

The Molecular Basis
of
Environmental Adaptation

SYMPOSIUM PROCEEDINGS

Gillian Renshaw

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International Congress on the Biology of Fish
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PREFACE

The successful exploitation of diverse aquatic habitats, from the abyssal depths to the poles, by over 20,000 species of fish has required sophisticated physiological and biochemical adaptations to solve the diverse challenges presented. Dynamic and sometimes extreme environments have provided the selection pressure needed to distil an array of solutions to the difficult task of maintaining homeostasis or achieving a new set point when there are severe fluctuations in food supply, oxygen levels, temperature and salinity.

This symposium begins by exploring some of the molecular switches required to effect a phenotypic change in response to decreased food, oxygen or temperature. Interestingly there are similarities and contrasts between the molecular switches activated in response to starvation and hypoxia in the common carp (*Cyprinus carpio*). Both of these physiological stressors turned on genes that were directed toward balancing the energy budget (Presented by Dave Randall). While metabolic depression is reversibly turned on in the common carp, by hypoxic exposure or starvation, the profile of genes up-regulated or suppressed during starvation differed from that of mammals challenged by the same stressor (Presented by Carrie Hung). Just as fish and mammals differ in their molecular response to perturbation, not all tissues exposed to hypoxia could be expected to behave in a uniform manner. The heart and brain have a limited capacity for repair accompanied by a large demand for oxygen. The next series of presentations explore tissue specific responses to hypoxic or anoxic challenge and reoxygenation.

An examination of cell death, cell proliferation and cell cycle in the liver of the common carp revealed that the molecular responses were dependent of the oxygen level rather than on the capacity of the tissue to regenerate (Presented by Winnie Poon). It is possible that selective cell sacrifice occurs under extreme conditions. Comparison of the activation of apoptotic pathways and the protective anti apoptotic gene product M-cl1 after anoxic preconditioning and reoxygenation in the tropical epaulette shark (*Hemiscyllium ocellatum*) indicated that both may be organ specific (Presented by Gillian Renshaw).

Grass carp (*Ctenopharyngodon idellus*) display tissue specific hypoxia-sensitive transcriptional co-activators from the CITED family (CBP/p300-interacting transactivator with glutamic acid [E] and aspartic acid [D] rich C-terminal domain), which may have the capacity to protect cells from hypoxia via their interactions with Hypoxia Inducible Factor (HIF) (Presented by Patrick Ng).

Similarly, a number of homologues of HIF occur in the killifish (*Fundulus heteroclitus*) that bind to and activate the hypoxia response element in hypoxia responsive genes (Presented by Bernard Rees).

In fish, HIF is not only responsive to low oxygen levels but its level of expression is also temperature sensitive, this may reflect its role in temperature acclimation (Presented by Eeva Rissanen). Sensitivity and adaptation to temperature is reflected in the expression of glucocorticoid mediated gene expression and correlates well with the data on selective adaptation of the killifish to different extremes of the thermal gradient present in its distribution (Presented by Patricia Schulte).

One of the greatest challenges presented by extreme cold is how to prevent the heart from entering a cardioplegic state. Fish have solved this problem a number of times during evolution. While previous chimeric and mutational studies failed to unambiguously identify the residues of the membrane protein NCX responsible for Ca²⁺ homeostasis in the heart of low temperature adapted fish, new studies using a variety of sequence analysis tools on NCX from puffer fish (*Fugu rubripes*) and Zebra fish (*Danio rerio*) have yielded candidate residues (Presented by Christian Marshall). Similarly the residues, responsible for increased sensitivity of cardiac troponin c at low temperatures have been identified in the trout and confer a functional advantage over mammalian isoforms (Presented by Todd Gillis).

The impetus for adaptation is not only provided by external factors such as dynamic environments or extreme environments but also by changes provided by the unfolding of the “hard wired” life history that provide a different set of challenges to development and growth. Anadromous species such as Atlantic salmon (*Salmo salar*) must be able to transform their osmoregulatory mechanisms to accommodate both salt and fresh water challenges, after smoltification and as breeding adults respectively, and several novel genes have been identified in gill lamellae that play a role in adaptation to hyperosmotic stress (Presented by Terry Bradley). However not all fish negotiate early developmental stages successfully, in Baltic salmon those with ‘maternally transmitted yolk-sac fry mortality syndrome’ downregulate genes that normally act to minimise oxidative stress resulting in the activation of apoptotic pathways (Presented by Kristiina Vuori).

During the battle for limited resources some rainbow trout (*Oncorhynchus mykiss*) wield a competitive advantage over others because of their position in

the dominance hierarchy, transcriptomics is providing the key to which genes enable them to do this (Presented by Lynne Sneddon). Surviving to reproduce presents fish with the challenge of matching reproductive effort with mate availability and selection. The blue gourami (*Trichogaster trichopterus*) has adapted its endocrine responses to ensure that reproductive effort is not wasted, oocytes are arrested until males are present to trigger the expression of gonadotrophins, injection of gonadotrophin-releasing hormone results in gonadotrophic gene expression mimicking male presence (Presented by Gad Degani).

The posters continue to explore molecular basis of environmental adaptation and commences with an analysis of expressed sequence tags in the brain of tambacui (*Colossoma macropomum*) (Poster by George Porto). The molecular biology of oxygen acquisition is examined: the conformation of oxygen-haemoglobin binding in catfish (*Hoplosternum littorale*) (Poster by Patricia Peres); hemoglobins in two species of Pacu (*Colossoma macropomum*) and (*Piaractus mesopotamicus*) (Poster by Patrician Caetano de Sousa); and phosphate binding to alter the conformation of haemoglobin in matrinxã (*Byron cephalus*) (Poster by Gustavo Bonilla –Rodriguez). Fish need a chemical defence against insults, one of the major chemical defences against hypoxia, altered salinity and xenobiotics is the liver enzyme glutathione S-transferase this has been sequenced for the pacu (Poster presented by Rodolpho Albano).

Symposium Organizers:

Gillian Renshaw, NHRC, Griffith University, Australia

Don MacKinlay, Fisheries and Oceans Canada

CONGRESS ACKNOWLEDGEMENTS

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

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

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

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

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

The Student Travel Award Committee of the Physiology Section of the American Fisheries Society, led by Michael Redding, evaluated 65 applications from 15 countries and awarded 40 Travel Grants, after an ambitious and trying fund-raising effort. Special thanks must go the US Department of Agriculture, the US Geological Survey, US National Science Foundation and the World

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

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

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RESPONSES TO HYPOXIA AND STARVATION IN FISH

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EXTENDED ABSTRACT ONLY - DO NOT CITE

Carp are both hypoxia and starvation tolerant. Trout are not very hypoxia tolerant but can easily survive a week of starvation. Hypoxia is associated with a decrease in food intake, as well as an inhibition of reproduction (Wu et al., 2003). Metabolism is reduced during both hypoxia and starvation (Zhou et al., 2001) and reproduction is inhibited. It is possible that the patterns of gene expression in response to hypoxia and starvation are also similar. There was no significant difference in rainbow trout muscle phosphofructokinase (PFK-1) activity during the hypoxic exposure (7 days at 12C and 30% oxygen saturation) but muscle PFK-1 was significantly reduced following 7 days starvation (Table 1). A significant decrease in the muscle citrate synthase (CS) activity was measured only at 7 days hypoxic exposure but there was no change in CS activity during starvation. There was no significant difference between control and experimental groups (hypoxia or starvation) in muscle and liver glycogen content throughout the experimental period, except a significant decrease in the liver glycogen content was measured at 7 days hypoxia.

Indicators	Control	Starvation
PFK-1	61.16±7.28	41.95±4.54*
CS	5.12±0.61	4.77±0.29
Glycogen: liver	161.73±20.98	152.57±27.05
muscle	5.17±0.24	6.43±0.91

Table 1. Effects of 7 days starvation on rainbow trout muscle phosphofructokinase (PFK-1) and citrate synthase (CS) activity and glycogen content of the muscle and liver. (Activity in $\mu\text{mol}/\text{min}\cdot\text{g}$ at 20°C , glycogen content as μmol glycosyl units/g. Mean \pm SE, n=6; *significant different from control, unpaired *t*-test, * $P<0.05$).

The changes in CS expression in the carp liver were similar to changes in CS enzyme activity in trout muscle during a week of either starvation or hypoxia. CS expression was reduced during hypoxia but there was no change during the first week of starvation. In the following five weeks of starvation, however, there was a marked increase in CS activity. Patterns of gene expression in carp subjected to either hypoxia or starvation are not similar. There is a reduction in the expression of genes associated with protein metabolism in both cases, but genes associated with anaerobic metabolism are up-regulated during hypoxia but down regulated during starvation. The target genes associated with hypoxia inducing factor 1 (HIF-1) showed some increase in activity, but this system appears to play only a minor role in the response to hypoxia. During starvation there was no indication of increased activity in HIF-1 target genes, if anything some of these genes were down regulated. Interestingly, hemoglobin genes were down regulated in both hypoxia and starvation. Uncoupling protein 2 & 3 genes showed increased activity during both hypoxia and starvation. The increase was more marked in carp exposed to hypoxia.

Although hypoxia and starvation are both associated with reductions in metabolism and inhibition of reproduction, the patterns of gene expression in response to these stresses are quite distinct.

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**A COMPARISON OF THE EFFECTS
OF STARVATION ON GENE EXPRESSION
IN FISH AND MAMMALS**

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EXTENDED ABSTRACT ONLY - DO NOT CITE

Starvation in Fish and Mammals

Much of vertebrate evolution has taken place in the aquatic environment, which is characterized by variable, and often low, oxygen levels and periods of reduced food availability. Many fish species survive in the absence of food for periods of several months. For example migrating salmon do not feed in freshwater when moving towards their spawning grounds and carp may over-winter without feeding. It is known starvation reduces growth rate, metabolism and hence respiration rate in fish. Thus, both hypoxia and starvation are associated with a fall in oxygen uptake as well as reduced food intake, protein turnover and growth. It appears that, when fish are starved, they are substrate limited and energy-saving strategies must be employed to maintain the supply of food to selected tissues, especially to the brain, to survive these adverse conditions. Reduction in metabolism has also been observed in mammals during starvation. Nevertheless, the post-starvation behaviour appears to be rather different between mammals and fish. Active hoarding of food is a common behaviour for starved mammals but fish, on the other hand, becomes rather inactive and many fish species employ “wait and see” strategy during food deprivation. Several papers have been published recently to investigate the gene expression profiles of starved mammals. On the contrary, the molecular mechanisms governing the survival of fish during starvation remains unknown.

Material and Methods

Common carp (*Cyprinus carpio*) were obtained from a commercial fish farm and kept in well-aerated water ($20\pm 1^\circ\text{C}$) for 3 months for acclimation. They were fed to *ad litum* until the onset of the starvation experiment. Fish were starved for 0, 4, 8, 16, 28 and 42 days and five fish were killed at each time point.

Total RNA was extracted and 10ug of RNA from each fish was reverse-transcribed separately into cDNA labeled with amino-allyl, followed by incorporation of Cy5 or 3 dyes. Individual Cy5-labelled cDNA was competitively hybridized to the carp cDNA microarray with a Cy3-labelled reference cDNA (which was a pool of equal amount of all the carp RNA samples). Dye-swap was carried out for all the samples. The microarrays were scanned, image analyzed by GenePix 4.0. Data was analyzed by GeneSpring. One-way ANOVA (false-discovery rate) was used to determine statistically significant results ($p < 0.05$).

Results and Discussions

Both mammals and fish have a general depressed metabolism during starvation. However, the gene expression profiles of rat / mice and fish are rather different. Genes involved in fatty acid / lipid breakdown and protein degradation/turnover were generally up-regulated in many tissues in mammals during starvation, with down-regulation of lipid biosynthesis-related genes (Bauzer *et al.*, 2004; Lange *et al.*, 2003; Jagoe *et al.*, 2002, Suzuki *et al.*, 2002). Other studies using subtractive suppression hybridization showed that energy metabolism, protein metabolism and intra-intercellular transporters were up-regulated in starved mammals (Zhang *et al.*, 2001). Whereas in carp, genes associated with various metabolic pathways, including fatty acid transport and utilization, gluconeogenesis and mitochondrial oxidative phosphorylation and ATP-synthesis were down-regulated during starvation. Expression of genes encoding oxygen-, iron- and other transporters were decreased also in fish. However, genes involved in growth arrest, DNA-damage-repair system, were up-regulated in both mammalian and fish during starvation.

The similarity and difference in the gene expression profiles of mammals and fish highlights the fact that growth is halted during starvation. However, mammals up-regulate a large number of genes during starvation in order to

sustain its body temperature and to spare protein as the last resort of food substrate. In the case of carp, gene expression and hence energy expenditure seems to be kept to a minimum until day 42 of starvation, when a number of genes showed increased expression. This strategy of conserving body substrate levels with reduced energy expenditure during food deprivation, but maintaining an efficient assimilation strategy for when food is available, help carp to survive adverse conditions for much longer periods than mammals.

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**CHANGES IN THE FISH LIVER DURING HYPOXIA AND
REOXYGENATION**

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Abstract

Increase cell death of less vital organs or tissues which have high regeneration power may be a strategy that occurs in fish during hypoxia. Liver is known to be an organ with high regeneration power in mammals. It is likely that fish liver also has this capacity. Energy can be re-localized to other vital organs to maintain survival. Levels of cell death and cell proliferation were measured by TUNEL (Tdt-mediated dUTP nick-end labeling, a method detecting DNA-nick ends) and PCNA (proliferating cell nuclear antigen, a marker for proliferating cell) during hypoxia and re-oxygenation. This study showed molecular responses were oxygen level dependent and, although there were DNA nick ends, there was no increase in apoptosis during hypoxia.

Introduction

The role of apoptosis (programmed cell death) in response to hypoxia has been studied *in vitro* (Carmeliet *et al.*, 1998 and Sowter *et al.*, 2001) but *in vivo* studies are limited. An *in vivo* study (Sollid *et al.*, 2003) limited to fish gills showed an increased area of the gill lamellae of crucian carp (*Carassius carassius*) resulting from increased apoptosis and decreased cell proliferation during hypoxia. Apoptosis occurs when old cells or damaged cells need to be recycled. Turnover rate of an organ is regulated by the balance between cell division and cell death. The normal turn over rate of liver cells is low, as long as

150 days in humans. However, the liver has a high capacity for regeneration, which can occur within few days. Not much is known in fish but it is possible that their turnover rate is low too. Increased cell death of less vital organs or tissues, with high regeneration power, may be one of the strategies that occur in fish exposed to hypoxia. As a result, energy can be re-located to more vital organs such as the heart and brain.

The objective of this study was to determine whether there were changes in carp liver during hypoxia with respect to cell death and proliferation and their recovery during reoxygenation. In other words, the aim of this study is to evaluate the effect of hypoxia on cell and organ turnover.

Materials and Methods

Common carp (*Cyprinus carpio L.*) were exposed to hypoxia (0.5mgO₂/L) for 4 days and the liver sampled. Levels of cell death were measured by TUNEL (Tdt-mediated dUTP nick-end labeling, a method detecting DNA nick ends; ROCHE, in situ cell death kit) and caspase (cystein aspartate proteases) 3 activity (molecular probes, caspase 3 activity assay kit). Cell proliferation rate was determined by the levels of PCNA (proliferating cell nuclear antigen; Dako).

Results and Discussion

Increase of the TUNEL signal was observed in hypoxic but not normoxic livers while there was no significant difference in caspase 3 activities between 4 day-hypoxic and normoxic livers. The level of cell death shown by TUNEL may not be apoptosis but only DNA nick ends or the TUNEL labeled cells underwent apoptosis via a caspase 3 independent pathway. Cell number, size and proliferation rate of the normoxic and hypoxic livers showed no significant difference. Thus it appears that hypoxia does not affect liver turnover rate.

Future Plans

TEM (transmission electron microscopy) will be used to confirm whether apoptosis occurred during hypoxia. DNA nick ends in hypoxic liver cells may be caused by Reactive Oxygen Species (ROS) and then restored by DNA repair systems. Increased ROS has been shown to occur during hypoxia and re-

oxygenation in other systems. Hydrogen peroxide (H₂O₂) is one form of ROS and its production rate will be measured by Amplex Red Hydrogen Peroxide Assay Kit. Patterns of H₂O₂ production and appearance DNA nick ends will be compared to evaluate their relationship.

In order to check the rate of recovery of the liver after hypoxic exposure, fish in hypoxic water for 4 days will be aerated again and levels of cell death measured and the recovery rate determined.

Conclusion

Increased DNA nick ends in liver during hypoxia were observed but the overall cell turnover rate was unchanged. DNA nick ends may not be an indication of apoptosis as the changes observed in the hypoxic liver were caspase 3 independent and there were no changes in cell numbers in the liver.

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**CHANGES IN GENE EXPRESSION
IN RESPONSE TO ANOXIC PRECONDITIONING.**

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EXTENDED ABSTRACT ONLY: DO NOT CITE

While most vertebrates are unable to withstand prolonged exposure to diminished oxygen, a few species of fish have adapted to survive periods of hypoxia or anoxia. They rapidly (Lutz *et al.* 2003) and reversibly (Hochachka *et al.* 1996) reprogram their metabolism to turn down their ATP consumption (Lutz *et al.* 2003) and produce molecular chaperones (Airaksinen *et al.*, 1998; Renshaw *et al.*, 2004) in order to prolong survival. The identity of genes responsible for successfully switching to such a “protected phenotype” is currently under investigation in a number of fish species. In order to examine the effect of low ambient O₂ without the confounding effects of lowered temperature we characterised the metabolic and respiratory response an anoxia tolerant tropical elasmobranch (Renshaw *et al.*, 2002).

