EPA United States Environmental Protection Agency

Fish Physiology, Toxicology, and Water Quality

Proceedings of the Eighth International Symposium Chongqing, China October 12-14, 2004

RESEARCH AND DEVELOPMENT

EPA/600/R-06/062
June 2006

FISH PHYSIOLOGY, TOXICOLOGY, AND WATER QUALITY

Proceedings of the Eighth International Symposium, Chongqing, China October 12-14, 2004

Edited By

David Randall and Dung Yuk Man Mandy
City University of Hong Kong

Published by

Ecosystems Research Division
Athens, Georgia 30605

U.S. Environmental Protection Agency
Office of Research and Development
Washington, DC 20460

NOTICE

The views expressed in these Proceedings are those of the individual authors and do not necessarily reflect the views and policies of the U.S. Environmental Protection Agency (EPA). Scientists in EPA's Office of Research and Development have authored or coauthored papers presented herein; these papers have been reviewed in accordance with EPA's peer and administrative review policies and approved for presentation and publication. Mention of trade names or commercial products does not constitute endorsement or recommendation for use by EPA.

ABSTRACT

Twenty-one participants from Europe, North America and China convened in Chongqing, China, October 12-14, 2005, for the Eighth International Symposium in Fish Physiology, Toxicology and Water Quality. The subject of the meeting was "Hypoxia in vertebrates: Comparisons of terrestrial and aquatic vertebrates". These Proceedings include 13 papers presented over the three day period and discuss the responses of fish, reptiles and mammals to hypoxia. These papers report on the responses of animals to hypoxia at the behavioural, physiological and molecular levels. Clearly hypoxia has wide ranging effects, the responses are complex and there are many similarities in the responses of all vertebrates to hypoxia. Organisms respond to hypoxia by reducing energy expenditure, in particular inhibiting reproduction, feeding and exercise. That is animals only expend energy that is absolutely necessary for survival. There is extensive reorganization of cellular machinery directed by hypoxia inducible factors (HIFs), proteins that increase during hypoxia and have a marked effect on the expression of many genes. As a result, anaerobic metabolism is up regulated and many aspects of aerobic metabolism are down regulated. Many cells enter cell cycle arrest. Thus organisms stop reproducing and growing during hypoxia and if the effects of hypoxia are severe, development of eggs and larvae is compromised.

CONTENTS

Page

NOTICE ii
ABSTRACT iii
ACKNOWLEDGEMENTS vi

Response of aquatic vertebrates to hypoxia
D.J. Randall, C.Y. Hung and W.L. Poon 1

Post-genomic approaches to the mechanisms of hypoxia response in the common carp, *Cyprinus Carpio*
E.J. Fraser, A.Y. Gracey and A.R. Cossins 11

Isolation and characterization of the VEGF-A gene of grass carp
Gallant K.L. Chan, Helen O.L. Mok, Patrick K.S. Ng, Minnie M.L. Wong and Richard Y.K. Kong 19

Molecular studies of the HIF transcription factors of grass carp
H.W. Law, Richard Y.C. Kong and Rudolf S.S. Wu 31

Gene expression profiling of raw 264.7 cell survival and apoptosis under oxidative stress
C.C. Fong, Y. Zhang, M.S. Wong, C.H. Tzang, W.P. Lai and M .Yang 39

Extreme adaptations to hypoxia and anoxia in crucian carp
Göran E. Nilsson 53

Suppressive effects of hypoxia on male rat reproductive function
Weigong Liao, Yuqi Gao, Mingchun Cai, Jian Chen, Yi Wu 59

Endocrine disrupting and teratogenic effects of hypoxia on fish, and their ecological implications
Rudolf S.S. Wu, Eva W.H. Shang and B.S. Zhou 75

Effects of hypoxia on lipid metabolism in teleosts
Guido van den Thillart 87

The influence of oxygen shortage on the development of the cardiovascular system in Zebrafish
Bernd Pelster 107

A biocomputational tool to measure cardiac rhythms in zebrafish
S.H. Cheng, Eric P.K. Chan and Alex C.C. Lin 121

Hypoxia in reptiles
Tobias Wang and Nini Skovgaard 127

Bioaccumulation and enantioselective biotransformation of fipronil by rainbow trout (*Oncorhynchus mykiss*)
Brad J. Konwick, Aaron T. Fisk, Jimmy K. Avants, J. MacArthur Long and Arthur W. Garrison 139

ACKNOWLEDGEMENTS

The 8th Symposium was organized by Professor Gao Yuki of the Institute of High Altitude Medicine, Third Military University, Chongqing, China and Professor David Randall of the Department of Biology and Chemistry, City University of Hong Kong, with the able help of Ms. Mandy Dung Yuk Man, also from Biology and Chemistry, City University of Hong Kong. The meeting was managed in Chongqing by the Third Military University, who provided excellent meeting rooms for the Symposium. Professor Gao and his associates were excellent hosts and showed us both the University and Chongqing. Funding for the symposium was provided by the U.S. Environmental Protection Agency and we were advised throughout by Dr. George Bailey and Dr. Rosemarie Russo of the Ecosystems Research Division, U.S. Environmental Protection Agency, Athens, Georgia. Ms. Dung Yuk Man assisted in the preparation of this document. Finally I attribute the success of this symposium to the many people who came to Chongqing to present and discuss their work.

David Randall
Department of Biology and Chemistry,
City University of Hong Kong

RESPONSE OF AQUATIC VERTEBRATES TO HYPOXIA

D.J.Randall[1], C.Y.Hung and W.L.Poon

ABSTRACT

The major effect of hypoxia on the individual is to reduce exercise capacity. Fish respond to hypoxia by inhibiting feeding and reproduction, and moving to lower temperatures, all of which lower energy expenditure. Oxygen delivery is augmented by increasing both gill ventilation and hemoglobin content and oxygen affinity. Aerobic metabolism is down-regulated whereas anaerobic metabolism is up-regulated. Steroid metabolism is reduced. Liver cells go into cell cycle arrest. Genes associated with cell growth and aerobic metabolism are down regulated but genes associated with anaerobic metabolism are up-regulated. Uncoupling proteins 2 & 3 are up-regulated and may play a role in reducing mitochondrial activity during hypoxia. Much of the response to hypoxia occurs in the first few hours and days. There are many similarities between fish and mammalian responses to hypoxia, presumably because they evolved in common aquatic vertebrate ancestors. Cardiovascular responses have been somewhat modified in terrestrial vertebrates and organic phosphate levels in the red blood cell decrease rather than increase in fish exposed to hypoxia. The relative role of temperature change, starvation, adenosine production, and HIF-1 expression in metabolic depression is not clear in any vertebrate.

