14 points located at different positions and depths. Three sea bass were simultaneously tagged with ultrasonic pressure transmitters (Vemco V16P). Transmitters were placed in an anterio-ventral position as described in Claireaux and Lefrançois (1998). The pulse rate of the acoustic tags was linearly related to the hydrostatic pressure (depth). Transmitters had a specific frequency and were sequentially monitored for 5 measurements each (approx. 5 s) during 2-3 weeks. Fish vertical distribution was analysed in relation with the thermal and oxygenation characteristics of the water column and their potential impact on metabolic scope.

Results

In all three species tested, the influence of water temperature and oxygenation on aerobic metabolism took place within a general framework (Figure 1) which we delineated through a simple analytical procedure.

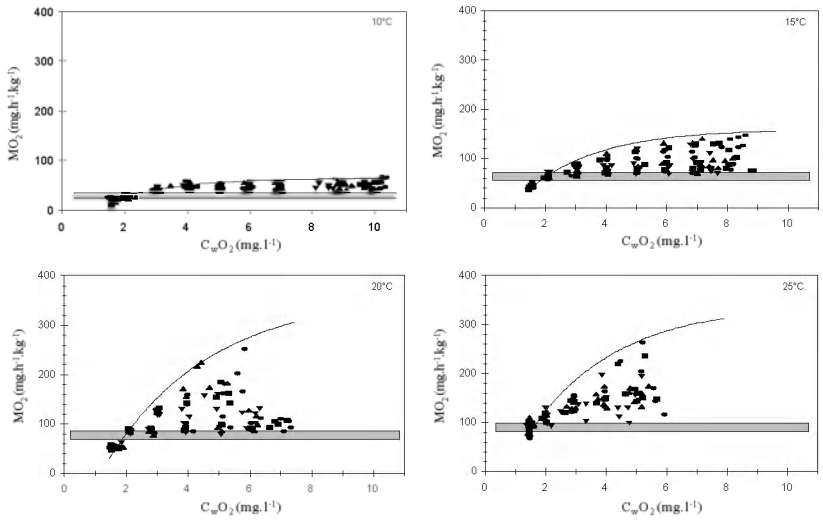

Figure 1. Sea bass routine metabolic rate in various oxygenation and temperature conditions. Solid lines: limiting oxygen concentration curves (LOC-curves; equation 2), shaded area standard metabolic rate.

In the first place, fish standard metabolic rate (SMR; shaded area on Figure 1) was modelled as a function of ambient temperature using the following equation:

$$SMR = Y_3 (1\text{-}e^{(\alpha_3 T^{\beta 3})}). \quad (1)$$

At each experimental temperature, we then established the relationship between the maximum rate of oxygen uptake measured (MO_{2max}) and the ambient oxygenation level (solid line on Figure 1). This relationship (LOC-curve), which indicates the limiting (critical) oxygen concentration for any given rate of oxygen consumption was modelled using the following equation:

$$MO_{2max} = Y_1 (1\text{-}e^{(\alpha_1 C_W O_2 + \beta_1)}) \quad (2)$$

The asymptote of the LOC-curve being the largest aerobic energy expenditure in normoxia, it corresponds to the active metabolic rate (AMR) of the species

at the temperature considered. AMR was then modelled as a function of temperature using the following formula:

$$Y_1 = \alpha_2 T^{(\beta_2 T+\delta_2)}+\varepsilon_2. \quad (3)$$

α_1 and β_1 showing no correlation with temperature, they were substituted with their averaged values (α_m and β_m). Equation 2 then became:

$$MO_{2max} = (\alpha_2 T^{(\beta_2 T+\delta_2)}+\varepsilon_2)(1-e^{(\alpha_m C_W C_2+\beta_m)}). \quad (4)$$

Finally, fish metabolic scope (MS) was modelled as a function of ambient temperature and oxygenation using the following equations (equation 4 - equation 1):

$$MS = ((\alpha_2 T^{(\beta_2 T+\delta_2)}+\varepsilon_2)(1-e^{(\alpha_m C_W C_2+\beta_m)}))-(Y_3 (1-e^{(\alpha_3 T^{\beta_3})})). \quad (5)$$

As an example, Figure 2 is a 3D representation of equations 4 and 5 for Atlantic cod.

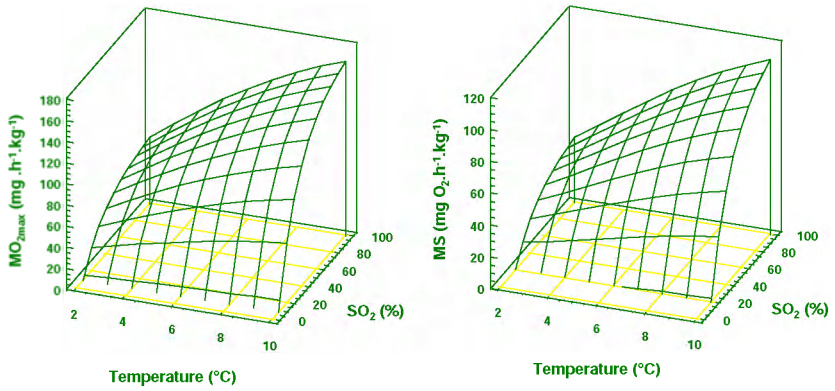

Figure 2. Left panel- 3D representation of the relationship between Atlantic cod maximum oxygen consumption (MO_2max), temperature and oxygen saturation (equation 4). Right panel- 3D representation of the relationship between Atlantic cod metabolic scope, temperature and oxygen saturation (equation 5).

Concurrently, the behavioural study showed that when faced with a stratified water column, telemetered sea bass displayed a great ability in adjusting their distribution pattern according to the environmental constraints imposed. Based on equation 5 and using the hourly oxygen and temperature profiles, we were able to map the experimental tank in terms of potential metabolic scope. The comparison of these maps with bass successive positions suggests that the observed changes in vertical distribution do contribute to the optimisation of their aerobic metabolic capacity (Figure 3).

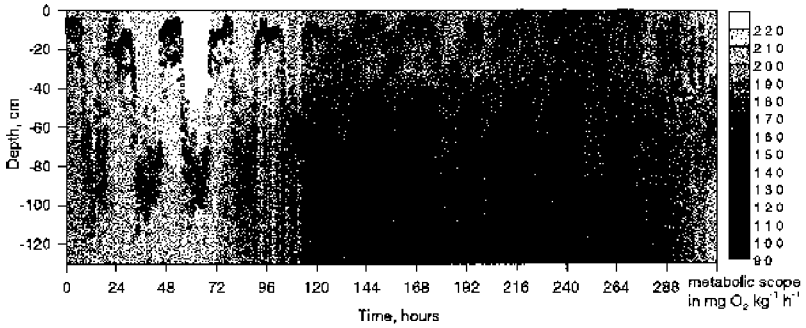

Figure 3. Vertical movements of a sea bass facing stratified and fluctuating thermal and oxygenation conditions. The colored background gives the metabolic scope of the animal as calculated using equation 5.

Conclusion

The experiments briefly presented above were design to fit within the context of the concept developed by Fry (1971) and later revisited by Neill *et al.* (1994). The followed experimental and analytical approaches allowed us to determine the standard and maximum metabolic rates of 3 ecologically distinct species in various combinations of temperature and oxygenation conditions. Through the modelling of the relationship between MO_2max and oxygen saturation, this procedure allowed the evaluation of fish active metabolic rate and the modelling of metabolic scope as a function of the 2 environmental factors tested. The experimental results presented herein also provided some insights on the energetic interactions which govern the relationships between coastal species and their environment. The strength of the constraints exerted by the environmental physico-chemical conditions on fish scope for activity is particularly crucial if one keeps in view that all things considered, the

magnitude of the metabolic potential is negatively related to mortality risk (Priede, 1977). Accordingly, the preliminary results presented here do suggest that through adaptative behavioural responses, sea bass optimise their aerobic metabolic scope, reducing energy budgeting conflicts and presumably increasing the probability of routinely operating away from lethal boundaries (Priede, 1985).

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MODELLING TOLERANCE TO TOXICANTS –

A COMPARISON OF FATHEAD MINNOWS AND RAINBOW TROUT

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Introduction

Toxicity testing on multiple species is often required because it is widely recognized that species have differential tolerances to toxicants, i.e. there is no one species that is universally sensitive to all toxicants. Two such species that are in common use and illustrate this principle are rainbow trout and fathead minnow. For example, fathead minnows are much more sensitive to low pH than rainbow trout but more tolerant of ammonia (Hickie *et al*, 1993; Thurston *et al*, 1983a; Thurston & Russo, 1983b). Thus the objective of our study was to understand the origin of this differential toxicity. Our approach was to examine a variety of physiological functions that may be correlated with the mechanism of toxicity of each toxin. For low pH, which acts by disrupting gill function, we examined differences between the two species in iono-regulation, focussing on the role of the gills, e.g. gill electrolyte permeability, ion uptake kinetics and regulation of ion balance under routine and challenge conditions. For ammonia, whose mechanism is thought to be internal and non-specific (i.e. no particular internal target tissue), we focussed on gill non-electrolyte permeability, cellular tolerance and detoxification.

Methods

Rainbow trout (RBT) were used as juveniles and fathead minnows (FHM) as adults to ensure a similar size range (1 to 3 g). Techniques for measuring

unidirectional and net fluxes of electrolytes and non-electrolytes, whole body and tissue levels under routine conditions, and in response to a variety of acute and chronic environmental challenges or internal dosing by intra-peritoneal (IP) injection was adapted from procedures outlined in Gonzalez and McDonald (1992), McDonald and Milligan (1997) and Linton *et al* (1997).

Ionoregulatory challenges consisted of the following: acute exposure to low Ca^{2+}, 400 ppb Cu in high and low Ca^{2+}, pH 4.1 in high Ca^{2+}, acclimation to low Ca^{2+}, acute exposure to pH 5.2 after low Ca^{2+} acclimation, confinement stress, osmotic shock and chronic temperature change. Challenges related to ammonia tolerance consisted of the following: exposure to high external ammonia, ammonia and monochlorobenzene dosing by IP, and uptake of radiolabelled urea and ethanol.

Results

Correlates of low pH tolerance

Overall, the gill surface-active challenges to ion balance (Cu, low pH, and low Ca^{2+}) had a greater acute impact on FHM than RBT as reflected in net Na^{+} losses (Table 1). However, Cu and low pH had greater effects on FHM only when combined with low external Ca^{2+} (Table 1). After two weeks of low Ca^{2+} exposure, both species had largely recovered ionic homeostasis although FHM exhibited a slight but significant depression in whole body Na^{+}. When both were acutely exposed to pH 5.2, FHM exhibited greater Na^{+} losses than RBT (Table 1).

TABLE 1: The effects of acute gill surface active challenges on net Na^+ losses in FHM and RBT expressed as % whole body Na h^{-1}. Values are means ± SEM. Challenges were 4h duration. Softwater (SW) = external Ca^{2+} of ~ 50 μeq L^{-1}. Hardwater (HW) = Ca^{2+} > 1000 μeq L^{-1}. Significant differences between species for each challenge are indicated by an *.

Acute Challenge	RBT	FHM
SW exposure	4.8 ± 1.4 % h^{-1}	7.8 ± 1.3 % h^{-1}
400 ppb Cu HW	8.6 ± 1.1 % h^{-1}	10.5 ± 1.4 % h^{-1}
400 ppb Cu SW	9.7 ± 2.0 % h^{-1}	18.7 ± 2.5 % h^{-1}*
pH 4.1, HW	6.5 ± 1.5 % h^{-1}	7.9 ± 3.0 % h^{-1}
pH 5.2 SW (SW acclimated)	1.4 ± 0.4 % h^{-1}	2.8 ± 0.3 % h^{-1}*

The other acute challenges to ion balance had even greater impact on FHM relative to RBT. Confinement stress for 4h provoked a 35% greater loss of Na^+ in FHM than RBT and substantially greater mortality (36 *vs* 12 %, FHM *vs* RBT). Similarly, exposure to 260 mM NaCl for 2h caused a 3 fold higher gain in Na^+ in FHM and much greater mortality (0 *vs* 100%).

Recovery from non-lethal challenges was also more prolonged in FHM. While both species recovered whole body ion balance after confinement stress, RBT significantly up-regulated Na^+ uptake during recovery while FHM did not. Similarly, FHM exhibited less regulation of Na^+ uptake and slower recovery after chronic temperature change.

Under routine conditions there were also significant differences in ionoregulation between the two species. Whole body Na^+ content was the same in both species and both were in Na^+ balance, i.e. $J_{in} = J_{out}$. However, Na^+ turnover, estimated from routine J_{in}^{Na}, was higher in FHM (22 *vs* 14 % day^{-1}).

Correlates of ammonia tolerance

Under routine feeding or fasting conditions, FHM had approximately 2 fold higher whole body ammonia levels and 30% lower ammonia excretion rates than RBT. When exposed to elevated ammonia in the water, tissue ammonia levels increase linearly in proportion to $[Am]_{ext}$ in both species with a slightly higher slope in FHM. Under these conditions, ammonia and urea excretion rates in FHM remained lower than in RBT. Rates of glutamine synthesis (an ammonia detoxifying mechanism) were similar in both species and unaffected by elevated $[AM]_{ext}$. Despite these similarities, ammonia loading by IP injection had a much greater impact on RBT. Time to 50% mortality (ET_{50}) in response to an ammonia dose of 15 $\mu M\ g^{-1}$ was 13.7± 10 min. In contrast, there were no mortalities in FHM at this dose, and by 4h 83% of the IP load had been excreted. Even at a 20 $\mu M\ g^{-1}$ dose there were no mortalities in FHM. At lower doses, where RBT survived, the rates of clearance of the ammonia loads were similar between the two species.

FHM were also more tolerant of IP injected doses of monochlorobenzene. For RBT, the ET_{50} in response to a 20 $\mu l\ g^{-1}$ dose was 1.3 ± 1.2 h. For FHM it was 4.1 ± 0.4 h. Also, FHM were significantly less permeable to two non-electrolytes, ethanol and urea.

Conclusions

The greater sensitivity of FHM to low pH is possibly related to one or more of the following properties of its ionoregulatory mechanism: intrinsically higher gill electrolyte permeability, less permeability control in the face of mild and severe electrolyte losses, a greater dependence on external Ca^{2+} for permeability control, greater cell shrinkage and slower, less effective cell volume regulation in the gills in response to osmotic shock and/or a lower capacity to up regulate gill Na^{+} transport in response to ion loss.

In contrast, the greater tolerance of FHM to ammonia appears to be primarily related to greater cellular tolerance rather than either lower gill permeability or detoxification. Furthermore, this tolerance is not confined to ammonia only, but includes the non-specific internal toxicant, monochlorobenzene. Nonetheless,

FHM exhibit lower gill permeability to other non-electrolytes which may convey an increased resistance to other internally acting toxicants.

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PHYSIOLOGICAL AND BEHAVIORAL MEASURES OF NEUROTOXICITY IN RAINBOW TROUT

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Neurotoxicant exposures can alter physiological functions resulting in subsequent abnormal behavioral patterns. For example, organophosphate insecticides (OPs) inhibit the enzyme, acetylcholinesterase, resulting in increased concentration of the neurotransmitter, acetylcholine, and overstimulation of cholinergic pathways. These events cause a number of physiological effects that can result in subsequent aberrant behavioral responses. Several physiological endpoints can be measured in this pathway such as cholinesterase activity (ChE), and the number and affinity of cholinergic receptors. A decrease in muscarinic cholinergic receptor (MChR) number reflects an adaptation by the cell to the increased neurotransmitter concentration. This study was designed to demonstrate correlations between changes in measurable cholinergic endpoints and resulting behavioral responses in aquatic organisms exposed to sublethal concentrations of two OP insecticides.

Larval rainbow trout (RBT) were exposed to the OPs, diazinon and malathion, in static renewal experiments. Behavioral measurements were taken at 24 and 96h of exposure and after 48h of recovery. RBT were videotaped individually using a motion analysis system to assess four locomotory behaviors, including swimming speed and swimming distance. Brain tissue from these fish was then used to measure the physiological endpoints, ChE and MChR number and affinity.

Cholinesterase activity was significantly decreased by exposure to both chemicals, with malathion exposure causing a greater effect. There was no significant effect on MChR number or affinity for either chemical, but there was a concentration-dependent trend of decrease in receptor number for malathion. Longer exposure durations may be needed for downregulation, or decrease in receptor number, to occur under this protocol. There were significant correlations between ChE activity and swimming speed for both chemical exposures and for ChE activity and swimming distance for malathion exposed animals. These results suggest that correlations between physiological and behavioral changes previously seen in mammals also occur in fish.

Acknowledgements

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EFFECTS OF TEMPERATURE ON THE BILIARY EXCRETION OF BENZO[A]PYRENE IN RAINBOW TROUT, ONCORHYNCHUS MYKISS

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Abstract

The excretion kinetics of [^{3}H]-benzo[a]pyrene (BaP) in rainbow trout were examined by toxicokinetic modelling in animals acclimated to 8 or 18 ° C and exposed at 8 or 18° C, following an intra-arterial injection of BaP *via* a dorsal aortic cannula. The tissue distribution of the parent compund and its metabolites was determined by HPLC and liquid scintillation chromatography. Blood concentration-time curve data were fit to an 2-compartment open toxicokinetic model and toxicokinetic parameters such as half-lives and total body clearance were determined from best-fit curves. A high degree of temperature compensation of BaP excretion was observed, i.e. animals acclimated to 8 or 18° C showed similar half-lives of BaP in blood. Acute temperature shifts resulted in changes in the half-life and total body clearance of BaP. There were no significant differences in the tissue distribution of BaP-derived radioactivity among the different test groups. Based on these results, it is postulated that the mechanisms of biliary excretion in aquatic ectotherms, such as rainbow trout, are adapted to compensate for intermediate-term changes in temperature in order to maintain xeniobiotic clearance capacity.

Introduction

As ectotherms, teleosts have adapted to their thermal environments by developing a host of physiological and biochemical alterations associated with acute and seasonal temperature change (Hochachka and Somero 1984). As well as altering the routine physiology and metabolism of the animal, adjustments to temperature can have a modifying influence on many events during the interaction between xenobiotic and organism. Phases in this process include: the exposure phase (initiation of interaction), the toxicokinetic phase (uptake, biotransformation, distribution and excretion of xenobiotic) and the toxicodynamic phase (xenobiotic-receptor interaction). Modification of xenobiotic-organism interaction at any one of these phases may alter the toxicity of the xenobiotic (Jimenez and Stegeman 1990). In order to investigate how temperature modifies the basic physiological mechanisms underlying the toxicokinetic phase of chemical carcinogenesis, researchers have employed the use of a model compound, benzo[a]pyrene (BaP), in studies involving whole-organism and isolated cell cultures from aquatic ectotherms such as fish. Investigators have determined that an acute increase in temperature can lead to an elevation in the rate of xenobiotic uptake due to increases in ventilation rate and in fluidity of the plasma membrane of the gill cells of the Gulf Toadfish, *Opsanus beta*. (Kennedy *et al.*, 1989a; Kennedy and Walsh, 1994). In addition, several studies have demonstrated temperature compensation in xenobiotic metabolism, such that biotransformation rates in animals acclimated to different temperatures are similar if they are exposed to the xenobiotic at their respective acclimation temperatures (Curtis *et al.*, 1990). However, acute temperature change appears to significantly alter xenobiotic metabolism (Kennedy *et al.*, 1989b). Increased temperature may also increase the rate of excretion of xenobiotics as well (Curtis 1983; Jimenez *et al.* 1987). However, these results are not conclusive due to the complicating effects of temperature on xenobiotic uptake, which had not been eliminated in these experiments.

The purpose of this study was to continue investigations into the effects of temperature on the toxciokinetics of xenobiotics in fish; specifically, to examine the effects of temperature on the excretion of benzo[a]pyrene. Information from these studies will be useful because they will provide information as to how an extremely important environmental variable, temperature, modifies the effects of xenobiotic exposure by altering the excretion phase of toxicokinetics.

Materials and methods

Rainbow trout, *Oncorhynchus mykiss*, were acclimated to 8 or 18° C for six weeks prior to an experiment. After dorsal aorta cannulation and recovery, animals were dosed intra-arterially with 5 mg/kg [^{3}H]-Benzo[a]pyrene (approximately 1 μCi) at a temperature of 8 or 18° C. Blood was sampled serially for 48 h and each sample was analysed for parent compound, benzo[a]pyrene, by reverse phase HPLC (Kennedy and Law 1990). Blood concentration-time curves were fitted to an open two-compartment pharmacokinetic model by non-linear least-squares regression using the computer application PCNONLIN (Metzler *et al.*, 1974; Metzler and Weiner, 1986, Kennedy and Law, 1990) and using the formula:

$$C=\sum_{i=1}^{n} A_i e^{-x_i t}$$

Bile was analysed by HPLC for Phase I metabolites and for conjugated metabolites following enzymatic digestion and acid hydrolysis with solvent extraction. Tissues were removed, homogenised, oxidised, and counted for total radioactivity by LSC.

Results and Discussion

The pharmokinetic modelling of the effects of temperature on excretion was performed in order to determine which aspects of excretion may be thermally modulated. A schematic representation of the assumed open two-compartment toxicokinetic model from which the toxicokinetic parameters were derived is shown in Fig. 1.

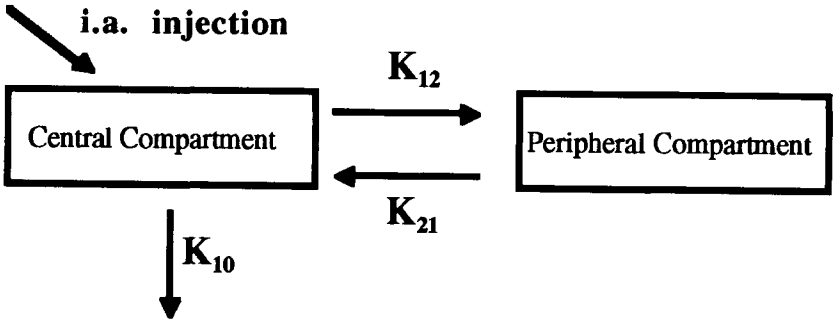

Fig. 1 Schematic representation of a two compartment model consisting of a central and peripheral compartment used to describe the diposition of BaP in rainbow trout after intra-arterial administration (Gibaldi and Perrier, 1975)

The two-compartments pictured are the central compartment, composed of the circulatory system, and the peripheral compartment which is composed of all of the other tissues in the animal.

Toxicokinetic parameters determined included: elimination rate constants; $K_{12}(min^{-1})$, from central to peripheral compartment, $K_{21}(min^{-1})$ from peripheral to central compartment, $K_{10}(min^{-1})$ the from central compartment to excretion; the area under the curve AUC (μg min/ml), used to determine the plasma clearance; and the total body clearance, Q_b (ml/min)

BaP concentration in blood declined biphasically with time (Fig.2) and toxicokinetic parameters were derived from the fitted regression curve (Table I).

Animals acclimated and exposed at the same temperature appeared to show similar terminal half-lives (X_i hl) and total body clearance rates (Q_b) of unchanged parent compound from the blood as can been seen from the parameters determined from an open two-compartment pharmacokinetic model.

However, differences were seen in these parameters in the animals which were subjected to an acute temperature change at the time of BaP administration. Animals acclimated at 8° C and exposed at 18° C showed an increase in clearance rates and a decrease in terminal half-lives of unchanged parent compound. Whereas, animals acclimated to 18° C and exposed at 8° C demonstrated larger terminal half-lives and a decrease in total body clearance.

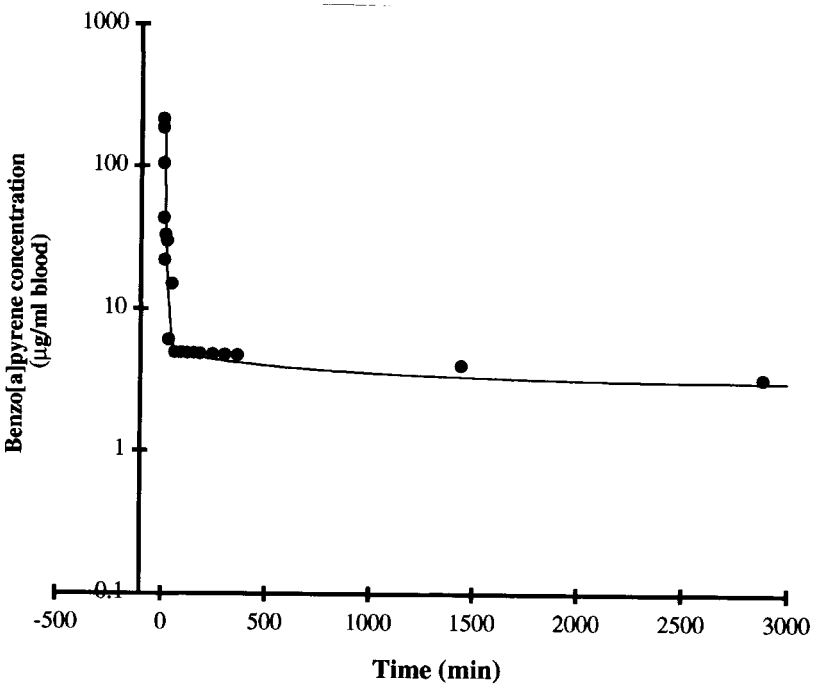

Fig. 2 Time course of unchanged BaP concentrations in the blood of rainbow trout after a dose of 10 mg/kg. Line represents the model-dependant curve derived from the toxicokinetic parameters for a typical exposure.