In its natural environment, the epaulette shark (*Hemiscyllium ocellatum*) can be subjected to intermittent hypoxia on low nocturnal tides if the water on shallow reef platform is prevented from mixing with the surrounding ocean by a fringing reef. The anoxia tolerant epaulette shark employs respiratory and metabolic strategies, including metabolic depression, in order to maintain brain energy homeostasis during anoxia (Renshaw *et al.*, 2002).

We used a prior exposure to a sub-lethal episode of anoxia (anoxic preconditioning) to elicit changes in gene expression in the epaulette shark to investigate cell survival. Briefly, sharks were exposed to a sub-lethal episode of anoxia followed by an anoxic challenge 24 hours later. Anoxic tanks, 60-litre glass aquaria, were prepared by bubbling N₂ through an air stone, at 4 lmin⁻¹

until the $[O_2]$ fell below 0.3 % of air saturation ($< 0.02 \text{ mg O}_2 \text{ l}^{-1}$). Sharks were transferred singly to anoxic tanks. Anoxic preconditioning was terminated when the righting reflex was lost, epaulette sharks were returned to the holding pool for 24 hours, then exposed to an anoxic challenge for 55 min, corresponding to the mean time taken for sharks to lose their righting reflex during anoxic preconditioning. The control sharks ($n=5$) were exposed to normoxia using an otherwise identical protocol. Five of the anoxic preconditioned sharks ($n = 10$) were sacrificed after the anoxic challenge, a further 5 sharks were returned to normoxic conditions for 30 minutes prior to sacrifice (re-oxygenated). Sharks were euthanased by excess benzocaine dissolved in seawater then heart and liver samples were taken and immediately frozen in liquid nitrogen.

No marked DNA fragmentation was observed in the tissues examined. Using western blot analysis, we examined caspase levels at two control points: caspase-3 and caspase-9.

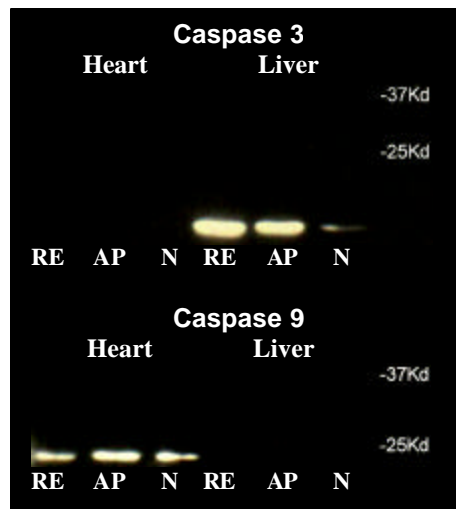


Figure 1. Activated caspase-3 and caspase-9 proteins in the cytoplasmic fraction of heart and liver samples: 30 minutes after reoxygenation (RE), anoxic preconditioning (AP) or normoxia (N).

Anoxic challenge resulted in the selective activation of apoptotic pathways. Cleaved caspase-3 was elevated above baseline in anoxic preconditioned and re-oxygenated liver revealing that caspase 3 was activated in the liver but not in the heart. A low level of cleaved caspase-9 was present in normoxic heart and was not markedly elevated in the hearts from anoxic preconditioned or re-oxygenated sharks, indicating that apoptotic pathways may not have increased during or immediately after an anoxic challenge.

Increased expression of Mcl-1, a member of the anti-apoptotic bcl-2 family, inhibits apoptotic cell death. Mcl-1 was elevated in the re-oxygenated heart but not in the liver of epaulette sharks subjected to an anoxic challenge after preconditioning.

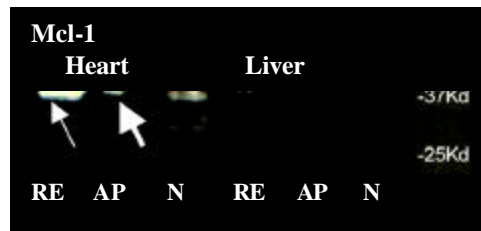


Figure 2 Mcl-1 protein in the cytoplasmic fraction of heart and liver samples: 30 minutes after reoxygenation (RE), anoxic preconditioning (AP) or normoxia (N).

The level of Mcl-1 was not detected in liver. Mcl-1 expression appeared to be decreased, relative to the normoxic control, in the anoxic preconditioned heart (thick arrow) and elevated in the re-oxygenated heart (thin arrow), indicating that the anti-apoptotic factor Mcl-1 may play a crucial role in the prevention of apoptosis in the epaulette heart subjected to an anoxic challenge.

Together, these findings support the notion that preconditioning elicits a cardio-protective response by affecting gene transcription (Hochachka *et al.*, 1996) and suggest that adaptations to survive oxygen deprivation may include the selective protection of vital organs such as the heart.

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**HYPOXIA-RESPONSIVE CDNAS OF THE CITED
COTRANSACTIVATOR PROTEINS FROM GRASS CARP**

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EXTENDED ABSTRACT ONLY – DO NOT CITE

The CITED (CBP/p300-interacting transactivator with glutamic acid [E] and aspartic acid [D]-rich C-terminal domain) proteins are members of a new family of transcriptional co-activators that are not only involved in the modulation of a variety of cellular and developmental processes (Dunwoodie et al., 1998; Yahata et al., 2001) but also in responses to various stimuli, such as hypoxia (Bhattacharya et al., 1999). In humans, similar to a number of hypoxia responsive genes, CITED2 (hCITED2) is transcriptionally activated by hypoxia inducible factor-1 (HIF-1). Conversely, CITED2 negatively regulates HIF-1 by competitive binding to the CH1 domain of the CBP/p300 coactivator (Bhattacharya et al., 1999). Recently, our group reported the isolation of a hypoxia responsive CITED3 cDNA (gcCITED3) from the grass carp, and Northern blot analysis showed that gcCITED3 was most highly expressed and responsive to hypoxia in the fish kidney, although hypoxic induction was also observed in gill, heart and liver albeit at a lower level (Ng et al., 2003).

Since CITED2 has been shown to negatively regulate HIF-1 activity in mammals, it is believed that HIF-1 activity in fish may also be negatively regulated by CITED proteins. We have demonstrated that the tissue expression pattern and hypoxia responsiveness of gcCITED3 is very similar to the CITED2 gene in mammals; and therefore it is hypothesized that gcCITED3 may modulate HIF-1 activity in a manner similar to hCITED2. In an attempt to better understand the role(s) of CITED proteins in the hypoxia signaling pathway in fish, comparative studies of the different CITED isoforms of grass carp were carried out.

To estimate the number of CITED isoforms that are encoded in various fish genomes, a bioinformatic search against the Fugu genome and zebrafish EST

databases was performed. Based on the putative CITED gene sequences obtained, primers were designed for degenerate RT-PCR cloning of the corresponding CITED cDNAs. The full-length cDNAs of two additional CITED proteins – 1.9 kb and 3.1 kb in lengths – were obtained by 5'- and 3'-RACE. Computer analysis indicated that the ORF of the 1.9-kb cDNA encodes for a 143 amino acid protein which shares high sequence identity (60%) to the hCITED1 protein, and hence the cDNA clone was named gcCITED1. On the other hand, the ORF of the 3.1-kb cDNA encodes for a 240 amino acid protein that showed high identity (>74%) with the CITED3 proteins from different vertebrate species including gcCITED3 of grass carp, and hence was named gcCITED3b.

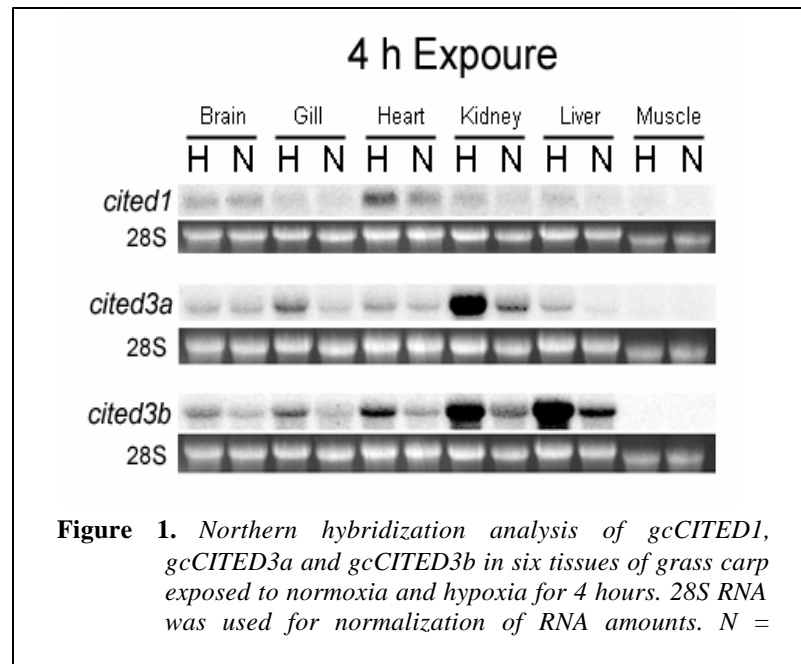
To study the tissue expression pattern and hypoxia responsiveness of *gcCITED1* and *gcCITED3b*, grass carps were exposed to hypoxia (0.5 mg/L DO; n = 3) or normoxia (7.0 mg/L; n = 3) for 4 hours and total RNA from 6 different tissues of each fish was isolated for Northern hybridization analysis (Figure 1). Under normoxia, expression of *gcCITED1* was detected in heart and brain, while expression of *gcCITED3a* and *gcCITED3b* was detected in most tissues examined except muscle. In addition, significant hypoxic induction of *gcCITED1* was observed in heart, while strong hypoxic induction of *gcCITED3a* was detected in kidney. Interestingly, induction of *gcCITED3b* was detected in a broader spectrum of tissues, although the most prominent induction by hypoxia was observed in kidney and liver. Similar to *gcCITED3a*, *gcCITED3b* expression was undetectable in muscle under both normoxic and hypoxic conditions.

In conclusion, besides *gcCITED3a*, we have isolated two additional CITED cDNAs from the grass carp using a degenerate RT-PCR and RACE PCR approach. Computer analysis indicated that they encode for the CITED1 and a CITED3-like protein. The tissue expression patterns of *gcCITED3a* and *gcCITED3b* are very similar, but are distinct from that of *gcCITED1*. All three CITED genes from grass carp are differentially responsive to hypoxia in various fish tissues. Whether the different CITED isoforms play a similar role in the different tissues would require further studies.

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**ELEMENTS OF OXYGEN-REGULATED GENE EXPRESSION
IN THE KILLIFISH, *FUNDULUS***

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EXTENDED ABSTRACT ONLY – DO NOT CITE

Introduction

Low oxygen is prevalent in many aquatic habitats and one response of fish to hypoxia is compensatory changes in gene expression (Gracey et al., 2001). In mammals, this phenomenon is mediated, in part, by the hypoxia-inducible factor (HIF). This transcription factor accumulates at low oxygen, binds to hypoxia response elements (HRE) of a variety of target genes, and ultimately increases gene expression (Semenza, 1999). In fish, homologs of HIF are present (Soitamo et al., 2001; Powell and Hahn, 2002). However, the targets of HIF regulation remain largely unknown. In the killifish, *Fundulus heteroclitus*, long-term hypoxic exposure leads to an increase in the activity of the liver isozyme of lactate dehydrogenase, LDH-B (Greaney et al., 1980). Here, we report on the functional characterization of an HRE in promoter region of the *F. heteroclitus* *Ldh-B* gene.

Methods

RTG-2 cells were obtained from the ATCC and cultured in HEPES-buffered DMEM containing 5% fetal bovine serum at 19°C and 1% CO₂ (Soitamo et al., 2001). Reporter gene plasmids were generated containing the coding sequence of firefly luciferase downstream of the wild-type *Ldh-B* promoter (plasmids N400, N250, N150) or an *Ldh-B* promoter mutated at two putative regulatory

sites (HIFmut and HASmut). Each reporter plasmid was transfected into the fish cells along with a plasmid to assess transfection efficiency (pRL-CMV, Promega) using Fugene-6 (Roche). Cells were exposed to various levels of oxygen (0.5-5%) for 12-96 h. Normoxic controls (21% O₂) were included in each experiment. Luciferase activity was measured using Promega's Dual-Luciferase Reporter Assay System. Data were corrected for background and transfection efficiency. Fold-induction was calculated as the ratio of hypoxic to normoxic reporter gene expression and compared to 1.0 (no induction) using one-sample t-tests.

Results

The *F. heteroclitus* *Ldh-B* promoter conferred hypoxia-sensitivity upon reporter gene expression in RTG-2 cells. Through a series of truncations of the wild-type promoter, the HRE was localized to a region of the promoter between 150 and 250 bp upstream of the translation start site (Fig. 1). In this region is a perfect inverted repeat of GATGTG. Mutation of either portion of this repeated sequence abrogated the hypoxic induction of reporter gene expression (HIFmut and HASmut).

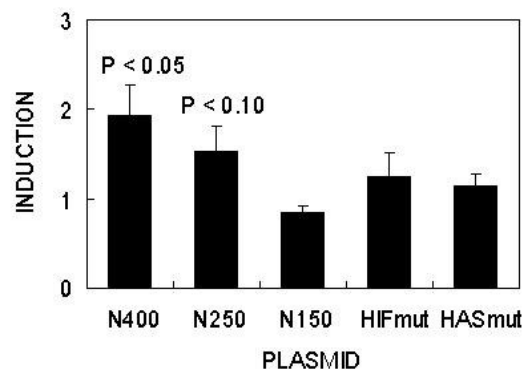


Figure 1. Hypoxic induction of reporter gene plasmids. RTG-2 cells were transfected and exposed to 1% O₂ for 16-20 h. Bars represent mean values from 5 experiments and error bars are one standard error of the mean. P values for the comparison to no induction are shown.

Further experiments evaluated the time and oxygen dependence of the expression of the full-length wild-type *Ldh-B* promoter (Fig. 2). Luciferase expression increased over time, reaching a plateau between 48 and 96 h. Between 0.5% and 5% O₂, reporter gene expression increased as oxygen

percentage decreased. At 0.5% O₂, reporter gene expression in RTG-2 cells peaked at values 4- to 5-fold higher than normoxic expression.

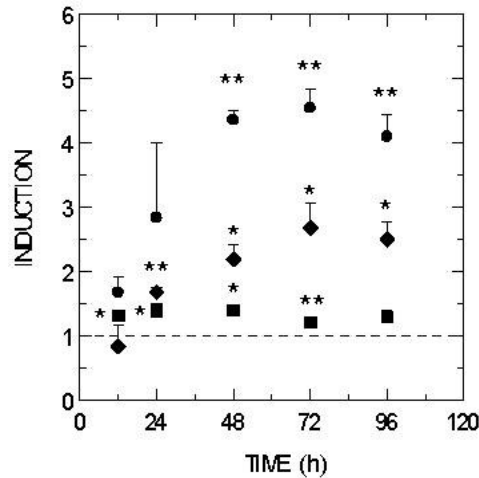


Figure 2. Time course of hypoxic induction of plasmid N400 at various percentages of oxygen (circles, 0.5%; diamonds, 1%; squares, 5%). Symbols represent mean values of 3 experiments. Error bars represent one standard error of the mean and are shown only in one direction. At 5% oxygen, the standard errors were smaller than the symbol. No induction is shown by the dashed line. Significant inductions are shown by * ($P < 0.05$) and ** ($P < 0.01$).

Discussion

We have identified a region of the promoter of the *F. heteroclitus Ldh-B* gene that is capable of driving reporter gene expression in an oxygen-dependent manner. The region is between 150 and 250 bp upstream of the translation start site and consists of a perfect inverted repeat of GATGTG, a sequence similar to the consensus HIF-binding sequence in mammalian genes (Semenza et al., 1999). Mutation of either portion of this repeated sequence abolished the hypoxia sensitivity of reporter gene expression. Thus, this region of the *F. heteroclitus Ldh-B* promoter has the characteristics of a functional HRE.

The hypoxic induction of reporter gene expression in the RTG-2 cell line was markedly affected by oxygen percentage. Hypoxic induction was robust at 0.5% oxygen and minimal at 5% oxygen. In contrast, greatest HIF protein accumulation occurs at 5% O₂ in the same cell line (Soitamo et al., 2001). This difference suggests that HIF protein stabilization represents only one step in the cascade of gene activation and that hypoxia may be important in regulating other steps in this cascade (e.g., DNA binding).

The putative HRE in the *Ldh-B* promoter may be involved in the increased LDH-B activity observed in liver tissue in *F. heteroclitus* exposed to hypoxia (Greaney et al., 1980). Furthermore, it is possible that this DNA element is important in other instances of developmental and physiological changes in *Ldh-B* gene expression in this fish.