INTRODUCTION

Hypoxia is a common feature of the aquatic environment. Oxygen levels in the water depend on photosynthetic activity in the water, exchange with the atmosphere, usage by aquatic organisms and oxidation reactions in the water. Photosynthesis requires light and, therefore, is restricted to surface waters and during daylight. Nocturnal hypoxia is common in tropical lakes and lagoons. Gas diffusion in water is slow and so hypoxia occurs at depth in unmixed waters. There is a minimum oxygen layer in oceans, blow the photosynthetic zone where a large number of organisms live on food dropping from above. Hypoxia is also common beneath the ice on frozen lakes and ponds. Although hypoxia is a natural and frequent event in the aquatic environment, it has been exacerbated by human actions. Eutrophication of waterways generally leads to hypoxia, and many regions of the world where there are large human populations have increasing levels of hypoxia. The movement of vertebrates onto land is relatively recent and much of vertebrate evolution occurred in water at lower oxygen levels than at present. It is possible that terrestrial vertebrates, living in an oxygen rich atmosphere, have lost some of their capacity to survive hypoxia.

EFFECTS OF INCREASING HYPOXIA ON AQUATIC VERTBRATES

Increasing aquatic hypoxia has led to changes in species composition, some leave, some die, what is left are those more tolerant of hypoxia. In general there is a reduction in species diversity and biomass (Diaz and Rosenberg 2001). Vertebrates respond to hypoxia by enhancing oxygen uptake, down regulating energy expenditure, and up-regulating anaerobic pathways. Fish move to colder

[1] Department of Biology and Chemistry, City University of Hong Kong, Kowloon, Hong Kong, SAR, China

waters in response to aquatic hypoxia (Petersen and Steffensen 2003) and this fall in body temperature reduces energy metabolism. In addition, because oxygen is more soluble in colder waters, this move may be associated with an increase in the oxygen content of water. The reduction in temperature will also enhance oxygen uptake because of increased hemoglobin oxygen affinity.

REDUCED FOOD INTAKE AND REPRODUCTION DURING HYPOXIA

Salmonids reduce food intake and stop growing when water oxygen saturation falls below 70% (Jobling 1994). Several studies in other fish have reported similar results, for example, cod exposed to hypoxia reduce food intake and growth (Chabot 2003) both in the laboratory and in the natural environment. The mechanisms by which hypoxia inhibits food intake are largely unknown.

Hypoxia is known to disrupt the endocrine systems in fish, retarding gonadal development and reducing success in spawning, fertilization and larval growth (Zhou *et al*. 2001; Wu *et al*. 2003). Reproductive success depends on multiple factors and control systems exist at several levels, including the hypothalamic-pituitary axis, steroid metabolism, and modulation of steroid receptors. Plasma steroid levels were low in hypoxic carp and gonad-somatic index (GSI) was reduced along with plasma testosterone levels (Zhou *et al*. 2001). Hypoxia appears to have a marked inhibitory effect on breeding and spawning behaviour of carp (Wang *et al*. unpublished data). Hypoxic (0.5-0.8mg/L) exposure of zebrafish reduced egg production to only 9/fish after the first day, compared to 52/fish in the control group. The egg number in the hypoxia treated group continued to drop over time.

In normoxia, fertilized zebrafish eggs hatch between 48 and 60 hours post fertilization (PF) and 93.8% of the eggs hatched. In hypoxia, fertilized eggs did not hatch until 96 to 260 hours PF, only 4.9% hatched and the rest died. Fertilized eggs developing under hypoxia were pale, indicating a lack of pigment. Growth was retarded and there were many abnormalities. Hypoxia exposure had little effect on the expression of the **A**ryl hydrocarbon **R**eceptor **N**uclear **T**ranslocator (Randall and Yang 2004) or the activity of the P450scc (CYP11A), the rate limiting enzyme controlling steroidogenesis via the conversion of cholesterol to pregnenolone (Yang unpublished data). Hypoxia increased vitellogenin gene expression in both zebrafish (Eva Shang unpublished data) and carp (C.Y. Hung unpublished data) but, surprisingly, this was associated with a marked reduction in vitellogenin levels in the fish.

OXYGEN DELIVERY DURING HYPOXIA

Most vertebrates maintain oxygen delivery during aquatic hypoxia by increasing ventilation and the diffusing capacity of the respiratory surface (Randall 1982). Fish skim the surface water, which usually has more oxygen, or may breathe air. Hypoxia due to breath holding in birds and mammals is associated with a reduction in heart rate and cardiac output and blood flows preferentially to the brain and heart rather than to the skin, gut, and muscles. Many fish, however, maintain cardiac output by increasing stroke volume to offset the decrease in heart rate. The number of circulating red blood cells increases due to release from the spleen (within minutes/hours), and due to increases erythropoietin levels (within days/weeks). Unlike mammals where increased organic phosphate levels decrease hemoglobin oxygen affinity during hypoxia, in fish hemoglobin (Hb) oxygen affinity increases due to the combined effect of decreased organic phosphate (NTP) levels and reduced temperature, as ffish move to colder water. The increase Hb-O_2 affinity facilitates oxygen uptake at the gills. The decrease in NTP/Hb ratios associated with hypoxia are rapid enough to compensate for circadian oscillations in dissolved oxygen seen in lagoons and tropical lakes (Val 2000). Anaemia in fish results in an increase in NTP/Hb ratio facilitating oxygen unloading to tissues, as seen in mammals (Val *et al*. 2002).

All-major modulators of vertebrate Hb-O_2 affinity, including IPP, GTP, ATP, 2,3DPG, (in decreasing order of effect) and IP_2 appeared during the radiation of fish (Val 2000). Thus, un-like mammals, RBC organic phosphates in fish decrease and Hb-O_2 affinity increases during hypoxia whereas the response to anemia is similar to that seen in mammals (organic phosphates increase).