The results obtained in this study are contradictory to previous findings (Curtis 1990) which demonstrated an increase in excretion rates in animals acclimated and exposed to warmer temperatures when compared to animals exposed and acclimated to colder temperatures. However, in those studies, administration of

xenobiotic was by intraperitoneal injection, which may cause the uptake of xenobiotic to be affected by the exposure temperature. The method of intra-arterial administration *via* dorsal aorta cannula used in this experiment precludes this possibilty and it is therefore a more reliable measure of the excretion rate of BaP.

Two-Compartment Model Parameters
Acclimation/exposure temperature

Parameters	8/8° C	8/18° C	18/8° C	18/18° C
X_1 hl (min)	2.78	2.47	3.42	3.06
X_2 hl (min)	4257.5	3789.2	5236.7	4683.3
$K_{12}(min^{-1})$	0.302	0.269	0.371	0.332
$K_{21}(min^{-1})$	0.008	0.007	0.010	0.009
$K_{10}(min^{-1})$	0.022	0.020	0.027	0.024
AUC (µg·min/ml)	40210.4	35787.3	49458.8	44231.4
Q_b (ml/min)	0.21	0.19	0.26	0.23

Table I: Model parameters describing the blood concentrations of BaP in rainbow trout following a single intra-arterial administration of 10 mg/kg. n=3.

The distribution of BaP-derived radioactivity in rainbow trout remaining after 48h is presented in Table II. Significant differences in tissue distribution were not seen between any treatment. There appear to be some trends towards increased deposition in liver, visceral fat, and kidney in the animals acclimated to 8° C and exposed at 18° C, however the relatively large standard errors preclude any definite conclusions. The highest body burdens were to be found in liver, visceral fat, kidney, and gill respectivley. Preliminary examination of BaP metabolites indicates a variety of Phase I and Phase II metabolites present in the bile and an alteration, with acute temperature, in the chemical species of BaP-metabolite present in the bile.

	Radioactivity (% body burden) Acclimation/exposure temperature			
Organs and Tissues	8/8° C	8/18° C	18/8° C	18/18° C
Liver	42.3±6.0	52.1±10.1	46.0±11.7	49.1±9.8
Visceral fat	10.6±2.7	15.4±4.7	12.1±4.3	12.7±3.7
Kidney	8.1±3.0	12.4±4.9	9.2±5.2	9.4±4.0
Gill	6.7±0.5	7.7±0.7	6.0±0.9	7.0±0.7
Intestine	4.1±1.3	3.1±1.9	2.9±1.2	4.5±1.3
Muscle	3.1±1.0	3.2±0.6	2.1±1.1	2.5±1.2
Stomach	0.5±0.1	0.5±0.1	0.6±0.1	0.5±0.1
Spleen	0.9±0.2	1.2±0.2	0.6±0.2	1.0±0.2
Heart	0.2±0.1	0.2±0.1	0.2±0.1	0.2±0.1
Skin	0.2±0.1	0.2±0.1	0.2±0.1	0.2±0.1

Table II: Percent of total body burden of BaP-derived radioactivity remaining in the animal at the end of the sampling period. (% Total radioactivity ± S.E.). n=3

The results obtained in this experiment appear to indicate temperature affects the excretion of benzo[a]pyrene in rainbow trout. Temperature compensation in the biliary excretion was seen as animals acclimated and exposed at the same temperatures had similar excretion kinetics. The biochemical and physiological changes associated with acclimation to temperature appear to confer differences in the ablity of the animal to rid itself of a xenobiotic, such as bezno[a]pyrene, especially when exposure to the xenobiotic is associated with acute temperature change. This finding may have significant consequences for the animal in the wild, if xenobiotic exposure is coupled with an acute temperature change as may often be the case with effluent discharge or surface-layer petrochemical spills. Incorporation of this sort of information into the assessment of contaminant effects on organisms in their environment is necessary in order to understand the nature of how xenobiotic events proceed in aquatic environments.

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SURVIVAL AND GROWTH OF ATLANTIC COD IN HYPOXIA

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Introduction

In the Gulf of St. Lawrence, cod frequently spend time in waters with low levels of dissolved oxygen (hypoxia). The present study was carried out to assess the tolerance of cod to acute hypoxia, as well as the impact of chronic hypoxia on their growth rate.

Methods

Experiments were conducted by placing 10 cod in each of six flow-through 800-L tanks. Oxygen saturation levels were 13.8, 17.8, 23.7, 29.5, 36.5, and 42.5%. Salinity was ~28‰. Tolerance was tested for two size classes (small cod, 45.2 cm ± 4.2 [mean ± SD] and large cod, 57.5 cm ± 3.8), and two water temperatures (2 and 6°C, representative of deep waters in the Gulf of St. Lawrence). Each experiment was repeated twice. Mortality was measured at 1, 3, 6, 12, and every 12 h thereafter until 96 h. Median and incipient (50% and 5% mortality) lethal thresholds were calculated for each observation period by probit analysis (Stephan 1977). Tolerance levels with non-overlapping 95% confidence intervals were considered different.

The growth experiment was conducted at 10°C and ~28‰ in the same tanks. We allocated 120 cod (length = 44.2 ± 3.1 cm, mass = 715 ± 188 g, condition (Fulton K) = 0.81 ± 0.1) among six oxygen conditions (45, 56, 65, 75, 84 and 93% saturation). Cod were fed capelin ad lib for one hour, three times a week, for 84 days. Each fish was tagged to allow determination of individual growth rates. Differences in growth were tested by Anova.

Results and Discussion

No cod survived more than a few hours at 13.8% saturation and only a few survived 96 h at 17.8% saturation. All fish survived 96 h at 36.5 and 42.5% saturation. Median lethal thresholds at 96 h differed significantly between the two replicates of large cod at 6°C (26.4 and 22.3% saturation). Because the first replicate was the only group of fish which was tested during reproduction and after a full year in captivity, it was dropped from further analyses. Median lethal levels (96 h) did not differ between replicates for the other 3 experiments, and replicates were pooled. Median lethal thresholds were 19.0%, 21.0% and 22.4% oxygen for small cod at 6°C, large cod at 2°C and small cod at 2°C, respectively. Size class and temperature had no detectable effect on 96 h median lethal thresholds, so experiments were combined, and new median and incipient lethal thresholds were calculated for each observation period (Figure 1). Overall, the 96 h median and incipient lethal thresholds were 21.0% (95% CI: 18.8-23.2%) and 28.1% saturation (23.4-32.8%), respectively.

Scholz & Waller (1992) observed a 24 h median lethal threshold of 39.7% saturation, and no cod survived 24 h at 20% saturation. Maybe the lower hypoxia tolerance of their fish was due to differences in frequencies of hemoglobin alloforms between cod from the North Sea and the Gulf of St. Lawrence (Karpov and Novikov 1980).

Cod were very tolerant to hypoxia in another study (Schurmann and Steffensen 1992), where the median lethal threshold at 5°C was 5% saturation. However these authors did not follow the normal procedure for a tolerance test (American Society for Testing and Materials 1988). All fish were subjected to continuously declining oxygen levels, and levels likely to kill cod according to our study were only reached in the last hour of their test. Therefore their lethal threshold ought to be compared to our 1 h median lethal threshold (8.2%,
Figure 1). Although agreement is good, this is a poor measure of hypoxia tolerance in cod because mortalities increase rapidly in the first 24 h of exposure (Figure 1).

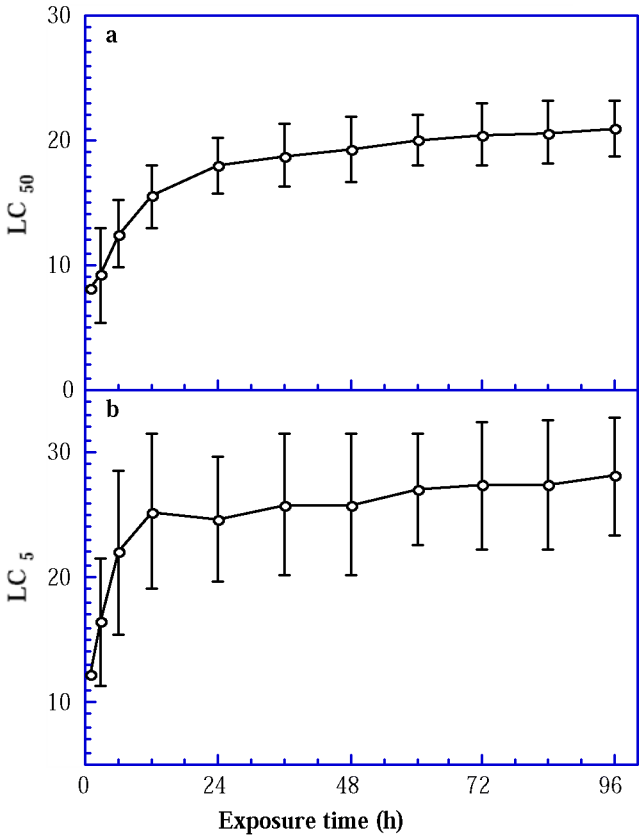

Figure 1 Median (a) and incipient (b) lethal hypoxic level (% O_2 saturation, with 95% CI) and exposure time in Atlantic cod from the Gulf of St. Lawrence. Significant growth in length and mass, as well as significant improvements in condition, occurred in all oxygen conditions (Fig.2). However changes in length and mass were significantly less at 45% and 56% saturation than at the highest levels of dissolved oxygen. Changes in condition were negatively affected by hypoxia as soon as oxygen saturation fell below 70%.

Cod are expected to avoid waters with less than 30% oxygen saturation, whereas growth is expected to be reduced in waters with less than 60% oxygen saturation. In the Gulf of St. Lawrence, waters deeper than 200 m are almost always below 60% oxygen saturation, whereas the deeper parts of the Gulf and the estuary are commonly below 30% oxygen saturation.

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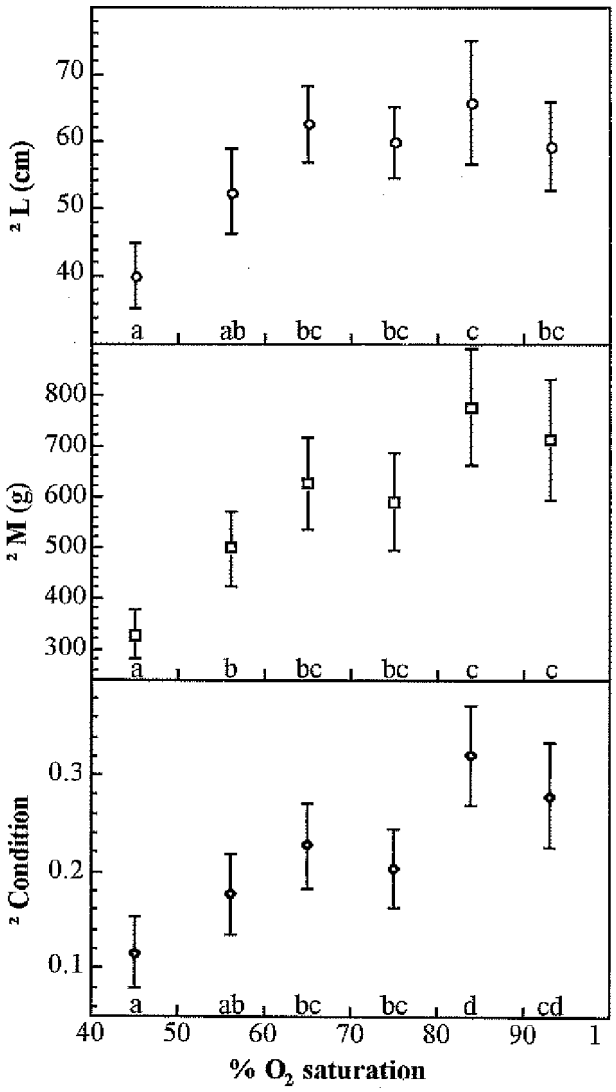

Figure 2. Growth in length and mass, and change in condition of Gulf of St. Lawrence cod according to level of dissolved oxygen. Error bars are 95% CI. Groups with different letters were different at p=0.05.

PHYSIOLOGICAL RESPONSES OF CENTRARCHID SPECIES THAT OCCUPY HYPOXIC SWAMP HABITATS

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The Atchafalaya Basin is a 8,345-km^2 distributary of the Mississippi River that flows through central Louisiana and contains the largest hardwood swamp in North America. Large expanses of the swamp can become hypoxic (dissolved oxygen "DO" < 2.0 mg/l and PO_2 < 45 mm Hg for > 12 hours each day) for several months during the annual spring-summer flood pulse. Centrarchids are among the fishes we most frequently observed in chronically hypoxic habitats.

During May-August, 1995, we electroshocked 91 largemouth bass *(Micropterus salmoides)* from hypoxic habitats (DO < 2.0 mg/l, > 12 hr each day) and 100 from normoxic habitats (DO always > 5.0 mg/l) in the Atchafalaya Basin, and collected 0.4 ml of blood from the caudal vein of each fish within six minutes of capture. Mean hematocrit and plasma cortisol concentrations did not differ among hypoxic and normoxic habitats (both $p > 0.39$, Table 1), but mean hemoglobin concentration was higher in hypoxic habitats, as was mean corpuscular hemoglobin concentration (MCHC). The mean hematocrit levels, hemoglobin concentrations, and plasma cortisol concentrations that we observed in largemouth bass were among the lowest that have been reported for the species, but the mean MCHC we observed was the highest reported (Clark et al., 1979; Carcichael et al. 1984; Gustaveson et al., 1991).

We also collected 120 largemouth bass from the Atchafalaya Basin and examined their blood after they were held at three temperatures (22°C, 27°C, and 32°C) and subjected to four rates of DO depletion from saturation to 1.5 mg/l (no depletion, six hours, 48 hours, and six hours with DO of 1.5 mg/l maintained for 7 days) thereby producing 12 different treatments , with 10 fish being subjected to each treatment. There were no significant differences among treatments in mean hematocrit, hemoglobin concentration, or MCHC (all

$p>0.11$).). Mean cortisol concentration was higher in control-group fish (1.74 μg/dl) than fish in treatment groups (1.26, 1.21, 1.20, all $p<0.05$), and mean concentration at 22°C (1.04) were significantly lower than at other temperatures (1.41, 1.60, both $p<0.05$). However, all measured plasma cortisol concentrations were well below reported concentrations for stressed largemouth bass (Carmichael et al., 1984).

From 7 April through 31 May, 1995, we collected female bluegill (*Lepomis macrochirus*), longear sunfish (*Lepomis megalotis*), and spotted sunfish (*Lepomis punctatus*) at two hypoxic and two normoxic sites in the Atchafalaya Basin. We collected more than 40 adult females of each species from each habitat. For both bluegill and longear sunfish, gonadosomatic indices were 2-3 times higher in normoxic habitats than in hypoxic habitats (all $p<0.05$), but did not differ between habitats for spotted sunfish. For all species, the percentage of females with yolked eggs was higher in normoxic habitats (Table 2, all $p < 0.02$). The mean number of yolked eggs did not differ between hypoxic and normoxic habitats for any species (all $p>0.1$). All three species produced smaller yolked eggs on average in hypoxic habitats than in normoxic habitats (all $p<0.03$).

Largemouth bass and bluegill have been reported to strongly avoid DO < 2.0 mg/l (Whitmore et al., 1960) and to die at DO < 1.0 mg/l (Moss and Scott, 1961; Smale and Rabeni, 1995). Our results indicate that they can adapt when exposed for several months to DO within that range. Largemouth bass may partially adapt by increasing the amount of hemoglobin in their red blood cells, but the increase we recorded was slight and the hematocrit and hemoglobin concentrations present in bass from this system were low compared to literature values. We suspect that these fish have a type of tetrameric hemoglobin with increased oxygen-binding capabilities. This adaptation appears to always be present, because bass acclimated to saturated DO were able to withstand rapid decreases in DO without exhibiting a hematological or stress response.

Table 1. Summary statistics of blood parameters of largemouth bass collected from hypoxic and normoxic habitats in the Atchafalaya Basin. Significant mean differences are indicated by * ($p < 0.01$) or ** ($p<0.0001$). Temperatures in hypoxic habitats ranged from 28-32°C, and temperatures in normoxic habitats ranged from 24-35°C.

	Hypoxic			Normoxic		
Blood parameter	*N*	Mean ± SE	Range	*N*	Mean ± SE	Range
Hematocrit (%)	91	28.0 ± 0.6	17.5-50.0	100	27.5 ± 0.6	9.5-47.5
Hemoglobin (g/dl)	49	6.7 ± 0.1*	4.5-9.0	52	6.2 ± 0.1*	4.8-8.0
MCHC (%)	49	25.5± 0.6**	12.8-37.5	52	22.1 ± 0.5**	14.9-34.2
Plasma cortisol (μg/dl)	49	0.4 ± <0.1	0.2-0.6	49	0.4 ± <0.1	0.2-0.6

Table 2. Mean number and size estimates for yolked eggs of three *Lepomis* species collected from hypoxic and normoxic habitats. Different letters under Tukey's Group indicates the estimates significantly differed based on ANOVA and post-ANOVA testing. Numbers next to the habitat type are the percentage of females collected in that habitat with ovaries that contained yolked eggs.

		Egg Number			Egg Size		
Species	Habitat	*N*	Mean	Tukey's Group	*N*	Mean (mm^2)	Tukey's Group
Bluegill	Hypoxic (24)	10	4665.5	A	12	0.200	A
	Normoxic (71)	29	7582.7	A	29	0.301	B
Longear Sunfish	Hypoxic (22)	12	1129.4	B	17	0.445	B
	Normoxic (93)	57	1458.7	B	57	0.687	C
Spotted Sunfish	Hypoxic (71)	36	1921.7	B	41	0.477	B
	Normoxic (99)	69	2030.2	B	70	0.656	C

Although bluegill and longear sunfish also appear able live in hypoxia, it appears that their metabolism was affected to the point that egg production was impaired. Moss and Scott (1961) reported that the standard metabolic rate of centrarchids decreased when exposed to long-term hypoxia. Egg production was least impaired in spotted sunfish, so their metabolic rate may be less affected by hypoxia. But even they produced fewer mature ovaries and smaller yolked eggs when they lived in low DO. Future studies on the effects of hypoxia on centrarchids should focus on how long term exposure affects their metabolism and consequently the population characteristics of these species in systems where hypoxia chronically occurs.

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LAMELLAR ADHESION AND IMPLICATIONS FOR GASEOUS EXCHANGE IN BROWN TROUT EXPOSED TO LOW LEVELS OF ALUMINIUM

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INTRODUCTION

Exposure of salmonids to aluminium in acidic soft waters has been shown to cause death by interfering with the ionoregulatory and respiratory processes of the gills, although species sensitivity varies. Our recent studies have shown the brown trout, *Salmo trutta* L., to be extremely intolerant of aluminium at low pH, with exposure to 12.5 μgl^{-1} in soft water of pH 5.0 causing severe respiratory disturbances, while 25 μgl^{-1} was lethal for 66 % of fish within 5 days (Brown & Waring, 1995). The present study continues this work and aims to link physiological responses and changes in blood parameters with morphological examination of the gill, the major target organ for aluminium toxicity.

EXPERIMENTAL ANIMALS

Brown trout (*S. trutta* L.) acclimated to soft water (Ca^{2+} 0.02 mM; Na^{+} 0.05 mM; K^{+} 0.01 mM) were exposed to control (pH 7.0, no aluminium, n = 6), acidic (pH 5.0, no aluminium, n = 8) or acidic with aluminium (pH 5.0, 25 μgl^{-1} aluminium, n = 6) conditions for five days in individual 6 litre darkened perspex boxes, after 2 days pre-acclimation to the boxes.

TISSUE AND BLOOD SAMPLING

After five days, fish were anaethesised with an overdose of benzocaine (40 ppm) and killed by a sharp blow to the head. Large blood samples were rapidly withdrawn from the caudal vascualture, completing the whole process of blood sampling in less than two minutes. Whole blood lactate concentrations were measured enzymatically (Sigma No. 826-UV).

GILL MORPHOLOGICAL STUDIES

The second gill arch from the right side of each fish was excised and fixed to assess gill morphology. Gills were processed appropriately for scanning electron microscopy (SEM) and light microscopy (LM).
Examination of gill structure revealed unusual levels of apparent lamellar fusion in fish exposed to aluminium. Lamellar fusion was quantified with light microscopy by randomly assessing 50 secondary lamellae on four different filaments per fish, classifying lamellae as unfused, fused as pairs, fused in groups of three, or fused in groups of four. Image analysis was used to measure the effect of lamellar fusion on the gill area available for gaseous exchange. Processing was done on a Macintosh 7200/90 computer using the public domain NIH-Image 1.59 program. Perimeters of fused secondary lamellae were measured, and because the epithelial layers between fused lamellae were visible, it was possible to determine the theoretical perimeters had the lamellae remained unfused. This enabled calculation of the percentage reduction in lamellar surface due to fusion.

RESULTS AND DISCUSSION

Exposure of brown trout to 25 μgl^{-1} aluminium in soft water of pH 5.0 resulted in respiratory stress inferred by the significant accumulation of blood lactate (4.07 $\pm$ 0.64 mM) compared with acid controls (1.67 $\pm$ 0.09 mM) and neutral controls (1.19 $\pm$ 0.19 mM). One potential cause of this was the apparent fusion of adjacent secondary lamellae. In aluminium-exposed fish, whilst the majority of lamellae remained unfused (80.9 %), there were 16.4 % fused as pairs, 2.4 % in groups of three, and 0.3 % of gill lamellae fused in groups of four (Fig. 1). No fish in either control group showed any of this type of lamellar fusion.

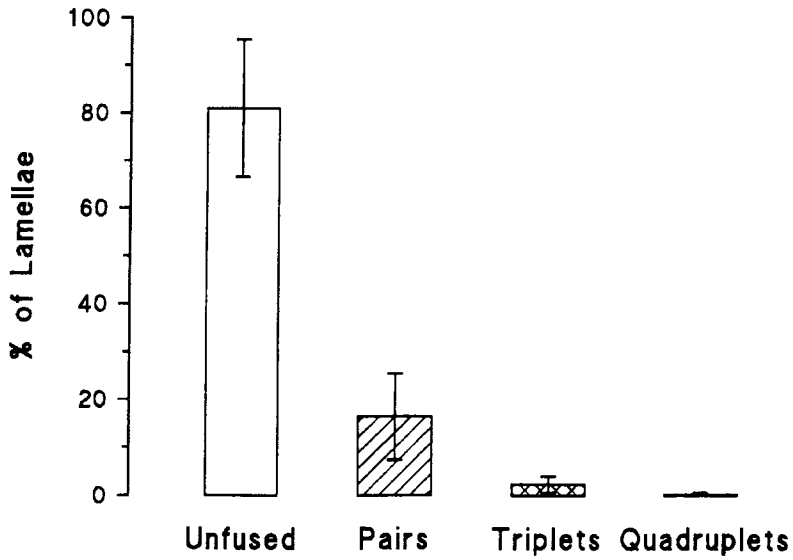

Figure 1. Quantification and characterisation of lamellar fusion after 5 days exposure to soft water of pH 5.0 plus 25 $\mu g l^{-1}$ aluminium.

Lamellar fusion was easily visible by SEM (Fig. 2), and was characterised by a gradual attachment of adjacent secondary lamellae. Fusion was always initiated at the trailing edge of the gill filaments, but was not always complete across the entire length or width of the lamellae. In some cases, SEM showed an epithelial layer between fusing lamellae, although sometimes fusion appeared complete. In the vast majority of the LM sections however, the adjacent epithelial layer was visible, suggesting the majority of fusions were incomplete. The lamellar fusion reported here is different to that reported by previous workers in that it was lacking hyperplasia of the primary filament that appears to 'engulf' the secondary lamellae (Mueller et al, 1991). The present study also used much lower aluminium levels.