Acknowledgements

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**TEMPERATURE-DEPENDENT EXPRESSION OF HYPOXIA-
INDUCIBLE FACTOR-1a (HIF-1a) IN CRUCIAN CARP
(*CARASSIUS CARASSIUS*)**

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EXTENDED ABSTRACT ONLY – DO NOT CITE

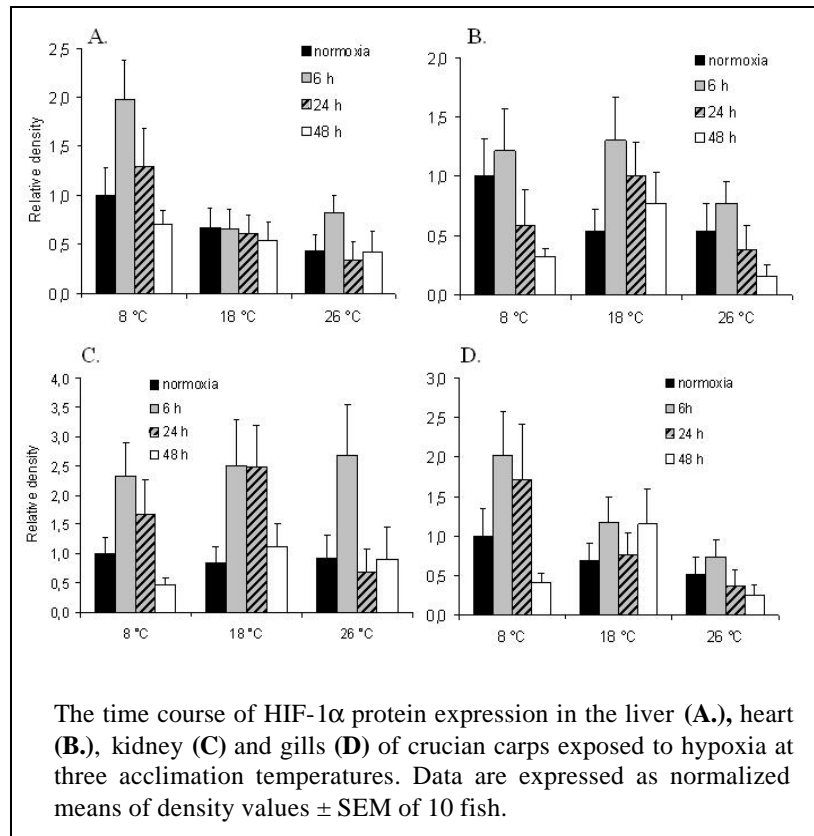
Crucian carp (*Carassius carassius*), inhabiting Northern shallow ponds, tolerates large variations in both ambient oxygen and temperature. The well known extreme anoxia tolerance of crucian carp is based on metabolic downregulation and on large glycogen stores of the liver which provide substrate for anaerobic glycolysis (Hyvarinen et al, 1985). Adaptive rearrangements in energy metabolism are also needed for temperature acclimation in eurythermic fish to compensate for the effect of temperature change on enzymatic processes (Hardewig et al, 1999).

Hypoxia-inducible factor 1 α (HIF-1 α) is an evolutionarily conserved transcription factor that upregulates the expression of several genes involved in oxygen transport and anaerobic energy metabolism. Previously it has been thought that HIF-1 α is activated only in response to low oxygen, not in other cell stresses. However, increased expression of HIF-1 α has been observed during heat-acclimation in rat (Maloyan et al, 2001) and it is required for heat-acclimation in *C. elegans* (Treinin et al, 2003). These studies suggest that HIF-1 could have an evolutionarily conserved function also in temperature acclimation.

To find out whether variations in HIF-1 α level are associated with temperature acclimation as well as in hypoxia adaptation in crucian carp,

we studied the expression of HIF-1 α in crucian carps under severe hypoxia (0.7 mg/L O₂) at acclimation temperatures 8, 18 and 26 °C. To reveal the time course of HIF-1 function, fish were sampled after 6, 24 or 48 hours of hypoxia. HIF-1 α expression in the liver, heart, gills and kidney was analysed by Western and Northern blotting and the DNA-binding activity of HIF-1 by electromobility shift assay.

In air saturated water the amount of HIF-1 α protein in the liver, heart and gills was highest at 8 °C and lowest at 26 °C, which is closest to optimum temperature for crucian carp (Figures A, B and D).



Under hypoxia HIF-1 α levels increased in 6 hours of hypoxia and thereafter declined during subsequent 42 hours. In fish acclimated to 8 °C this time course of HIF-1 α expression was evident in all tissues examined (Figure). The DNA binding activities of HIF-1 α in different tissues followed mainly protein levels. In addition, HIF-1 α mRNA levels increased under hypoxia in gills of fish acclimated to 26 °C.

These results suggest that in addition to hypoxia adaptation, HIF-1 α has a role in temperature acclimation, at least in cold acclimation, in crucian carp. This supports the view that HIF-1 α is not activated specifically in response to low oxygen but has also an evolutionarily conserved function in temperature acclimation. In further studies, we will examine whether HSP-90 is involved in temperature regulation of crucian carp HIF-1 α and carry out experiments at 32 °C, which is above the optimum temperature for crucian carp.

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**GLUCOCORTICOID -DEPENDENT GENE EXPRESSION IN
TEMPERATURE ADAPTATION**

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EXTENDED ABSTRACT ONLY – DO NOT CITE

Acclimation and adaptation of fishes to local temperature regimes involves a series of coordinated changes at the physiological, biochemical, and molecular levels. We have been using several geographically distinct populations of the mummichog, *Fundulus heteroclitus*, as a model to explore the molecular mechanisms involved in this process of local adaptation. Mummichogs are distributed along the East Coast of North America, from Newfoundland to northeastern Florida. There is a steep thermal gradient along this coast, and mummichogs at the extremes of the species range experience a greater than 10°C difference in environmental temperatures.

Associated with these differences in temperature, there are two distinct genotypes of mummichogs at the northern and southern ends of the species range, with a broad zone of admixture between the two genotypes in intermediate latitudes. There are substantial differences in performance, developmental rate and survival between the northern and southern genotypes at either temperature extreme, suggesting that these genetic differences have impacts on a variety of fitness correlates. In fact, selection experiments in the laboratory have indicated very high selection coefficients associated with population-specific allozyme patterns, particularly with genotype at the glycolytic enzyme LDH-B (lactate dehydrogenase-B). There are also differences in the expression of the Ldh-B gene between genotypes. Northern fish express ~2-fold more Ldh-B in both heart and liver than do southern fish, and several lines of evidence suggest that this difference in gene expression is of adaptive significance (reviewed in Schulte, 2001).

We have performed an extensive series of experiments investigating the molecular basis of this adaptively significant difference in gene expression. Sequence and population genetic analysis of the promoter region of the Ldh-B gene suggested that mutation in a putative glucocorticoid responsive element were responsible for the differences in gene expression between populations (Schulte et al., 1997). Subsequent functional analyses in cell culture and in transiently transgenic fish confirmed the effects of these mutations (Schulte et al., 2000). In addition, we have recently completed site-directed mutagenesis experiments using the Ldh-B promoter. These experiments indicate that several sites associated with this glucocorticoid responsive element are responsible for the differences in expression between the two populations. Overall, the southern fish appear to possess a more sensitive glucocorticoid-responsive element within the Ldh-B promoter than do northern fish.

When mummichogs are exposed to handling stress or injected with high doses of cortisol, LDH-B activity and expression increase in the livers of fish from the southern population, but not the northern population, consistent with the predictions of the molecular experiments described above. Injection of cortisol into fish transiently transfected with a construct containing the Ldh-B promoter coupled to a luciferase reporter gene had similar results (Schulte et al., 2000), strongly implicating this glucocorticoid responsive element in the differential regulation of the Ldh-B gene between populations of mummichogs.

LDH-B is the terminal enzyme in anaerobic glycolysis. To determine whether stress hormones similarly affect the expression of other glycolytic genes, we assayed the activity and expression of all of the glycolytic genes in the livers of fish exposed to cortisol injection or handling stress. Only two enzymes (in addition to LDH-B) changed in activity in stressed fish, and only in fish from the southern population, not the northern population. Only these two enzymes differed in activity between populations in resting fish, and the net effect of stress-exposure was to remove these differences in activity (Dekoning et al., 2004). This pattern is very similar to that observed for LDH-B, and thus these data suggest that there is a coordinated response of the glycolytic enzymes to stress, and that this response differs between northern and southern fish.

We next used differential-display PCR to investigate whether this pattern held for other (randomly selected) genes. Although several genes conformed to this pattern, we also discovered several genes that differed in expression between populations, but which did not respond to stress, suggesting that there is a

complex interaction between the stress-response and population-specific differences in gene expression in these fish (Picard and Schulte, 2004).

To further explore the role of the stress-response in shaping adaptively significant variation in mummichogs, we also examined features of the stress response itself. We found that southern mummichogs experience greater increases in cortisol in response to a standardized stressor than do northern mummichogs, and that northern mummichogs have higher cortisol turnover than southern mummichogs (DeKoning and Schulte, 2004). These data suggest that there are profound differences in the stress-response between these two populations, and that these differences in the stress-response are associated with differences in liver gene expression between populations.

Taken together, these studies at the molecular, biochemical and physiological level are providing new insights into the mechanisms underlying adaptation to local environmental conditions.

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Acknowledgments

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**NOVEL GENES INVOLVED IN ADAPTATION OF SALMON
TO THE MARINE ENVIRONMENT**

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Anadromous salmonids contend with hyperosmotic stress as juveniles during seaward migration from their natal freshwater stream. This osmotic stress is more acute in routine aquaculture practice, in which juveniles are directly transferred from freshwater hatcheries to netpens in full salinity seawater (\approx 1100 mOsm). Plasma osmolality can rise from 316 mOsm/kg to $>$ 500 mOsm/kg in 24 – 48 hours. Individuals incapable of regaining osmotic/ionic homeostasis die, or grow at a reduced rate. A variety of osmoregulatory mechanisms have been demonstrated to be involved in adaptation of salmon and other teleosts to the marine environment. The most well documented of these is the branchial Na⁺/K⁺ ATPase antiport pump (Zaugg and Wagner, 1973). Activity of this enzyme increases markedly during parr-smolt transformation and adaptation to seawater, facilitating branchial elimination of Na⁺ and maintenance/reattainment of osmotic homeostasis. More recently, the activity and quantity of another transporter, the Na⁺-K⁺-2Cl⁻ cotransporter, has been observed to increase during adaptation to the marine environment (Pellis et al., 2001).

Here we report, on the upregulation of several novel genes in tissues of Atlantic salmon (*Salmo salar*) exposed to osmotic stress in vitro or in vivo. Previous investigations in our laboratory demonstrated upregulation of heat shock protein (hsp)70 following exposure of salmon to hypersaline conditions (Smith et al.1999). Additionally, mRNA coding for hsp90 accumulated in branchial lamellae, but not in kidney, in response to osmotic stress in vitro and in vivo (Pan et al., 2000).

Several novel cDNAs were isolated from branchial lamellae exposed to osmotic stress using differential expression analysis of mRNA and cloned. Nucleotide sequence analysis of the first clone indicates that the cDNA codes for a 21 kDa protein (Shop21; Pan et al., 2002) with 98% identity to Rbx1, an E3 ubiquitin ligase; the protein also contains a novel 81 amino acid domain at the NH terminus not found in Rbx1. Moderate hyperosmotic stress (24h at 550 mOsm/kg) increased Shop21 transcript 10-fold in branchial lamellae whereas no upregulation was observed under more severe stress (>800 mOsm/kg). Expression of the gene also was observed in heart and kidney. Replacement of NaCl with mannitol, but not glycerol, also elicited an increase in shop21 mRNA. Inhibition of the MAPK and MEK signal transduction pathways failed to blunt the Shop21 response during hyperosmotic stress. Shop21 mRNA also accumulated during thermal stress, but to a lesser extent than hsp70 mRNA.

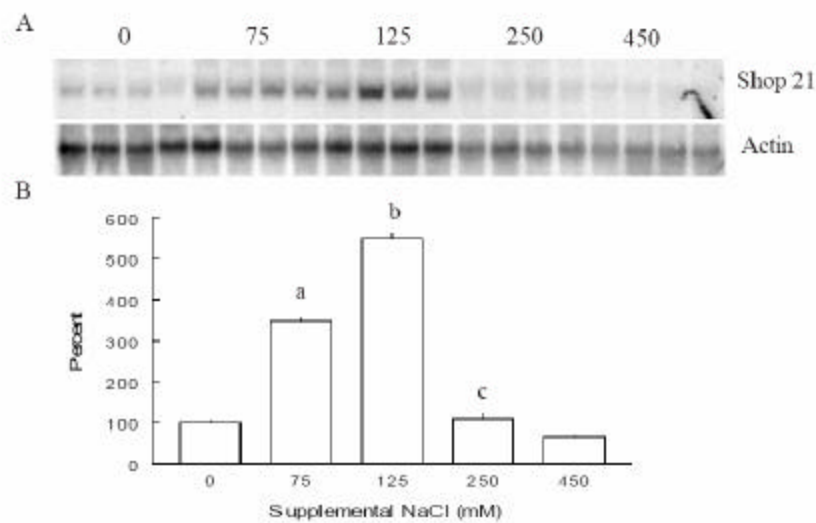


Fig.1. Northern blot showing upregulation of Shop21 in isolated branchial lamellae cultured in media supplemented with NaCl for 24 h.

A second clone contained a 1.3 kb transcript with an open reading frame of 618 nt coding for a 205 amino acid protein with a molecular weight of 21.5 kDa.

The putative protein possesses a high degree of identity (>70%) with cold inducible RNA binding proteins (CIRP) of mammals and amphibians and contains a single RNA recognition motif (RRM), high glycine content and conserved flanking motifs (Pan et al., 2004). The gene coding for salmon glycine rich RNA binding protein (SGRP) was upregulated in response to hyperosmotic stress of branchial tissue. Maximum levels of expression were observed at 24 h in vitro and 48 h in vivo. Exposure of isolated lamellae to heat stress did not stimulate accumulation of SGRP transcript. Of significance was the absence of an increase in expression of SGRP in response to cold stress ($\Delta T = 5$ and 12° for 12 and 24 h). These findings suggest that endothermic salmon inhabiting boreal waters lack CIRPs similar to those of mammals and amphibians and that SGRP likely has a more prominent role in adaptation to hyperosmotic stress.

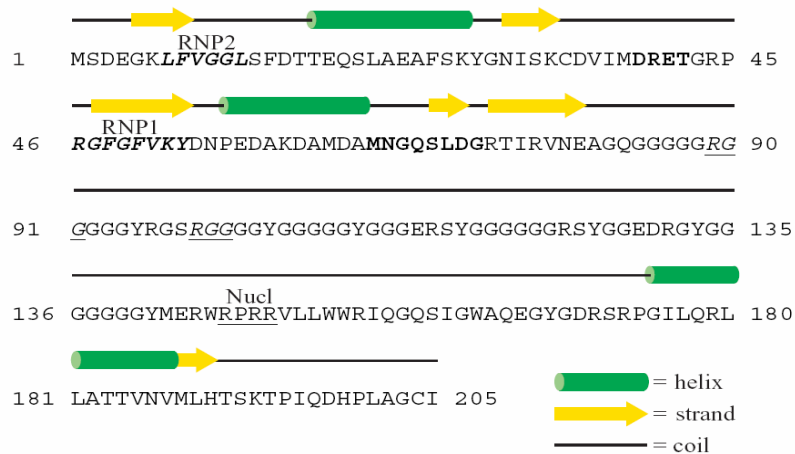


Fig 2. Deduced amino acid sequence of SGRP illustrating features: RNP1 and RNP2 (RNA Recognition Motif elements) in **bold italics**, RRM associated motifs in **bold**, RGG boxes underlined and *italics*, nuclear localization sequence (Nucl) underlined. Predicted secondary structure is designated in the key.

The results of these investigations suggest that a complex array of branchial mechanisms, similar to those observed in the renal system of terrestrial mammals, are involved in adaptation of salmon to seawater. Ongoing research

in our laboratory seeks to further characterize the functions of these and other genes using a variety of approaches including identification of binding proteins with a yeast-two hybrid system and localization of GFP-fusion proteins.

Acknowledgements

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MOLECULAR MECHANISMS OF EARLY MORTALITY SYNDROME

(M74) IN BALTIC SALMON

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EXTENDED ABSTRACT ONLY - DO NOT CITE

Baltic salmon (*Salmo salar*) suffer from abnormally high, maternally transmitted yolk-sac fry mortality. In 1990's 50-90% of newly hatched salmon from wild parents in Sweden and Finland died before feeding. The occurrence of the syndrome is highly variable, and only 5-10 % mortality was observed in 2003. M74-affected fry are characterized by low thiamine content and show signs of oxidative stress. At the morphological level diseased fry appear to have impaired vascular development, hemorrhages, reduced number of circulating erythrocytes, hepatocellular lipid accumulation, dilatation of hepatocytic endoplasmic reticulum and degeneration of skeletal muscle. Necrotic cells are observed in the brain. At the behavioural level, various neurological disorders such as lack of light sensitivity, passivity and spiral swimming have been reported (Bengtsson et al., 1999). Since, despite intensive investigations, the proximate course of the disease has remained elusive, the aim of the ongoing

project is to clarify the molecular and cellular mechanisms involved in the developmental disturbances associated with the mortality.