To enhance oxygen delivery during hypoxia, hypoxia-inducible factor 1 (HIF-1) is stabilized, resulting in an increase in erythropoietin, heme oxygenase and transferrin to enhance red blood cell production in the mammals (Semenza 2000). In carp, however, heme oxygenase, is down-regulated (Hung unpublished results), whereas in goby fish *Gillichthys mirabilis*, a moderate-hypoxia tolerant species, heme-oxygenase is up-regulated but expression of transferrin is suppressed (Gracey *et al*. 2001). A variety of genes which may be independent of HIF-1, namely hemopexin precursor, a heme-binding protein and haptoglobin alpha & beta chains, hemoglobin-binding molecules, are all induced whereas ferritin was downregulated. Hemoglobin alpha and beta chains remained unchanged during hypoxia in carp kidney whereas expression of myoglobin gene increased by about 3 fold during long term hypoxia exposure (Hung unpublished data).

MUSCLE OXYGEN LEVELS

A fish body consists of about 60-70% muscle, made up of highly vascularized, red, oxidative, slow twitch fibres and poorly vascularized, white, glycolytic, fast twitch fibres. The red muscle of a trout has a much higher oxygen tension than that of a rat as measured by an oxygen sensitive (Presens) micro-optode, tip diameter <10 μm, into red muscle of intact trout (McKenzie *et al*. 2004) and rat. Interestingly, the arterial and venous oxygen tensions of rat and trout are similar (Table 1), however, muscle oxygen levels were below mixed venous levels in the human, rat, and dog whereas in trout muscle oxygen levels were midway between arterial and mixed venous levels, that is fish muscle oxygen levels are higher than that of rat.

Species	Human, Rat, or Dog	Trout
PaO_2 mmHg	100	100
PvO_2 mmHg	40	40
Muscle mmHg	25-35	61

Table 1. Oxygen tensions in arterial (PaO_2) and venous (PvO_2) blood and in red muscle in humans, rats, dogs and trout (adapted from McKenzie *et al*. 2004)

Many fish hemoglobins have a Root shift where reduced pH decreases Hb-O_2 saturation, even at very high O_2 levels. This Root shift is important in maintaining swimbladder inflation using very high levels of oxygen. McKenzie *et al*. (2004) suggest that the Root shift leads to the high muscle tissue oxygen levels they reported in trout. They argue that, as CO_2 transfer is more rapid than oxygen transfer, CO_2 can enter capillary blood, reduce RBC intracellular pH, and drive oxygen from the hemoglobin raising PO_2 in both blood and tissues (Root off shift). Subsequently, deoxy-hemoglobin will bind protons, raising pH, causing oxygen binding to hemoglobin as the blood leaves the tissue capillaries (Root on shift), reducing PvO_2 to levels below that in the tissues. Thus fish tissue oxygen levels are much higher than that seen in terrestrial mammals. This mechanism ameliorates the decrease in tissue oxygen levels during hypoxia (Figure 1) but the fish must maintain hemoglobin oxygen

saturation during hypoxia if this system is to work. Thus, this mechanism is coupled to the increased Hb-O_2 affinity during hypoxia seen in fish.

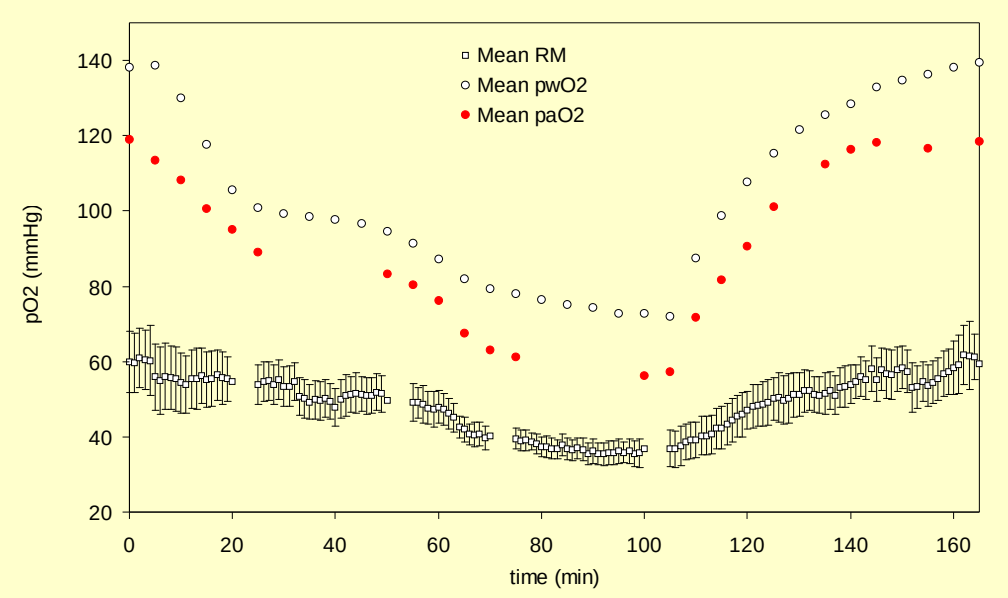


Figure 1. The decrease in muscle pO_2 is ameliorated and the blood:muscle pO_2 gradient is decreased during hypoxia in the rainbow trout (from McKenzie *et al*. 2004). RM = muscle pO_2, pwO_2 = water pO_2, paO_2 = arterial blood pO_2.

METABOLIC ADJUSTMENTS TO HYPOXIA

Fish reduce energy expenditure by moving to a lower temperature, reducing or stopping swimming activity, feeding and reproduction. As a result growth slows and eventually becomes zero or negative. All of these factors reduce energy expenditure. Adenosine levels are increased, as in mammals, protecting the animal against hypoxia and balancing energy usage and production (Bernier *et al*. 1996 a, b; Nilsson 2002). Many studies have shown an up-regulation of anaerobic enzymes during hypoxia with increased glucose transport, utilization of liver glycogen (muscle glycogen used during exercise) with increased lactate production. Lactate accumulation is ameliorated in carp and goldfish by ethanol production from lactate (ref).