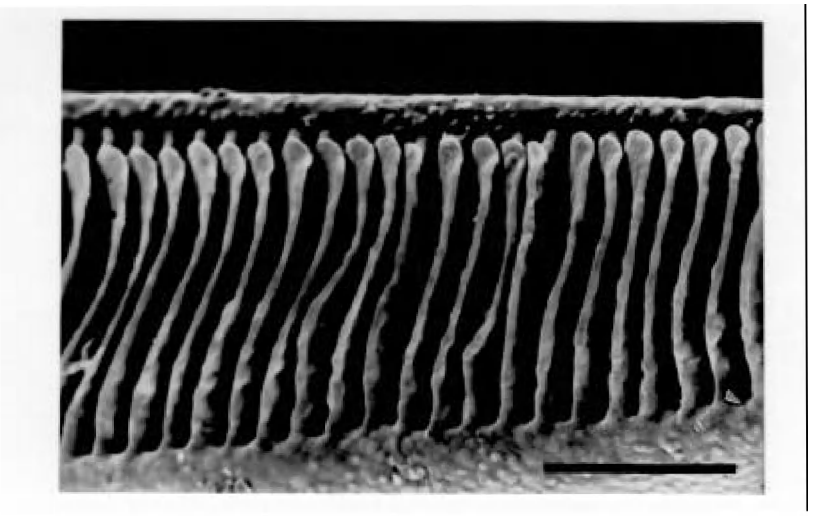

Figure 2a.

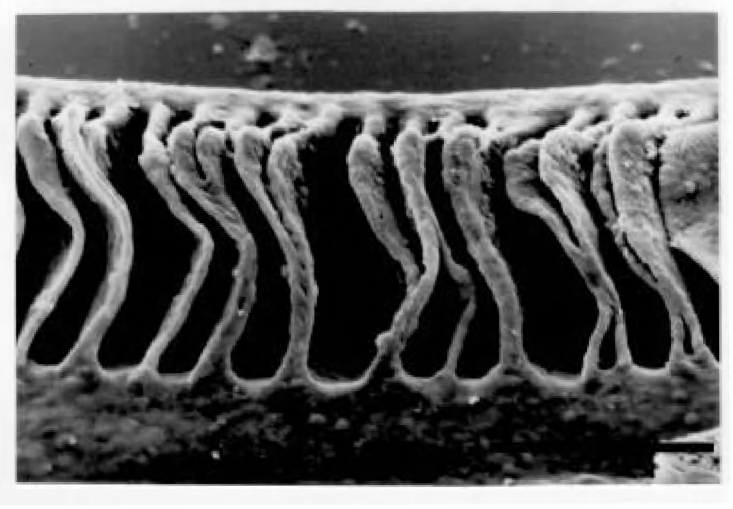

Figure 2b.

Figure 2. Scanning electron micrographs of brown trout after exposure to (a) soft water of pH 7.0 (b) soft water of pH 5.0 containing 25 μgl^{-1} aluminium. Bars denote 200 μm.

The net effect of lamellar fusion of the type reported here is a reduction in the maximum area of gill available for gaseous exchange. In gills from the aluminium-exposed fish, the available respiratory gill surface area was reduced on average by 5.7 %. Fusion will also interfere with the laminar flow of water across the gill, potentially further adversely affecting gaseous exchange. Although lamellar fusion was not apparent in all aluminium-exposed fish, elevated blood lactate levels were. Thus, mucous release in response to aluminium precipitation/binding (Handy & Eddy, 1989) seems likely to be more important than lamellar fusion as the primary cause of the respiratory stress.

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EFFECTS OF O, P′ DDT ON STEROID HORMONE METABOLISM BY RAINBOW TROUT, *ONCORHYNCHUS MYKISS*, EMBRYOS

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Introduction

Sublethal effects of DDT have been reported in many species. Such effects include egg shell thinning, feminization, demasculinization, impaired embryo development and induction of vitellogenesis in fish. The ability of DDT to bind to estrogen receptors appears to be one mechanism leading to reproductive impairment (Fry and Toone, 1981; Cross and Hose, 1988; Donohoe and Curtis, 1996; LeBlanc et al., 1997) but other routes of action are suspected. This study was aimed to examine whether the action of o, p′ DDT on the development of fish embryos is related to impaired steroid metabolism.

Materials and Methods

The effect of o, p′ DDT (111-trichloro-2-[o-chlorophenyl]-2-[p-chlorophenyl]ethane;) on the metabolism of [^{3}H]pregnenolone ([^{3}H]P5) by rainbow trout, *Oncorhynchus mykiss*, embryos during embryonic developmental stage was studied *in vitro*. Hatched embryos (48 day post fertilization[dpf]) were reared in plastic screen cages in a trough supplied with constantly running aerated water at 8-9° C. Embryos were exposed by means of immersion for one hour per day in vehicle control (0.01% ethanol), 0.01 and 1.0 ppm of DDT solution (Chem Service, West Chester, PA) for a single or ten consecutive days; six replicates, three embryos per incubation were used. The yolksac was removed and the embryos killed by severing the head. Embryos (49, 53 and 58 dpf) were randomly sampled from each group and incubated medium 199 with Hank's salts, glutamine, sodium carbonate, bovine albumin and β-glucose at pH 7.2 (Khan et al., 1997) using 1 nmol (21.1 μ Ci) of [7-^{3}H(N)]-P5 (21.1 Ci mmol^{-}

[1], NEN) as substrate. Embryos were also co-incubated with DDT at 0.01 and 1 ppm. The incubation vessels were gently agitated on a shaker at 10°C for 16 hours, the medium + embryos was then stored frozen at -20°C until extracted and analyzed as described in Khan et al. (1997).

Results and Discussion

In both naive and vehicle control, [^{3}H]P5 was metabolized to an as yet unidentified metabolite and some conjugated steroid form (Figure 1). For embryos immersed in DDT, there was no discernable change to the conversion pattern of either free or conjugated metabolite in relation to age of embryos (49, 53, and 58 dpf), dose (0.01 and 1.0 ppm) and number of exposures (single and ten exposures), nor was the amount of [^{3}H]P5 converted to the unknown metabolite and conjugated form affected. However, when embryos were co-incubated with DDT, although the conversion pattern was similar, the amount of conversion was severely impeded (Figure 2).

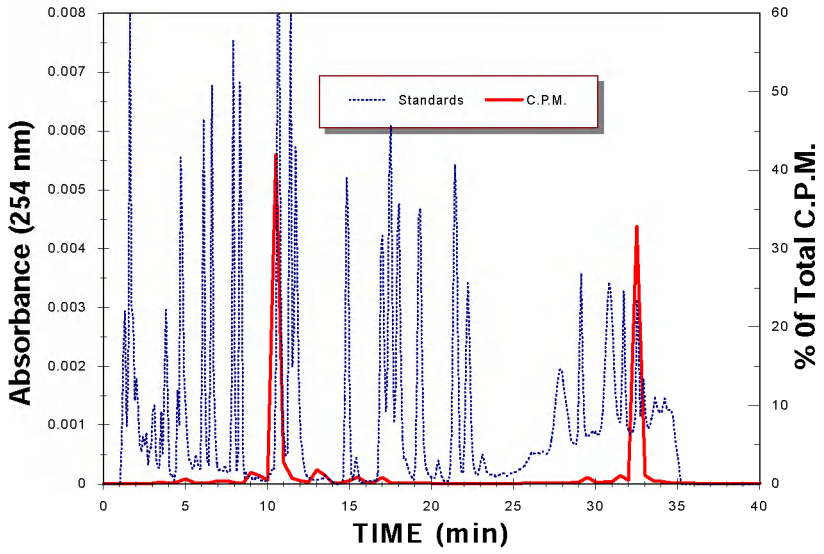

Figure 1. A representative HPLC profile of radiolabelled free steroid hormone metabolite produced by 58 dpf embryos after incubated as described above. The dotted line is the elution of 21 authentic steroids as internal

standard. The solid plot represent the radioactivity (CPM) found in naive control sample, fractions collected at 0.5 min. The abscissa is the retention time (min) where the substrate ([^{3}H]P5) eluted between 32-33 min and the unknown metabolite eluted between 10-11 min.

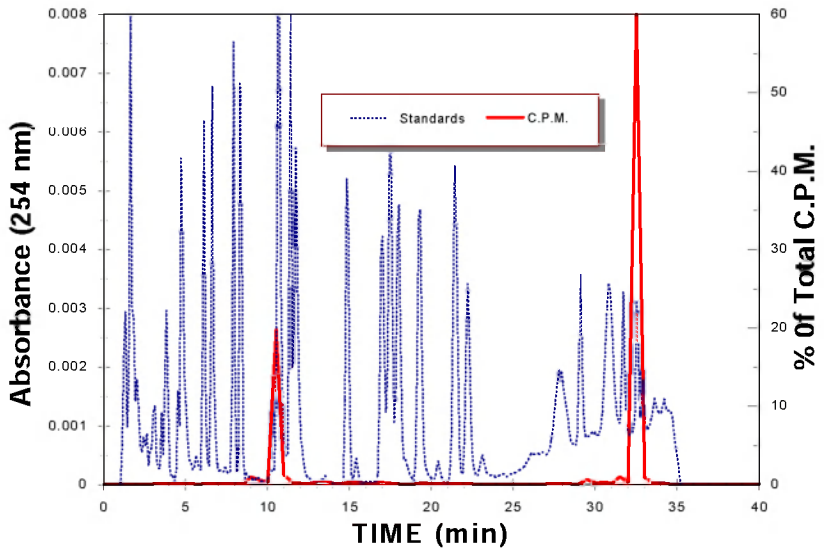

Figure 2. A representative HPLC profile of radiolabelled free steroid hormone metabolite produced by 58 dpf embryos after co-incubated as described above. The dotted line is the elution of 21 authentic steroids as internal standard. The solid plot represent the radioactivity (CPM) found in the co-incubation sample (0.01 ppm of DDT), fractions collected at 0.5 min. The abscissa is the retention time (min) where the substrate ([^{3}H]P5) eluted between 32-33 min and the unknown metabolite eluted between 10-11 min.

There was no difference among treatments and no pattern of changes related to dose and age. This may suggest that DDT was not taken up by the embryos due its high lipophilicity. On the other hand, the conversion of [^{3}H]P5 to the unknown metabolite, tentatively identified as 17,20β[^{3}H]P5 based on its elution time, is obviously inhibited by the presence of DDT. This may indicate that the enzyme which catalyze the conversion is inhibited. Similarly, 3β hydroxysteroid dehydrogenase and 17β hydroxysteroid dehydrogenase are inhibited by DDT in

Oreochromis mossambicus (Bhattachary and Pandey, 1989, after Kime, 1995). The analysis of the unknown metabolite is presently being undertaken. Further work will be undertaken to mimic the natural contamination pathways and existing concentrations of DDT found in wild fish and we propose to incubate embryos with other precusors to explore the possible effects of DDT on other steroidogenic enzymes.

In summary, although there was no response to exposure to DDT in embryos following immersion, the co-incubation results suggest that DDT at both low and high concentrations has an inhibitory effect on the metabolism of [^{3}H]P5 by rainbow trout embryos (58 dpf).

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HEMATOLOGICAL EFFECTS IN RAINBOW TROUT SUBJECTED TO A CHRONIC SUBLETHAL CONCENTRATION OF LEAD

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Compared to terrestrial animals, erythrocytes in the peripheral circulation of teleosts are dynamic (Houston 1990). The structure of the erythrocyte population is heterogeneous and represented by a variety of stages at any one time including mature, juvenile, intermediate ages, dividing, and senescent cells. The age structure of the erythrocyte population in teleosts, however, is affected by environmental stressors and will shift in favor of certain stages. Respiratory stresses due to elevated temperatures and low dissolved oxygen levels result in erythropoiesis. The peripheral red blood cell population shifted in favor of juvenile and dividing cells in goldfish (*Carassius auratus*) subjected to elevated temperature and hypoxia (Murad et al. 1990). In contrast, the number of dividing cells decreased while karyorrhectic (degrading) cells increased in goldfish subjected to sublethal levels of cadmium (Houston et al. 1993).

The enzyme -aminolevulinic acid-dehydratase (ALA-D) has been used as an indicator of lead exposure in fish, however, its effects on hematological and physiological responses are inconsistent and not clearly understood. ALA-D is essential in the synthesis of porphobilinogen, a precursor of heme (Schmitt et al. 1993). Although ALA-D is profoundly affected by low concentrations of waterborne lead in fish, there does not appear to be a causal link between ALA-D activity and hematological responses in fish.

Juvenile rainbow trout (120-200 mm total length) were subjected to sublethal chronic concentrations of lead for 8 weeks and subsequently allowed to recover

in lead-free water for an additional 8 weeks. Blood was collected prior to lead exposure, and on days 14, 28, 56, 70, 80, and 112. Despite a pronounced inhibition of ALA-D activity (80% of the controls) in fish subjected to 89 ug $Pb \cdot L^{-1}$, a suite of hematological variables (hematocrit, hemoglobin, total erythrocyte number, mean corpuscular hemoglobin, mean corpuscular volume, mean corpuscular hemoglobin concentration) were not significantly affected. The peripheral erythrocyte population reflected a slight shift in favor of juvenile cells which may confer an adaptive advantage for transport and delivery of oxygen. Within 56 days in lead-free water, ALA-D returned to pre-lead levels.

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BEHAVIOURAL AND METABOLIC EFFECTS OF CHRONIC EXPOSURE TO ALUMINIUM IN ACIDIC SOFT WATER IN JUVENILE RAINBOW TROUT

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Introduction

Acid deposition, principally as a result of the combustion of fossil fuels, is a serious pollution problem in the northern hemisphere. When acidity is deposited onto soils with low buffering capacity the H^+ ions may displace and mobilise metal ions, including aluminium. Aluminium in the aquatic environment is known to be detrimental to organisms at all trophic levels and has been cited as the cause of fish kills (Howells *et al*, 1990).

Wilson *et al.* (1994a) found that juvenile rainbow trout exposed to sublethal pH (pH 5.2) and aluminium (30 μg l^{-1}) experienced appetite depression to about 90% of pre-exposure levels, but paradoxically had higher food conversion efficiencies (% food consumed converted into dry mass gain) than control groups of fish. It was anecdotally noted that the fish exposed to aluminium were less active, and it was hypothesised that the reduced activity may permit a greater proportion of the energy consumed to be diverted to growth. The data described here includes sensitive patterns of swimming behaviour measured concurrently with metabolic rate to clarify if changes in these parameters upon exposure to aluminium could account for the apparent energetic economisation.

Methods

Triplicate groups of 10 artificial soft water acclimated juvenile rainbow trout (*Oncorhynchus mykiss*) between 13 and 28g were allocated to one of three treatments; pH 6.5 with no added aluminium (6.5/0), pH 5.2 with no added aluminium (5.2/0) and pH 5.2 with 30 μg l^{-1} added labile aluminum (5.2/ Al). The aluminium dose was sub-lethal and lasted for 34 days.

All fish were fed to satiation every second day prior to the exposure. As predicted, upon exposure to aluminium fish experienced appetite reduction, and from this point onwards fish in all treatments were fed the same equivalent ration as the fish exposed to aluminium (gram food per gram fish basis). This ensured that the food conversion efficiencies and metabolic rates were directly comparable between treatments. Activity was recorded on video using a camera suspended above the tanks. The swimming behaviour of five randomly chosen fish from each tank was analysed during a two minute period for three mutually exclusive and distinct swimming behaviours; position holding, slow swimming and burst episodes. Metabolic rates were measured concurrently with the behavioural determinations using a closed respirometry system. Statistical significance was tested using a nested design ANOVA at $p < 0.05$. All data expressed as percentages were *arc sin* transformed to obtain a normal distribution and homogeneity of variance.

Results and Discussion

The fish exposed to aluminium experienced appetite depression, consuming a minimum ration at day 15 (Figure 1). All groups of aluminium exposed fish experienced some subsequent degree of appetite recovery, however, the degree of recovery was not homogeneous amongst the replicates. Plasma glucose concentrations measured upon terminal sampling revealed a linear inverse relationship between plasma glucose and appetite. This suggests that elevations in plasma glucose content may have reduced appetite in the fish exposed to aluminium.

FEEDING RESPONSES OF REPLICATE TANKS OF FISH EXPOSED TO ALUMINIUM

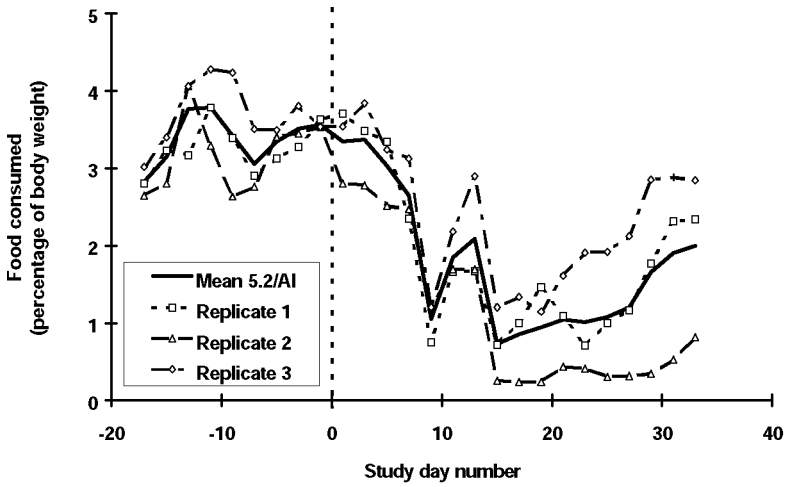

Figure 1 Food consumption of the three replicate tanks of fish exposed to aluminium. Day 0 signifies the start of the exposure. The solid dark line represents the mean of the three replicates and hence the ration fed to the two control groups.

No increase in food conversion efficiency or significant change in routine metabolic rate was observed in the fish exposed to aluminium. Exposure to aluminium in acidic soft water did however have a pronounced effect on swimming behaviour, producing hypo-activity which was statistically distinct from the control groups from day one throughout the study in all swimming behaviours analysed (see Figure 2 for position holding data).

It is proposed that the characteristic respiratory stress displayed by fish exposed to aluminium in acidic soft water (Wilson *et al*, 1994b) limited the locomotor activity of the fish. Upon terminal sampling further support for this hypothesis was found in the haematological parameters measured. The blood of fish exposed to aluminium had lower haematocrit values, haemoglobin content and red blood cell counts, all of which would impair gas transport.

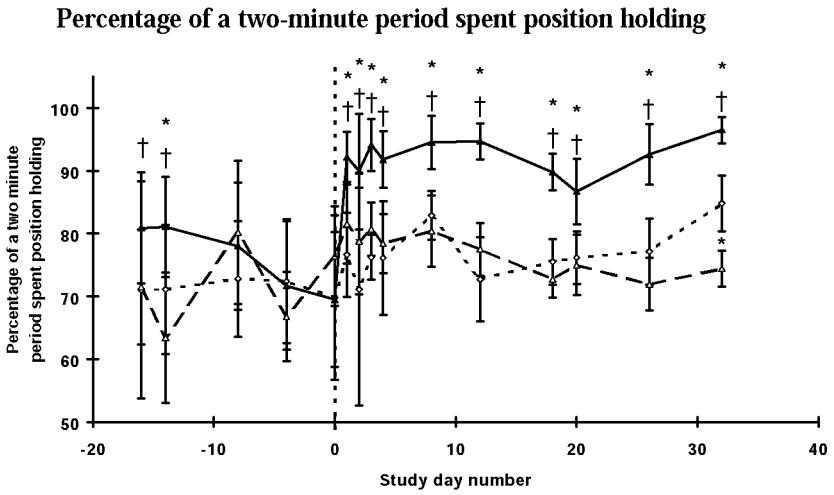

Figure 2 Effect of exposure to aluminium in acidic soft water on the percentage of time spent position holding. Error bars represent the mean standard deviation of the three replicates in each treatment.
* denotes behaviour significantly different from 6.5/0 group at $p < 0.05$.
† denotes behaviour significantly different from 5.2/0 group at $p < 0.05$.

Conclusions

Exposure to acid and aluminium caused no changes in food conversion efficiency or routine metabolic rate when all fish were fed the same ration. Swimming behaviour was the most sensitive indicator of exposure to aluminium. Hypo-activity was recorded in the fish exposed to aluminium which was significantly different from the activity of the control fish from day 1 onwards. It is proposed that the respiratory stress and hence reduced ability to extract oxygen from the water limited the locomotor activity of the fish exposed to aluminium. Behaviour represents a sensitive tertiary stress response of ecological relevance. If fish exposed to aluminium in the wild were to respond

in a similar manner it would affect their viability by influencing their ability to feed, avoid predation, migrate and reproduce.

Acknowledgements

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MATERNAL EFFECT ON CADMIUM TOLERANCE IN LARVAL TILAPIA, *OREOCHROMIS MOSSAMBICUS*

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Maternal inheritance can be classified into genetic and non-genetic effects. The maternal transfer of non-genetic cytoplasmic materials is known to affect offspring performance in their early life stages. This study examines the possible cytoplasmic transfer of Cd, mRNA of metallothionein (MT-mRNA), or MT itself from mother to offspring in tilapia, *Oreochromis mossambicus*. This transfer may explain the observation that tilapia larvae from cadmium pre-exposed mothers bear a higher cadmium tolerance.

To test the hypothesis that hatchlings from Cd-treated mothers were more tolerant to Cd pollution, mature female tilapia around 100 g were subjected to 0 (control) and 500 μg/kg (treatment) $CdCl_2$ by intraperitoneal injection. Hatchlings from mothers of both groups exposed to environments with or without 150μg/l $CdCl_2$. Therefore, the four treatment combinations were: (1) Cd-treated hatchlings from Cd-treated mothers (Cd^{Cd}), (2) non-Cd-treated hatchlings from Cd-treated mothers (N^{Cd}), (3) Cd-treated hatchlings from non-Cd-treated mothers (Cd^{N}) and (4) non-Cd-treated hatchlings from non-Cd-treated mothers (N^{N}). The time to 50% mortality (LT_{50}) were estimated and compared among treatment combinations. Egg size, Cd content in egg, hatching time and hatching success were measured as indicators for egg quality

To investigate the possible mechanisms for the increase in Cd tolerance, we analyzed the amount of Cd, MT-mRNA, and MT at three different developmental stages of tilapia. The three stages were oocytes collected for ovaries of female fish, newly fertilized eggs (F_0), and newly hatched offspring (H_0). Cadmium content was analyzed by graphite atomic absorption spectrophotometry. MT-mRNA was detected by dot blot hybridization and quantified by an image analysis program (Image Pal Plus) and MT was by ELISA.

The LT_{50}s' of Cd^{Cd} group (mean ± SE = 10.9 ± 0.5 days, n = 4) were statistically greater than those of Cd^{N} group (6.9 ± 1.2 days, n = 4). Thus, hatchlings from females pre-treated with Cd had a higher tolerance to Cd. There was no mortality in either N^{Cd} or N^{N} groups within 10 days of observation. This implies that exposing female tilapia to Cd did not affect either female reproduction and egg quality or the survival of their offspring in Cd-free environment (Table 1).

Significant amounts of MT-mRNA were found in both oocytes and newly hatched larvae (Table 2). However, MT-mRNA was low in newly fertilized eggs. The amount of MT-mRNA was very

Table 1. Comparison of egg quality between Cd-treated and control female tilapia.

	eggs from female	Cd-treated eggs from control female
egg weight (mg, mean ± SE)	0.58 ± 0.04	0.59 ± 0.04
Cd content (ng/egg)	<1	<1
hatching time (day)	4	4
hatching success (%)	>80	>80

Table 2. The ratio of relative amount of MT-mRNA in different stages of tilapia.

	from Cd-treated female/from control female
oocytes	8.4-10.1
newly fertilized eggs	around 1
newly hatched larvae	7.0-8.27

low (close to background value) at all the three stages in the control (no Cd) group. Cadmium could not be detected (<1μg/l) at any stage for either control or Cd-treated group. Metallothionein was also very low (MT/total protein < 5%) in all groups. The source of MT-mRNA in oocytes remains to be determined. The early appearance of MT-mRNA in newly hatched larvae from Cd-treated females may explain their tolerance of Cd (higher LT_{50}s').