As a starting point of the research we have hypothesized that the developmental disturbances in Baltic salmon are caused by oxidative stresses, which may be either a result of changes in the natural food web or caused by human action. Since the disturbances of both vascular, erythrocyte and neurological development in M74 affected salmon fry resemble those observed in mammalian embryos, in which the function of the hypoxia-inducible transcription factor HIF-1 α and consecutive gene expression is disturbed, and since the stabilization and DNA-binding of salmonid HIF-1 α is redox sensitive (Nikinmaa et al., 2004), the initial studies have focussed on the possible role of HIF-1 α in the development of the syndrome. We have shown that the vascular defects observed in fry suffering from M74 are associated with reduced DNA-binding of HIF-1 α and subsequent downregulation of its target gene vascular endothelial growth factor (VEGF), involved in angiogenesis (Vuori et al., 2004). In addition, we have observed that also the DNA binding of xenobiotically induced transcription factor AhR (Aryl hydrocarbon receptor), which is upregulated in healthy Baltic salmon fry, is reduced in fry suffering from M74 (Vuori et al., 2004). This suggests that man-made dioxin-like substances may not be directly involved in the development of the syndrome in yolk-sac fry.

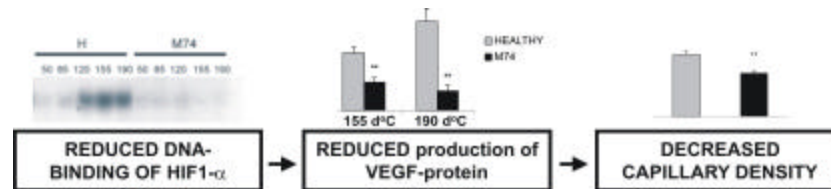


Figure 1. The vascular defects observed in fry suffering from M74 are associated with reduced DNA-binding of HIF-1 α and subsequent downregulation of vascular endothelial growth factor (VEGF), involved in angiogenesis.

To evaluate which genes are differentially expressed between M74-affected and healthy fry, we have used cDNA microarray approach. Based on the microarray data, the fry dying during the first third of the yolk-sac stage are characterized by a strong stress response and upregulation of stress- and extracellular matrix-associated signalling transcripts. The fry dying at the halfway of yolk-sac stage suffer from pronounced downregulation of hemoglobin biosynthesis, occurring

at the period of hemoglobin switching from embryonal to adult types. This finding agrees with the results showing reduced DNA-binding of HIF1- α , which in mammals regulates erythrocytic differentiation. The fry dying at the last third of yolk-sac stage show gene expression responses to prolonged stress, inhibition of cell proliferation and loss of cell contacts. At terminal stages, all M74-fry show similar downregulation of chromatin components and upregulation of genes involved in apoptosis, indicating that cell growth and proliferation are diminished and cell death is increased.

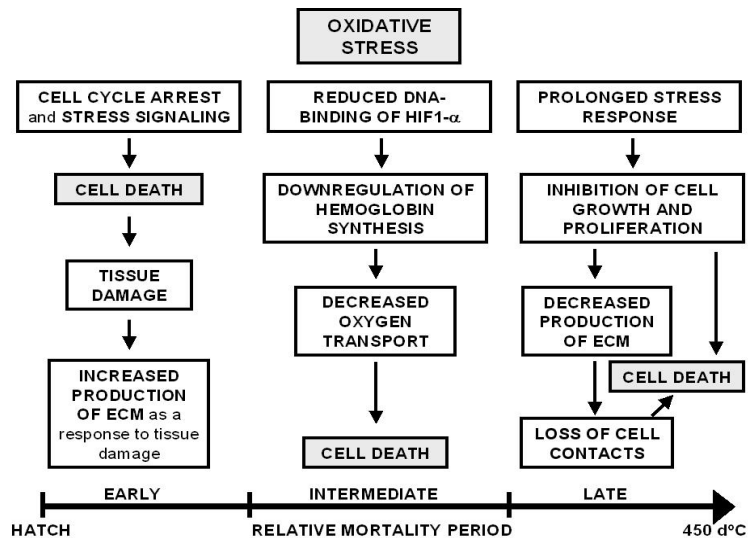


Figure 2. Working hypothesis of molecular events leading to mortality in fry dying at different periods of yolk-sac fry development.

In conclusion, the data hitherto gathered suggest that early mortality syndrome in Baltic salmon yolk-sac fry results from oxidative stresses disturbing developmental expression of several different genes depending of the severity of the M74-syndrome, which eventually leads to cell death and mortality by activation of similar mechanisms cell proliferation arrest and death gene expression. In future studies we will be investigating the mechanisms by which oxidative stresses develop in yolk sac fry, including the possible involvement of the natural food of Baltic salmon.

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**USING TRANSCRIPTOMICS TO PROFILE RANK POSITION
WITHIN DOMINANCE HIERARCHIES OF RAINBOW TROUT**

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EXTENDED ABSTRACT ONLY – DO NOT CITE

Functional approaches have been successful in elucidating the role of behaviour as a component of fitness, but it is now evident that a full understanding of how natural selection acts on such behavioural traits requires information about the mechanisms that cause them. Competitive interactions between conspecifics contributes to individual fitness and survival and is of fundamental theoretical interest. Many animals form dominance hierarchies or pecking orders where the dominant individual obtains exclusive or priority access to resources. Other studies have shown profound behavioural and physiological differences between individuals with differing dominance status. Dominance hierarchies can be problematic in aquaculture with one or a few dominant animals obtaining the majority of food and showing higher growth rates than animals lower in the status and, therefore, they have limited access to food, show poor growth and suffer stress. This study was designed to understand the phenotypic correlates of dominance position by using microarray screening technology to define genes whose expression differs between dominants, subdominants and subordinates within rainbow trout dominance hierarchies.

Methods

Size matched rainbow trout were kept in groups of three in uniform conditions. The behaviour of each fish was assessed to record number of attacks and

number of retreats to calculate the dominance score for each individual in order to discern a linear dominance hierarchy for each group. Only fish which showed a clear pattern of behaviour with the dominant, rank 1, having the highest score, rank 2, the subdominant, having an intermediate score and the subordinate, rank 3, having the lowest score were used in the study. Once the hierarchy was stable for 7 days the fish were killed and brain, liver and muscle were removed. These tissues were stored at -80°C and RNA was extracted using a Trizol method. The RNA was reverse transcribed to cDNA with amino allyl labelling. Two fluorescent dyes, Cy3 and Cy5, were coupled to either an individual sample or to a common reference and then these are hybridised to a rainbow trout microarray in a forward and reverse reaction (dye swap). The library used for the array comprises cDNAs collected from rainbow trout. Data analysis is performed by the GeneSpring software using hierarchical cluster analysis and ANOVA with a multiple testing correction factor

Results

The results for brain gene expression were assessed and approximately 1165 genes were consistently found to be differentially expressed between the ranks. Two major patterns of expression were seen with genes either showing up-regulation in the subdominant and down-regulation in the subordinate relative to the dominant, or a profound down-regulation in the subdominant and an up-regulation in the subordinate relative to the dominant. The majority of genes were significantly different in expression between rank 2, the subdominant, and rank 3, the subordinate. These gene expression profiles were similar between the hierarchies with fish of the same rank showing the same profile across hierarchies.

Discussion

Major differences were consistently observed in the gene expression profiles of brains from fish with differing dominance status. This demonstrates that these behavioural states give rise to discernible transcript phenotypes at least in the brain, and probably in other tissues. This indicates that the microarray screening technique will prove useful as a profiling and phenotyping tool. These gene expression differences may be correlated with documented differences in behaviour and physiology of individuals of different rank, and might provide insights into the functional significance of the physiological differences between hierarchical states. By identifying these genes it may be possible to manipulate

these in future studies and unravel the causes and consequences of dominance in future studies.

Acknowledgements

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**TRANSCRIPTION OF FSH- β , LH- β AND GROWTH HORMONE IN
FEMALE *TRICHOGASTER TRICHOPTERUS* (PALLAS 1770)
TREATED WITH GnRH ANALOG**

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Abstract

In this work, the effect of [D-Ala6,Pro9Net]-LHRH (GnRH α) on the expression of mRNA, the follicle-stimulating hormone (FSH- β), the luteinizing hormone (LH- β) and the growth hormone (GH) was examined in female blue gourami during advanced vitellogenesis.

The levels of FSH- β and LH- β transcripts in females injected with GnRH α were significantly higher than those of the control. However, no significant difference was found between the GH mRNA expression of saline (control) and females injected with GnRH. In the ovaries of GnRH injected fish, some vitellogenic oocytes reached final oocyte maturation, as manifested by nuclear migration and central lipid drop formation. The different effects of GnRH on the transcription of FSH- β , LH- β and GH are discussed.

Introduction

Oogenesis in teleosts, as in other vertebrates, is induced by increasing levels of gonadotropins (GtHs) via the mediation of ovarian steroids, starting from the early growth of primary oocytes to vitellogenesis and through maturation to ovulation (Yaron et al., 2003). Two distinct gonadotropins exist in the pituitary gland of teleosts: follicle-stimulating hormone (FSH) and luteinizing hormone (LH).

Pituitary growth hormone (GH), a single-chain polypeptide, plays an essential role in the regulation of growth and development by promoting the division, differentiation and enlargement of cells. The biological activities of GH have been examined in many fish species (Degani et al., 2003).

It is well known that gonadotropin synthesis and secretion are regulated by the gonadotropin-releasing hormone (GnRH), a hypothalamic decapeptide that is synthesized in the hypothalamus and released by the axonal terminations at the neurohypophysis (Zohar et al., 1995). Fourteen different forms of GnRH have been isolated from the brain of different vertebrates, all of them consisting of 10 amino acid peptides, as reviewed by King and Millar (1995).

GH synthesis and release are regulated by the peptide growth hormone-releasing hormone (GHRH), which is stimulatory, whereas somatostatin has an inhibitory effect (Melamed et al., 1998). GnRH can also cause GH release, as was demonstrated in fish (Melamed et al., 1996). Morphological features support the existence of direct stimulatory actions of GnRH on GH release: GnRH neurons were found to project the growth hormone cells in steelhead trout (Taniyama et al., 2000); specific gonadotropin-releasing hormone receptors were detected on goldfish somatotrophs (Cook et al., 1991). However, the influence of GnRH on GH production is still controversial: GnRH increases GH mRNA content in goldfish (Mahmoud et al., 1996) and sockeye salmon (Taniyama et al., 2000), but direct exposure to sGnRH in tilapia did not alter the transcript levels (Melamed et al., 1996).

Blue gourami, *Trichogaster trichopterus*, belongs to the Anabantidae family. This multi-spawning and male-dependent fish has an asynchronic ovary development (Degani, 1993). In high vitellogenic females injected with GnRH_a, cGnRH-II or sGnRH, FOM was reached without the male presence (Degani et al., 1995). The above GnRHs also caused an increase in LH content in a primary cell culture (Degani et al., 1997). Jackson et al. (1999) cloned and sequenced the

cDNAs of blue gourami FSH- β and LH- β , and quantified the mRNA of these hormones during the gonadal cycle. GH cDNA was cloned using RACE PCR. The expression of blue gourami GH mRNA was measured during spermatogenesis and male sexual behavior (Degani et al., 2003).

The purpose of the present study was to examine the effect of GnRH α on the gene expression of FSH- β , LH- β and GH in female blue gourami in a state of advanced vitellogenesis.

Materials and Methods

Fish

Female blue gouramis, maintained and bred at MIGAL Laboratories in northern Israel, were used in this study. The fish were grown in containers measuring 2 x 2 x 0.5 m at a temperature of 27°C and under a light regime of 12L 12D. The fish were fed an artificial diet (45% protein, 7% fat), supplemented by live food (*Artemia salina*).

In the experiment, females (10-20 g) with oocytes in advanced vitellogenesis were treated with GnRH α dissolved in saline (0.7% NaCl) and injected i.m. with doses of 0.3 μ g/g B.W. and a final volume of 100 μ l. Ten females were treated with either [D-Ala⁶,Pro⁹Net]-LHRH (GnRH α) or saline (control). Forty-eight hours after injection, pituitaries were collected for RNA extraction (see below) and ovary samples processed for light microscopy.

Measurement of FSH- β , LH- β and GH mRNA levels by RT-PCR

The relative levels of FSH- β , LH- β and GH mRNAs were measured by reverse transcriptase (RT-PCR) using the 18S rRNA as an internal standard, as previously described by Jackson et al. (1999). The RT-PCR method was employed because the very small size of the blue gourami pituitary gland precluded the use of Northern blotting, which requires relatively large amounts of RNA (Kawasaki, 1990). The 18S rRNA was used as an internal standard of the measurements since it is an abundant RNA and its expression has been found to be uniform throughout the gourami female reproductive cycle (Jackson et al., 1999).

Total RNA was extracted from freshly excised pituitaries by means of the Rneasy® total RNA kit (QIAGEN, Hilden, GR). Usually 40 ng/μl of total RNA was obtained from a single pituitary. The cDNA pools were synthesized as described by Frohman (1990) using an AMV reverse transcriptase (Promega, Madison, WI, USA). The primers for the cDNA synthesis consisted of an oligo dT (17 bases), linked to a unique 17mer adapter (Table 1: dT17 adapter) and a mixture of random decamers (Bio-Technology General, Rehovot, Israel).

Amplification of the cDNAs of FSH, LH and GH

Each amplification of the three cDNAs was coupled to an amplification of the internal standard, 18S rRNA. In each case, the 18S rRNA cDNA and the hormone cDNA were amplified in separate tubes since many attempts to use multiplex PCR resulted in considerable interference between the two reactions. The concentration of each primer pair and the number of cycles were carefully calibrated so that the amplifications would be within the linear range and would depend on the initial concentration of the target cDNA.

The FSH-?, LH-? and GH cDNAs were amplified with gene-specific primers (see Table 1) at a concentration of 6.25 pmol/reaction. The 18S rRNA cDNA was amplified with specific primers, obtained from Ambion as part of its Quantum RNA kit, at a concentration of 5 pmol/reaction. The reagents, except for the cDNA and the primers, were prepared as a batch for each set of reactions to minimize variations. The PCR amplifications were carried out in 50 μl reactions, using 1.25 units of Taq polymerase (Promega, Madison, WI, USA), and a suitable reaction buffer (1.5 mM MgCl₂, 0.2 mM of each nucleotide, 5 μl of cDNA pool and 20 μl mineral oil). Cycling parameters were 3 min denaturation at 94°C, followed by 28 cycles, comprising 1 min denaturation at 94°C, 1 min annealing at 54°C and 1 min extension at 72°C.

Table 1. Primers used in this study

Primer Name	cDNA Target	Use	Nucleotide Sequence
P1	FSH	Complete cDNA cloning	5'GTCTGTACAGATGTTTAGAGAG
P2	FSH	Complete cDNA cloning	5'AACGTGGGATGAATGATGAGTG
P3	LH	Complete cDNA cloning	5'CTGGCTAACCTGCCGCTGACAC
P4	LH	Complete cDNA cloning	5'TTGCTTTTGGTTTGCTGTGCA
P5	GH	Complete cDNA cloning	5'AGAAGTGAACCTGAACCTGTATC
P6	GH	Complete cDNA cloning	5'CATTGTGCTGGAACCTGG
dT ₁₇ -Adapter	Universal	cDNA synthesis	5'GACTCGAGTCGACATCGA(T) ₁₇
Adapter	Universal	RACE PCR	5'GACTCGAGTCGACATCG

Quantification of PCR products

The PCR products (½ of the PCR reaction) were electrophoresed on 2% agarose gel at 120 mA for 45 min. The DNA was then stained with ethidium bromide (0.03 mg in 100 µl distilled water) and imaged by Eagle Eye II Image analysis (Stratagene, La Jolla, CA, USA). The gels were imaged at three different exposure times to prevent a possible saturation of the images. The resulting images were analyzed with the aid of the PCBAS-2 densitometry software (Raytest, Straubenhardt, Germany). The significance of the differences in the amount of PCR products was analyzed by Student's t-test.

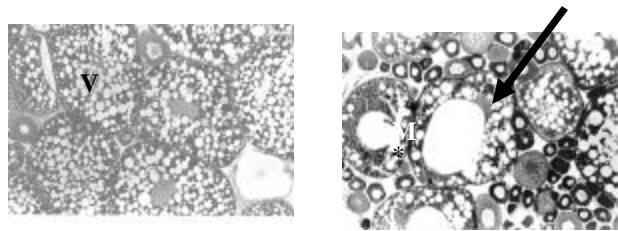
Histological examination

Histological sections of 6 μm of ovary were obtained by means of a Reichert (Austria) microtome and stained with trichrome of Mallory.

Results

The ovaries of saline injected fish contained oocytes at different developmental stages, but mainly large vitellogenic oocytes. In the ovaries of GnRH α injected fish, some vitellogenic oocytes reached FOM, as manifested by nuclear migration and central lipid drop formation (see Fig. 1).

Figure 1. Histology of fish ovaries injected with saline (right) or GnRH α (left). Legend: V=Vitellogenic oocytes, M=oocytes at final maturation. Note germinal vesicle breakdown (arrow) and the formation of the central droplet (*).



The amplification of FSH- β cDNA produced a 306 bp product, that of LH- γ cDNA a 284 bp product, and the amplification of the internal standard (cDNA of 18S rRNA) a 430 bp product. The identity of each PCR product was confirmed by DNA sequencing.

The relative levels of FSH- γ , LH- γ and GH expressions are given in relation to the 18S rRNA. (Fig. 2; Table 2). The levels of FSH- γ and LH- γ transcripts in females injected with GnRH α were significantly higher than those of the control. However, no significant difference ($p > 0.05$, t-test) was found between the GH expression of saline and GnRH injected females (Fig. 2; Table 2).

Figure 2. Levels of (A) FSH-? mRNA, (B) LH-? mRNA, (C) LH-? mRNA in *T. trichopterus* after saline (control) or GnRH α injection. Each histogram represents the average of five independent measurements (mean \pm SE).