We hypothesized that programmed cell death (apoptosis) was one of the strategies that help common carp (*Cyprinus Carpio* L.) survive hypoxia (0.5mgO_2/L). Although there were DNA nick ends shown by Terminal transferase mediated dUTP Nick End Labelling (TUNEL) staining in liver of carp exposed to hypoxia, it appeared that there was no apoptosis because there was no change in caspase 3 activity assay (Figure 2), DNA fragmentation assay, and anti-ssDNA staining (Figure 3). In addition there was no significant difference in the hepatosomatic index between 42-day hypoxic exposure in carp. Subsequent Electron Microscope (EM) analysis also indicated no change in the level of apoptosis between control and hypoxic carp livers. Results of an analysis using flow cytometry supported the absence of apoptosis. The cell cycle inhibitor, p27, which prevents cells from going into the S phase if there is DNA damage, was up-regulated in the 4-day hypoxic samples showing a low

TUNEL signal and indicated that the liver cells were in cell cycle arrest. Cell death activator CIDE-B was found to be down-regulated during hypoxia along with transmembrane 4 superfamily, member 8, whereas the anti-apoptotic gene, Bcl-2 was up-regulated during hypoxia (Figure 4). Thus the carp liver cells seem to be going into cell cycle arrest, rather than apoptosis, during hypoxia.

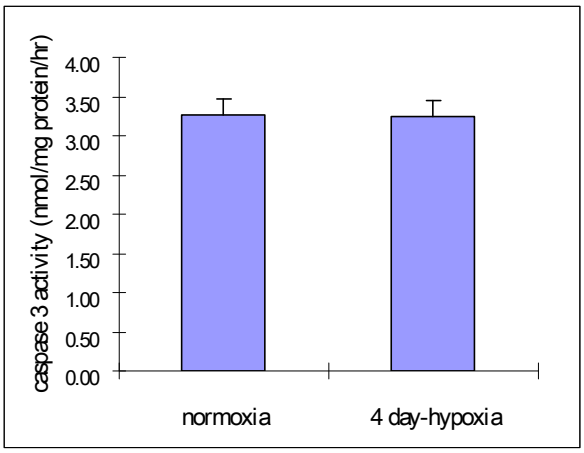


Figure 2. Caspase 3 activity of liver exposed to 4-day hypoxia (0.3mgO_2/L) and control. No significant different between two groups. Mean+SE with n=6.

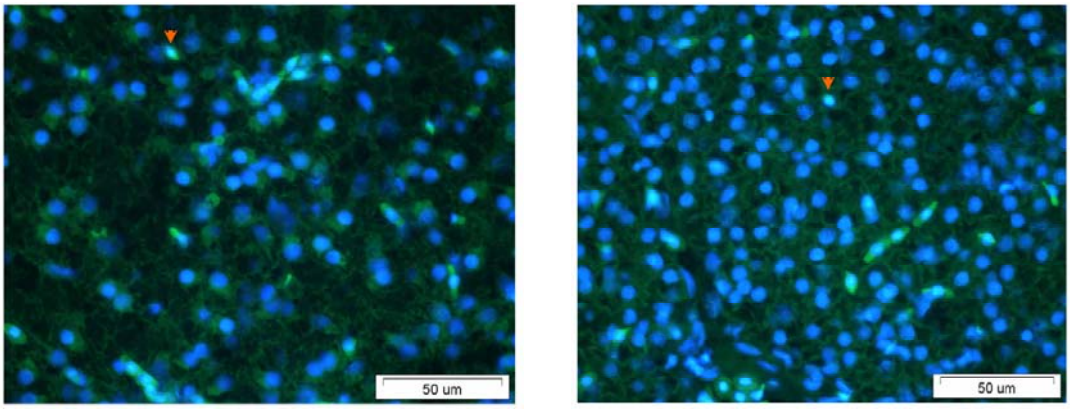


Figure 3. (A) Normoxia (B) 4-day hypoxia (0.5mgO_2/L). Apoptotic cells stained by both anti-ssDNA monoclonal antibody and DAPI (chromosome stain) were marked with ↓ . There was no observable difference between groups.

CHANGES IN GENE EXPRESSION DURING HYPOXIA

Changes in gene expression play a significant role in the response to hypoxia with the major changes occurring at the onset of hypoxia. The following is a description of the changes in gene expression in the liver of carp subjected to 42 days hypoxia. In general genes associated with aerobic metabolism and cell growth were down regulated but genes associated with anaerobic metabolism were up-regulated.

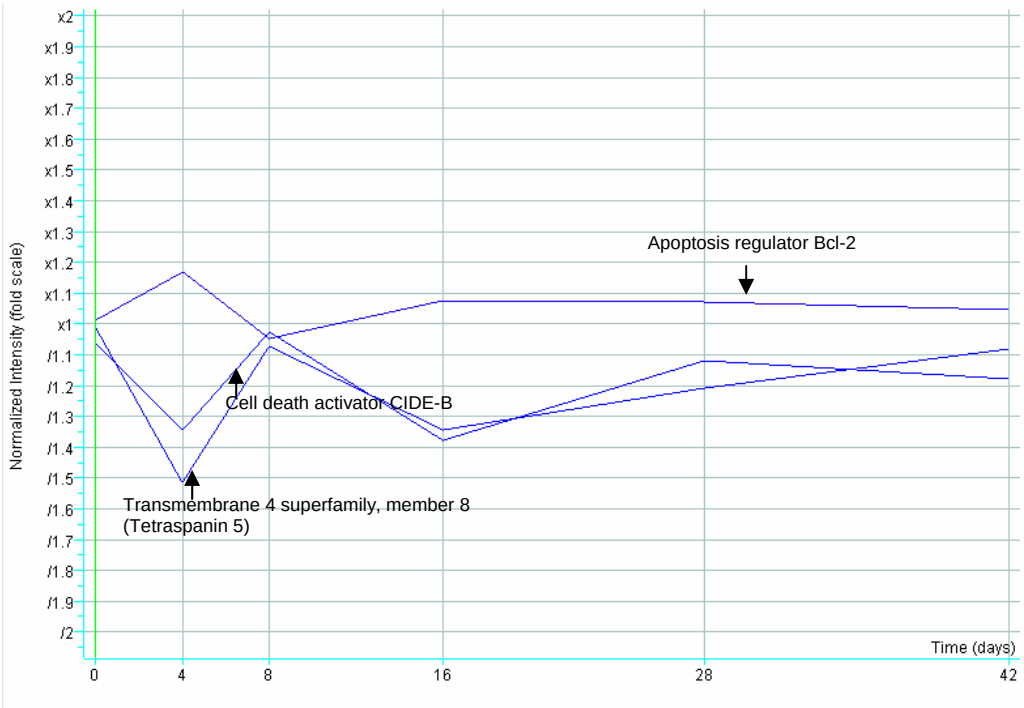


Figure 4. Expression of apoptosis-related genes during hypoxia in carp (One-way ANOVA, $p<0.07$).