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FACTORS AFFECTING DIETARY COPPER BIOAVAILABILITY TO RAINBOW TROUT

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Introduction

In polluted environments, rainbow trout may be consuming invertebrates containing copper (Cu) concentrations as high as 1000 $\mu g\ g^{-1}$, whereas laboratory studies suggest that Cu contaminated diets containing only 730 $\mu g\ g^{-1}$ may be toxic to rainbow trout. This contradiction may be explained by lower Cu bioavailability in a wild invertebrate diet. Bioavailability is defined by the amount of copper that is taken up across the gut tissue and enters the blood and internal organs. Mammalian research suggests that the major factors influencing the bioavailability of dietary Cu are dose and diet composition. For example, Cu bioavailability can be reduced by a competing ligand (e.g. zinc) or by complexation to a non-soluble dietary constituent (e.g. fibre), or increased by complexation to a nutrient (e.g. amino acids). This investigation aimed to examine the effect of a selection of different ligands on copper bioavailability. We have developed an *in vivo* technique to dose juvenile rainbow trout (200-250 g) via a surgically implanted stomach catheter with 2.4 μmol of radio-labelled Cu in the presence or absence of ligands. Using this method Cu uptake was partitioned into fractions bound in or on the gut tissue, absorbed internally (the bioavailable fraction), remaining in the gut lumen, and excreted.

Methods

Our control treatment was a solution of Cu and glucose (pH = 5.44) which exposed Cu to the stomach as the free Cu^{2+} ion (i.e. no ligand). The glucose control was compared to four different treatments, Cu + nitrilotriacetic acid (NTA) (pH = 8.41), Cu + calcium (Ca) (pH = 2.99), Cu + zinc (Zn) (pH = 5.05) and Cu + cysteine (pH = 1.33). Both cysteine and NTA are metal chelators, and the Cu complexes were expected to be more bioavailable than the free Cu^{2+} ion. Both Ca and Zn were expected to compete with Cu for uptake.

Results

Absorption of copper from the control dose was exceptionally slow compared to the absorption of glucose which was complete by 4h at 16° C. After 48 h only 5.0 % of the Cu dose was absorbed, i.e. was bioavailable (Fig. 1). The largest fraction of this was found in the liver. Both NTA and Ca increased bioavailability of the dose (to 9.1% and 7.5% respectively). Although NTA and Ca had similar effects on bioavailability, with NTA there was less copper bound to the gut tissue than with Ca. Moreover, with NTA, Cu was distributed primarily to liver, kidney and plasma, whereas with Ca, Cu was distributed mainly to the liver and bile.Thus these two treatments appear to have resulted in uptake via different pathways.

Both Zn and cysteine reduced Cu bioavailability (to 3.7% and 3.1% respectively) and both decreased Cu uptake into the gut tissue, plasma, bile, kidney and liver of the fish.

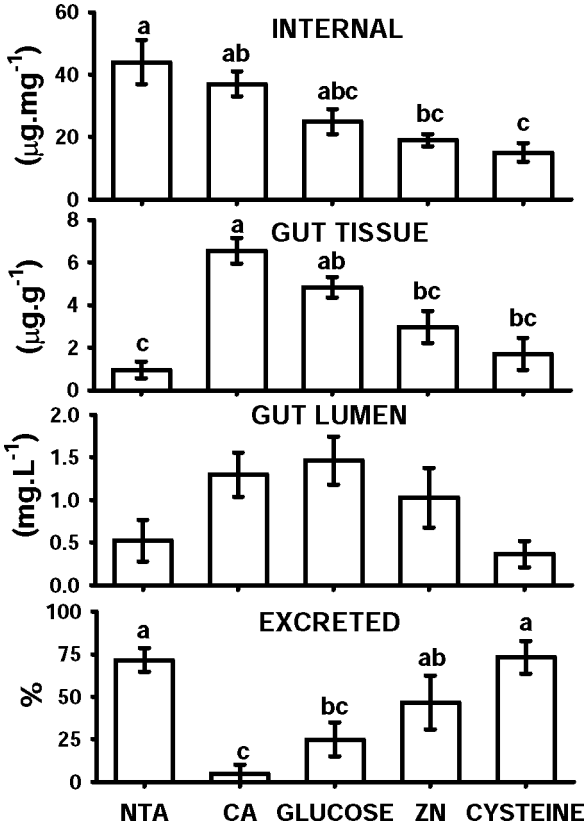

Figure 1. Effect of ligands on Cu (2.4 μmole) uptake after 48 h at 16°C.

Discussion

The Cu-NTA complex was more bioavailable for dietary uptake than the Cu^{2+} ion. The Cu-NTA complex may pass more readily through the gut tissue than Cu^{2+} and thus be more bioavailable for uptake into the plasma. Zinc probably decreased the bioavailability of the Cu^{2+} ion, as has been observed in many other organisms, by direct competition for uptake at the gut surface (Cousins, 1985). The presence of cysteine at a low pH unexpectedly resulted in low Cu bioavailability. One possibility is that the Cu-cysteine complex may have been insoluble at the alkaline pH of the lower digestive tract. Alternatively, cysteine may have reduced Cu^{2+} to Cu^{1+} which is thought to be a less bioavailable form of Cu (Wapnir, 1995). The presence of Ca^{2+} increased copper bioavailability, however the mechanism by which this may have occurred is currently unknown, although similar results have been found in mammals (Davis and Mertz, 1987). These data allow us to explore the different pathways by which dietary copper uptake occurs and to understand the important factors that influence copper bioavailability.

Acknowledgements

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A COMPARISON OF THE INTESTINAL METAL BIOAVAILABILITY OF CD AND ZN IN RAINBOW TROUT (*ONCORHYNCHUS MYKISS*)

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Introduction

The uptake and toxicity of metals in the water has been a subject of intense research in fish but until recent years the importance of metal-contaminated diets as a potential for toxicity to fish has received little attention. Zinc is known to be an essential micronutrient which must be present in the diet of all vertebrate species but can be toxic at higher levels. Cadmium is a toxic metal that arguably has no essential function in the body but is also prevalent in the environment. Both of these metals can be found in high concentrations in water, sediments and benthos of polluted areas. Aquatic invertebrates may contain up to 1290 Tg g^{-1} dw of zinc (Dallinger and Kautzky, 1985) while recent estimates have shown cadmium levels in aquatic invertebrates to be as high as 56.6 Tg g^{-1} dw (Handy, 1996).

Therefore the objective of this study was to determine the uptake and distribution of Zn and Cd from the diet. Our hypothesis was that Zn uptake and distribution would be very different from that of Cd, given its essential role. Our approach was to use a single oral dose protocol for infusing radio-labelled metal into the stomach. This technique allows us to trace the uptake, storage

and distribution of Zn and Cd in the tissues of rainbow trout over various time courses and temperatures.

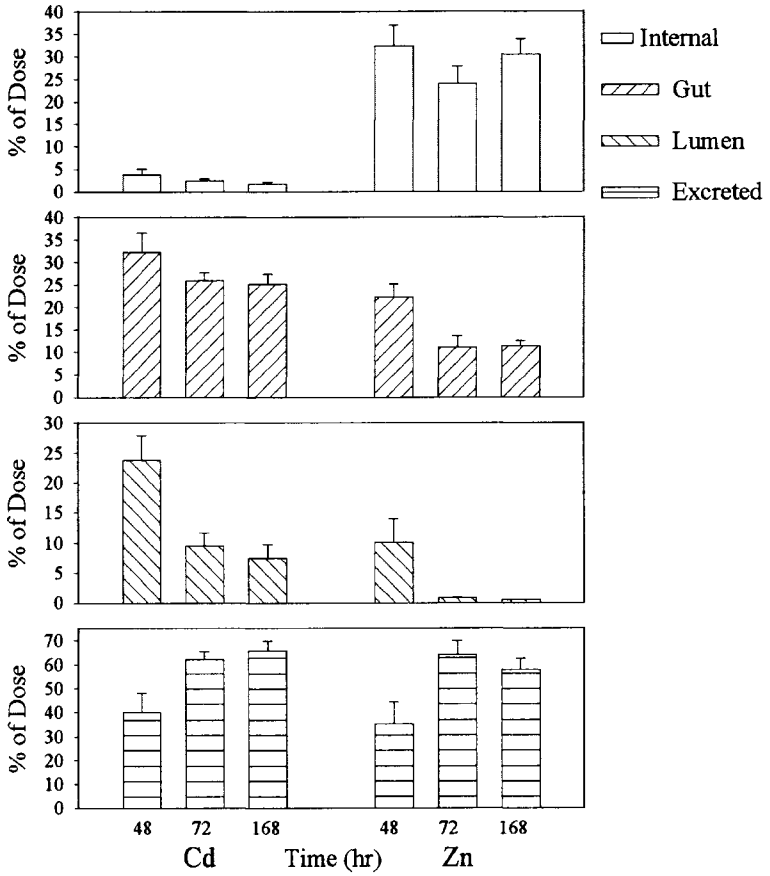

Figure 1. The fate of infused Cd^{109} and Zn^{65}(0.5 μmol) in rainbow trout after exposure times of 48, 72 and 168 hr.

Methods

Rainbow trout (*Oncorhynchus mykiss*), 0.3 kg were acclimated to 150C for at least one week and then fitted, under MS-222 anaesthesia, with an indwelling stomach catheter. After 48 h recovery each fish was dosed with a single bolus of 0.5 Tmol of radio-labelled Zn^{65} or Cd^{109}. After 2, 3, or 7 days, fish were terminally anaesthetized and all internal organs and carcass were individually counted for radioactivity. Each of the gut compartments (stomach, pyloric caeca, mid-intestine and distal intestine) was rinsed of all contents and individually counted. Rinsed contents were also counted separately.

Results

By 48 h, the infused dose of Zn^{65} was distributed as follows: 32.4% was absorbed, 22.3% was bound up in the gut tissues and the remainder was either excreted (35.2%) or was present in the gut lumen (10.1%; Figure 1). By five days later, there was no further absorption and the gut Zn had declined to only 11.8% of the initial dose (bound + luminal contents). The remainder was excreted (57.7%). Cadmium showed a much different pattern of uptake. By 48 h, only 3.8% of the dose had been absorbed, with the remainder found either in the gut (56.0%, bound + luminal) or excreted (40.2%). There was no further uptake of Cd after longer exposure. In fact, after 7 days, only 1.7% of the dose remained in the body with 65.7% being excreted.

Discussion

Clearly there are substantial differences in the intestinal handling of Cd and Zn even though the amounts infused were similar and well below toxic thresholds for either metal. One explanation for the difference is the possible presence of carrier-mediated pathways for Zn uptake in the intestine whose activity may be controlled by the amount of Zn present in the diet. Cadmium, however, is not essential in the diet. Thus, it is unlikely that specific carriers are present in the intestine for the uptake of Cd into the body. Moreover, the high Zn radioactivity in certain tissues, most notably muscle and bone, probably reflects the turnover of the Zn pool in these tissues rather than net accretion. This may also explain why Zn^{65} is also detected in the gills and plasma in high amounts. Cadmium distribution is limited in the body and is largely retained within the gut tissues, in agreement with the findings of others (Harrison and Klaverkamp, 1989; Handy, 1992). It is thought that metallothionein plays a role in the binding of

Cd at the gut to prevent its uptake into the body and thus protect the fish from the toxic effects of Cd (Handy, 1996). Cadmium is also adsorbed to the intestinal mucosa (Handy, 1996).

One other striking feature of these results is the very slow turnover of both metals at 150C with significant gut retention even after 7 days post-infusion. In contrast, the nutrient constituents of the diet would be completely absorbed within 40 h at this temperature (Fänge and Grove, 1979).

Acknowledgements

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FEEDING, PROTEIN SYNTHESIS AND GROWTH

IN RAINBOW TROUT EXPOSED TO SUBLETHAL COPPER

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Although dietry sources, (*i.e.* prey items containing pollutant levels reflecting both the contamination within the surrounding water and the trophic level of the prey item itself) are recognised as being the most significant route of copper uptake, understanding the effects of waterborne exposure is still valuable since a physiological response often precedes significant copper accumulation and the limitations of tissue contaminant analysis have been recognised (Handy, 1992). In addition if applied to a commercial situation, where husbandry practices would be aimed to ensure exposure to dietry pollutants is negligable, pertubations in fish growth, caused by changes in water quality, have a particular importance. This study therefore addresses the fundamental question: how is fish growth affected by exposure to sublethal levels of waterborne copper.

One of the biggest challenges, when isolating the effect of waterborne pollutants on growth is to distinguish the influence of differential feeding arising from social dominance. However the social structure of rainbow trout populations can be assessed from individual variation in feeding rates (e.g. Winberg *et al*, 1993). Groups of individually marked rainbow trout, of similar size, and originating from the same stock, were exposed to 15 or 56 1g l^{-1} Cu at 10°C. Consumption, of a commercially available diet, by each individual, was monitored daily and, after 3 weeks exposure, each fish was labelled (i.p.) with a flooding dose of ^{3}H-phenylalanine. White muscle fractional rates of protein synthesis (ks, % day^{-1}) were then determined from the specific radioactivity levels in the unbound and protein-bound amino acids (Garlick *et al*, 1980). By repeating this basic procedure data representing groups, displaying a variety of hierarchical characteristics, have been integrated.

In all cases whole body specific growth rate (SGR, % day^{-1}) and feeding rate (% body mass day^{-1}) were linearly correlated. Whole body growth is largely the result of white muscle protein growth but, when these data, from all fish in all trials, were combined, neither white muscle SGR or white muscle protein synthesis rates were significantly affected by waterborne copper, up to 56 Tg l^{-1}. However, a knowledge of the individual feeding rates allowed the fish in these experiments to be arbitrarily categorised into dominant (*i.e.* those consuming more than the mean share of meal) and subordinate (*i.e.* those consuming less than the mean share of meal) animals. Although all fish classed as dominant, irrespective of copper treatment, exhibited positive white muscle SGR, there was a reduction in white muscle growth in dominant fish exposed to 56 Tg l^{-1} Cu. In these trials, not only were white muscle protein growth rates in subordinate fish lower than in dominant fish, but were in fact were universally negative; *i.e.* there was a net loss in white muscle protein throughout the trial period. However, in subordinate fish, there was no additional effect attributable to copper exposure (Fig.1.).

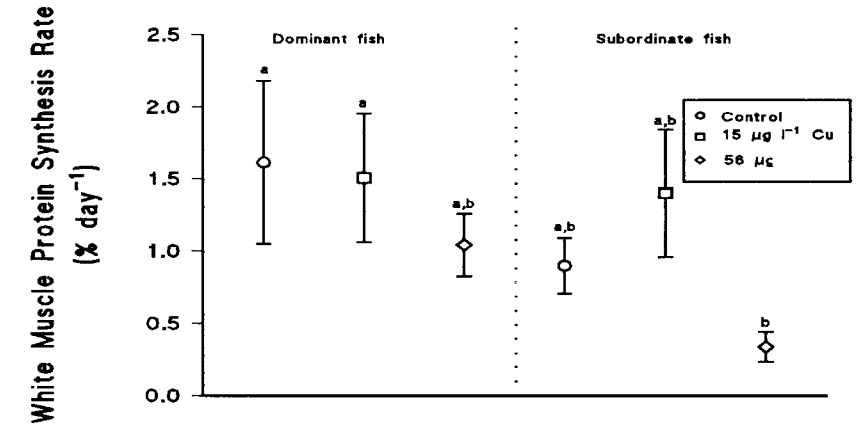

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Figure 1. White muscle protein growth rates in rainbow trout exposed to sublethal copper.

In contrast neither social rank, nor exposure to 15 Tg l^{-1} Cu, was found to effect on white muscle protein synthesis. However, whilst the white muscle protein synthesis rates of dominant rainbow trout, exposured to 56 Tg l^{-1} Cu, were also unaffected, this level of waterborne copper did induce a reduction in the white muscle protein

synthesis of subordinate fish (Fig.2.). White muscle RNA, relative to protein, was unaffected by copper: 1.4±0.1, 1.6±0.1 and 1.6±0.2 Tg RNA mg^{-1} protein in for fish exposed to 0, 15 and 56 Tg l^{-1}, respectively. Consequently the reductions in protein synthesis could be exclusively accounted for by reductions in translational efficiency (mg protein synthesised Tg^{-1} RNA day^{-1}): 2.9 ± 0.7^{a}, $2.2\pm0.4^{a,b}$ and 1.4 ± 0.3^{b}, (superscripts indicate similarities and differences).

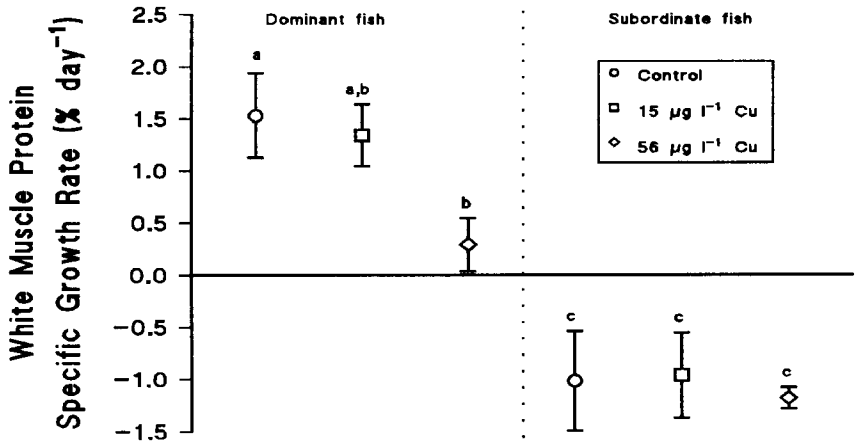

Figure 2. White muscle protein synthesis rates in rainbow trout exposed to sublethal copper.

Clearly growth rates in rainbow trout are influenced by more than one variable and, as this study demonstrates, to understand the effects of a waterborne metal, such as copper, one must account for the other growth related determinants; at these copper levels, social position and feeding rate are more significant than the metal itself. Furthermore the effect of a given copper concentration was dependent on nutritional /hierarchical status. However these data do suggest a mechanism by which waterborne copper could act to inhibit white muscle protein growth. In rainbow trout which receive an adequate food supply, the effects of copper appear to be restricted to post-translational events. Although synthesis occurs normally, there is a reduction in the efficiency of the deposition of newly synthesised proteins or those obtained directly from the food source. Such a toxicological mechanism

also applies to nutritionally compromised fish but, additionally in these animals, there is also an adverse effect on the translational aspects of protein metabolism.

Acknowledgment

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EFFECT OF ACUTE EXPOSURE TO COPPER ION ON GILL EPITHELIA OF *Prochilodus scrofa* (PROCHILODONTIDAE)

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Abstract

Juveniles *Prochilodus scrofa* were exposed to 0.020, 0.025 and 0.029 mg Cu L^{-1} during 96 h and the changes on gill epithelia were analyzed. Several pathological changes were identified in all copper concentration exposure. Filament cell proliferation, lamellar cell hypertrophy, lamellar fusion, epithelial lifting, aneurysm and cellular necrosis were the main gill damages and their frequency increased with the increase in copper concentration in water. Ultrastructural changes were also observed on pavement and chloride cells. Microridges reduction were significant in fish exposed to 0.029 mg L^{-1} Cu and also the number of chloride cells in apoptotic and necrotic stages. All gill structure damage found in *P. scrofa* may contribute to cause severe respiratory and ion regulation impairment depending on copper concentration.

Introduction

Copper ion is an essential metal that plays an important role in cellular metabolism and usually fish take their copper needs from food or water. Copper

levels in unpolluted waters vary from 0.5 to 1.0 μg L^{-1} however, after the industrial revolution, a significant increase in copper levels have been found in natural waters making this metal a serious polluter (Moore and Ramamoorthy, 1984). The toxic effects of copper on fish have been documented in several studies and the vulnerability of gills to copper and others metals have been demonstrated (Laurén and McDonald, 1985; Mallat, 1985; Benedetti et al., 1989; Pratap and Wenderlaar Bonga, 1993; Sola et al., 1995).

The gills are the first target organ of several heavy metals because of their very large interface area between external and internal fish environments. Performing vital functions such as gas exchange, ion- osmoregulation and N_2 excretion, the gills are particularly sensitive to adverse environmental conditions, and the changes on gill epithelia have been considered good indicators of the effects of xenobiotics on fish.

In this context, the present study has examined the effects of acute exposure to copper on gill morphology in the Brazilian freshwater fish, *Prochilodus scrofa*.

Materials and Methods

Juveniles *P. scrofa* (W = 15-25 g; L = 10 - 15 cm) were obtained from the Hydrobiology and Pisciculture Station of Furnas Hydroelectric Power Plant, Furnas, MG, Brazil, and were maintained in tanks with continuous dechlorinated waterflow and constant aeration at 25 ± 1 °C for, at least, one month prior to the experiments. The laboratory photoperiod was 12D:12L. Fish were fed with balanced fish food for this species provided by Aquaculture Research and Training Center CEPTA/IBAMA. Feeding was suspended 24 h before experiments.

Experimental design

Ten fish were placed into four tanks (200 L) in a way that the relation 1 g fish L^{-1} would not be exceed, 24 h before copper exposure. Fish from of the one tank did not receive copper and served as controls. Fish from the remaining tanks were exposed to 0.020; 0.025 and 0.029 mg Cu L^{-1} (the latter concentration is the LC_{50} - Mazon, 1997) during 96 h. Copper agent was $CuSO_4.5H_2O$ and copper concentration in water was determined at the beginning and end of each experiments using Atomic Absorption Spectrophotometry. Water tanks were

kept at 25 ± 1 °C, total hardness (as $CaCO_3$) 24 mg L^{-1} and pH 7.3 ± 1. After exposure, fish were removed, anaesthetized with 0.01% benzocaine and the gill filaments excised and processed for light and electron microscopy.

Light microscopy

Gill filaments were fixed with McDowell, dehydrated in crescent ethanol series and embedded in historesin (LKB). Sections were stained with toluidine blue and fucsin acid and observed under an Olympus-Micronal photomicroscope.

Electron Microscopy

Gill filaments were fixed in 2.5% glutaraldehyde buffered to pH 7.3 with 0.01 M phosphate buffer at 4 °C. Tissue samples for scanning electron microscopy were dehydrated by a graded ethanol and acetone series, critical point dried using CO_2 as transition liquid, coated with gold in a vacuum sputter and examined in a DSM 940 ZEISS Scanning Electron Microscope. Tissue samples for transmission electron microscope were post-fixed with 1% osmium tetroxide in 0.1 M phosphate buffer pH 7.3, dehydrated by graded acetone series and embedded in Araldite 6005 (Ladd Research). Semi-thin sections were stained with toluidine blue and examined under an Olympus-Micronal photomicroscope. Ultra-thin sections were stained with uranyl acetate and lead citrate and examined with a JEOL 100 CX Transmission Electron Microscope.

Results

The gill of control *P. scrofa* had a stratified filament epithelium between lamellae in which there were scattered chloride cells and some mucous cells. Lamellae epithelium consisted of two cell layers, pavement cells on the outer layer and undifferentiated cells on the inner layer, lined on the basal lamina which cover pillar cells and blood channels formed by their flanges.

After exposure to copper, several pathological changes such as proliferation of filament epithelial cells, lamellar pavement cell hypertrophy, epithelial lifting, aneurysms and necrosis were recorded on gill epithelia and their frequency increased with increasing copper concentration in water (Table 1).

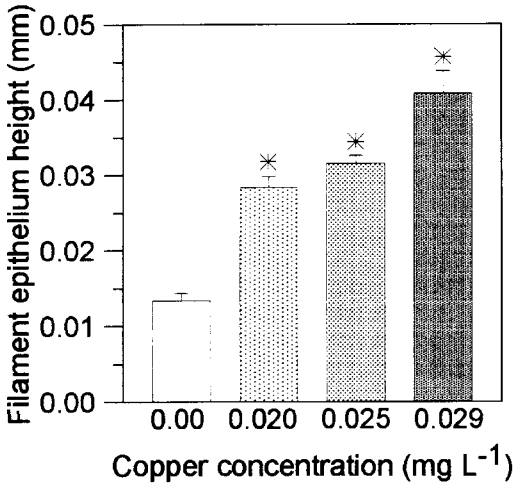
Filament cell proliferation were quantified by the height of filament epithelium showing significant increase in all copper concentration exposures (Fig.1). Such

cell proliferation together with pavement cell hypertrophy reduced the interlamellar space until complete lamellar fusion depending on copper concentration in water.