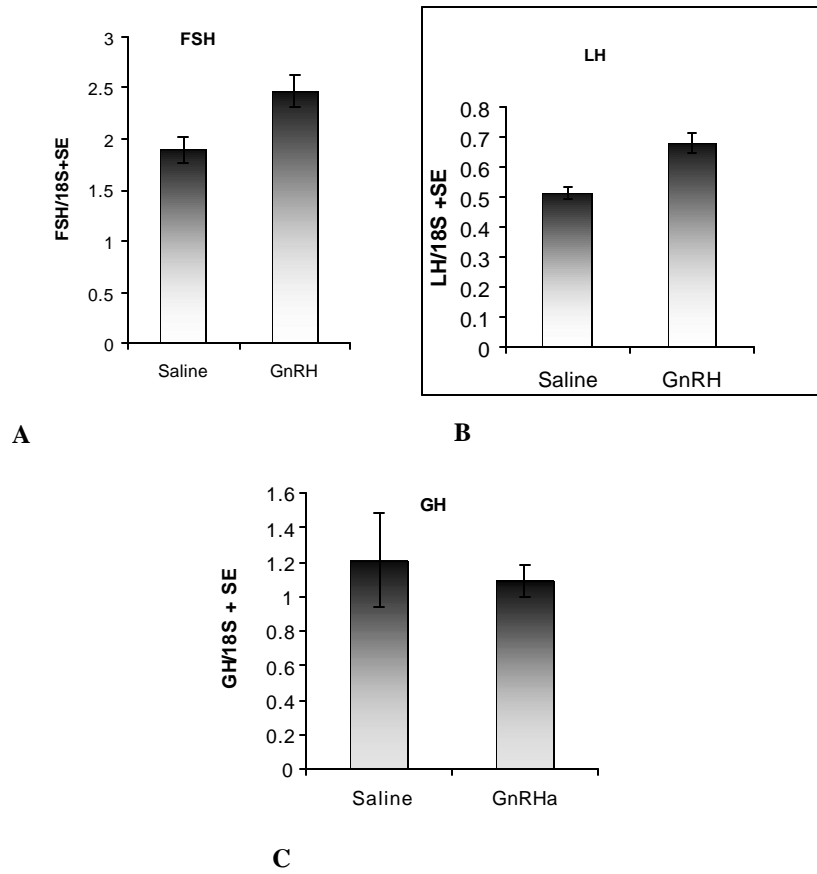


Table 2. Comparison between FSH, LH and GH of saline and GnRH injected fish (t-test)

Group	t-test	P (probability)
FSH- β control	2.70	< 0.05
LH-? control	2.40	< 0.05
GH control	1.78	> 0.05

Discussion

The effect of different GnRHs on LH release in blue gourami has been previously described (Degani et al., 1997). The three forms of GnRH tested (GnRH, aGnRHs and c-II GnRH) stimulated LH release from the pituitary *in vitro* and *in vivo* in *T. trichopterus* (see Fig. 2) Injection of aGnRH induced FOM in post-vitellogenic females kept without a male (Degani et al., 1995).

The results of this study indicate that GnRH enhances the transcription of LH and FSH in blue gourami. The effect is presumably at the transcription level. Interpretation of the results suggests that FSH and LH are regulated differently by GnRH since the effect on LH is much more pronounced than on FSH. Nevertheless, a direct effect of GnRH α on FSH transcription, though not on LH, was demonstrated in a cell culture of maturing salmon (Dickey and Swanson, 2000).

Together, these results indicate that the effect of GnRH on the two gonadotropins depends on the reproductive state of the fish, as previously noted (Swanson and Dickey, 1996). Mateos et al. (2003) found that injection with a GnRH analogue to sea bass (*Dicentrarchus labrax*) had no effect on FSH- β mRNA levels but stimulated pituitary α and LH- β mRNA levels.

At the end of vitellogenesis, the ovaries in blue gourami contain oocytes arrested at advanced stages of vitellogenesis. Only when males are added to the tank do these oocytes reach FOM (Degani, 1993). Based on results from several studies performed in our lab, our explanation is that LH is produced during vitellogenesis in the pituitary but not released to the plasma. During this phase, the expression of LH-? and FSH-? mRNA is higher than in younger fish (Jackson et al., 1999). High vitellogenic females transferred to the presence of a male reach FOM. During this period, LH is secreted to the plasma, probably due

to GnRH action (Degani et al., 1997). The levels of FSH- α and LH- β expression remained high in FOM females and could be a consequence of GnRH action (Jackson et al., 1999). The effect of GnRH before FOM is not fully understood, but it is possible that during this phase, other factors in addition to GnRH are involved in the control of LH- β and FSH- α mRNA expression in multi-spawning and male-dependent fish.

The effect of GnRH on GH release has been studied intensively, and today GnRH is also considered a GH secretagogue in several teleost species (Degani et al., 1999; Marchant et al., 1989). However, the effect of GnRH on GH transcription, as examined in the present study, is less clear. In goldfish, the injection of sGnRH resulted in an increase in GH transcript levels (Marchant et al., 1989). In other species, such as tilapia and sockeye salmon, it did not alter the transcript levels (Melamed et al., 1996; Taniyama et al., 2000). In the present study as well, GnRH α did not alter GH transcription levels in vitellogenic females. Recently, GH transcription has been studied during gonadal development in female gouramis (Degani et al., 2003) and was found at a high level during growth vitellogenesis and maturation. Both sGnRH and cGnRH-II, using cultured dispersed goldfish pituitary cells of pituitary a, stimulated GtH, FSH- α , LH- β and GH mRNA levels, however cGnRH-II appeared to have a more pronounced effect (Klauson et al., 2001). According to Klauson et al. (2001), sGnRH or cGnRH-II treatments also stimulated GtH and GH gene expression. Growth hormone-releasing hormone (GHRH) and pituitary adenylate cyclase-activating polypeptide (PACAP) control GH secretion in fish and other vertebrates (Fradinger and Sherwood, 2000).

Unlike FSH- α and LH- β , GH transcription was practically uniform during the entire cycle. This situation was found not only in females but also in males during sexual behavior and spermatogenesis (Degani et al., 2003).

In conclusion, we suggest that in female blue gouramis in high vitellogenesis, LH- β , FSH- α and GH regulations are controlled by GnRH, but in different ways. GnRH affects the transcription of LH- β and FSH- α but not of GH and the release of all the hormones (Degani et al., 1999).

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HYDRATION AND ALLOSTERIC EFFECT IN REGULATES OXYGEN

BINDING IN CATFISH (*Hoplosternum littorale*) HEMOGLOBINS

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Summary

Catfishes are considered to present accessorial air oxygenation and isoforms that exhibit a greater variety of functional behavior compared with mammalian hemoglobins (Hb), in terms of cooperative ligand binding and allosteric control exerted by a variety of chemical factors, such as H⁺, Cl⁻, CO₂ and organic phosphates. In order to determine the existence of different conformational states in *H. littorale* hemoglobins, we used the osmotic stress proposed by Colombo et al., (1992) to the purified isoforms. We demonstrate that water potential does affect oxygen affinity of *H. littorale* hemoglobins in the presence of an inert solute (sucrose). Oxygen affinity increases when water activity increases, indicating that water molecules stabilize the high-affinity state of the hemoglobin. This effect is the same observed in tetrameric vertebrate (human, bovine, snake) hemoglobins. We show that eletrophoretically-anodic and cathodic hemoglobins show an oxygenation pathway similar to human hemoglobin. Addition of anionic effectors strongly increases the number of water molecules bound, and accordingly we propose, for both Hbs, that the deoxy-conformations coexists in two anion dependent allosteric states, as occurs for human hemoglobin. We did not found evidences to confirme the proposal of an additional phosphate binding site to the cathodic hemoglobin.

Introduction

Water plays a unique and ubiquitous role in biology. Folding, stability, and function of protein molecules are all strongly influenced by their interactions with water molecules (Royer Jr. *et al.*, 1996). A central role for water in determining structure and regulating function of proteins is becoming increasingly evident, since water molecules act as allosteric effectors, by preferentially binding to a specific protein conformation (Colombo *et al.*, 1992). Significant changes in protein hydration are conveniently studied by the osmotic stress method (Colombo and Seixas, 1999), where water activity of the solution is altered by changing the concentrations of solutes (polyols, sugars and amino acids).

Fish Hbs, that show a wide range of oxygen binding properties and allosteric effects and are characterized extensively structurally and functionally, are excellent candidates for such an analysis. Besides, fish have several iso-Hbs with marked functional differentiation.

The present study analyses the water effect in the cathodic and anodic Hbs from catfish *H. littorale*, which present different oxygen affinities and sensitivities to allosteric effectors (Weber *et al.*, 2000).

Generally, only a molecule of organic phosphate is linked to the desoxi-Hb molecule, although additional binding sites for ATP have been proposed (Amiconi *et al.*, 1985; Weber *et al.*, 2000; Tamburrini *et al.*, 2000; Riccio *et al.*, 2001). For the cathodic Hb^{ct} of the fish *H. littorale*, Weber *et al.* (2000) suggested the possible existence of one additional phosphate binding site.

Materials and Methods

Hemolysate preparation

Blood was collected by caudal vein puncture from adult specimens at the Central Animal Facility of the State University of São Paulo (IBILCE-UNESP) at São José do Rio Preto SP (Brazil). The animals were anaesthetized using benzocaine (1g per 15 L of water). Subsequent purification procedures were carried out at low temperature (around 4°C). Red blood cells were washed by centrifugation four times with saline buffer (50 mM Tris-HCl pH 8.0 containing 0.2% D-glucose, 0.9% NaCl and 1mM EDTA). Hemolysis was accomplished

with buffer (30 mM Tris -HCl pH 9.0), followed by clarification by centrifugation (1000xg for 1 h). Using the previous buffer, purification was performed by gel filtration on Sephacryl S-100 (Sigma) on a 2.6 X 30 cm column equilibrated with the same buffer, and subsequently on Q-Sepharose using a linear saline gradient between 0-100 mM of NaCl. The isolated components were concentrated by centrifugation on Amicon microconcentrators. Analytical isoelectric focusing was performed in agarose gel. The Hb solutions were stored at -80°C in aliquots that were thawed immediately before oxygen binding studies.

Osmotic stress experiments

Water activity was varied by addition of inert solutes (sucrose). In the osmotic stress method changes in the Hb-oxygen affinity are related to changes in water activity that can be converted to changes in protein hydration by use of linkage equations (Colombo et al., 1992; Feuerlein et al., 1996). Oxygen binding experiments were performed with 60 μM (heme) hemoglobin solutions in 30 mM Hepes buffer, pH 7.5 in the presence and absence of ATP and NaCl, as described by Colombo and Bonilla-Rodriguez (1996). All equilibrium measurements were carried out at 20°C by tonometric method (Giardina and Amiconi, 1981). The functional parameters P50 (O₂ partial pressure at half saturation) and cooperativity (n₅₀) were calculated from the Hill plots by linear regression around half saturation. Hemoglobin and methemoglobin concentrations were estimated using the extinction coefficients of Benesch et al., 1965. At the end of the experiments, methemoglobin concentration was below 5%.

The solution osmolalities (Osm) of Hb samples were determined after binding experiments from freezing point depression measurements using a Osmette A model 5002 osmometer (Precision Systems Inc.). The Osm was converted to the natural logarithm of water activity through the following relationship (Colombo and Bonilla-Rodriguez, 1996):

$$\ln a_w = \frac{\Delta}{K_f M_w} = \frac{-Osm}{M_w} \quad (1)$$

where Δ is the freezing point depression, K_f = 1.86 K Kg mol⁻¹ is the cryoscopic constant, M_w is the molarity of pure water (55.56 mol.L⁻¹) and Osm is the solution osmolality.

The effect of water as a single heterotropic ligand on oxygenation is typically analyzed with the linkage equation (Wyman, 1964; Wyman and Gill, 1990),

$$\frac{d \ln(P_{50})}{d \ln(a_w)} = \frac{\Delta n_w}{4} = -(n_w^{oxi} - n_w^{desoxi})$$

(2)

where a_w is the water activity. The slope of the linkage plot $\ln(P_{50})$ vs. $\ln(a_w)$ gives the apparent difference in number of water molecules bound to the oxy and deoxy structures of Hbs, Δn_w .

Calculation of the Association Constants of ATP to the Forms Oxygenated and Deoxygenated of the Hb^{Ct}

The 'x' number of molecules of ATP differentially bound per heme between the deoxy and oxy-Hb was calculated using the linkage equation of Wyman (Amiconi *et al.*, 1985):

$$-x = \Delta \log P_{50} / \Delta \log [\text{ATP}] \quad (3)$$

The association constants with ATP were calculated using a nonlinear regression fitting using the program SigmaPlot (Jandel Scientific, USA), according to the equation below (Szabo and Karplus, 1976):

$$\log(P_{50})_p = \log(P_{50})_a + \frac{1}{4} \log \left(\frac{1 + K_D \cdot X_i}{1 + K_O \cdot X_i} \right) \quad (4)$$

where $\log(P_{50})_p$ is the logarithm of P_{50} measured in the presence of ATP, $\log(P_{50})_a$ is measured in the absence of ATP, K_D and K_O they are the association constants to the deoxygenated and oxygenated forms respectively, and X is the free molar concentration of ATP.

Results

O₂ equilibria of hemoglobin at various osmolalities

Cathodic Hb

We tested the oxygen affinity of the cathodic Hb as a function of water activity, in different experimental conditions: for the stripped Hb (in a ATP/Cl⁻-free buffer solution), in the presence of 0.1 mM and 1 mM of ATP, in a buffer containing 100 mM NaCl, and a last set containing 100 mM NaCl + 1mM ATP. The plots in Figure 1, show that $\ln(P_{50})$

varies linearly with changes in the water activity (a_w), in agreement with Colombo *et al.*, (1992) and Hundahl *et al.*, (2003). According to the Wyman linkage equation (equation 2), the rate of change of $\ln(P_{50})$ on $\ln(a_w)$ gives the apparent number of water molecules (Δn_w) involved with the allosteric transition from the fully deoxygenated to fully oxygenated state of Hb.

The analysis of the data according to the Wyman equation shows that Hb^{ct} in the stripped form, binds 41 ± 9 extra water molecules in the T-R transition. In the presence of 0,1 mM and 1mM of ATP these numbers increase to 73 ± 8 and 65.6 ± 12 , and in the presence of chloride increase to 85 ± 12 water molecules. In the presence of 100 NaCl mM and 1mM of ATP Δn_w corresponds to 4 ± 16 water molecules. Increasing water activity also increases O_2 affinity of the cathodic Hbs, similarly to trout HbI and eel HbC (Hundall *et al.*, 2003).

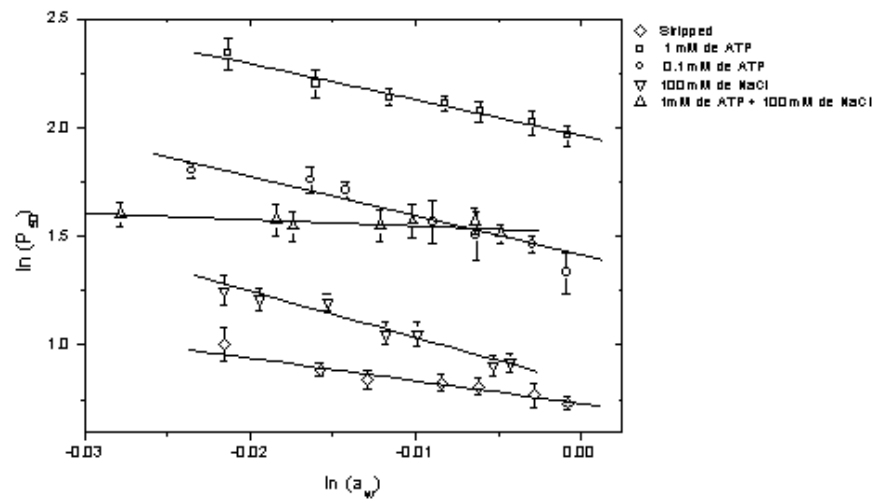


Figure 1. Relative shift in P50 as a dependent on solution water activity at different conditions to Hbct. Experimental conditions: 30 mM Hepes pH 7.5 buffer, room temperature.

Anodic Hb

The other fraction here studied, Hban, has a similar behavior when compared to the Hbct and other fish Hbs (Hundahl et al., 2003), indicating preferential binding of water molecules to the R state. The oxygen-affinity modulators pronouncedly affect water and O₂ binding (figure 2). Curve fitting of the data showed a Δn_w of 58 ± 8 water molecules of the stripped form, increasing to 68 ± 12 , in the presence of 0.1 mM and 1 mM of ATP. In the presence of NaCl, Δn_w raised to 116 ± 16 water molecules, close to that found for the anodic eel Hb in the presence of KCl and GTP (~ 118) (Hundahl et al., 2003). In the presence of 1 mM of ATP and 100 mM of NaCl Δn_w decreased to 28 ± 8 .