Uncoupling proteins 2 & 3 were up-regulated in hypoxia and may play a role in reducing mitochondrial activity during hypoxia (Hung, unpublished data). In mammals, hypoxia inducing factor (HIF-1α) levels increase due to decreased rates of degradation, and results in the up-regulation of a large number of genes (>40 in mammals) controlled by HIF-1. HIF-1α is a constitutively expressed gene but its protein is also rapidly degraded under normoxic conditions. During hypoxia, 26S proteosome complex degradation of HIF-1α is inhibited. HIF-1α, together with HIF-1β, form a dimer that functions as a transcriptional factor (HIF-1) that up-regulates the gene expression of many target genes including VEGF, erythropoietin and glucose-transporters (Semenza 2001). One crucial function of HIF-1 transcription complex is to increase oxygen delivery and enhance anaerobic respiration during hypoxia by turning on oxygen delivery and glycolytic gene expression. HIF-1 expression was up-regulated at day 4 of hypoxia in carp and returned to control value thereafter, even though hypoxia persisted until day 42 (Figure 5). A similar pattern of glycolytic gene expression was observed in common carp during hypoxia at day 4 (glyceraldehydes 3-phosphate dehydrogenase, phosphoglycerate mutase 1 and 2, beta enolase, fructose-bisphosphate aldolase A, B and C, hexokinase) but the expression remained higher (about 2-fold) than time 0 during prolonged exposure to hypoxia (Figure 6).

HIF-1a is translationally regulated during hypoxia in mammalian systems but it appears that HIF-1a expression also increases in fish during hypoxia (Gracey 2001; Hung, unpublished data). The increase in carp HIF-1a expression could be driving the large increase in the expression of glycolytic genes at day 4. After 4 days of hypoxia, carp might have become better adapted to hypoxia and shift to solely translation control of HIF1a, as seen in mammals.

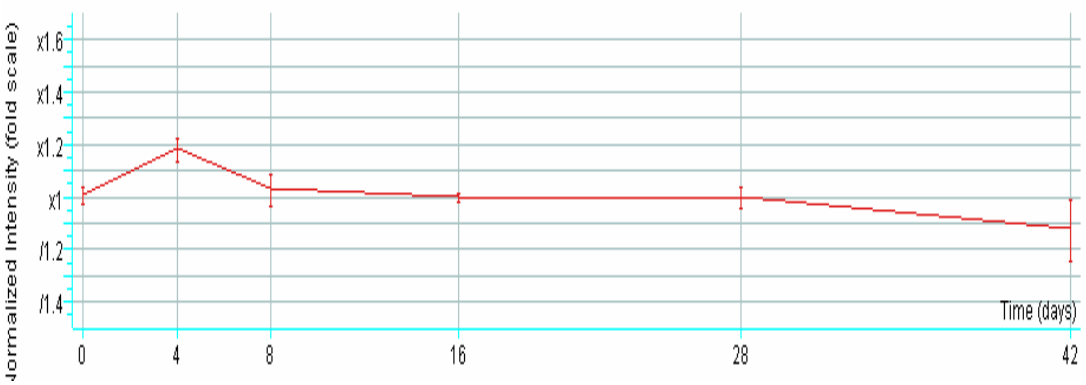


Figure 5. Expression profiles of carp HIF1a during hypoxia exposure. Day 4 is significantly different from all the time points (One-Way ANOVA, $p<0.05$).

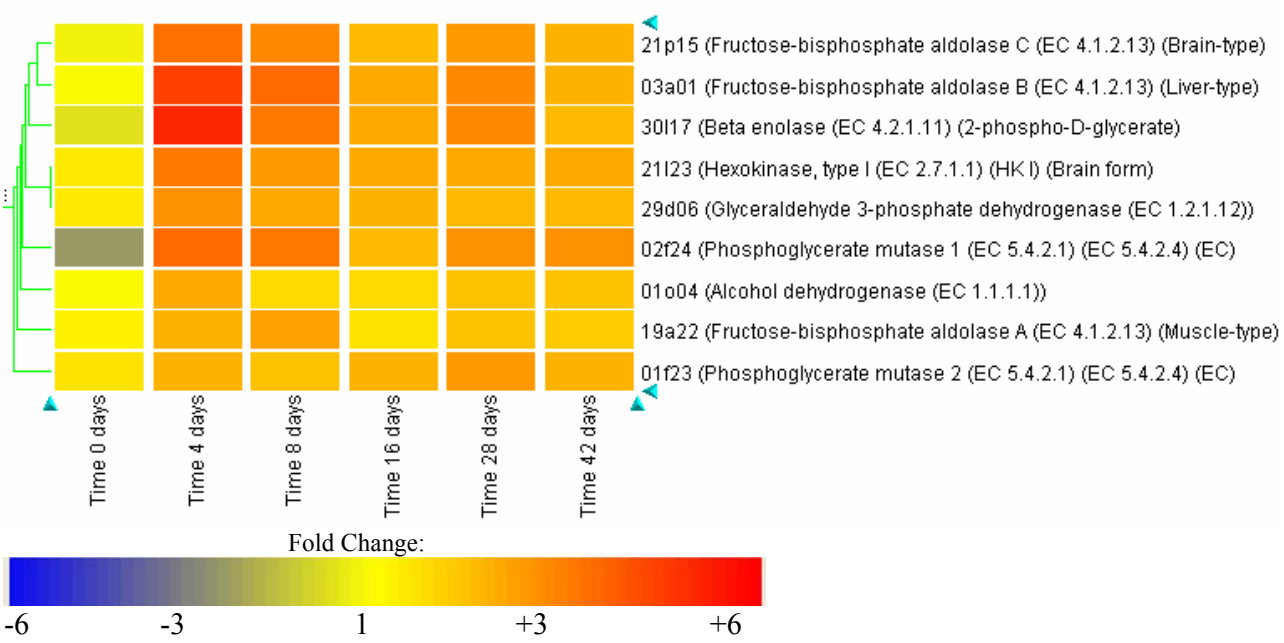


Figure 6. Expression profiles of some glycolytic genes during hypoxia in common carp. Most genes are up-regulated, especially at day 4.