Table 1. Histopatological changes on gill epithelia of *P. scrofa* exposed to copper in water

Gill Histophatology	[Copper] in water (mg L^{-1})			
	0.0 (control)	0.020	0.025	0.029 (LC_{50})*
Filament cell proliferation	0	++	++	+++
Lamellar cell hypertrophy	0	-	+	+++
Lamellar fusion	0	-	+	++
Epithelial lifting	0	+	++	++
Aneurysm	0	+	++	++
Necrosis	+	++	+++	++++

(0) absence; (+) rarely present; ++ moderate; (+++) frequent; (++++) very frequent
estimated by Mazon (1997).\



.Fig. 1. Interlamellar epithelium height of *P. scrofa* gill filament from control group and groups exposed to copper in water. Values are mean ± SEM. * indicates significant difference from control fish ($p<0.05$)

Pavement cells microridges from filament epithelium changed from fingerprint-like to almost smooth cell surface and the circular microridges which delimited cell boundary were also reduced in fish exposed to 0.025 and 0.029 mg Cu L^{-1} (Fig 2).

The number of mucous cells did not change following exposure to copper although a significant increase in mucus secretion was observed. The chloride cell apical surface increased after exposition to copper and the chloride cell number in apoptotic and necrotic stages increased.

Discussion

Cell hyperplasia and hypertrophy, epithelial lifting, aneurysm and increase in mucus secretion have been reported after exposure of fish to a variety of noxious agents in the water, such as pesticides, phenols and heavy metals (Mallat, 1985).

All these lesions may impair respiratory function. Filament cell proliferation and lamellar pavement cell hypertrophy reduce the interlamellar space and may cause complete lamellar fusion reducing the total surface area for gas exchange such as found in *P. scrofa* and also in *Hepteroneutes fossilis* (Rajbanshi and Gupta, 1988), *Ictalurus nebulosos* (Benedetti et al., 1989), *Cyprinus carpio* (John and Jayabalan, 1993) and *Oncorhynchus mykiss* (Wilson & Taylor, 1993). Otherwise, they increase the distance of the water-blood barrier, which together with epithelial lifting and the increase in mucus secretion may drastically reduce the O_2 diffusion capability (Pinkey et al., 1989). The aneurysm in the lamellae also contribute to decrease O_2 uptake and, if the damaging agent is not removed, it can lead to the rupture of blood vessels with small hemorrhage focus.

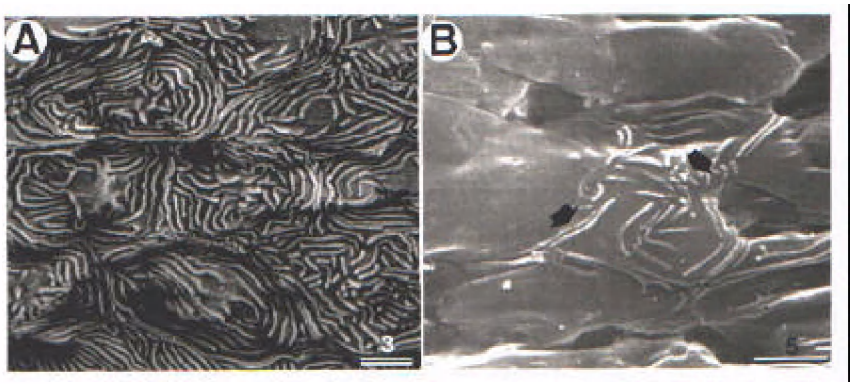


Fig. 2. Scanning electron photomicrograph of filament epithelium of *P. scrofa*. A. Control fish, B. Fish exposed to 0.029 mg Cu L^{-1}. Note the reduction of microridges (arrows) on pavement cell surface. Scale bar = μm.

Reduction of microridges on pavement cell surface have been earlier described for fish exposed to other metals such as cadmium, iron, chromate and mercury in water (Temmink et al., 1983; Pereira, 1988; Pratap & Wenderlaar Bonga, 1993) however, the physiological significance of this change was still unknown.

Chloride cell proliferation have been reported in fish exposed to metals (Pratap & Wenderlaar Bonga, 1993) and Sardet et al.(1979) suggested a possible role of these cells through the gills. In *P. scrofa* chloride cell number showed only a slighty increase however, the number of chloride cells in apoptotic and necrosis stages increased which may indicate an impairment on ion regulation. It corroborates with the studies carried out by Pelgrom et al. (1995) in *Oreochromis mossambicus* exposed to copper and cadmium in which the chloride cell number increased but it was followed by an increase of the number of these cells exhibiting degeneration signs and, in this case, they were unable to participate of active ion transport.

In conclusion, the effects of acute exposure to copper include most of gill lesions reported by Mallat (1985) and can be classified as the first and second reversible toxic stages for gills described by Poleksic and Mitrovic-Tutundzic (1994). Considering that *P. scrofa* have large respiratory surface area (Mazon et al., 1998), this species may maintain oxygen cascade in spite of the reduction of the O_2 uptake by gills, at least, in fish at rest. However, on the other side , its

large respiratory surface may also facilitate the copper transference to blood stream.

Acknowledgments

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MODELLING CHRONIC THRESHOLDS FOR TOXICITY – PHYSIOLOGICAL EFFECTS OF CHRONIC COPPER EXPOSURE TO RAINBOW TROUT

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Introduction

Impairment of growth is one of the most commonly used endpoints for the evaluation of the chronic toxicity of environmental contaminants and for the setting of chronic no-effect thresholds. In this study, we test the hypothesis that growth reduction is not necessarily the most sensitive indicator of chronic toxicity, at least for copper. Juvenile rainbow trout (*Oncorhynchus mykiss*) were exposed to sublethal waterborne copper, at levels either above or below the threshold for acclimation (i.e. increased lethal tolerance), for 30 days in moderately hard and soft water to evaluate six endpoints: acclimation, tissue residues, whole body electrolytes, growth, swim performance, and gill copper binding characteristics.

Materials and Methods

Juvenile rainbow trout, with an initial weight of 3-5 g, were held in 200L tanks supplied with either moderately hard dechlorinated Hamilton tap water (Ca^{2+} 1 mM, hardness 120 mg/L as $CaCO_3$, pH 8, 14°C) or synthetic soft water (Ca^{2+}

0.13 mM, hardness 20 mg/L as $CaCO_3$, pH 7.2, 17°C). Fish were exposed for at least 30 days and metal concentrations were maintained by the metered addition of copper stock ($CuSO_4.5H_2O$) into diluent H_2O in mixing head tanks.

In hard water (HW), fish were exposed to either 20 or 60 µg Cu/L and in soft water (SW), fish were exposed to only 1 and 2 µg Cu/L due to the increased toxicity in soft water. Fish were fed 3% of their wet body weight daily, distributed over 3 meals. At Day 0, 2, 10, 20 and 30, subsamples of fish were sacrificed and target organs removed for analysis of metal burden and electrolyte content. Growth was assessed from whole tank measurements throughout the exposure period. At the end of 30 days, swim performance was measured using a fixed velocity sprint test. Acclimation was tested by acute lethal metal challenges (96 h LC50s).

Results

Waterborne copper is more toxic to juvenile rainbow trout in soft water than in hard water. For naïve trout (1-2 g), copper was approximately 30 times more toxic. After 30 days of Cu in hard water, only the high Cu test group showed significant acclimation (see Table 1). These fish were approximately 2 times more resistant to copper than control fish. Sub-lethal exposure to copper in soft water resulted in no significant increase in copper tolerance.

Of the six endpoints, only four were affected by 30 day copper exposure. There was no significant effect on either growth or swimming performance in either HW or SW. Initial mortality (Day 1-10) occurred only in HW at high Cu, and occurred before there was any significant Cu accumulation in target tissues, principally the liver. However, only the high exposure groups (in HW and SW) showed any elevation in gill and liver Cu. The gills accumulated 4 times more Cu in HW than SW (2000 *vs* 500 ng/g) even though the water Cu levels were 30 fold higher (60 *vs* 2 µg/L, HW *vs.* SW), indicating a lower Cu permeability in HW. In contrast, Cu levels in the liver were the same in HW and SW fish and were several fold higher than in the gills. Only Cu in HW had an effect on whole body electrolytes (Na^+ and Cl^-) and it was not proportional to Cu level; 15% reduction in high Cu and 40% in low Cu.

An acute exposure to radiolabelled copper (^{64}Cu), was used to assess Cu permeability of the gills and confirmed a higher permeability to Cu in SW vs. HW. After 3 h at 9 µg/L, newly accumulated gill Cu in SW was 5 fold higher

than in HW. Furthermore, this test revealed a bimodal response to chronic copper exposure in both HW and SW fish. Low Cu fish showed a significant increase in newly accumulated Cu compared to controls whereas high Cu fish showed a signficant decrease in gill Cu compared to controls.

Discussion

In this study we have shown that growth impairment is, in fact, not the most sensitive indicator of chronic copper exposure. The most sensitive indicator was a change in gill copper permeability. This was the only response exhibited by all 4 exposure groups, followed by increased tissue burdens (2 of 4 groups), electrolyte losses (2 of 4) and acclimation (1 of 4). Indeed, our results show that a variety of effects occurs below the threshold for impairment of growth.

This absence of a growth effect is in contrast to an earlier chronic Cu exposure study by Waiwood and Beamish (1978) who showed inhibition of growth in rainbow trout at similar Cu levels to the present. The discrepancy between the two studies could be the result of a lower ration level in the earlier study. The importance of nutrition in toxicity modification has been largely overlooked, even though it can alter the uptake, metabolism and depuration rates of toxicants (Lanno et al., 1989).

Thus the broad implication of the present study is that chronic toxicity regulations or guidelines based on growth impairment alone may be too conservative. This leads to the question of whether there is an endpoint that is a universally more sensitive indicator than growth (e.g. acclimation), or will it be necessary to define a unique suite of indicators specific for each new toxicant?

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Pre-exposure	Acclimation	Growth	Swimming Performance	Gill Tissue Burden	Liver Tissue Burden	Whole Body Burden	Whole Body Na^+	Whole Body Cl^-	Newly Accumulated Gill-Copper
Hard Water									
20 µg/L	no	no effect	enhanced	no effect	no effect	no effect	decreased	decreased	greater than control
60 µg/L	yes	no effect	enhanced	increased	increased	increased	decreased	decreased	less than control
Soft Water									
1 µg/L	no	no effect	no effect	no effect	no effect	no effect	no effect	no effect	greater than control
2 µg/L	no	no effect	enhanced	increased	increased	no effect	no effect	no effect	less than control

Table 1. Presence or Absence of a Physiological Effect After 30 Days Copper Exposure.

PHYSIOLOGICAL MECHANISMS OF ACCLIMATION TO CHRONIC SUBLETHAL CU OR CD EXPOSURE IN RAINBOW TROUT

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Introduction

While the majority of heavy metals exert their primary toxic action at the gills of freshwater fish, specific mechanisms of toxicity vary (McDonald and Wood 1993). The development and validation of simple models to predict chronic toxicity are not as advanced as those for acute toxicity where currently, the "Gill Receptor Loading Model" (Bergman and Dorward-King 1997) has been used successfully. Predicting chronic toxicity is complex as responses of fish to long term metal exposure can be variable, for example gill metal burden can change considerably, and as well, many metals are reported to induce acclimation. Acclimation is an increased tolerance to an otherwise toxic level of a metal as a result of chronic sublethal exposure.

This study examines the influence of a pre-exposure to chronic sublethal waterborne metal on the subsequent uptake of Cd in rainbow trout to determine some of the physiological mechanisms involved in acclimation.

Materials and Methods

Juvenile rainbow trout were reared in dechlorinated Hamilton tap water (120 mg/L as $CaCO_3$) and then exposed to nominal concentrations of either 3 µg/L Cd ($Cd(NO_3)_2 \cdot 4H_20$) or 75 µg/L Cu ($CuSO_4 \cdot 5H_20$) for a month. These levels of

Cd and Cu exposure have previously been shown to induce acclimation (Hollis et al. 1998; Taylor et al 1998). A control group of fish were treated similarly but without metal.

Once acclimated, trout were tested for Cd uptake using ^{109}Cd. Each group (control, Cd or Cu acclimated) were exposed to 3 µg/L Cd with 1 µCi/L ^{109}Cd for up to 72 h in a 140 L recirculating system with aeration and constant temperature (13°C). Samples of 6 fish were removed at 3, 6, 10, 24, 48 and 72 h, placed in a solution of 3 µg/L Cd (without ^{109}Cd) for 5 min, transferred to a lethal concentration of MS222 (200 mg/L), euthanized and weighed. A gill sample was collected, washed in deionized water for 10 sec, blotted dry and then saved. Gills and whole bodies were analyzed for gamma radioactivity. Uptake of Cd was calculated using water specific activity with Cd content of water measured by graphite furnace atomic absorption spectrophotometry.

Results and Discussion

Chronic exposure Cd or Cu resulted in few mortalities (< 3% overall) and there were no differences in specific growth rate among exposed and control fish. Significant accumulations of Cd or Cu occurred in the gill, liver and kidney with Cd accumulations being highest in the gill and kidney while those for Cu were greatest in the liver.

When trout were exposed to 3 µg/L Cd for 72 h, fish acclimated to Cd had reduced uptake of Cd in both gills and whole body compared to controls (Table 1) and this reflects a mechanism of protection against the deleterious effects of Cd.

The results in Figure 1 show the time course of accumulation of Cd into the gills and illustrate that there were 2 distinct phases of Cd uptake; an initial rapid loading lasting about 12 to 24 h, and then a slower rate of continuing and constant uptake. It is likely that the initial uptake occurs into a labile pool of Cd (termed the "fast pool") which fills quickly and may be the source for transfer into other body compartments for storage (particularly the gill and kidney). The fast pool has been interpreted by Aslop et al (1998) using Zn, as a dynamic pool which binds to high affinity sites and may provide for temporary storage and detoxification.

Table 1. Mean and SEM of accumulation of new Cd in gills and whole body of rainbow trout exposed to 3µg/L Cd with added ^{109}Cd for 72 h.

	Treatment		
Accumulation of New Cd (ng Cd·g^{-1})	Unexposed Controls	Cd 3 µg · L^{-1}	Cu 75 µg · L^{-1}
Gills after 72 h	370 ± 28	233* ± 19	363 ± 30
Whole body after 72 h	2.4 ± 0.8	1.5* ± 0.2	6.5* ± 0.9

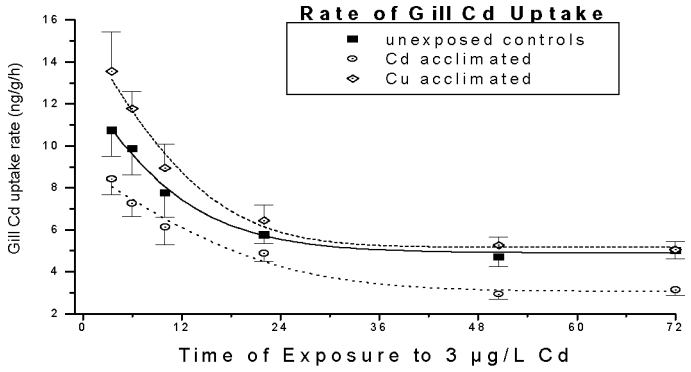

Figure 1. Rate of uptake of Cd into the gills of rainbow trout exposed to 3 µg/L Cd for up to 72 hours

The flattening of the curves (see Fig 1) after 24 h shows that Cd uptake falls to a constant level and this likely represents the transfer of Cd into storage pools occurring relatively slowly compared to initial uptake. This "slow pool" uptake rate is clearly reduced in Cd acclimated fish, results similar to those found for Zn uptake in Zn acclimated fish (Alsop et al. 1998).

Cu acclimated fish had no change in the rate of uptake into the slow pool in the gill (Fig 1) but had higher uptake into the body compared to controls (Table 1). The mechanism(s) associated with this elevated rate of transfer through the gill and into the body is unknown but may reflect an alterated gill fast pool for Cd in Cu acclimated fish.

The possibility of fast and slow accumulation pools in the gill and the incorporation of these parameters into current aquatic toxicity models is deserving of further study.

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EFFECT OF TEMPERATURE ON COPPER TOXICITY IN *Petenia kraussii* (PISCES: CICLIDAE) JUVENILES

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Abstract

Static biotrials were conducted at 22°C and 30°C, exposing copper sub lethal doses of 0.2 and 1.2 ppm in *Petenia kraussii* juveniles for 4 weeks and for same depuration period. The results obtained showed that temperature, metal concentration and exposing time are a determining factor in copper accumulation and depuration processes. Copper concentrations of 0.2 ppm at 22°C are usually regulated by the fish, but at 30°C the metal is not accumulated. The Cu concentrations of 1.2 ppm at 22°C and 30°C yield a progressive accumulation of the metal, reaching its highest concentration after 3 to 4 weeks of exposure. The accumulation was higher at 30°C. Copper depuration process in juveniles occurs 14 days after exposure to doses of 0.2 and 1.2 ppm of Cu at both temperatures tested.

Key words: Accumulation, Copper, *Petenia kraussii.*

Introduction

Trace metals are present in aquatic ecosystems as a natural element. Nevertheless, several dumps of anthropogenic origin have caused a progressive increase in their concentrations, creating several environmental problems in coastal zones, lakes and rivers. In most of these cases, it has been a consequence of untreated industrial and sewage dumps. The concentration of these elements above tolerable levels in the ecosystems is a disturbing factor in the survival of the species and the stability of the ecosystems.

The toxic effect of these metals is directly influenced by several environmental factors, such as salinity, pH, water hardness and temperature (Forstner &

Wittman, 1981). This is a very important factor, since it affects the metabolic rate of the organism (Lemus *et al.*, 1993), as well as its orientative responses, including its distribution inside the ecosystem (Coutant, 1987).

The effects of temperature variation on metal toxicity is a complex subject, since it influences the toxic potential, modifying the degree of lethality for one species and its survival rate at determined doses (Boada *et al.*, 1991), as the temperature affects the metal availability in the ecosystem and propagates reactions between this and the sediment.

The depuration of organisms exposed to toxic elements is a very important matter, since it allows to find out the time needed for a species to be detoxified. If it can not depurate the metal, and in this sense, potentially commercial species could not be recovered for human consumption. Boada (1984) reported that *Mugil curema* expunges Cu and Cd in 14 days and Zn in 21 days, after being exposed to 0.06 and 1.00 ppm of Cu, 5.00 ppm of Zn and 8.00 ppm of Cd.

Petenia kraussii, a fresh water fish living in the Maracaibo Lake basin, was introduced to Campoma Lake (Sucre, State) and others lakes in Venezuela as an edible species, since its meat has a high nutritional value (Carvajal, 1983). This species is eurithermal, showing ample tolerance to temperatures ranging between 15°C and 38°C, making it suitable to study the effect of temperature changes on copper muscular accumulation and detoxification (Segnini,1990).

The objective of this research is to establish the possible thermal effects on Cu bioaccumulation and detoxification in *Petenia kraussii* juveniles.

Materials And Methods

Specimen collection

Petenia kraussii juveniles were collected in "El Aguá" lagoon, 10° 30" north and 63° 41" west, near Chiguana, (Sucre State, Venezuela) during November 1990 and May 1991, using 52 feet long and 10 feet wide net with 3 mm mesh. The specimens were placed in coolers with aerators and transported to the laboratory for their acclimation. The content of dissolved oxygen in the field water ranged between 8.2 - 9.2 mg/l, pH between 7.8-8.0 and temperature between 26-32°C.

Acclimation

Petenia kraussii juveniles with an average weight of 3.91 ± 1.2 g were acclimated in 100 l tanks, at 22°C and 30°C for 15 days. During this period the fish were fed a universal commercial diet, ingesting as much food as they could for 5 minutes, three times a day.

Determination of 96-h CL_{50} (mean tolerance limit)

The determination of the mean lethal concentration at 22 °C and 30 °C was made through a static system, replacing both water and metal every 24 hours, using 6 tanks of 40 liters each, with concentrations of 0, 2, 4, 8, 16 and 32 mg/l of Cu, prepared with copper sulfate. The 8 fish were placed in each tank, with their respective duplicates, for a 96-hour exposition period. Observations were made at intervals of 12, 24, 48, 72 and 96 hours. The fish were not fed during this period. Determination of 96-h CL_{50} was made using Probit's Method (Stephan, 1977).

Experimental treatment

Copper accumulation and detoxification essays were performed at 22°C and 30°C, using 0.20 and 1.20 ppm doses, after the mean tolerance limit was established for such temperatures. Each condition was set by duplicate with their respective temperature controls at 22°C and 30°C. The exposition time to the metal was 30 days, with observation intervals of 7, 14, 21 and 30 days; then 8 specimens were sacrificed each time. The fish left were placed in clean water (without metal) for 30 days, sampling them after 7, 14, 21 and 30 days of detoxification. The 50% of the water and the metal were renewed. The pH readings were taken every 2 days, showing a mean value of 8.1 ± 0.15. Water hardness during this investigation was 55 μg/l for calcium carbonate and temperatures ranged between 22.0 ± 0.5 ~ 30.0 ± 0.75 °C.

Sampling

Every week 8 fish were weighed and beheaded, washed with distilled water, frozen and stored to establish total copper content.

Determination of Copper content

After being dehydrated, the specimens were pulverized in a mortar and submitted to digestion with 10 ml of HNO_3 (70% v/v) for 1 hour at 60°C until drying off completely. Later, a mixture of nitric acid, perchloric acid and sulfur acid (HNO_3, $HCLO_4$, H_2SO_4:10:4:1) was added to complete digestion for 1 hour and was later evaporated. The processed samples were filtered using Watman N° 42, adding 50 ml of distilled water. Determinations were made using a Perkin Elmer atomic absorption spectrophotometer, Model 403, and the concentrations were expressed as μg of Cu/g of dry weight.

Statistical treatment

Copper accumulation and detoxification were determined using a multifactorial variance analysis to establish both the individual and group effect of temperature (22°C and 30°C), copper concentration (0.20 and 1.20 ppm) and exposure time (7, 14, 21 and 30 days) (Hicks, 1973).

Results

Mean tolerance limit (96-h CL_{50})

Copper mean lethal concentration (96-h CL50) in *P kraussii* juveniles was 2.84 ppm at 30°C, with confidence limits between 1.82-4.46, while at 22°C the concentration was 4.85 ppm, with confidence limits between 3.28 - 7.16 (Table 1).

COPPER BASAL LEVELS

Copper basal levels in *P kraussii* juveniles at 22°C showed concentration values ranging between 6.54 ± 0.946 and 7.33 ± 0.560 μg/g, presenting no significant differences during the four weeks of exposure. An increase in the metal concentration was observed at 30°C, with values ranging between 8.55 ± 1.401 and 10.31 ± 1.536 (Table 2).

Table 1.- Copper mean lethal concentration (96-h LC_{50}) in *Petenia kraussii* juveniles, after 96 hours of exposure at 22°C and 30 °C.

N (number of specimens)	Temperature (°C)	LC_{50}	Confidence limits (95%)	Total weight (g)
48	22	4.85	3.28-7.16	3.61±1.10
48	30	2.84	1.82-4.46	3.88±0.70

Table 2.- Content of basal copper (μg/g of dry weight) in *Petenia kraussii* juveniles exposed to 22 and 30°C.

	TIME (days)				
Temperature	7	14	21	30	Fs
22°C	6.86±0.626	7.33±0.560	7.31±0.767	6.54±0.946	2.647ns
30°C	10.31±1.53	8.92±1.312	9.41±1.908	8.55±1.401	1.965ns

COPPER ACCUMULATION

The results obtained during the accumulation process proved that temperature, metal concentration and exposure time significantly affected the accumulation patterns of this species ($P<0.001$). Similarly, these three factors had an interactive effect on the copper concentration of this species (Table 3).

Table 3.- Variance analysis between copper concentrations (μg/g of dry weight) in *Petenia Kraussii* juveniles exposed to concentrations of 0.2 ppm and 1.2 ppm at 22 °C and 30 °C after 4 weeks of accumulation and depuration.

Source of variation	Accumulation Fs	Depuration Fs
Temperature	223.426***	54.548***
Concentration	386.724***	107.314***
Time	53.198***	142.942
TexC	80.592***	8.957***
TexTi	15.214***	2.647ns
CxTi	86.636***	53.767***
TexCxTi	12.751***	1.721ns

COPPER ACCUMULATION AT 22°C

Petenia kraussii juveniles exposed to copper doses of 0.2 ppm for 4 weeks showed a significant accumulation after 7 days of exposure (17.48 ±0.993 μg/g of dry weight), decreasing to 50% of this value after 14 days (8.19 ±0.601), and remaining constant in the following 2 weeks (Fig.1A).