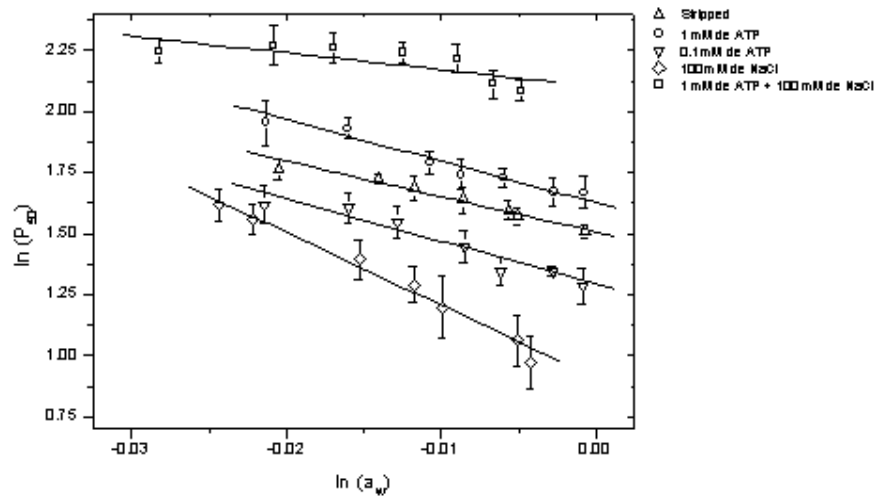


Figure 2. Relative shift in P50 as a dependent on solution water activity at different conditions to Hban. Experimental conditions: 30 mM Hepes pH 7.5 buffer, room temperature.

Calculation of the Association Constants of ATP to the Oxygenated and Deoxygenated forms of the Hbct

Using the equation 3 (figure 3), we calculated the value of Δx as 0.23 ± 8.10^{-5} ATP molecules/heme to Hb^{ct}, confirming the binding of only one molecule of ATP per Hb tetramer.

It was possible to calculate the ATP association constants to the oxygenated and deoxygenated Hb according to the equation 4. The value of the binding constant in the deoxygenated form (K_D) for Hb^{cl} was $2.2 \times 10^5 \pm 1.3 \times 10^4 \text{ M}^{-1}$, and for the oxygenated form (K_O) was $2.6 \times 10^2 \pm 3.3 \times 10^1 \text{ M}^{-1}$.

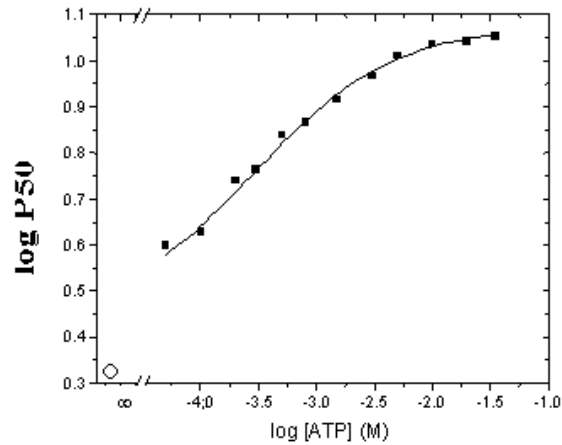


Figure 3. The shift in P_{50} , related to ATP concentration. The symbol \circ show the value of $\log P_{50}$ in absence of ATP.

Discussion

Hemoglobin O_2 equilibria as a function of water activity

The Hbs here studied respond to an increase in water activity with an increase on oxygen affinity, indicating preferential binding of water molecules to the R state, also observed in other vertebrate Hbs (Colombo et al., 1992; Hundahl *et al.*, 2003).

Concerning the values found for Δn_w during oxygenation, for both Hbs, in the stripped condition the value is smaller than in the presence of saturating levels of Cl⁻ or ATP, suggesting that in absence of anions, the Hbs assume a new conformational state, different from the classical T state (T_x), adopting the intermediary state, denominated T_0 , more hydrated than the T_x . This fact is in

agreement with the findings reported by Colombo and Seixas (1999) for human and bovine Hbs.

In the presence of ATP, the variation of the number of water molecules with the oxygenation is smaller (~65.6), suggesting that at high phosphate concentrations, occurs a stabilization of the Hb structure in a conformational state similar to the deoxygenated state.

The fact of the O₂-affinity of Hb^{an} increased in the presence of effectors was first reported by Weber et al. (2000), when in the presence of low concentrations of NaCl and 2,3-BPG at pH 7.5. The unexpected increase in the O₂ affinity could be interpreted as a result of binding to the R state, but could also suggest an excess of negative charges in the Hb central cavity (alpha₁beta₂ interface).

Taken together, human Hb, Hbct and Hban show similar overall sensitivities to changes in water activity, despite the diversity in their allosteric responses, and seem to exist some basic structural differences in anion-induced conformational changes.

In conclusion, we showed that both Hbs here investigated respond to an increase in water activity by stabilizing the R state conformation, and that the presence of an intermediate conformational state in the oxygenation process is similar amongst Hbs (Hundall *et al.*, 2003).

Calculation of the ATP Association Constants to the Oxygenated and Deoxygenated Forms of Hb^{ct} and Hb^{an}

The value obtained for the association constant of ATP to the deoxygenated form (K_D) of Hb^{ct} is about ten larger times regarding 2,3-BPG binding to human hemoglobin (K_D = 3,6 x 10⁴ M⁻¹) (De Rosa *et al.*, 1998) showing that ATP binds to Hb^{ct} more strongly than 2,3-BPG to human hemoglobin. The value for Hb^{ct} is similar to found for Hb-II of the fish *Piaractus mesopotamicus* (3,05 x 10⁵ M⁻¹) (Poy, 2001).

The value obtained for the ATP association constant to the oxygenated form (K_O) is according to the related for the of oxygenated Hb human (K_O = 3.5 x 10² M⁻¹), and greater than for *P. mesopotamicus* Hb (2.7 x 10¹ M⁻¹).

We did not observe the presence of additional phosphate binding sites to Hb^{ct}, as suggested by Weber et al. (2000).

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COMPARATIVE ANALYSIS OF THE HEMOGLOBINS
FROM TAMBACU, TAMBAQUI (*C. macropomum*)
AND PACU (*P. mesopotamicus*).

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Abstract

The present work performed a comparative analysis of the hemoglobin pattern by the analysis of the distribution pattern of iso-hemoglobins of the parental species Tambaqui and Pacu in the hybrid Tambacu, as well as a functional characterization. The hemoglobin pattern and oxygen binding properties from the hybrid Tambacu are analogous to those from Tambaqui, showing five bands, against three fractions for the hemolysate from Pacu. For Pacu we were able to purify the main hemoglobins (Hb-I and Hb-II), which show different functional properties.

Introduction

Vertebrate blood has been constantly studied with several purposes, such as basic knowledge of its composition, and mainly for auxiliary parameters of biochemical, hematological and systematic determinations. Molecular evaluation of fish hemoglobins, analysis of leucocytes, oxygen carrying properties, heterogeneity, comparative studies, etc., are some of the many aspects analyzed, frequently trying to establish correlations with different

species, environment, developmental stage, sex, temperature, season, or many other variables (Verde and di Prisco, 2004; Hundahl et al., 2003; Affonso et al., 2002; Panepucci et al., 2001; Val, 1995; Powers, 1980; Ellis, 1976).

Hemoglobin (Hb) is a globular oligomeric protein encapsulated in the red blood cells (RBC). It is composed by four polypeptidic chains named globin (a tetramer), each one carrying a prosthetic group Heme. The globin chains have been classified according to their genetic similarities to human globins as alpha or beta. The main function of vertebrate Hb is oxygen transport from the gills, lungs or other modified gas-exchange tissues, and at least two functional properties: O₂-affinity and cooperativity are effected by binding of a variety of chemical substances, non-heme ligands (allosteric effectors) such as protons, chloride, phosphates (Riggs, 1972), which exert a tuning of O₂ delivery. For example, changes of proton concentration or pCO₂ can decrease Hb O₂-affinity, a process called Bohr effect. In fact, two kinds of this effect can be considered: the alkaline or normal (when proton binding decreases oxygen affinity) and the acid or reverse Bohr effects (when the opposite occurs: H⁺ binding increases affinity) (Perutz et al., 1980).

The existence of Hb heterogeneity is a common characteristic among fishes (Pérez et al., 1995; DeYoung, 1994; Fyhn et al., 1979), which frequently includes functional differences as well (Powers et al., 1978), characteristics that have been discussed from different points of view (Fyhn et al., 1979; Bossa et al., 1982; Petersen et al., 1989; Val et al., 1992; DeYoung et al., 1994). A frequent opinion, supported by the literature, is that heterogeneity can provide an adaptive advantage to the specimen, giving different physiological responses than if there were a single Hb (Brunori, 1975), whereas could also be evolutionary remnants (Weber et al., 1976). Accordingly, heterogeneity or polymorphism would have a selective value in unstable environments, and seems to have a significant plasticity, as demonstrated by Houston and Cyr (1974), working with *Carassius auratus*, showing that the Hb pattern can change in response to acclimation to varying temperatures.

Evolution of different hemoglobins from a common protein ancestor (a primitive Hb) must have been guided by the necessity of finding a mechanism able to meet the metabolic needs (Brunori, 1975). Generally, the number of components found not only in fishes but also among reptiles and amphibians is higher than that found for mammals or birds. This multiplicity of components rises doubts concerning their origin and possible functional purposes.

The Amazon basin presents a variety of aquatic environments isolated by geographical barriers that limit the genic flux among individuals, but produces a high intraspecific heterogeneity within fish populations. The ability of an organism to adapt to an unstable environment can be related to its biological and genetic variability (Ramirez-Gil et al., 1998).

Frequently the iso-hemoglobins found in fish blood have intrinsic properties that differ from each other concerning the response to allosteric effectors. For example, the isoforms can present a different behavior when proton or phosphate concentration changes, and blood O₂-transport reflects both the relative concentration of each component and its functional properties, what could, at least theoretically, help the animal to cope fluctuations of O₂ availability.

The present work intended to analyze comparatively the iso-hemoglobins present in the hybrid fish known as “tambacu” with those present in the parental species, female “Tambaqui” (*C. macropomum*) and male “Pacu” (*P. mesopotamicus*), analyzing the number and pattern of iso-Hbs, and their functional properties. The hybrid and the other two species are largely cultivated in Brazil.

Materials and Methods

Adult specimens of Tambaqui (*Colossoma macropomum*), Pacu (*Piaractus mesopotamicus*) and Tambacu were obtained from a local “fish-and-pay” and identified by Dr. Francisco Langeani Neto, from the Dept. of Zoology and Botany. Fishes were anesthetized by immersion in a benzocain solution (1 gr. for 15L of water). Blood was obtained from the caudal vein using a disposable syringe containing 0.2mL of buffered saline (0.9% NaCl in 50mM Tris-HCl pH 8.0, containing 0.2% D-glucose and 1mM EDTA, and all subsequent purification procedures were performed at 4°C. Erythrocytes were washed three times by centrifugation in a similar buffered saline without glucose, and hemolysis was achieved by the addition of 50mM Tris-HCl pH 9.0 buffer to the pellet. Hemoglobin purification was performed using Sephacryl S-100 HR and anion-exchange chromatography in DEAE-Sepharose, using a salt gradient. The pure Hb was concentrated using Amicon Centriprep-50 and kept in liquid nitrogen until use.

Purity of the isolated hemoglobins was checked by isoelectric focusing in agarose (Naoum, 1997) using samples stabilized by carbon monoxide. The

present work was performed using the partially purified hemolysates from Tambacu and Tambaqui, and the purified hemoglobins I and II from Pacu.

Functional studies of 60mM (heme) Hb-II were performed at 20°C using tonometers as described (Colombo and Bonilla-Rodriguez, 1996, Bonilla et al., 1994), using ultra-pure water (ELGA Sci.) and suitable buffers (TAPS, HEPES and ADA purchased from Sigma) to cover the tested pH range. The parameters P_{50} and n_{50} were obtained from Hill Plots from samples containing up to 5% methemoglobin at the end of the experiments.

Results and Discussion

Hemoglobin pattern

The analysis by isoelectric focusing was done using CO stabilized samples, except for the hemolysate from Pacu (Fig. 1). We added a sample of human blood containing Hbs A and S. For the CO-bound samples we can observe a similar pattern between Tambacu and Tambaqui, which present five distinct components, whereas Pacu showed only three (Fig. 2, first well).

The electrophoretic pattern suggests that the hybrid fish inherits its Hb pattern only from *C. macropomum* (Tambaqui).

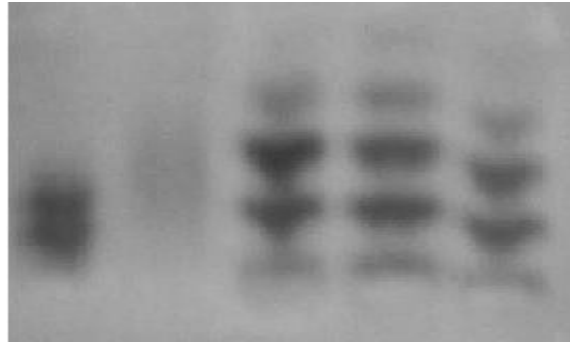


Figure 1. Electrophoretic pattern of hemoglobins. From the left to the right: human HbAS, Pacu (oxidized sample), Tambacu, and two samples of Tambaqui

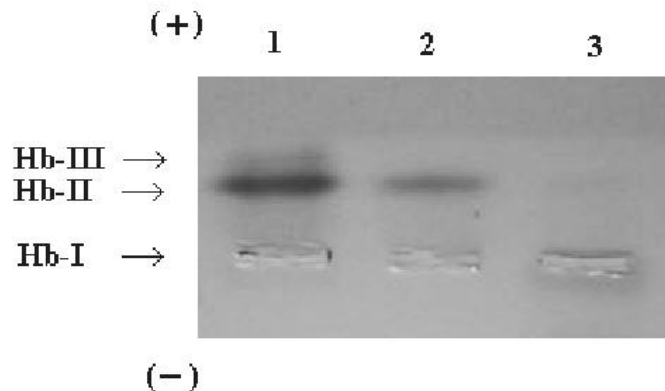
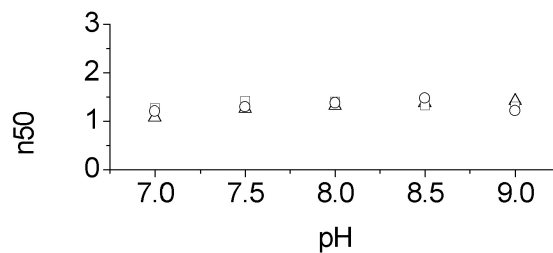


Figure 2. Isoelectric focusing: From the left to the right: Pacu (hemolysate), and purified Hb-II and Hb-I.

Functional studies

Hemoglobin purification was accomplished for Pacu, allowing to obtain two fractions, named Hb-I and II. For Tambaqui and Tambacu we were not able to perform a suitable purification, and accordingly worked with the stripped hemolysate (without phosphates or other low molecular mass components), partially purified by passage by Sephacryl S-100 HR. Functional properties were determined for Hbs in the stripped form and in the presence of 1 mM ATP or 100mM Chloride at 20°C.

Figure 3. Pacu Hb-I oxygen affinity (expressed as P_{50}) and cooperativity as a function of pH.



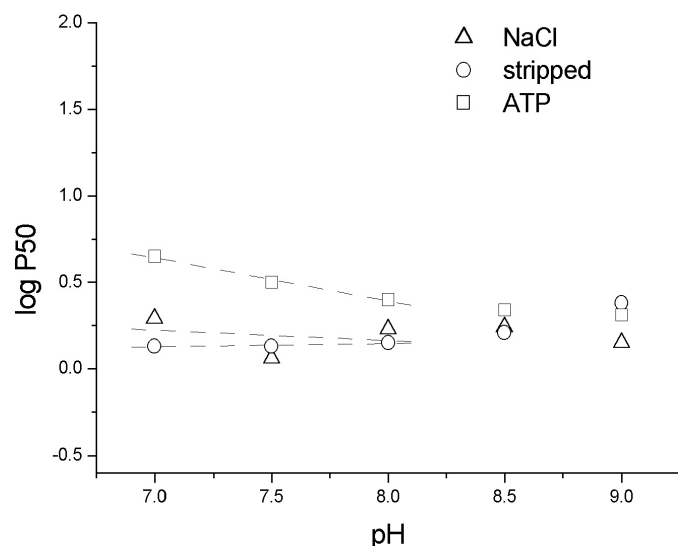


Figure 3 shows the Bohr effect ($\Delta \log P_{50} / \Delta \text{pH}$) for the minor fraction (Hb-I) from Pacu. The slope calculated by linear regression corresponds to the number of protons (H^+) per heme that are released during the oxygenation process. In the pH range from 7.0 to 8.0 there is no proton binding for the stripped Hb ($\Delta \text{H}^+/\text{heme} = 0.02$) nor in the presence of Chloride ($\Delta \text{H}^+/\text{heme} = -0.09$), and accordingly, there is no variation of O_2 -affinity with the pH or, in other words, there is no Bohr effect. The addition of 1 mM ATP raises a normal Bohr effect ($\Delta \text{H}^+/\text{heme} = -0.25$). We found a similar behavior in Hb-I from matrinxã (*Brycon cephalus*) (Honda, 2000), and the absence of Bohr effect has been reported also for other hemoglobins.

The most investigated, among those proteins, is trout Hb-I (Brunori, 1975). In human hemoglobin the main residues which are responsible for the alkaline Bohr effect are the Histidines beta 146, the C-terminal amino-acids. Some fish cathodic hemoglobins found in trout, eel, matrinxã and *Hoplosternum littorale* have a substitution for that residue, and a Nterminus of the alpha chains blocked. Those hemoglobins do not have Bohr effect or a reverse one (Weber et al., 2000).