The increase of glycolytic genes at day 4 was accompanied by increase of glyconeogenic genes fructose-1,6-bisphosphatase and fructose-1,6-bisphosphatase isozyme 2 and genes involved in the phenylalanine catabolic pathway and the expressions remained slightly higher than time 0 throughout the exposure period. Hepatic glycogen decreased rapidly at day 4, presumably as a result of increased anaerobic respiration. At day 16, carp had replenished its liver glycogen content to pre-hypoxia exposure (day 0) (Figure 7). Unfortunately, only one glycogen synthesis gene, glycogenin, is represented on our carp microarray and liver expression of this gene was too low to be detected. In contrast to liver, muscle glycogen remained unchanged during hypoxia, indicating that liver, but not muscle, glycogen is the main substrate for anaerobic respiration during hypoxia (Figure 8).

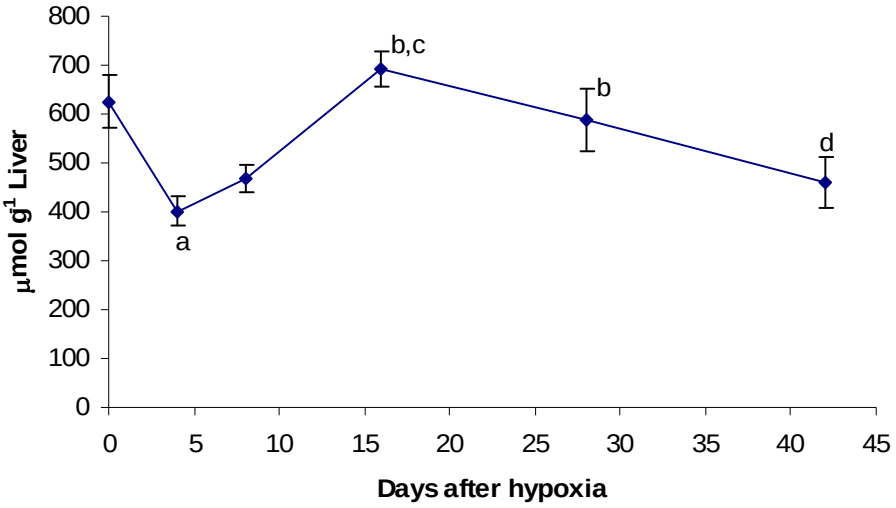


Figure 7. Hepatic glycogen levels of carp during hypoxia. Data shown are presented as (Mean ± SE), One-way ANOVA, $p<0.05$, a: significantly different from Day 0, b: significantly different from Day 4, c: significantly different from Day 8, d: significantly different from Day 16.

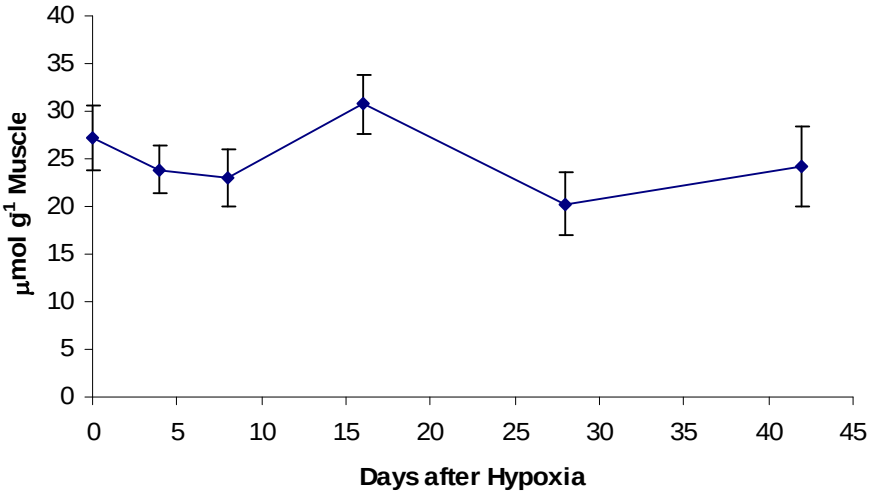


Figure 9. Muscle glycogen level of carp during hypoxia. Data shown are presented as (Mean ± SE), One-way ANOVA, $p<0.05$. No significant change during hypoxia.

SUMMARY

The major effect of hypoxia on the individual is to reduce exercise capacity. Fish respond to hypoxia by inhibiting feeding and reproduction and moving to lower temperatures, all of which lower energy expenditure. Oxygen delivery is augmented by increasing both gill ventilation and hemoglobin content and oxygen affinity. Aerobic metabolism is down-regulated whereas anaerobic metabolism is up-regulated. Steroid metabolism is reduced. Liver cells go into cell cycle arrest. Cell growth and

aerobic metabolism genes are down regulated but genes associated with anaerobic metabolism are up-regulated. Uncoupling proteins 2 & 3 are up-regulated and may play a role in reducing mitochondrial activity during hypoxia. Much of the response to hypoxia occurs in the first few hours and days. Four days seemed to be critical for carp, *Cyprinus Carpio* if they survived day 4 they continued to live under hypoxic conditions. There are many similarities between fish and mammalian responses to hypoxia, presumably the response evolved in vertebrate aquatic ancestors. Cardiovascular responses have been somewhat modified in terrestrial vertebrates and organic phosphate levels in the red blood cell decrease rather than increase in fish. The relative role of temperature change, starvation, adenosine production, and HIF-1 expression in metabolic depression not clear in any vertebrate.

Major effects of hypoxia on the individual is to reduce exercise capacity, if prolonged there is reduced growth. Finally there is reduced reproduction. At the ecosystem level hypoxia results in reduced species diversity and biomass. There is differential survival; some species are much more tolerant of hypoxia.

ACKNOWLEDGEMENTS

The work described in this paper was substantially supported by grants from the Research Grants council of the Hong Kong Special Administrative Region, China Project # CityU 1096/01M, CityU 1244/02M and by the Areas of Excellence Scheme established under the University Grants Committee of the Hong Kong Special Administrative Region, China (Project No. AoE/P-04/2004)

REFERENCES

Bernier, N.J., J. Harris, J. Lessard, and D.J. Randall. 1996a. Adenosine receptor blockage and hypoxia-tolerance in rainbow trout and Pacific hagfish. I. Effects on anaerobic metabolism. Journal of Experimental Biology 199: 485-495.

Bernier, N.J., J. Fuentes, and D.J. Randall. 1996b. Adenosine receptor blockage and hypoxia-tolerance in rainbow trout and Pacific hagfish. II. Effects on plasma cathecholamines and erythrocytes. Journal of Experimental Biology 199: 497-507.