Exposure to copper doses of 1.2 ppm showed the species progressively accumulated the metal, reaching its highest concentration during the last 2 weeks (18.77 ± 3.775 and 18.34 ± 4.007 μg/g of dry weight in the 3rd and 4th week, respectively) (Fig. 1B), thus demonstrating that the accumulation pattern for this species using copper doses of 1.2 ppm is inverse to the one observed with a concentration of 0.2 ppm during the 4 weeks of exposure.

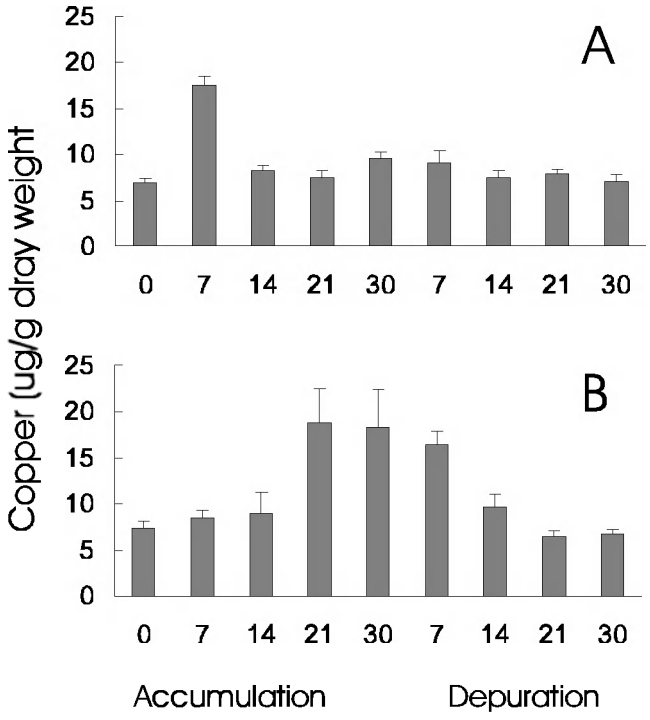

Figure 1.- Accumulation and Depuration of Copper in *Petenia kraussi* Juveniles Exposed to: A 0.2 ppm And B. 1.2 ppm of Cu and 22ºC.

COPPER ACCUMULATION AT 30 °C

Fish exposed to 0.2 ppm showed no greater variations in metal accumulation during the 4 weeks of exposure to copper, with concentrations ranging between 11.38 ±1.468 ~13.26 ±2.48 μg/g of dry weight (Figure 2A). When the dose was increased to 1.2 ppm, the accumulation was progressive, until reaching a maximum concentration after 3 weeks, showing values of 33.51± 3.457 μg/g, nd remained stable until the 4th week (33.78 ± 1.930 μg/g of dry weight) (Fig. 2B).

COPPER DEPURATION

Statistical tests for the copper concentrations in *Petenia kraussii* juveniles are shown in Table 1, attesting that temperature, metal concentration and exposure time definitively affected the copper elimination process ($P<0.001$). An interactive effect between the concentration and the exposure time ($P<0.001$) was also observed and it lasted 14 days. In this 2nd week, fish reached copper concentrations within the basal concentration ranges at the temperatures tried (Fig.1 and 2).

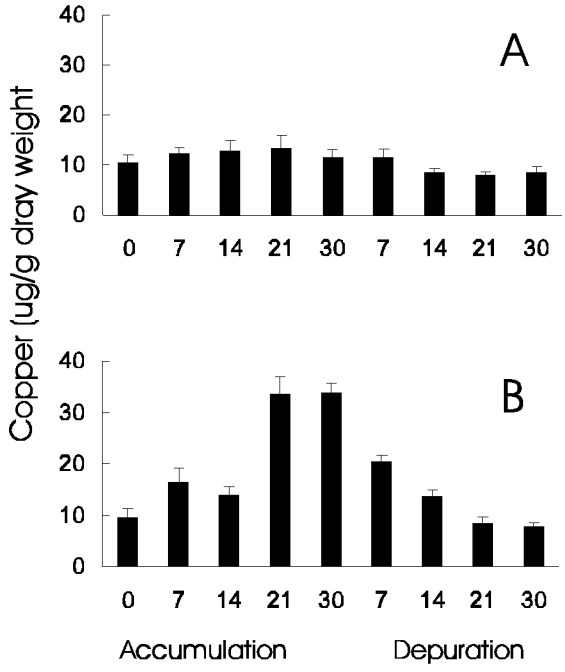

Figure 2.- Accumulation and depuration of copper in *Petenia kraussi* juveniles exposed to: **A** 0.2 ppm and **B**. 1.2 ppm of Cu and 30°C.

Discussion

Mean tolerance limit (96-h LC_{50})

Determination of copper mean lethal concentration after 96 hours in *Petenia kraussii* juveniles with a wet weight of 3.88 ± 0.98 g was 4.93 ppm at 22°C and 2.79 ppm at 30°C, suggesting that temperature is a determining factor in the survival of the species (Table 1). This indicates that copper has a more toxic effect in juveniles exposed to warmer temperatures.

These results were similar to those obtained by other authors for several other metals and indicated that for many species temperature is a determining factor influencing heavy metal toxicity (Sprague, 1973; Sullivan, 1987). Boada (1984), however, pointed out that 96-h LC_{50} for copper, cadmium and zinc in *Mugil curema* juveniles were slightly higher at 28°C than at 22°C.

At the present time, there is no substantial evidence supporting the joint effect of temperature and metal toxicity, mostly due to its complexity (Hodson & Sprague, 1975; Thomas, 1990), even though it has been suggested that warm temperatures could increase the toxicity of one contaminant, since its absorption is somehow accelerated (Sprague, 1973) due to changes in the membrane permeability (Bryan, 1976). Temperature also affects the fish's breathing rate, as suggested by Segnini (1990). In *Petenia kraussii*, juveniles presented a higher breathing metabolic rate at 30°C than at 22°C. This response implies a higher water flow toward the gills that would somehow facilitate a higher intake of the metal available in the water through breathing. To summarize, it can be suggested that warm temperatures and metal act synergically, lowering the fish's survival rate.

Many authors point out that temperature is a factor influencing the organisms' metabolism. They also suggest that fish have a higher energetic expense at warm temperatures than at lower ones, which implies a more active metabolism (Savitz, 1969; Graham, 1970; Bulow *et al.*, 1978; Lemus *et al.*, 1993).

Moreover, the high metabolic expense generated by high temperatures makes the organism diminish its physiological condition, turning it more susceptible to the metal. Copper itself has many side effects on the intermediate metabolism, affecting the activity of many enzymes. As a consequence, the energetic load in the hepatic and muscular tissues can also decrease (Heath, 1984). Many other effects are caused by sub lethal doses, but chronical exposure can be accounted

for direct effects on growth and reproduction (Beisinger & Christense, 1972; Barron & Adelman, 1984; Rengel, 1990).

The 96-h CL_{50} results obtained through probit-logarithm tests for this research yield a very wide range for the confidence limits (95%). This would indicate that 96-h CL_{50} parameter is a major feature indicating the investigator if a concentration is lethal or not within a given time period, but never reflecting the fish's physiological condition (Sprague, 1973) and consequently keeping the researcher from inferring the toxic effects on the population.

Similarly, it is difficult to set comparisons between the mean tolerance limits obtained in this research and those of other authors, since the mean tolerance of the pollutants is directly related to physicochemical conditions used to determine the parameter (Miller & McKay, 1980; Campbell & Stokes, 1985).

COPPER ACCUMULATION AND DEPURATION

Petenia kraussii juveniles exposed at 22°C showed lower copper basal concentrations than those exposed to 30°C, indicating that this essential bioelement is subject to modifications in its concentration when the fish are changed between temperatures. So, temperature is an important factor in redistributing the metal inside the organism (Delpledge & Rainbow, 1990; Davalli *et al.*, 1990; Lemus *et al.*, 1993). Likewise, it has been suggested that concentrations of bioessential elements such as copper and zinc are subject to metabolic requirements depending on the physiological condition, maturity level and post-capture stress (Kevin & Thomas, 1990).

Accumulation is directly related to copper levels in the environment; specimens exposed to doses of 1.20 ppm at 22°C accumulated the metal progressively during the 4 weeks of exposure, while the specimens exposed to doses of 0.2 ppm showed its peak accumulation after seven days, decreasing then to concentrations slightly above those basal levels for that temperature with slight fluctuations. This could suggest that the fish exposed to lower doses are able to regulate the concentration in their tissues.

This mechanism allowed the intake of high concentrations during the first 7 days, and later decreased them after 14 days, keeping a stable 0.2 ppm copper concentration similar to the control group. It was not maintained when the metal dose was 1.20 ppm, since there was a significant accumulation due to the

exposure period, allowing it to reach concentrations of up to 18.34 ± 4.07 μg/g of dry weight after 4 weeks.

When the bioassay temperature was 30°C, copper concentrations of 0.2 ppm had little effect on the accumulation processes, remaining almost stable during the 4 week period, a behavior opposite to the one observed at 22°C. This could indicate that warmer temperatures facilitate the metal regulation processes in a way that the specimen is unable to accumulate concentrations of the pollutant in its body.

When the concentration was increased to 1.2 ppm at 30°C, a very definite response was observed, confirming the fact that the fish accumulated concentrations of up to 33.78 ± 3.930 μg/g dry weight, and this is the direct influence of temperature on the metal availability and possibly on the membrane permeability to the pollutant.

It has also been suggested that high concentrations of the metal can be stored in the organism in a non toxic way, through specific proteins known as metalothionine, which can strongly link to copper and other metals (Brady, 1982) depending on the metal affinity: Hg>Cu>Cd>Zn (Engel & Brower, 1989). Rainbow & White (1989) suggest that this mechanism for metaloprotein forming admits intracell bioavailability, since the active points can be metabolically regulated, even though an overall body or tissue analysis could show extremely high concentrations.

The depuration process in juveniles exposed to both chronic doses and temperatures was achieved after 14 days. During this period, metal accumulation of the fish reached a concentration similar to the control group. Similar results were obtained by Boada (1984) during the copper depuration process (0.06 and 1.00 ppm) in *Mugil curema* juveniles exposed to 22 and 30 °C.

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OIL PRODUCED WATER: CHRONIC IMPACTS

ON JUVENILE TURBOT

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Background

Oil and gas extraction generates 'produced water' (PW), a mixture of formation and injection waters. Marine platforms, such as those in the North Sea, discharge large volumes of PW containing dispersed and dissolved oil, metals and treatment chemicals (Roe et al 1996). Our studies of turbot larvae on a North Sea oil platform indicate acute sub-lethal effects in fish exposed to 0.1-10% PW (Stephens et al 1996; Stephens 1997). While acute effects may be limited to the immediate vicinity of discharge perhaps extending 50-100m, chronic effects are likely to extend further from the outfall, although they are largely unknown. We have therefore studied longer-term effects of 0.001%-1% PW on juvenile turbot.

Methods

Produced water, supplied approx. weekly by Amerada Hess from a North Sea oil platform (AH001) had osmolality 2065-2492 mOsm/kg, but at the dilutions used seawater osmolality was not significantly elevated. Total oil content of stock PW (IR spectrophotometry pre-dispatch) varied (24-101 ppm); GC-MS

analysis in Exeter indicated a consistent range of aliphatic (C11-C32), aromatic and polycyclic aromatic hydrocarbons. Solid-phase micro-extraction (Supelco

fibres) and GC of 1% PW gave: benzene (2.2±1.1ppb), xylene (1.5±0.7ppb), naphthalene (0.5±0.3ppb), C14 (0.7±0.4ppb), C17 (0.3±0.2ppb) and C18 (0.2±0.1ppb), declining to 'undetectable' between water exchanges.

Turbot (≈ 53 days old), from Mannin Sea Farms, Isle of Man, were held as separate groups in 0.001%, 0.01%, 0.1%, 1% PW and clean seawater for 1-6 weeks and fed dry pellets (Mannin Sea Farms; 0.5% total body wt fish/tank/day). Survival was monitored daily. The proportions of fish swimming were monitored daily from day 10. At the end of experiments fish were killed and either fixed for scanning electron microscopy of gills (Cambridge Stereoscan 100) or frozen. Growth was determined from wet weights.

Individual fish were used to determine hydrocarbon accumulation (chrysene equivalents) by pentane-extraction and fluorescent spectrometry, EROD activity (Stagg et al 1995), and cortisol content (index of corticosteroid stress responses; Stephens et al 1997).

Results and Discussion

Survival, swimming activity & growth

Turbot survival was unaffected by PW-exposure. At least 78% of fish survived 6 weeks' exposure (Fig1).

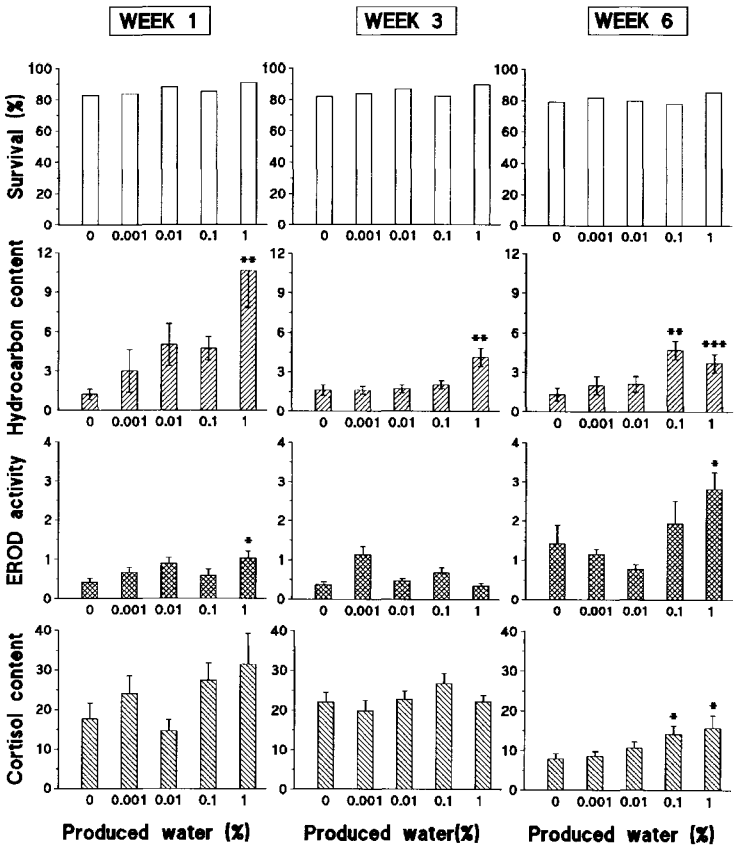

Figure 1. Survival, hydrocarbon content (μg g body wt^{-1}), EROD activity (pmol min^{-1} gbody wt^{-1}) and cortisol content (ng g body wt^{-1}) of turbot juveniles after 1, 3 or 6 weeks in 0.001-1% PW or clean seawater. *P<0.05,**P<0.01,***P<0.001 compared to controls (5-10/group/parameter).

In 0.01% PW the mean proportion of fish swimming at any time was elevated (51±2%, P<0.05) compared to control fish (34±2%). However, 1% PW

depressed swimming activity (21±1% active, $P<0.01$), probably reflecting narcotic actions of aromatic hydrocarbons.

Growth was initially low in all fish and apparently unaffected by PW-exposure. Between weeks 3-6, growth increased dramatically to 1.6-3% wet wt/day (calculated over 3 weeks), but was unrelated to PW-exposure. Over the 6 week study, growth was lower in the more physically active fish held in 0.01% PW (wet wt (mg): 573 ±17; controls: 656±25, n=10/group). However, all fish were fed equal and restricted rations; in the wild, food availability and its capture will influence growth.

Whole body hydrocarbon accumulation & EROD activation

After 1 week's exposure to 1% PW, ≈8-fold hydrocarbon accumulation was observed (Fig 1). Thereafter, hydrocarbon accumulation declined implying increased xenobiotic metabolism.

Whole body EROD activity was significantly elevated after 1 or 6 week exposure to 1% PW (Fig 1), but not after exposure to more dilute PW, despite some hydrocarbon accumulation, which declined as time progressed. Complex time-relations between hydrocarbon accumulation and metabolism are implied.

Gill morphology

Gill damage in fish exposed to PW increased with time until, after 3 weeks, lamellar fusion was common and severe in turbot exposed to 1% PW. Even in fish exposed to 0.01 or 0.001% PW damage was apparent and persistent (Fig 2) and could disturb ion and/or acid/base balance and/or cause respiratory stress.

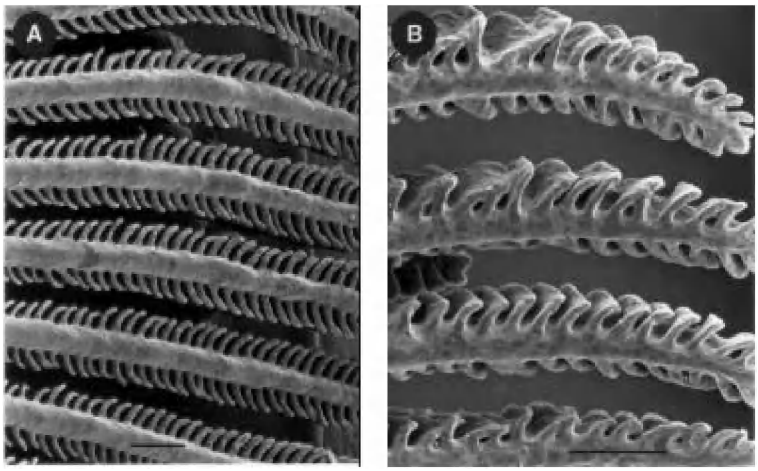

Fig 2. Scanning electron micrographs of gills of turbot A: control; B: 6 weeks in 0.001% PW. Bars: 100 μm.

Whole body cortisol content

Despite gill damage and implied physiological stress, fish held in 0.001% or 0.01% PW showed no significant increase in cortisol content (Fig 1). However, cortisol content of turbot exposed to 0.1% or 1% PW for 6 weeks was significantly higher than in control fish.

These investigations have demonstrated that in the immediate vicinity of PW discharges, depressed physical activity could influence prey capture. North Sea hydrocarbons, measured around platforms, suggest at least 10,000x and possibly 50,000x dilution of PW 1000m from the discharge (Stagg et al 1996). However, prolonged experimental exposures of juvenile turbot to even 10,000-100,000x-diluted PW caused gill damage and the functional implications of these changes should be investigated.

Acknowledgments

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UPTAKE, INHIBITION, AND DEPURATION OF NITRITE TO SHORTNOSE STURGEON ACIPENESER BREVIROSTRUM FINGERLINGS

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The objectives of this study were to characterize nitrite uptake rates, determine the ability of chloride to inhibit the uptake of nitrite, and to determine the effectiveness of chloride as a treatment for shortnose sturgeon fingerlings *Acipenser brevirostrum* exposed to high levels of environmental nitrite. All experiments were conducted in glass aquaria, each containing one shortnose sturgeon fingerling (16.5 ± 4.85 g; 174.5 ± 12.15 mm TL) and 30 L of continuously aerated test water (1.8 mg Ca/L; < 1.0 mg Cl/L; 18.0 ± 0.13 °C). Plasma nitrite concentrations of shortnose sturgeon fingerlings increased with exposure time. Nitrite concentrations in plasma were elevated > 63 times the environmental concentration after 5 d of exposure. Shortnose sturgeon fingerlings are strong concentrators of nitrite compared to other warmwater fishes (Tomasso 1986). Plasma nitrite concentrations of shortnose sturgeon increased with increasing environmental nitrite concentrations. Nitrite concentrations were significantly higher in those fish exposed for 48 h to 4 mg/L than those exposed to 1 or 2 mg/L nitrite-N. Palachek and Tomasso (1984) found similar results with channel catfish *Ictalurus punctatus* and tilapia *Tilapia aurea*, but not largemouth bass *Micropterus salmoides*. Chloride was more effective at inhibiting the uptake of environmental nitrite when added as calcium chloride rather than as sodium chloride. Chloride is effective at reducing the uptake of nitrite in several fishes, especially in the form of calcium chloride (Tomasso 1994). The ability of calcium chloride to prevent the uptake of environmental nitrite increased with increasing concentrations of calcium chloride. After 2nd exposure, blood plasma concentrations were significantly

lower in those fish treated with 20 mg/L chloride than those treated with 5 mg/L chloride as calcium chloride. Plasma nitrite concentrations did not continue to increase when exposed fish were treated with calcium chloride or moved to fresh water. There was a significant difference in plasma nitrite between those fish that remained in nitrite solutions without chloride protection and those that received either calcium chloride or were transferred to freshwater. Also, there was no difference between the calcium chloride and fresh water treatment. The addition of calcium chloride to water with an elevated concentration of nitrite is similar to moving exposed shortnose sturgeon fingerlings to nitrite-free water. Plasma nitrite concentrations in shortnose sturgeon fingerlings were significantly reduced each day after 40 mg/L chloride (as calcium chloride) was added to the test water. There was a strong linear relationship (r^2 = 0.8598) between plasma nitrite concentration and exposure time to chloride. Although plasma nitrite concentrations did not reach control levels (0.40 ± 0.440 mg/L nitrite-N) after 3 d exposure to the calcium chloride treatment, the linear model (plasma nitrite-N = 137.225 - 33.96 * days of exposure) predicts that plasma nitrite-N concentrations should reach zero in about 4 days for shortnose sturgeon fingerlings exposed to 2.14 ± 0.069 mg/L nitrite-N for 48 h (a molecular ratio of 18.7 chloride ions to 1 nitrite ion). Nitrite is taken up from the environment and concentrated in the plasma of shortnose sturgeon fingerlings to many times the environmental concentration, presumably by the chloride uptake mechanism. Although several warmwater fishes are able to concentrate nitrite in their plasma above environmental levels (Tomasso 1986, 1994), shortnose sturgeon fingerlings tend to concentrate nitrite in their plasma to greater levels, similar to salmonids (Margiocco et al. 1983). Environmental nitrite can be competitively excluded by chloride and calcium chloride is more effective than sodium chloride. The addition of calcium chloride to the environment appears to be an effective means of preventing nitrite uptake and treating nitrite toxicity in shortnose sturgeon fingerlings.

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DIETARY EXPOSURE TO PCB 126:

INFLUENCE ON INTERRENAL STRESS RESPONSE

AND INDUCTION OF P450 SYSTEMS

IN RAINBOW TROUT

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Introduction

Environmental (lipophilic) pollutants have been shown to induce the cytochrome P450 (CYP1A) system, involved in biotransformation of xenobiotics (Gøksoyr, 1995), with some having endocrine disruptive potencies (McKinney, & Waller, 1994). Influences of PCBs and related organic chemicals on the stress responsiveness of fish have been demonstrated in field studies (for review see Hontela 1998). In a laboratory study, we have shown that in Mozambique tilapia the stress responsiveness, i.e. interrenal cortisol release, is impaired after 5 days-dietary exposure to PCB126 (Quabius et al. 1997). In the present work, we extend our observations to another species, the rainbow trout (*Oncorhynchus mykiss*), and consider a possible involvement of the PCB-inducible CYP1A in the impaired endocrine stress response. To this end, trout were fed control food or food containing either 0.5µg (low) or 50µg (high) PCB 126 per kg body weight per day for 5 days. To study the influence on the interrenal stress response groups of fish were sampled either directly, were subsequently exposed to handling (confinement) or starvation with an additional

handling treatment at the end of the starvation period. Treatment effects on the interrenal stress response were estimated from alterations of plasma cortisol levels; influence on CYP1A was estimated by immunohistochemical analysis of CYP1A and by measuring the CYP1A-associated catalytic activity, 7-ethoxyresorufin-O-deethylase EROD.

Material and Methods

Juvenile rainbow trout (n=96) with an average bodymass of 25.6±1.2g were kept in duplicate tanks and received either control, high or low PCB food. After 5 days of feeding 8 fish from one control, low and high diet tank respectively were sampled immediately, and the remaing fish were confined in a net for 4h in their original tanks and sampled afterwards. The fish in the second group of tanks remained undisturbed, were starved for 3 weeks and then sampled either directly or after 4h confinement. Fish were bled and plasma was analysed for cortisol by RIA. Headkidney, gill, liver and intestinal tissues were either placed in Bouin's fixative for immunohistochemical investigation or were fractionated by ultracentrifugation into microsomes for fluorometric EROD analysis.