Oxygen binding for Pacu Hb-I was always cooperative ($n_{50} > 1.0$), varying between 1.2 and 1.4, a behavior that sometimes is described as “low cooperativity”. For comparison, human Hb has an n_{50} around 2.8.

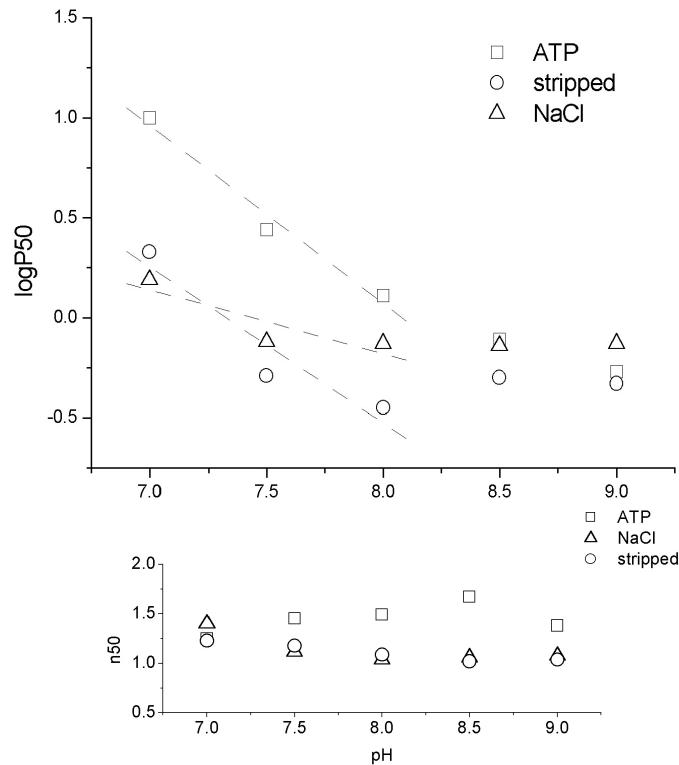


Figure 4. Pacu Hb-II oxygen affinity (expressed as P_{50}) and cooperativity as a function of pH.

Figure 4 shows the Bohr effect and cooperativity for the major component of Pacu's hemolysate, Hb-II, that in the stripped form has a normal Bohr effect ($\Delta H^+ = -0.78$) in the pH range from 7.0 to 8.0. In the presence of 1mM ATP displays an alkaline Bohr effect, with $\log P_{50}$ close to 1.0 at pH 7.0, decreasing to -0.27 at pH 9.0 ($\Delta H^+ = -0.89$). Chloride seems to affect proton binding, since the Bohr effect decreases to -0.32 H^+ /heme. Oxygenation is cooperative in the

presence of ATP, being lower for the other experimental conditions, reaching almost non-cooperative values.

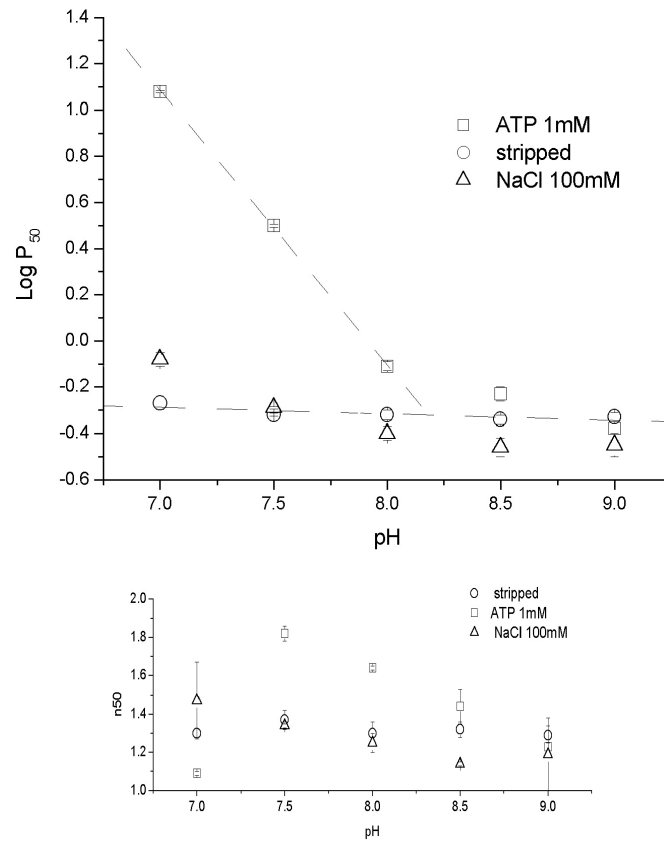
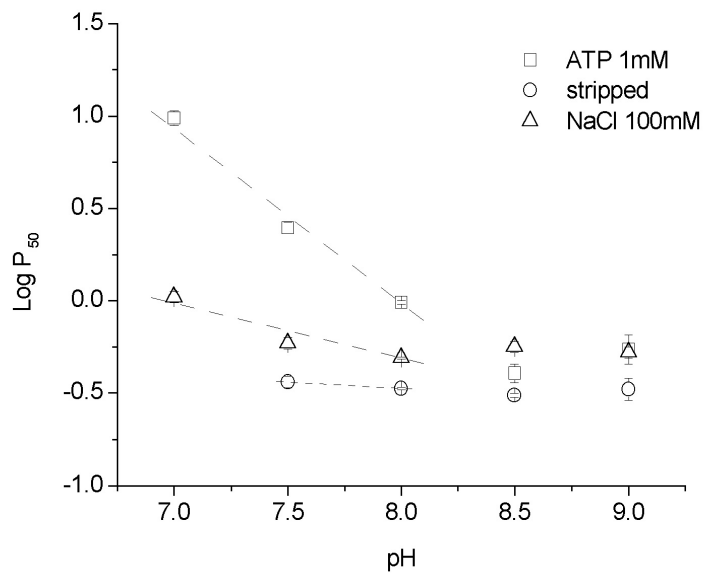


Figure 5. Oxygen affinity and cooperativity of the hemoglobins from Tambacu at 20°C.

Figure 5 show the Bohr effect and cooperativity of the hemolysate from Tambacu. In the presence of ATP there is a strong alkaline Bohr effect ($\Delta H^+/\text{heme} = -1.17$) in the pH range from 7.0 to 8.0. $\text{Log}P_{50}$ decreases abruptly from 1.1 at pH 7.0 to -0.1 at pH 8.0. For the stripped form there is no proton binding ($\Delta H^+/\text{heme} = -0.03$), but in the presence of chloride there is also a normal Bohr effect ($\Delta H^+/\text{heme} = -0.32$). Oxygenation is always cooperative in



the presence of ATP, although n_{50} reaches higher values in this condition.

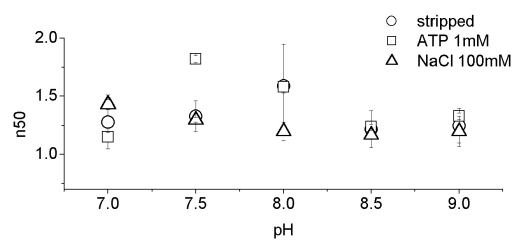


Figure 6. Oxygen affinity and cooperativity for the hemolysate from Tambaqui at 20°C.

Figure 6 shows the functional parameters calculated for the hemolysate (partially purified) from Tambaqui. In the presence of saturating ATP there is a strong Bohr effect ($\Delta H^+/\text{heme} = -1.00$) in the pH range from 7.0 to 8.0, with P_{50} decreasing 10 times, from 10 to 1 mmHg. In the absence of anions there is no proton binding ($\Delta H^+/\text{heme} = -0.03$), but the presence of chloride raises a normal Bohr effect ($\Delta H^+/\text{heme} = -0.30$). Cooperativity is higher in the presence of ATP, reaching 1.8.

Taking into account the experimental error, there is no significant difference of the functional properties between Tambacu and Tambaqui on the basis of the data gathered so far. This confirms the similarity found concerning the electrophoretic pattern, discussed above.

The present work will continue including molecular studies of the globins, globin chain analysis, temperature effect and osmotic stress determinations, trying to depict a comprehensive framework of the hemoglobin systems of the hybrid and the parental species.

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**THE COMBINED EFFECT OF PHOSPHATE BINDING TO TWO SITES
AND PROTONS CAN LOCK THE MAJOR HEMOGLOBIN FROM
BRYCON CEPHALUS (MATRINXÃ) IN A T-STATE**

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Abstract

The major hemoglobin from *B. cephalus* presents a fast partial deoxygenation even in the presence of oxygen, that is induced by phosphate binding at pH below 7.0. Accordingly, saturation decreases around 50% at pH 6.5, having an estimated P_{50} around 200mmHg. The variation of $\log P_{50}$ with increasing ATP concentration displayed a bell shaped curve, reaching a maximum at a phosphate concentration around 5mM. The T-R transition was evaluated at the UV range using IHP, and phosphate binding to the oxygenated form was also demonstrated by gel filtration experiments.

Introduction

Vertebrate hemoglobins (Hb) evolved optimizing its main function: oxygen transport, thanks to their ability to respond to environmental changes by a chemical fine tuning, mainly performed by binding of chloride, organic phosphates and protons (Riggs, 1972), although also water molecules should be considered (Colombo et al., 1992). Following the canonical MWC model (Monod et al., 1965), preferential binding to the low-affinity T or the high affinity R states displace oxygen affinity according to the stabilized conformation. For fish hemoglobins, external and internal oxygen availability is

a constant challenge (Val, 1995), and frequently display an heterogeneous hemoglobin system, a supposed advantage if the several isoforms have different functional properties (Fonseca et al., 2003).

Proton binding can produce a substantial decrease of the O₂-affinity, a phenomenon known as the Bohr effect, but the largest decrease is produced by a mechanism that occurs at low pH values, named the Root effect, and decreases blood capacity to transport oxygen. The first mechanism has a presumable purpose of ensuring oxygen unloading for hypoxic tissues, whereas the second would allow O₂ pumping to the choroid rete mirabile of the eyes, and probably also to the fish swimbladder (Pelster and Decker, 2004; Val and Almeida-Val, 1995). Concerning the Root effect, a mechanism proposed by Mylvaganam et al. (1996) has been criticized by Mazzarella et al. (1999), suggesting that several combinations of structural characteristics could lead to explain the drastic decrease of O₂-affinity (Pelster and Decker, 2004).

The fish known as matrinxã, a teleost from the Amazon basin, presents aquatic surface respiration, where lips' swelling helps to trim the upper layers, that have more dissolved O₂ (Almeida-Val and Farias, 1996). For the specimens, identified as *Brycon cephalus*, we found two hemoglobins: a minor cathodic component (Hb-I) without proton binding and weak phosphate effect (Honda, 2001, Honda et al., 2000), and a major fraction (Hb-II) that will be functionally described below.

Materials and Methods

Adult specimens of *Brycon cephalus* (Matrinxã) were obtained from a 'fish-and-pay' at São José do Rio Preto, SP, Brazil, and identified by Dr. Francisco Langeani Neto, from the Dept. of Zoology and Botany. Fishes were anesthetized by immersion in a benzocain solution (1 gr. for 15L of water). Blood was obtained from the caudal vein using a disposable syringe containing 0.2mL of buffered saline (0.9% NaCl in 50mM Tris-HCl pH 8.0, containing 0.2% D glucose and 1mM EDTA, and all subsequent purification procedures were performed at 4°C. Erythrocytes were washed three times by centrifugation in a similar buffered saline without glucose, and hemolysis was achieved by the addition of 50mM Tris-HCl pH 9.0 buffer to the pellet. Hemoglobin purification was performed using Sephacryl S-100 HR and anion-exchange chromatography in DEAE-Sephacryl, using a salt gradient. The pure Hb was concentrated using Amicon Centriprep-50 and kept in liquid nitrogen until use.

Purity of the isolated hemoglobins was checked by isoelectric focusing in agarose (Naoum, 1997) using samples stabilized by carbon monoxide. The present work was performed using the major hemoglobin component, named Hb-II.

Functional studies of 60mM (heme) Hb-II were performed using tonometers as described (Colombo and Bonilla-Rodriguez, 1996, Bonilla et al., 1994a), using ultra-pure water (ELGA Sci.) and suitable buffers (TAPS, HEPES and ADA purchased from Sigma) to cover the tested pH range. The parameters P_{50} and n_{50} were obtained from Hill Plots from samples containing up to 5% methemoglobin at the end of the experiments.

ATP binding constants were determined using a nonlinear regression program using the model proposed by Szabo and Karplus (1976) for two binding sites, with more phosphate molecules binding to the liganded form:

$$\log P_{50} = \log P_{50}^0 + \frac{1}{N} \log \frac{(1 + x K_d^1 + x^r K_d^2)}{(1 + x K_o^1 + x^s K_o^2)} \quad (1)$$

where $\log P_{50}$ and $\log P_{50}^0$ are the values in the presence and the absence of phosphate, N is the number of binding sites, K_d superscripts 1 and 2 are the binding constants for both sites in the deoxygenated form, K_o superscripts 1 and 2 refer to the binding constants to the oxygenated form, r and s are the number of phosphates bound. It is worthy to note that Szabo and Karplus suppose that $s > r$. Since at this time we don't know the value for s , we used 2 for both of them.

We evaluated the effect of IHP on the T to R transition using 60mM heme at pH 6.5, 7.0 and 8.5, performing spectral scanning in the UV range (Enoki and Tyuma, 1964) using a Varian Spectrophotometer Scan 100. We carried out three spectral readings: a first one for the oxygenated form without any phosphate, a second with IHP added to the oxygenated Hb, and a third for the deoxygenated Hb with IHP. The first spectrum was subtracted from the later two.

ATP binding to the R state was also analyzed by gel filtration in Sephadex G-50 pH 6.5 and 8.0 as proposed by Giardina and Amiconi (1981) in a Varian Scan 100 Spectrophotometer, recording absorbance readings at 280nm. To determine ATP concentration we used an extinction coefficient of 2580 M^{-1} .

Results and Discussion

Isoelectric focusing of CO bound Hb-II showed a single band (not shown) as previously described by Honda (2001). Figure 1 shows that in the presence of 1mM ATP, Hb-II has a very high alkaline Bohr effect in the pH range between 7.0 and 8.0 ($\text{DH}^+/\text{heme} = -1,19 \pm 0,07$), decreasing in the the stripped form or in the presence of 0.1M chloride ($-0.62 \pm 0,21$). A sharp change occurs above pH 7.5, showing no proton binding ($\text{DH}^+/\text{heme} = -0,18 \pm 0,11$). At pH 6.5 O_2 -affinity is low, with a P_{50} around 14mmHg, but at pH 7.5 affinity increases 10 fold, as P_{50} falls to 1.4mmHg.

Below pH 7.0 the ATP effect is remarkable, decreasing oxygen affinity (P_{50}) to around 200mmHg at pH 6.5, a pressure higher than partial O_2 pressure at 1 atm. Increasing pH to 7.0 decreases P_{50} to around 15mmHg, a drastic proton driven change.

Oxygen binding is cooperative ($n_{50} > 1$), except for the data gathered in the presence of ATP below pH 7.0, where cooperativity (n_{50}) falls below 1.0.

These results for Hb-II differ from the characterization of the cathodic Hb performed by Honda (2001), that showed a virtual absence of proton effect and a small change of O_2 -affinity induced by phosphates.

A previous work performed presumably with this species (Val and Almeida-Val, 1988) pointed out similar results for *Brycon* cf. *cephalus* in the range above pH 7.0, but below that pH, we found higher P_{50} values due to the induced Root effect.

At pH below 7.0 Hb-II undergoes a fast deoxygenation in the presence of ATP, decreasing its saturation to about 45% (Fig. 2). That effect operates up to pH 7.2, and apparently reaches a limit of deoxygenation around pH 6.5. Such an effect would represent a variation of the well described alkaline Bohr and Root effects, being regulated by RBC organic phosphates (NTP).

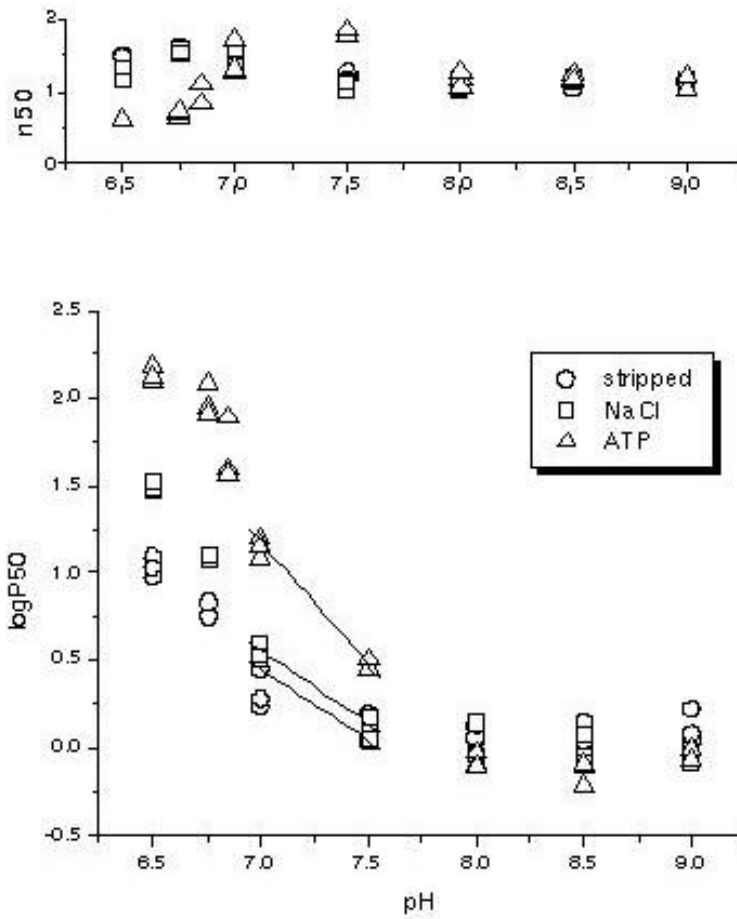


Figure 1. Oxygen affinity (expressed as P50) and cooperativity (n50) of Hb-II at 20°C.