Chabot, D. 2003. Chronic non-lethal levels of hypoxia limit distribution and growth of Atlantic cod (*gadus morhua*) in the northern gulf of St. Lawrence, Canada. Proceedings of the Seventh International Symposium, Tallinn, Estonia, May 12-15, 2003. E.P.A., Environmental Research Laboratory, Athens, Georgia, USA. (ed) R. Gretchen and D.W. Michelle, EPA/600/R-04/049. pp. 183-206.

Diaz, R.J. and R. Rosenberg. 2001. Overview of anthropogenically-induced hypoxia effects on marine benthic fauna. *In:* Coastal Hypoxia Consequences for Living Resources and Ecosystems, N.N. Rabalais and R.E. Turner (eds.), American Geophysical Union, Washington, D.C. pp. 129-146.

Gracey, A.Y., J.V. Troll, and G.N. Somero. 2001. Hypoxia-Induced Gene Expression Profiling in the Euryoxic Fish, Gillichthys mirabilis. Proceedings of National Academy of Sciences of the United States of America 98: 1993-1998.

Jobling, M. 1994. Fish Bioenergetics. Volume 13: 1-44 Chapman and Hall. London.

McKenzie, D.J., S. Wong, D.J. Randall, S. Egginton, E.W. Taylor and A.P. Farrell. 2004. The effects of sustained exercise and hypoxia upon oxygen tensions in the red muscle of rainbow trout. J. Exp. Biol. 207: 3629-3637.

Nilsson, G.E. 2002. Adenosine – A Universal Promoter of Survival under hypoxic conditions. Proceedings of the Sixth International Symposium, La Paz, B.C.S. Mexico 22-26 January 2001. E.P.A., Environmental Research Laboratory, Athens, Georgia, USA. (ed) R.V. Thruston, EPA/600/R-02/097. pp. 1-10.

Petersen, M.F. and J.F. Steffensen. 2003. Preferred temperature of juvenile Atlantic cod Gadus morhua with different haemoglobin genotypes at normoxia and moderate hypoxia. Journal of Experimental Biology 206: 359-364.

Randall, D.J. 1982. The control of respiration and circulation in fish during exercise and hypoxia. Journal of Experimental Biology. 100: 275-288.

Randall, D.J. and H.P.Yang. 2004. The role of hypoxia, starvation, β-Naphthoflavone and the arylhydrocarbon receptor nuclear translocation in the inhibition of reproduction in fish. Proceedings of the Seventh International Symposium, Tallinn, Estonia, May 12-15, 2003. E.P.A., Environmental Research Laboratory, Athens, Georgia, USA. (ed) R. Gretchen and D.W. Michelle, EPA/600/R-04/049. pp. 253-261.

Semenza, G.L. 2000. HIF-1: mediator of physiological and pathophysiological responses to hypoxia. Journal of Applied Physiology 88: 1474-1480.

Semenza, G.L. 2001. Hypoxia-inducible factor 1: oxygen homeostasis and disease pathophysiology. Trends in Molecular Medicine 7: 345-350.

Val, A.L. 2000. Organic phosphates in the red blood cells of fish. Comparative Biochemistry and Physiology 125: 417-435.

Val, A.L., B. Wicks, and D.J. Randall. 2002. Anemia and polycythanemia affect levels of ATP and GTP in fish red blood cells. Proceedings of the Sixth International Symposium, La Paz, B.C.S. Mexico 22-26 January 2001. E.P.A., Environmental Research Laboratory, Athens, Georgia, USA. (ed) R.V. Thruston, EPA/600/R-02/097. pp. 11-20.

Wu, R.S.S., B.S. Zhou, D.J. Randall, N.Y.S. Woo, and P.K.S. Lam. 2003. Aquatic hypoxia is an endocrine disruptor and impairs fish reproduction. Environmental Science and Technology 37: 1137-1141.

Zhou, B.S., R.S.S. Wu, D.J. Randall, and P.K.S. Lam. 2001. Bioenergetics and RNA/DNA ratios in the common carp (*Cyprinus carpio*) under hypoxia. Journal of Comparative Physiology B 171: 49-57.

POST-GENOMIC APPROACHES TO THE MECHANISMS OF HYPOXIA RESPONSE IN THE COMMON CARP, *CYPRINUS CARPIO*.

E.J. Fraser[2], A.Y. Gracey and A.R. Cossins

ABSTRACT

Hypoxia is an important environmental stressor for many fish species, particularly in freshwater where it interacts with thermal stress. The cyprinid fish in general and the common carp (*Cyprinus carpio*) in particular are resilient to hypoxia and some species can withstand anoxia for long periods of time. We have explored large-scale responses to hypoxia in the common carp, and interactions between temperature and hypoxia, using transcript screening techniques. Fish were subjected at either 17°C or 30°C to progressive reduction in PO_2 down to 0.8mg O_2/l where they were held for up to 8 days. Replicate fish were removed on days 0, 1, 3, 5 and 8 and tissues rapidly dissected and stored for subsequent extraction of RNA. In reference-based, dual label experiments, RNA was hybridised to ~700 carp cDNA microarrays comprising 13,349 separate carp probes which were obtained from a series of cDNA libraries enriched for hypoxia- and thermally-responsive genes. The resulting large-scale data set, including >5 fold biological replication, has been analysed using the *GeneSpring/GeNET* package and interpreted using the annotated gene lists provided by the *EST-Ferret*. We have defined extensive lists of genes from the 5 different tissues that respond to hypoxia, and whose hypoxia response is affected by temperature of exposure. These lists have been assessed using advanced statistical techniques to identify patterns in expression that relate to underpinning biological responses to single and combined stressors.

INTRODUCTION

Terrestrial mammals experience high levels of environmental oxygen and their tissues consequently have a restricted ability to withstand prolonged hypoxia. Vascular pathologies in humans can cause tissue hypoxia and the limited resistance to hypoxia is the cause of major clinical conditions (Michiels 2004), including cerebral ischemia (stroke), myocardial ischemia and tumour angiogenesis. By contrast, freshwater organisms inhabit an environment where oxygen levels routinely fluctuate, sometimes profoundly, and survival demands a degree of resistance to hypoxia. Some species, such as salmonids are highly sensitive and short periods of hypoxia can be lethal, while others can survive several months of extreme hypoxia (common carp) and even anoxia (goldfish, crucian carp, bitterling) (Nilsson *et al*. 1993). These hypoxia-resistant fish typically respond to declining oxygen levels through a tissue-specific reorganisation of energy metabolism, which preserves ATP levels and thereby prolongs survival time. During the initial stages of hypoxia, a down-regulation of metabolism and shutdown of nonessential cell functions occurs (Hochachka et al. 1996; Smith *et al*. 1996). As the duration of exposure increases, anaerobic ATP-producing pathways are up regulated coupled to a mobilisation of stored glycogen and a down-regulation of oxygen-dependent pathways.