Results and Discusion

Directly after exposure no significant differences in plasma cortisol levels were obtained between groups. Confinement, used as an additional stressor, resulted in similar increases of plasma cortisol in all groups (fig1a). Starvation remained without effect on basal plasma cortisol levels (fig 1b), which suggests that this was not stressful. However, when starved fish were subjected to confinement stress, the cortisol response was depressed in a dose-dependent manner (fig1b), indicating an impaired ability of the PCB-fed fish to cope with an additional stressor, similar as described earlier for tilapia in a laboratory study (Quabius et al.1997) and in field studies on yellow perch (for review see Hontela, 1998). An alternative explanation, i.e. exhaustion of the interrenal cells due to starvation, as suggested by Hontela (1998) was considered unlikely because confinement induced a similar rise of plasma cortisol levels in starved and unstarved fish (fig 1a,b). Immunohistochemical examinations and indicated measurement of EROD activities revealed basically congruent results, namely CYP1A-induction at both concentrations in all tissues (Table1, figs.1c-h) except for the intestine, where no EROD activity could be measured. In trout headkidneys, significant EROD activities were induced by PCB126. As by immunohistochemistry, this increase was not correlated with CYP1A induction in the interrenal cells, but with headkidney immune cells. Similar results were obtained in immune organs,

including headkidney, in 3-methylcholanthrene-exposed carp (Marionnet et al. 1997). After three weeks starvation in none of the tissues investigated the induction response was altered (Table 1, figs 1c-h). Although food deprivation is frequently associated with reduced EROD levels, circulating or organ levels of PCB 126 in starved fish were still sufficient to support CYP1A induction. For fish hepatocytes, a potentiating role of cortisol on EROD activities has been described (Devaux, 1992); such an effect was indicated in the headkidneys of non-starved, high PCB-treated fish (figs 1a,c) in the liver and headkidneys of starved, low PCB-treated fish (figs 1b,d,f.) and in liver and gills of control fish (figs 1b,f,h). However, this relation between plasma cortisol and EROD activities was not consistent and the overall inverse relationship between plasma cortisol levels and EROD activities in confined starved fish makes it likely that other factors or synergistic effects (nutritional state in combination with nontoxic stress i.e. handling) contribute to the increase in EROD activities.

Conclusions

From the data presented we conclude that PCB 126 impairs the interrenal stress response in trout and thereby the ability of the fish to cope with additional stressors. We found no consistent evidence supporting the assumption that reduced plasma cortisol levels could be ascribed to an induction of CYP1A. Our data on EROD activities, in particular in starved fish, suggest that the physiological condition can modulate the CYP1A response in fish.

TABLE 1: Semiquantitative analysis of CYP1A protein expression in different trout tissues after oral exposure to PCB 126, subsequent confinement and starvation; with "-" indicating no expression up to "++++" indicating very strong expression, and "$^{+}$" indicating little or intermediate expression between i.e. "+" and "++"

	headkidney (n=8)		liver (n=4) note: bile ducts and arteries showed the same staining intensity under all conditions		intestine (n=4)		gill (n=4)	
control	endocrine cells leukocytes blood vessels	- +++ +	hepatocytes bile canaliculi	$^{+}$ +	epithelial cells lamina propria	+ -	filament lamellae	$^{+}$ $^{+}$
control confined	endocrine cells leukocytes blood vessels	- ++++ ++	hepatocytes bile canaliculi	+ ++	epithelial cells lamina propria	$+^{+}$ -	filament lamellae	+ +
control starved	endocrine cells leukocytes blood vessels	- +++ +	hepatocytes bile canaliculi	+ +	epithelial cells lamina propria	+ -	filament lamellae	$^{+}$ $^{+}$
control starved + confined	endocrine cells leukocytes blood vessels	- +++ +	hepatocytes bile canaliculi	++ +	epithelial cells lamina propria	$+^{+}$ -	filament lamellae	+ +
low PCB	endocrine cells leukocytes blood vessels	- ++++ ++	hepatocytes bile canaliculi veins	++ ++ +	epithelial cells lamina propria	++ +	filament lamellae	++ ++
low PCB confined	endocrine cells leukocytes blood vessels	- ++++ +++	hepatocytes bile canaliculi veins	$+++^{+}$ ++ +	epithelial cells lamina propria	$++^{+}$ +	filament lamellae	++ ++
low PCB starved	endocrine cells leukocytes blood vessels	- ++++ +	hepatocytes bile canaliculi veins	$+++^{+}$ +++ ++	epithelial cells lamina propria	++ ++	filament lamellae	+++ ++
low PCB starved + confined	endocrine cells leukocytes blood vessels	- +++ +	hepatocytes bile canaliculi veins	++++ +++ ++	epithelial cells lamina propria	$++^{+}$ ++	filament lamellae	+++ ++
high PCB	endocrine cells leukocytes blood vessels	- ++++ ++	hepatocytes bile canaliculi veins	++++ +++ +	epithelial cells lamina propria	+++ ++	filament lamellae	+++ ++

high PCB confined	endocrine cells leukocytes blood vessels	- ++++ +	hepatocytes bile canaliculi veins	++++ +++ +	epithelial cells lamina propria	$+++^{+}$ ++	filament lamellae	+++ ++
high PCB starved	endocrine cells leukocytes blood vessels	- ++++ ++	hepatocytes bile canaliculi veins	++++ ++ ++	epithelial cells lamina propria	++++ $++^{+}$	filament lamellae	$+++^{+}$ $++^{+}$
high PCB starved + confined	endocrine cells leukocytes blood vessels	- ++++ ++	hepatocytes bile canaliculi veins	++++ ++ ++	epithelial cells lamina propria	++++ $++^{+}$	filament lamellae	+++ ++

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CALIFORNIA RICE FIELD PESTICIDES:

SUBLETHAL RESPONSES OF LARVAL FISH

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Introduction

Several species of fish, including striped bass (*Morone saxatilis*), spawn in the Sacramento River in the central valley region of California. Water flowing from extensive adjacent rice culture activities carry the pesticides methyl parathion, molinate, and carbofuran, into the river at approximately the same time as the spawning occurs in late spring. After hatching, striped bass larvae drift for approximately 4 days down the river in the contaminated water to the Sacramento - San Joaquin ("Delta") Confluence/Estuary where subsequent growth and development occurs. We simulated these conditions in the laboratory using striped bass larvae, and larvae of medaka (*Oryzias latipes*) and fathead minnows (*Pimephales promelas*) as surrogates for the striped bass. Whereas striped bass larvae are only obtainable during a limited period during the year, medaka and fathead minnows are continuously culturable in a laboratory. Our objectives were to assess the sublethal responses of these species to rice field pesticides and to assess the potential usefulness of medaka and fathead minnow as striped bass surrogates for further testing.

Methods

Larvae were exposed for 4 days to pesticides at concentrations of 1/2 the LC-50 (high dose) or at a lower level approximating ambient levels in the Sacramento River (low dose), or in non-contaminated water (control) after which samples were taken for sublethal motor function and growth rate responses. The motor

function measurements included total animal acetylcholinesterase (AChE) activity and (forced) swimming performance, whereas the growth rate measurements included changes in dry body weight and RNA/DNA ratios. A subsample of larvae was then allowed to recover in non-contaminated water for 10 days and the same measurements were made to assess persistence of the effects. Heath et al. (1993a, b, 1997) describe the chemical concentrations and methods in more detail.

Results and Discussion

Larval striped bass generally showed decreased AChE activity and decreased swimming performance after (both immediately after and after 10 d in non-contaminated water) exposure to methyl parathion at both high and low doses, and decreased swimming performance after the high molinate exposure, compared with the controls (Heath et al. 1993a). Medaka decreased AChE activity after high molinate exposure and swimming performance only after high carbofuran (both immediately after and after 10 d in non-contaminated water) exposure (Heath et al. 1993b). Fathead minnows decreased swimming performance immediately after high carbofuran exposure, but not after the subsequent 10-d period in non-contaminated water (Heath et al. 1997). They also decreased their swimming performance after (both immediately after and after 10 d in non-contaminated water) exposure to the high molinate concentration. Comparative immediate responses (AchE, swimming performance) are shown in Table 1. Thus, the three fishes show species-specific patterns of sublethal motor responses to these concentrations of rice field pesticides. Growth responses, including dry body weight changes and RNA/DNA ratios were more variable.

Table 1. Mean (±SE) responses of striped bass[1] (sb), medaka[2] (m) and fathead minnows[3] (fm) immediately after 4-d exposure to three rice field pesticides.

AchE	(ug/mg prot.)			Swimming Performance (lines/min)		
Pesticide/ Dose	sb	m	fm	sb	m	fm
Methyl parathion						
Control	487±33	1651± 167	na	20±1	32±2	na
Low	*389±20	*664± 82	na	*16±2	26±2	na
High	*356±29	*604± 72	na	*16±2	24±3	na
Molinate						
Control	409±19	1464± 164	1615±354	22±1	18±1	57±3
Low	498±38	1336± 239	840±145	22±2	18±1	58±4
High	465±21	*942± 195	938±188	*17±1	16±1	*39±3
Carbofuran						
Control	286±37	na	1459±194	23±2	16±1	48±2
Low	253±25	na	990±97	20±2	13±1	48±2
High	202±16	na	1261±214	17±2	*11± 1	*20±2

Sources of data: [1]Heath et al. 1993a, [2]Heath et al. 1993b, [3]Heath et al. 1997

Unfortunately, both the medaka and fathead minnow larvae's responses to rice field pesticide exposures were not close enough to those of striped bass, limiting their usefulness as surrogates. Thus, medaka and fathead minnow larvae, which are easily obtainable from fish culturists during any month of the year, should not be relied upon to give sublethal test results comparable to those of striped bass larvae. So far, striped bass are available only during a several week "spawning window" during the spring months. Fortunately, current Sacramento Valley rice culture practices have reduced pesticide impacts on Sacramento River fishes. For example, water containing pesticides is held longer on the rice paddies to facilitate chemical breakdown, and methyl parathion is now used very little in Sacramento Valley rice culture.

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EFFECTS OF CREOSOTE-TREATED WOOD ON DEVELOPMENT IN PACIFIC HERRING

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Summary

The effects of creosote-treated wood on early development in Pacific herring (*Clupea pallasi*) embryos were investigated in the laboratory and from a natural spawning site in San Francisco Bay. Embryos exposed to creosote-treated wood exhibited a variety of developmental abnormalities, including delayed development, degeneration of embryos, edema, decreased heart rate, cardiac arrhythmia, and alterations in embryonic movement within the chorion. In both laboratory and field exposed embryos, the hatching success of exposed embryos was significantly decreased, with hatched larvae manifesting severe morphological deformities (scoliosis) and death shortly after hatching.

Introduction

The Pacific herring (*Clupea pallasi*) is a marine teleost that tends to seek out brackish water and spawns in bays and estuaries (Alderdice and Velsen, 1971).

For the San Francisco population at the southern end of its range, the optimal salinity for fertilization and early development is approximately 12-20 ppt (Griffin et al., 1998). At spawning, herring eggs adhere to a variety of substrata; the preferred substrate is submerged vegetation, however rocks, wharves and pilings are also utilized in urban estuaries lacking natural substrata. As such, environmental and/or anthropogenic factors associated with such substrata during the 9-10 day developmental period may have a profound impact on early embryonic development, successful hatching, and survival of larvae.

Creosote compounds have been extensively used to protect wood in both terrestrial and aquatic applications. Creosote is a mixture of polycyclic aromatic hydrocarbons (PAHs), cresols and phenols (U.S. Public Health Services, 1990) and the PAH constituents are considered to present the majority of toxicity to organisms. The genotoxic, mutagenic, and carcinogenic effects of creosote have been demonstrated in a number of organisms, both in the laboratory and in the field (reviewed by von Burg and Stout, 1992). However, only limited studies on the effects of creosote on early reproductive events (Iyer et. al., 1992) particularly in the marine environment, have been performed.

Materials and Methods

All assays were performed according to Griffin et al. (1998) with fish collected from San Francisco Bay. Fertilized embryos were exposed to 1/2 FSW (control), untreated wood (control) creosote-treated wood, or water incubated for 24 hours with creosote-treated wood. Some embryos were also exposed to varying salinities with or without creosote. Embryos from a natural spawning event were collected from the field and assessed in a similar manner as laboratory exposures.

Results

Creosote exposed embryos and larvae manifested a variety of abnormalities. The majority of embryos exposed directly to creosote-treated wood underwent early degeneration with the presence of edema in the yolk sac. Embryos that continued to develop showed delayed development, as evidenced by lack of optic vesicle pigmentation compared to controls. Exposed embryos also exhibited severe cardiac abnormalities, including a significant reduction in heart

rate from approximately 120 contractions/min in controls, to less than 10 contractions/min in exposed embryos. In addition, mild to severe arrhythmia was observed in many of the exposed embryos.

From day 5 post fertilization, normal embryos were observed to undergo vigorous movements within the chorion, decreasing in frequency until just prior to hatching. Creosote exposed embryos exhibited a decrease in normal embryonic movements, and an increase in abnormal movements consisting of periodic to almost continuous tremors.

Embryos exposed to creosote, both in the laboratory and at the field site had significantly decreased hatching rates as compared to the controls (fig.1) and, of the creosote-wood exposed embryos that hatched, 100% exhibited scoliosis, lack of vigorous swimming movements, and expired shortly after hatching.

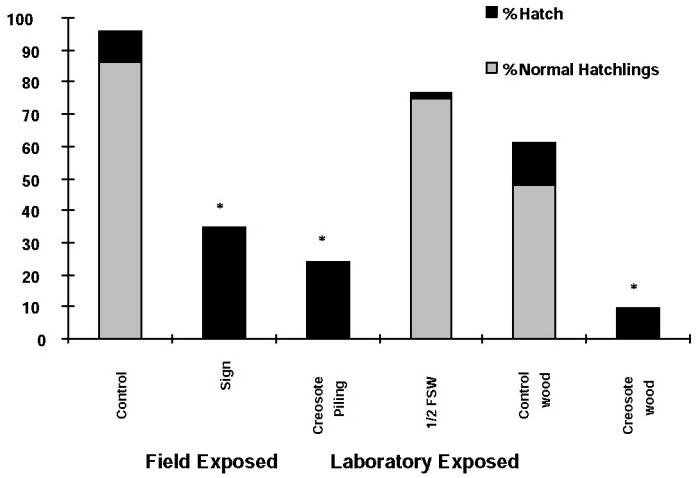

Fig. 1 Effect of creosote on hatching and normal morphology on embryos exposed in the laboratory and from the field site. Asterisks indicate significant decrease in hatching ($p<0.05$)

Salinity stress combined with creosote exposure was found to increase abnormal development in herring as compared to exposure at optimal salinity. Although exposure to reduced (8 ppt) and elevated (28 ppt) salinities alone resulted in

decreased hatching rates and abnormal morphology, creosote exposure at these salinities resulted in even lower rates and greater abnormal morphologies.

Conclusion

Since creosote pilings continue to be used in bays and harbors along the west coast of the U.S., there is a significant risk to organisms which utilize these substrata as spawning habitat. As natural spawning habitat has decreased due to urbanization of estuaries, fish such as herring have utilized man-made structures to a greater extent. Since estuaries experience periods of both low (during droughts) and high (during years of heavy precipitation) freshwater input, the combined effects of salinity stress and contaminants such as creosotes may have dramatic impacts on successful recruitment.

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HEPATIC ALANINE AND ASPARTIC AMINO TRANSFERASES OF THE FRESHWATER TELEOST *BRYCON CEPHALUS* (MATRINCHÃ) EXPOSED TO THE ORGANOPHOSPHOROUS METHYL PARATHION (FOLIDOL 600®)

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Introduction

The commercial production of *Brycon cephalus* has increased in the last years. In the aquaculture conditions, tropical fish are mostly affected by parasites and organophosphorous are commonly employed as treatment. Besides, the application of this pesticides in agricultural area, often located in proximity to culture ponds, has resulted in toxicity to non target species including fish. (Carr and Chambers, 1996; Strauss and Chambers, 1995).

Despite the immediate benefits, organophosphorous pesticides bring several disturbances among vertebrates reflecting on metabolism. Little attention has been paid to their possible impact on energetic metabolism associated enzymes.

Parathion has been found to cause changes into phosphorilases, which are required for the rapid mobilization of glycogen (Rao and Rao, 1983 apud Heath, 1995). It also causes increase in aldolase contents and, since this is a key enzyme on glycolysis, an increase of glucose utilization should be expected in the liver. Others organophophorous has been shown to suppress some oxidative enzymes (Koundinya and Ramamurthi, 1979 apud Heath, 1995). Such fact is attributed to gill damages resulting in histotoxic anoxia (Natarajan, 1984 apud Heath, 1995).

Amino transferase stimulation should be associated with gluconeogenesis, which is stimulated by glucocorticoids (Gill *et al.,* 1990 apud Heath, 1995). Plasma amino transferase may be as well used as biological indicators of tissue lesions caused by chemical aggressors.

Hepatic cells are rich in amino transferase because liver is the major organ for foodstuff interconversion. Enzymes such as aspartate amino transferase (ASAT) and alanine amino transferase (ALAT) are usually found in low blood concentration. Hence, if chemical aggressors damage some organs they will release those enzymes toward the plasma followed by increase of their catalytic activity. Red blood cells, kidney and heart tissues are source of ASAT and the increase of such enzyme into plasma may reflect some damage in those organs (Heath, 1995).

In the present work, we report some changes of amino transferase activities from *B. cephalus* submitted to different concentrations of the organophosphorous methyl parathion. Variations of plasma glucose and ammonia, and liver glucose and glycogen are also reported.

Material and Methods

Experimental design

Twenty-four young specimens of *B. cephalus* obtained from Research and Training in Aquaculture Center (CEPTA - IBAMA) - Pirassununga - SP, Brazil, averaging 180 g, were divided into four equal samples (A, B, C and D). After acclimated for forty-eight hours in aerated dark glass chambers of 80 liters the animals of the aquaria A, B and C were submitted to 2, 5 and 7 ppm of methyl parathion along six hours. Aquarium D was used as a control. After drug exposition the animals' blood were drawn in heparinized syringes and hematimetric values were determined. Plasma was separated from red cells by centrifugation and used as enzyme source. After puncturing blood the animals were immediately killed by a head blow and liver, gill and cephalic kidney were excised. The organs were transferred to liquid nitrogen and kept at -25° C until the analysis.

Aspartate amino transferase and alanine amino transferase assay

These enzyme activities were colorimetricaly estimated. Liver and kidney were mechanically homogenated in 10mM phosphate buffer pH 7.0 containing 50% glycerol. Extract was centrifuged at 7,000 g and the supernatant was used as enzyme source. The alanine amino transferase reaction mixture contained 400 mM alanine; 210mM α-keto-

glutarate; 2.5mM arsenate; 20mM TRIS-HCl pH7.5. Aspartate amino transferase was essayed in the same way replacing the substrate by 210mM aspartate. The reaction was killed by 0.1% dinitrophenyl hydrazine in 2N HCl. After that, suitable aliquot was transferred to NaOH 1.3N and the absorbance was read at 440nm.

Glycogen and glucose

Liver glycogen were determined, after alcoholic precipitation, by the acid hydrolytic procedure described by Duboie *et al.* (1956) and adapted by Bidinotto *et al.* (1998). Free protein acid extract made by mechanic homogenization and using 100 mg of tissue per ml of 20% TCA was employed to determine glucose by Duboie's method (Duboie *et al.*, 1956).

Ammonia analysis.

Ammonia was estimated by nesslerization and the product of reaction was determined at 420nm.

Hematologic determinations

Total hemoglobin was determined diluting 10μL of whole blood into 2.0ml of Drabkin solution. Absorbance was read at 540 nm. Microhematocrit was done with heparinized microtubes and expressed as percent values.

Data analysis

All data were analyzed by no parametric method of Mann-Whitney (Zar, 1984). The accepted level of confidence was 5%.

Results and Discussion

Young forms of matrinchã have shown an interesting response pattern when exposed to 2, 5 and 7 ppm of Folidol ® for six hours. These employed concentrations of methyl parathion were nearly the LC50 for several fish. After exposure, the animals presented evident morphological alteration of the macroscopic aspect of liver. Some tail scales became loose and muscles were abnormally rigidified. Similar responses are reported for the fish *Callichthyis callicthys,* which presented liver necrosis, nuclear degeneration, cellular vacuolization and decrease of hepatic glycogen (Silva *et al.,* 1993). Organophosphorous seemingly cause a variety of changes of the carbohydrate metabolism

of fishes. In the present work, when *B. cephalus* was exposed to high concentrations of methyl parathion, it was observed an increase of plasma glucose (fig.1). In the liver glucose remained constant and glycogen was reduced for the sample submitted to 5ppm (fig.2). Glycogen mobilization is reported for other fish exposed to sub lethal concentrations of organophosphorous compounds (Koundinya and Ramamurthi, 1979 apud Heath, 1995; Sastry *et al.*, 1982 apud Heath, 1995; Awasthi *et al.*, 1984 apud Heath, 1995; Rani *et al.*, 1990; Gill *et al.*, 1990 apud Heath, 1995). It was reported significant decrease of glycogen as well as glucose elevation in several tissues of *Clarias batrachus* exposed to methyl parathion. Moreover, inhibition of LDH, ICDH, SDH and MDH was also observed (Rani et al., 1989). It is supposed that sublethal doses of organophosphorous compounds may suppress the aerobic metabolism in tissues of fish causing mobilization of hepatic glycogen as glucose toward the blood (Gill *et al.*, 1991 apud Heath, 1995; Ramamurthi, 1979 apud Heath, 1995). Natarajan (1984) has attributed those alterations to gill damage by organophosphorous insecticides following histotoxic anoxia and consequent hematological hypoxic-like responses. However, such responses were not observed in matrinchã (fig. 3).

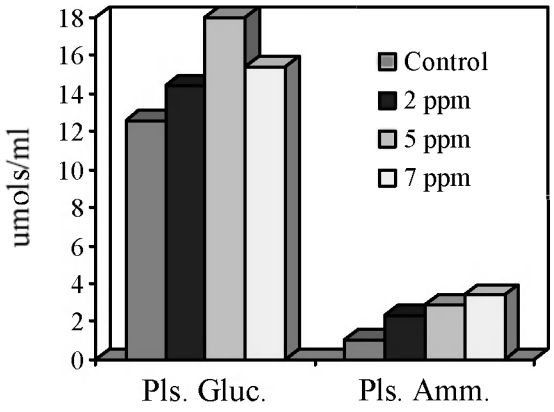

Figure 1. Glucose and ammonia concentration in the plasma of *B. cephalusv* (matrinchã) exposed to three concentrations of methyl paratinon for six hours. Amonia and gucose are expressed in umols / ml of plasma.

Pant and Singh (1983 apud Heath, 1995) found that acute exposure to the organophosphate compound dimethoate produced a hyperglycemia and glycogenolysis. This acute response

is explained as a classical response to stress, mediated by adrenergic and possibly cortisol stimulation. An increase of glucocorticoids, particularly cortisol, has been found in fish submitted to stress (Mazeaud and Mazeaud, 1981 apud Heath, 1995). Those hormones stimulate glycogenolysis and thus cause an increase of blood glucose. The glucose and glycogen profile observed in matrinchã, submitted to methyl parathion, is much more close to the chemical response to stress than to hypoxic-like.

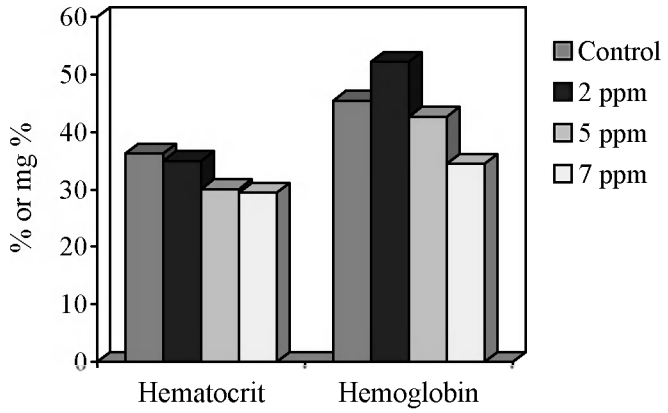

Figure 2. Liver glycogen and glucose of *B. cephalus* (matrinchã) exposed to three concentrations of methyl paratinon for six hours. Glycogen is expressed in umols of glucosil-glucose per gram of wet tissue.

Organophosphorous compounds should also affect the nitrogen metabolism. It was observed increase of plasma ammonia in *B. cephalus* exposed to methyl parathion (fig. 1). According to Wood, (1993 apud Heath, 1995) great amounts of ammonia may be produced from deamination of amino acids due to food inter conversion. Damage of enzymes, directly related to nitrogen metabolism as aminotransferases, also might be responsible for increase of ammonia. The plasma ALAT and ASAT activities of *B cephalus* decreased with elevation of methyl parathion concentration. However, the liver showed a significant increase of ASAT (fig. 4). Transaminases of several fishes were not affected by phosphamidon. (Gill et al., 1990 apud Heath, 1995). Increase of such enzymes could be associated to gluconeogenesis, which is also affected by glucocorticoids. Therefore is not

surprising that liver ASAT or even ALAT have been increased by parathion, particularly considering some stressing response as the most relevant for *B. cephalus*. Decrease of liver ALAT activities to the highest methyl paration concentrations should be attributed to other factors not identified. However, it is tempter to assume the enzyme extrusion from such tissue as responsible for picture. This assumption is reinforced by the larger concentration of both aminotransferases in plasma if compared to liver.