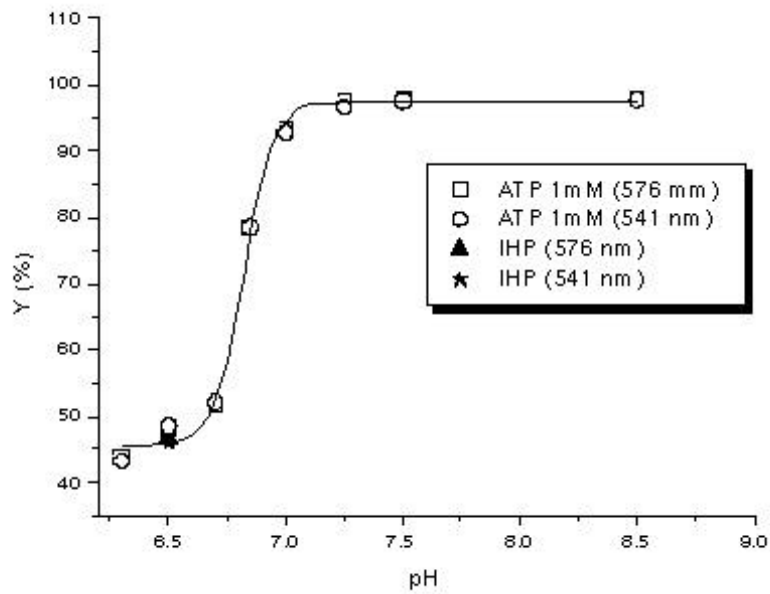


Figure 2. Hb-II saturation (Y%) as a function of pH of Hb-II in the presence of 1mM ATP or IHP at 20°C.

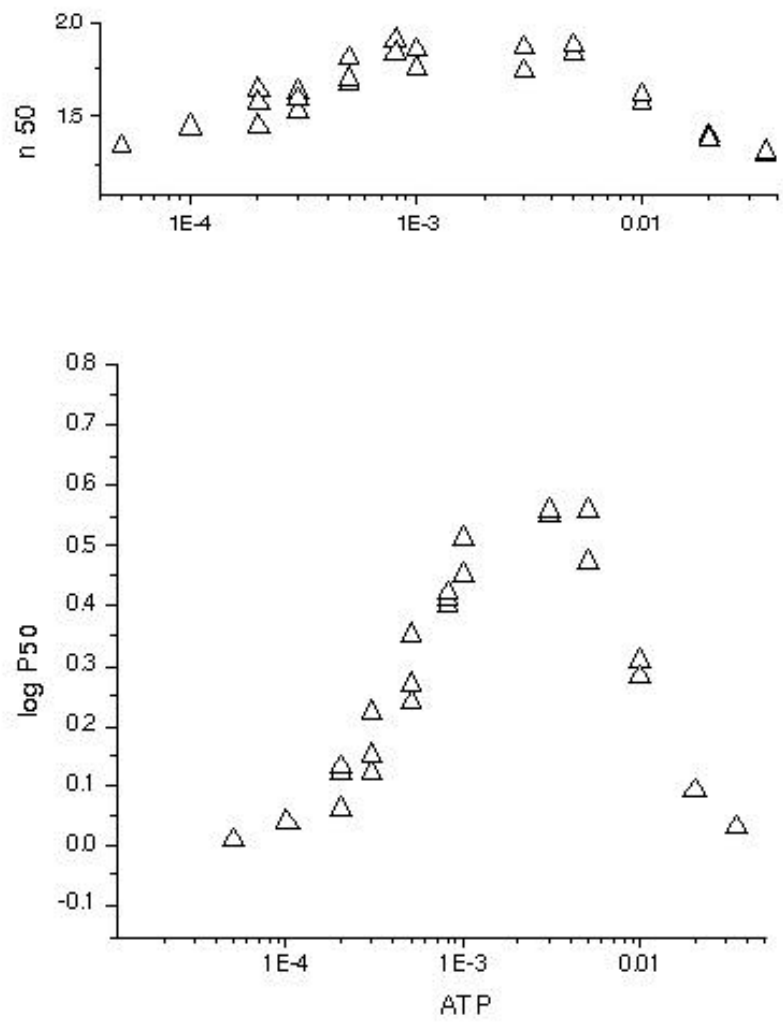


Figure 3. Oxygen affinity and cooperativity of Hb-II as a function of ATP concentration, at pH 7.0 and 20°C.

A similar effect induced by phosphates was described for the anodic hemoglobin of the brown moray (*G. unicolor*) by Tamburrini et al. (2001), and other hemoglobins (Val and Almeida-Val, 1995; Pelster and Weber, 1990).

The O₂-affinity characteristics would allow to classify it as an NTP-induced Root effect, being completely reverted by an increase of the pH (data not shown).

NTP binding to the oxygenated form, that occurs significantly below pH 7.0, would take Hb to a T sub-state, locking the protein into this conformation, following the proposal made by Colombo and Seixas (1999).

For further evidences concerning phosphate binding, we carried out an analysis of increasing ATP concentrations on oxygen affinity (Fig. 3) at pH 7.0.

The slope $D\log P_{50}/D\log[ATP]$ in the raising portion of the curve, that indicates the number of ATP molecules differentially bound between the deoxy and oxy conformations (Wyman, 1964) per heme is 0.5, twice the expected number for a 1:1 relationship, since indicates 2 ATP molecules bound per tetramer. The bell shaped curve shows that 5mM ATP produces the largest decrease of O₂-affinity ($P_{50} \sim 4\text{mmHg}$), but for higher concentrations there is a clear decrease of P_{50} reaching 1mmHg, as expected for strong phosphate binding to two sites (Amiconi et al., 1985) with a higher affinity for the oxygenated form (Szabo and Karplus, 1976). Cooperativity follows a similar behavior, reaching a maximum value of about 2, decreasing progressively to 1.3 when ATP increases.

A preliminary estimation of the binding constants as described, showed that the best fitting corresponds to stronger binding to the oxygenated protein, although the data were obtained at pH 7.0, where the effect of phosphate is relatively small. This estimation results are very different from those obtained for dromedary Hb by Amiconi et al. (1985), since they found higher values for K_d^1 than for K_d^2 . The binding constant to the first site in the deoxygenated form (K_d^1) has been estimated (Rsqr=0,885) as $8.1 \cdot 10^3 \pm 1.9 \cdot 10^3 \text{ M}^{-1}$, whereas the corresponding constant for the 2nd site in the oxygenated form (K_o^2) was $1.6 \cdot 10^5 + 5.1 \cdot 10^4 \text{ M}^{-1}$. The other two constants, K_o^1 and K_d^2 tend to zero.

Other evidences of phosphate binding to the R state included analysis by gel filtration, performed using oxygenated and CO-bound samples (Figure 4). The valley observed after Hb elution corresponds to the bound ATP. Clearly ATP binds to the R state, both to the oxygenated and CO-bound Hbs, although

binding is higher for the first condition, a relationship around 3:1. More experiments will be performed to quantify properly this binding and its dependence on pH and temperature.

Since phosphate binding would take the structure to a locked T-like state, we searched for evidences in the UV range of an R to T transition induced by ATP binding to oxygenated Hb.

We had to use 1mM IHP to avoid interference with absorbance readings, and the profile obtained at pH 6.5 (Figure 5) agrees with such a phenomenon, showing an absorbance increase around 293 nm that is larger and sharper than the spectral change obtained at pH 8.0.

This method is based on the absorbance change of the aromatic residues from the $\alpha_1\beta_2$ interface (Enoki and Tyuma, 1964) during the transition T->R, and the increased absorbance at 290nm reflects a displacement towards the T state. Seixas (2002) found similar evidences of locking of the oxy-Hb in a T-like state induced by phosphate binding to human Hb. The author applied osmotic stress determinations and found that after reaching a maximum value of the differential number of water molecules bound to the oxy state, higher phosphate concentrations decreased that number. The explanation found was a significant binding to the oxy Hb.

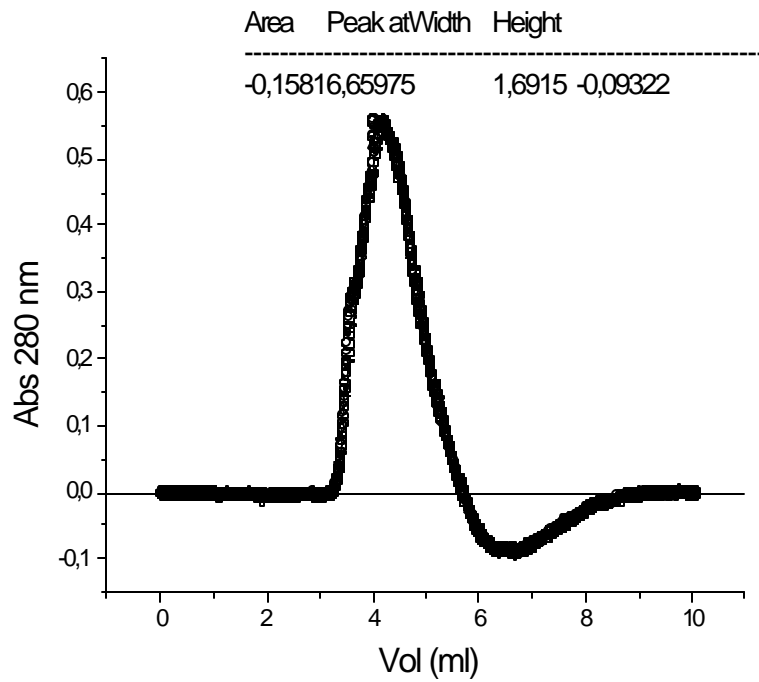


Figure 4. Example of a profile obtained for ATP binding analysis by gel filtration in Sephadex G-50 at 25°C, pH 6.5.

An analysis including the induced deoxygenation, the high estimated binding of phosphates to the oxygenated Hb-II at pH below 7, the spectral data and the effect of ATP both on the O₂-affinity and cooperativity show that there is a locking mechanism that takes the protein to a T state and prevents Hb to reach the R state.

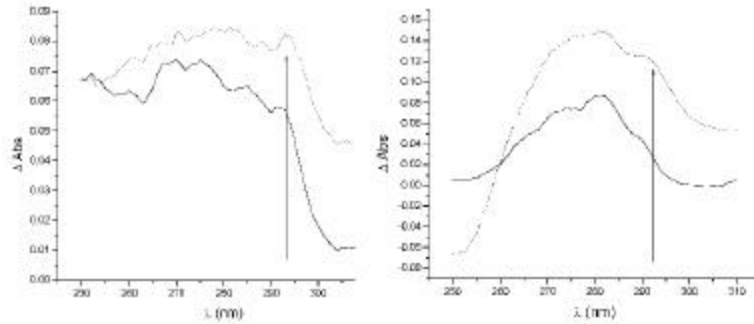


Figure 5. R to T transition of Hb-II analyzed in the UV range, induced by IHP binding to the oxy form (continuous line) and deoxy form (dashed line) at pH 6.5 (left) and 8.0 (right) at 20°C. The vertical line shows the region that reflects the conformational change.

This hemoglobin has been crystallized (Fonseca et al., 2003), and the next step will include obtaining its primary sequence and perform osmotic stress analysis, for a full analysis of the structural-functional relationships, since the Root effect deserves further research, as proposed by Mazzarella et al. (1999). This induced Root effect, under control exerted by protons and NTP would not help oxygenation under hypoxia. According to Pelster and Decker (2004), under stress conditions, such as hypoxia, RBC pH increases due to b-adrenergic stimulation of the Na^+/H^+ exchanger. For *B. cephalus*, ATP was identified as the organic phosphate present in the RBC, being its concentration related to oxygen availability (Val and Almeida-Val, 1995, 1988), and the interplay of proton and ATP binding could ensure oxygenation for the retina and swimbladder.

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**SEQUENCING cDNA OF ALPHA-GLUTATHIONE S-TRANSFERASE
FROM LIVER OF *PIARACTUS MESOPOTAMICUS***

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EXTENDED ABSTRACT ONLY – DO NOT CITE

Abstract

During characterization of glutathione S-transferases (GST) from pacu (*Piaractus mesopotamicus*) we found an unexpected high activity of a hepatic cytosolic alpha-GST. We thought that alpha-GST from pacu could have structural domains different from other alpha-GST because it did not bind to ligands of affinity columns. Then, we turned to get the cDNA sequence for pacu alpha-GST, with the aim of inferring its amino acid sequence. The amino acid sequence inferred from a nucleotide sequence obtained from an amplified cDNA fragment was found to be 70% similar to the homologous sequence of *Danio rerio*.

Introduction

Glutathione S-transferases isoenzymes of the alpha class (alpha-GST) are important cytosolic isoenzymes that catalyze one of the redox reactions in which electrons of two molecules of reduced glutathione are transferred to peroxides, yielding glutathione disulphide and reduced innocuous products. These reactions play an important role in detoxification because they are an alternative pathway for reduction of peroxides, which could otherwise produce oxygen reactive species capable of spontaneous harmful oxidative modification of proteins, lipids, and nucleic acids in the cell.

Considering the relevance that oxygen has to a variety of physiological processes in fish (Nikinmaa, 2002) we have been carrying out experiments to characterize alpha-GST isoenzyme activities from species of Neotropical fish adapted to changing oxygen tension in water. We have measured a high activity level of hepatic cytosolic alpha-GST in pacu (*Piaractus mesopotamicus* Holmberg, 1887), a characid found originally in rivers of the Paraguay-Paraná basin that has acquired economic importance to Brazilian fish culture. This is in contrast to what has been reported for teleostean liver, in which the pi class of GST was indicated as the major form of this isoenzyme (Dominey et al., 1991; Pérez-López et al, 1998; Pérez-López *et al.*, 2002). In order to detail the kinetic properties of this alpha glutathione S-transferase from liver of pacu we tried to purify this enzyme using preparative biochemical methods. Pacu's hepatic alpha-GST however exhibited no affinity with ligands coupled to the chromatographic resins commonly used to purify alpha-GST from other vertebrates. This suggested that pacu's hepatic alpha-GST has structural domains missing in alpha-GST isoenzymes already purified. Then, in order to infer the amino acid sequence of pacu's hepatic alpha GST isoenzyme we turned to molecular biology techniques to acquire its cDNA sequence.

Methods

We selected conserved amino acids by ClustalX alignment of several vertebrate alpha-GST sequences to produce a pair of oligonucleotides that allowed the amplification of a pacu homologue of this enzyme. Total RNA was extracted from pacu liver by mixing liver homogenates with a Trizol commercial reagent, followed by centrifugation. To selectively precipitate the RNA, removing glycogen contamination, the supernatant suspensions obtained were treated with 4 M lithium chloride and the mixture kept for 1h at 4°C. The suspension was then put at -80 °C overnight. After centrifugation, the deposit was washed with 3 M sodium acetate and 100% ethanol. After centrifuging the resulting supernatant, the pellet was washed with double-distilled water and centrifuged to produce a RNA suspension free of glycogen. Poly-A+ RNA was also prepared by oligo-dT column chromatography. Total and poly-A+ RNA were used in a reverse transcription (RT) reaction using random primers. The cDNA produced was used for PCR amplification; the products were separated by agarose gel electrophoresis and visualized by ethidium bromide staining. To be expressed, a bp-PCR product was gel purified and incorporated into a plasmid vector that was inserted into bacteria. The cDNA fragment cloned was sequenced in a MegaBACE apparatus. We tested the cDNA fragment homology to other GST molecules by a BLAST search.

Results and Discussion

Figure 1 shows the amino acid sequences of alpha-GST isoenzymes inferred from cDNA fragments obtained from liver of chicken (*Gallus domesticus*), zebrafish (*Danio rerio*) and pacu (*Piaractus mesopotamicus*). Sequences from chicken and zebrafish were obtained from a BLAST search in the internet <http://www.ncbi.nlm.nih.gov/BLAST/>.

We were able to clone a fragment of hepatic alpha-GST from pacu and found that it was about 70% similar to alpha-GST sequences from both zebrafish and chicken.

Figure 1. Primary amino acid sequence of alpha-GST segments.

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Chick  RGKMESIRWLLAAAGVEFEEVFLTREQYKLLQSGILMFQQVPMVEIDGM
Danio  RGKMESIRWLLAVAGVQFEEVFLTEKEQFDKLLSDGALTFQQVPLVEIDGM
Pacu   RGIMERIRWLLAVAGVEFEEVYLTREEYKVMVNDGALLFHQLPMVEIDGM
      ** ** ***** .***:****:*  :*::*:... * * *:.*:*****
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“*” Means that amino acids in that column are identical in the aligned sequences.

“:” Means that conserved substitutions have been observed.

“.” Means amino acid positions with semi-conserved substitution.

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