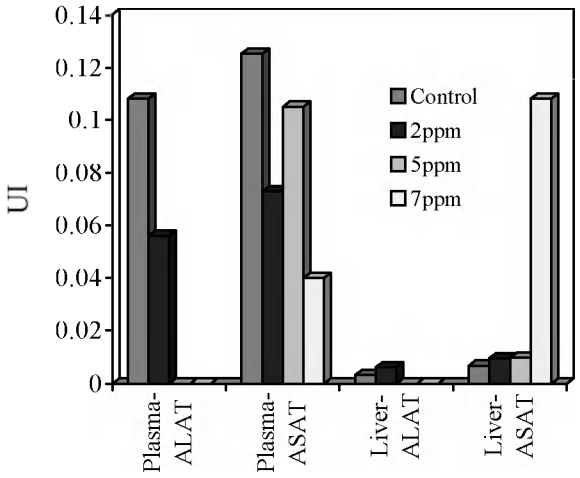

Figure 3. Hematocrit % and hemeglobin in mg % of of *B. cephalus* (matrinchã) exposed to three concentrations of methyl paratinon for six hours.

Different kinds of responses concerning aminotransferases are observed in fishes pursuant to aggressor. Increase of plasma ALAT and ASAT has been described in fish submitted to carbon tetrachloride (Inui, 1969 apud Heath, 1995). Decrease of plasma ALAT and ASAT activities has been described in *Carassius auratus* exposed to polluted waters by lead (Fantin *et al.*, 1989). We suppose that the absence of ALAT, both in plasma and liver, for the highest concentrations of Folidol, are related to enzyme inhibition.

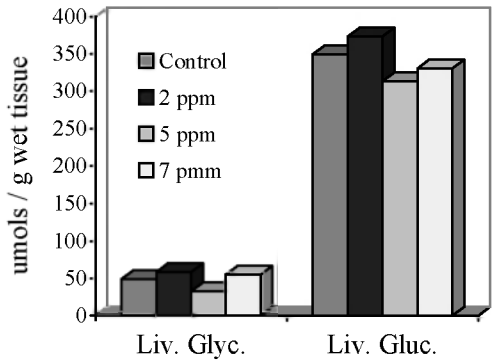

Figure 4. Alanine amino transferase (ALAT) and aspartate amino transferase (ASAT) from liver and plasma of *B. cephalus* (matrinchã) exposed to three concentrations of methyl paratinon for six hours. Activities of ALAT and ASAT are expressed in UI.

According Natarajan, (1984) and Lal *et al.,* (1986) organophosphate insecticides mostly cause increases in hematological variables because hypoxia resulted from gill damage. As pointed before, the hematological parameters (fig.3) suggest that Folidol, for the present concentrations, do not affected the gill and the respiratory system of matrinchã, since a decrease of hematocrit and hemoglobin concentration were observed. Reduction of erythropoiesis by methyl parathion is less probable since the exposure time is too short. Recent work (Bollard et al., 1993 apud Heath, 1995) has shown that artificially elevation of cortisol level may cause a drop in the mean cellular hemoglobin content. Considering that cortisol is frequently elevated in fish under a variety of stressors and the kind of blood and metabolic responses of *B. cephalus* facing Folidol, in the present experimental conditions, we are bent to assume that such kind of response is prevalent.

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METABOLIC AND BLOOD RESPONSES

OF *HOPLOSTERNUM LITTORALE*

(SILURIFORMES, CALLICHTHYIDAE)

EXPOSED TO ACUTE HYDROGEN SULFIDE

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Abstract

Hoplosternum littorale were subjected to 12, 24, 48 and 96 h of hydrogen sulfide (H_2S) after which samples of blood, heart, brain, liver, and white muscle were removed for hematological, blood pH, ions (Na^+ and K^+) and metabolite determinations. Hematocrit, hemoglobin concentrations, red blood cell counts decreased after 12 to 48 h sulfide exposure, probably in function of effect to sulfide. The increase in these parameters after 96 h can be caused by plasma volume expansion. The maintence of high blood pH in *H. littorale*, can be involved in the detoxification of sulfide by hemoglobin and these high values were not dependent of Na^+ and K^+ concentrations. The high sulfide tolerance of *H. littorale* can be explained by its air-breathing and metabolic depression.

Introduction

Many organisms live in environments which exhibit extreme flutuations in physical and chemical properties. The low oxygen availability, due to periodic or sazonal variations, associated with anaerobic decomposition of organic matter and bacterial reduction of sulfates (Fenchel & Riedl, 1970; Jorgensen, 1984) is a favourable condition to the production of hydrogen sulfide (H_2S). This substance is highly toxic to aerobic organisms due to the binding of sulfide to cytochrome *c* oxidase. In the Amazon region, besides oxygen deficit, the increase of sulfide concentration in the water column, as happens during the "friagem" (cold front), has been implicated in mass fish kills (Santos, 1979; Junk *et al.*, 1983, Affonso & Waichman, 1998). Although many species of fish die, another species can survive in rich-sulfide water as in the Amazon lakes (Affonso & Waichman, 1998), deep-sea hydrotermal vents (Cohen *et al.*, 1990) and salt marshs (Bagarinao & Vetter, 1989).

Hoplosternum littorale is a callichthyid catfish which inhabits shallow waters that are stagnant, poor in oxygen and rich in hydrogen sulfide. This species uptakes air using its intestine as an acessory air-breathing organ. Affonso & Rantin (1997) suggested that this behavior increases the survival probability of such species as it is forced to endure periodic exposure to unsuitable water. This should explain its wide distribution into severe environmental conditions. The sulfide tolerance limit of *H. littorale* (50μM H_2S) is high enough to match the potencial sulfide levels detected in the Amazon floodplain (24 μM H_2S), a regular habitat of this species. Moreover, this one is highly sulfide tolerant if compared with other marine and freshwater fish described in literature (Bagarinao, 1992).

Our study investigated metabolic responses of different tissues, changes of hematological parameters, blood pH and, ions (K^+ and Na^+) of *Hoplosternum littorale* during acute H_2S exposure.

Material and Methods

Hoplosternum littorale (60-180 g) were obtained from the Piracicaba river, São Paulo State of Brazil. Prior to experimentation, fish were maintained in tanks with aerated flow-through water supply at 28° ± 1°C during 3 weeks. They were fed on alternate days and fasted at least 24 h prior to the experiment.

Two groups of 24 fish (the control and the exposed to H_2S) were placed in plastic tanks of 200 L, 24 h before the experiment. Sulfide concentration ranged from 13 to 19 μM measured using a deoxygenated sulfide stock solution 250 mM. This solution was prepared from throughly washed and dried crystals of $Na_2S.9H_2O$. It was introduced into the plastic tank through a peristaltic pump (3 ml/min.) and mixed with 1000 mL of water.

During the experiment, water was sampled for sulfide analysis, collected in 25 ml plastic tube and fixed by adding 1 ml of the 2N zinc acetate. Hydrogen sulfide concentration was determined by the methylene blue method (Cline, 1969), with flow injection analysis (FIA), using a Micronal model 320 spectrophotometer. Water samples were also collected to measure pH, which varied between 7.2 and 7.8.

At 12, 24, 48 and 96 h, six control fish and another six fish exposed to sulfide were removed. Blood samples were withdrawn from the caudal vein into heparinized serynges, and kept immediately on ice until all analyses were completed. An aliquot of each blood sample was separated for immediate measurement of blood pH (pHe) and hematological analyses, which were completed alone 2 h from the blood withdrawal. Determination of pHe was done in a pHmeter Hanna instruments HI8521. Hematocrit (Ht) values were determined by microhematocrit centrifugation technique. The red blood cell count (RBC) was done with a Neubawer chamber. Hemoglobin concentration (Hb) was measured spectrophotometrically with Drabkin's reagent. Mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) were determined using classical equations. A second aliquot was centrifuged, and the plasma was used for glucose, sodium and potassium determination. A third aliquot of the blood sample was assayed for lactate determination. Afterwards, fish were killed by a cephalic blow. White muscle, liver, heart, brain and blood were used for analysis of glucose and lactate. The samples were deproteinized by 8% (w/v) percloric acid (PCA), homogenized under ice-cold bath, mechanically and centrifuged at 6000 rpm for 10 min. The supernatants were withdrawn and stored at -80°C until analysis. Lactate was enzymatically determined by Sigma procedure n° 826-UV on a MRX microplate reader (Dynex Technologies). Glucose was measured by Sigma Kit procedure n° 635-6 on a Spectronic Genesys 5 spectrophotometer.

Comparison between control and sulfide exposure groups in different times and within the groups was carried out by the Mann-Whitney U test. Differences were considered significant at $P<0.05$.

Results

Hematological parameters:

Changes of hematological parameters are indicated in Table 1. Significant decrease in Ht, RBC and Hb were observed in sulfide exposed fish compared to the control, except the RBC for 96 h of sulfide exposure. When the mean values for all three parameters were compared between different times of sulfide exposure, the values returned to normality after 96 h of exposure. Only hematocrit values presented progressive and significant increase according to time. Calculation of the corpuscular constants are also shown in table 1. The MCHC for 96 h sulfide exposed fish showed a significant difference from control ($P<0.05$). When comparing these parameters during 96 h exposure to hydrogen sulfide, there were significant differences after 24 h until 96 h of exposed to sulfide in MCH and MCHC, whereas stable values are recorded in MCV.

TABLE 1. Hematological parameters of *H. littorale* exposed to sulfide for different times. Values are means ± SEM, n = 6.

Time (h)	Ht(%)	RBC(x10^6)	Hb(g%)	MCV(μm^3)	MCH(pg)	MCHC(%)
Control 12 h	40.6± 1.5	1.99± 0.11	7.0± 1.2	250.0± 10.4	34.6± 9.1	21.2± 3.9
H_2S	20.7± 2.6*	1.1± 0.15*	6.3± 0.5	203.4± 23.9	63.7± 10.3	31.8± 4.4
Control 24 h	32± 2.2	1.8± 0.11	10.9± 1.6	176.5± 12.9	59.2± 7.8	33.8± 4.2
H_2S	19.2± 3.4*	1.1± 0.21*	5.8± 0.9*	218.4± 10.2	100.5± 36.5	45.1± 14.8
Control 48 h	37.8± 2.7	2.16± 0.13	11.4± 1.1	175.4± 10.4	52.6± 4.1	30.4± 2.6
H_2S	21.1± 3.5*	1.3± 0.19*	6.6± 0.9*	167.0± 16.7	53.1± 4.5	32.5± 2.9
Control 96 h	40.1± 1.5	2.2± 0.2	13.5± 0.5	191.6± 20.2	64.4± 4.8	33.8± 1.5
H_2S	33.0± 1.9*	1.8± 0.2	7.9± 0.2*	191.6± 15	46.2± 5.3	24.1± 0.8*

* significant differences from control values. ($P<0.05$)

Metabolic changes:

The concentrations of glucose and lactate measured for different tissues after 12, 24, 48 and 96 h of sulfide exposure and from control fish are shown in figures 1 and 2.

Glucose: Plasma glucose showed a decrease up to 50% for 48 and 96 h of sulfide exposure if compared to control, 12 and 24 h after sulfide exposure (figure 1). In contrast, the muscle glucose increased in with times of exposure if compared with control. Significant differences were obtained between 96 and 12 h sulfide exposure. No significant differences were observed in liver and heart glucose between the sulfide exposed and control fish. Brain glucose values obtained in 48 h sulfide exposure were significantly different of the control and higher than other times of sulfide exposure. We did not have the 12 h control values.

Lactate: Blood lactate was higher for 48 h of sulfide exposure (figure 2). Blood lactate concentration of 96 h sulfide exposure was significantly different from the other times of exposure. Although it was not different from its own control. In the liver and heart a progressive increase of lactate level occurred until 48 h after sulfide exposure and declined after 96h. Significant differences in the liver lactate values were obtained between control and sulfide exposure fish for all times, except for 12 h. Only 12 h sulfide exposure was significantly different ($P<0.05$) from 24, 48 and 96 h of exposure. Brain lactate did not show significant differences between different times of sulfide exposure.

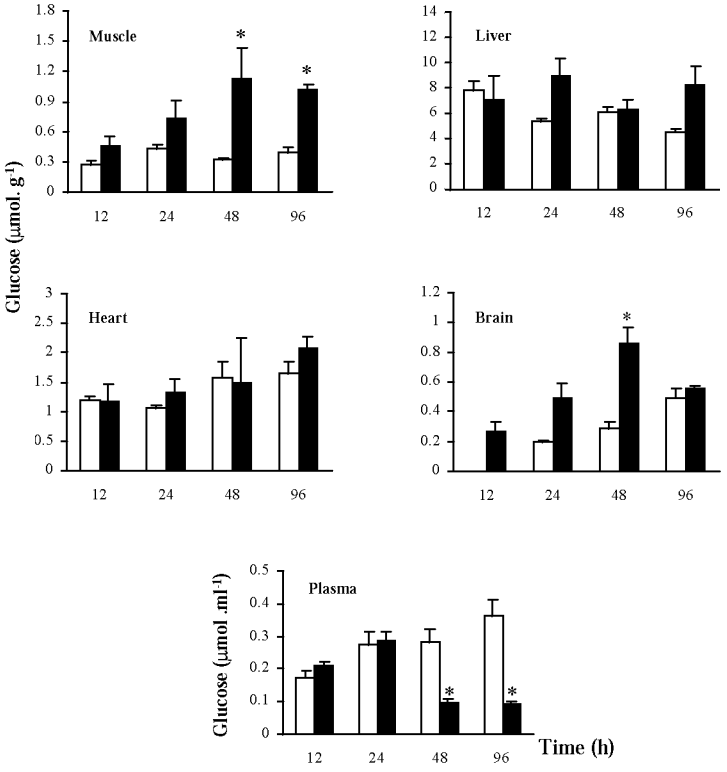

Figure 1. Glucose levels in the muscle, liver, heart, brain and plasma of the *Hoplosternum littorale* in control (□) and exposed to sulfide ( ) in the different times. $P<0.05$. * significant difference from control values. Mean ± SEM; n = 6.

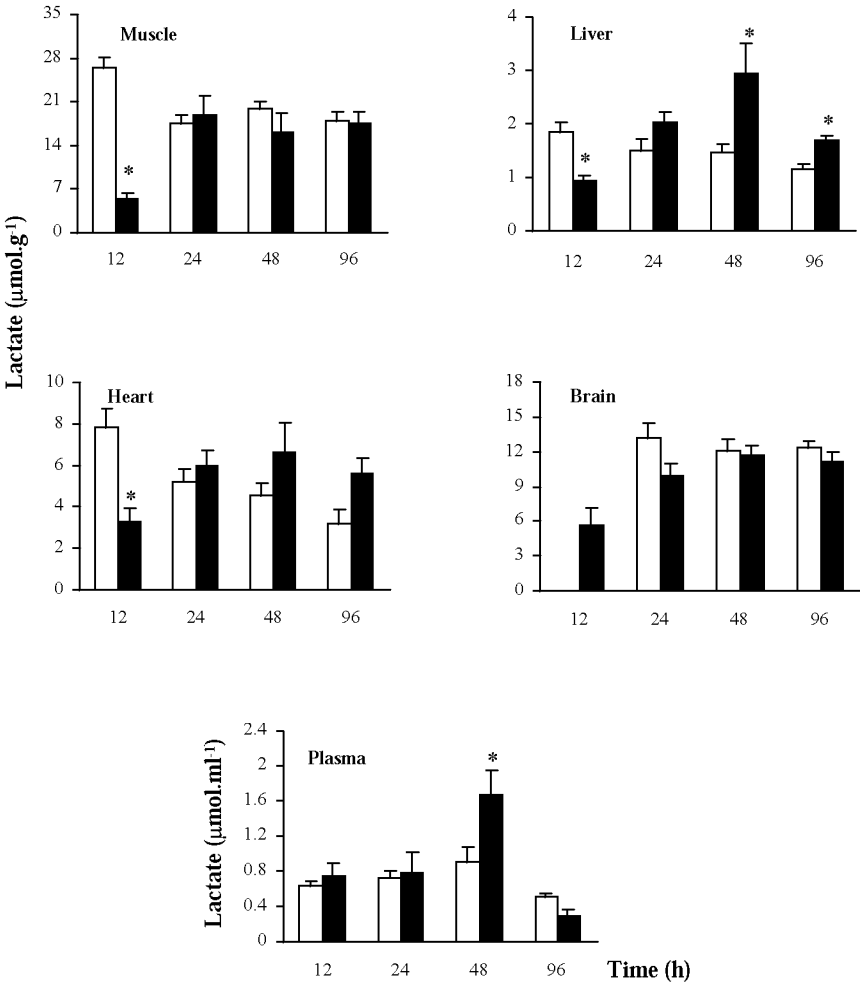

Figure 2. Lactate concentrations in different tissues of the *H. littorale* during different times sulfide exposure ( ) and control (□). $P<0.05$. *significant difference from control values. Mean ± SEM; n = 6.

Ions (Na^+ and K^+) and Blood pH:

Potassium and sodium concentrations are shown in Table 2. Plasma sodium concentration in the control and sulfide exposed fish did not differ, except in 12 h exposured to sulfide. There were no significant differences in Na^+ concentrations between the fish exposed to different times of sulfide. Potassium concentrations showed significant differences from control for 12 h and 24 h. Blood pH values increased significantly in all the times it was exposed to sulfide.

Discussion

The measurement of hematological parameters has become widely employed as an index of environmental stress in fish (Dheer, 1988; Tort & Hernandez-Pascual, 1990; Ginneken *et al.*,1997). Changes of these parameters may appear after acute exposure to different pollutants (Svobodová, *et al.*, 1994). Variations in the hematological parameters of *Hoplosternum littorale* were observed during exposure to sulfide. Hematocrit, hemoglobin concentrations and red blood cell counts were significantly low after 12 to 48 hours exposure to sulfide. After 96 hours, these parameters recovered and returned to values similar to that of the control. Some fish, within them bimodal breathers such as *H. littorale,* have a tendency to increase hematocrit and hemoglobin concentrations during hypoxia, and so they can increase blood oxygen content (Val, 1993). Bagarinao & Vetter (1994) detected significant increase for these parameters in *Fundulus parvipinnus* after 2 h of sulfide esposure. A short term compensatory response could be responsible for the RBC increase in the blood stream of *H. littorale* before 12 h of sulfide exposure. But the reduction of these parameters after 12 h appear to be a direct effect of sulfide.

TABLE 2. Plasma potassium and sodium concentrations of *H. littorale* exposed to sulfide for different times. Values are means ± SEM. n = 6.

	12 h		24 h		48 h		96 h	
	Control	**H_2S**	**Control**	**H_2S**	**Control**	**H_2S**	**Control**	**H_2S**
K^+	8.6± 1.5	4.8 ± 0.7*	4.9 ± 0.3	8.0 ± 1.3*	6.1 ± 0.6	8.6 ± 1.7	4.5 ± 0.1	4.0 ± 0.6
Na^+	150.2±5.9	122.2±2.9*	145.0±3.5	140.7±10.4	146 ±3.7	136.2±5.1	131.5±4.2	135±8.5

* significant differences from control values (P<0.05)

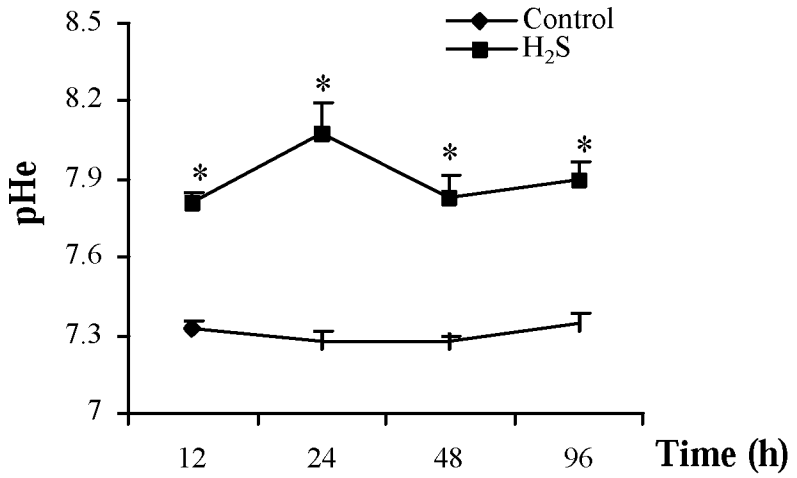

Figure 3. Blood pH of *Hoplosternum littorale*. Control values and exposed to sulfide in different times. * $P<0.05$, significant differences from control values. Data are expressed as mean ± SEM; n = 6.

Preliminary studies on erythrocytes comparing control and sulfide exposed fish showed microscopic showing variations of cells, either quantitative or qualitative ones. A decrease in the numbers of cells and the appearance of immature red cells was observed. Variations obtained in the Ht, [Hb] and RBC counts, associated with sulfide exposure, did not change the hematimetric indices. These results indicate a quantitative variation in red blood cells without volume alterations of erytrocytes. Such results agree with Pagés *et al* (1995) which suggested the development of anaemia in *Sparus aurata* under daily management stress causing plasma volume expansion and no rheological alterations.

The constant values in plasma sodium concentrations indicated that sodium is not affected by H_2S and so, the electrochemical potencial can be mantained. Plasma potassium variations from both, sulfide exposure and control, demonstrated that this cation can be active in stress condition, for example: exposure to sulfide and confinement (control). Nikinmaa (1990) discussed that

potassium and chloride transport occur via coupled potassium/proton and chloride/bicarbonate exchanges when the pHe increases. There were, however, no indications that this was the case of *H. littoralle*. Blood pH was maintained high for all times of sulfide exposure and it was not dependent of Na^+ and K^+ concentrations.

One of the major factors involved in the control of red cell pH and volume are the intracellular impermeable anions/buffers, as hemoglobin. The biding of sulfide to hemoglobin or other blood proteins reduces toxicity (Smith et al., 1977; Torrans & Clemens, 1982). According to Bagarinao & Vetter (1992) the hemoglobin of *Fundulus parvipinnis* is relatively insensitive to sulfide and so, sulfhemoglobin can not protect nor cause the sulfide poisoning. Goffredi *et al.* (1997) studying the hydrothermal vent tubeworm *Riftia pachyptila* demonstrated that this species is able to control sulfide movement, while keeping the extracellular pH stable and alkaline. Although this invertebrate has two extracellular hemoglobins and contains internal bacterial symbionts, we suggest that the maintenance of an alkaline extracellular pH in *H. littorale*, can be involved in the detoxification of sulfide by hemoglobin, in contrast to *Fundulus parvipinnis*. Future studies of the interation sulfide-hemoglobin will further clear this question.

The reliance of anaerobic metabolism have been described in the literature as one of the possible mechanisms employed for some organisms able to tolerate high sulfide levels in their habitats (Powell & Somero, 1986; Bagarinao & Vetter, 1989; 1994). The constant lactate values, inmuscle, heart and brain of *H. littorale*, except after 12 hours of sulfide exposure, indicate that these tissues are still receiving most of their oxygen requirement. Affonso & Rantin (1997) detected a high air-breathing frequency in this species when it was esposed to sulfide. Although the air-breathing should maintain a constant supply of oxygen to these tissues, the increase in blood lactate concentration after 48 h of exposure to sulfide showed signs of glycolytic activation. However, the anaerobic capacity in *H. littorale* is not clear. In the majority of tissues and blood lactate concentrations did not correlate with glucose concentrations. The decrease of glucose concentrations for 48 and 96 h should be involved with disturbances caused by sulfide, rather than energy provision. During all the time of exposition to sulfide, we observed the fish behaviour and concluded that *H. littorale* inicially uses its air-brething to maintain the oxygen uptake. However, after 48h and, particularly at 96 h, the air-breathing stopped and the fish stayed in the bottom almost without locomotor activity. Although this fish increases the air uptake in the surface when exposed to sulfide, it takes sulfide through the gills

which cause inhibition of cytochrome *c* oxidase. We suppose that the strategy of *H. littorale* in sulfidic habitats is to use metabolic depression, as the most effective survival strategy, instead of anaerobic metabolism.

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