The common carp survives long periods of hypoxia by metabolic depression (Zhou *et al*. 2000), cardiac depression (Stecyk and Farrell 2002) and increased blood oxygen affinity (Weber and Lykkeboe 1978) amongst other responses. However, the data are fragmentary, and in some cases,

2 School of Biological Sciences, University of Liverpool, Biosciences Building, Crown Street, Liverpool L69 7ZB, UK

contradictory, and the broader tissue-specificity and integration of these various response mechanisms has not been fully defined. Moreover, hypoxia sensitivity and the underpinning response mechanisms may be substantially modified by temperature, since hypoxia tolerance is better expressed in the cold. To date the underpinning mechanisms have generally been identified by the conventional process of hypothesis-experiment-deduction. This is most elegantly developed at the level of candidate gene whose role is inferred through physiology, though the breadth of the understanding is necessarily restricted to the limited number of genes that can be investigated simultaneously. New technologies developed recently within the context of genomic science now provides the means of exploring the expression of many thousands of genes simultaneously by means of massively parallel DNA sensors, laid in arrays on a solid surface such as glass slides. These so-called 'microarrays' not only offer breadth of analysis but also allow open screening of responses as a means of discovering novel responses and novel genes. We have recently deployed this technique to show that cold has profound effects on many thousands of transcripts. Moreover, we were able to identify core responses that exist in all tissues, as well as tissue-specific responses (Gracey *et al*. 2004).

Here we describe preliminary attempts to use the microarray technique to investigate the mechanism of response to deep hypoxia in multiple tissues of the common carp. We explore responses in liver at two different temperatures to uncover responses at different levels of stress.

METHODS

Common carp (*Cyprinus carpio* L.) were obtained commercially and held for 2 months at either 17±0.5°C or 30±0.5°C in large 2,000-liter aquaria. For hypoxic exposure the fish were transferred to 1,000-liter tanks and pre-conditioned for 2 weeks. Immediately prior to hypoxic exposure (Day 0) 5 fish were removed, killed, and liver, brain, kidney, heart, skeletal white muscle, gill and intestinal mucosa were rapidly dissected and frozen on dry ice. After this sampling the oxygen level was rapidly decreased over a 3 hr period until the oxygen level was maintained at 0.8 mg/l/O_2 (10% normoxia). Five replicate fish were sampled on days 1, 3, 5 and 8.

Due to the absence of genomic resources for the common carp, we prepared 14 full length, directionally cloned cDNA libraries. These were normalised (Carcini *et al*. 2000), subtracted (Diatchenko *et al*. 1996) and serially subtracted yielding 13,349 clones. 12,960 clones were end sequenced, and 9461 of which resulted in high quality reads. These were assembled by 2 passes of CAP-3 using an in-house informatics package, *EST–Ferret,* yielding 6806 singletons or subgroups. Clones were identified by BLAST homology searching and functionally annotated using the Gene Ontology and KEGG databases. The resulting data is presented in *carpBASE*, a MySQL database that is available at http://legr.liv.ac.uk.

We used amplicons of these 13,349 clones to fabricate a high-density carp cDNA microarray on glass slides with an in-house modification to the surface chemistry. Fluorescence-labelled target from the tissue samples and a common reference cDNA was hybridised to the array using standard techniques (www.microarray.org), and the images quantified to provide data files. Thus, for each sampling time we collected data from 5 replicate animals, each being measured twice in each dye combination to address problems of dye bias and provide a technical replicate. Data was analysed using the *GeneSpring* package (Agilent).

RESULTS

We have conducted two hypoxia exposure experiments, one at 17°C and the other at 30°C. Each consisted of a rapid reduction in oxygen tension to a sustained low level, with sampling of replicate specimens at regular intervals for an 8-day period. Tissue was archived at –80°C for subsequent analysis. With microarray experiments one of the major considerations is the sheer volume of data. Using 5 time points, each with 5 tissues analyzed and 5 replicates gives rise to 125 different RNA samples, each of which was analyzed twice both for the tissue sample and a common reference sample used for normalization purposes. Together these experiments required over 700 arrays resulting in ~19,000,000 data points. Raw data files were first subjected to a quality control procedure to interrogate the concordance of the technical replicates and then a normalised ratio was calculated by combining the expression data for each pair of fluor-reversed slides (Fang *et al*. 2003). Visualization of the data was performed using the *GeneSpring* data analysis package. We have sequence characterised most of the probes on the carp microarray, identified the genes by BLAST homology searching and annotated each identified gene using the Gene Ontology and KEGG systems (see supplementary information with Gracey *et al*. (2004)).

Discerning biologically meaningful information from such large, multi-dimensional data sets is difficult. We have used Principal Components Analysis (PCA), an exploratory multivariant statistical technique, for simplifying complex data sets (Basilevesky 1994) and to provide an overview of the main patterns in the data. PCA reduces high dimensionality to a manageable number that can be displayed on orthogonal axis. The final number of variables will seek to include as much variance as the original data set, whilst remaining mutually uncorrelated. In essence PCA gives us an overview of what is happening with the data without having to consider single genes. The other advantage of PCA is that because it investigates movement within statistical space no biological bias is ascribed to the interpretation.

Figure 1. illustrates the utility of PCA using the data from skeletal muscle. The 1st and 2nd components of PCA are shown which together account for 80% of the total variance in gene expression. There is a clearer separation between temperature than over time. For both temperatures there is movement across the two components although the degree of movement is greater for the fish held at 30°C than 17°C. Finally, the pattern of movement over time is very similar for the two temperatures. Thus, regardless of the water temperature the muscle responds to hypoxia in a similar manner though the degree of response appears different between the two temperatures. PCA can also reveal patterns between all the tissues to provide overviews of conserved and differentiated responses as illustrated for cold exposure in Gracey *et al.* (2004).

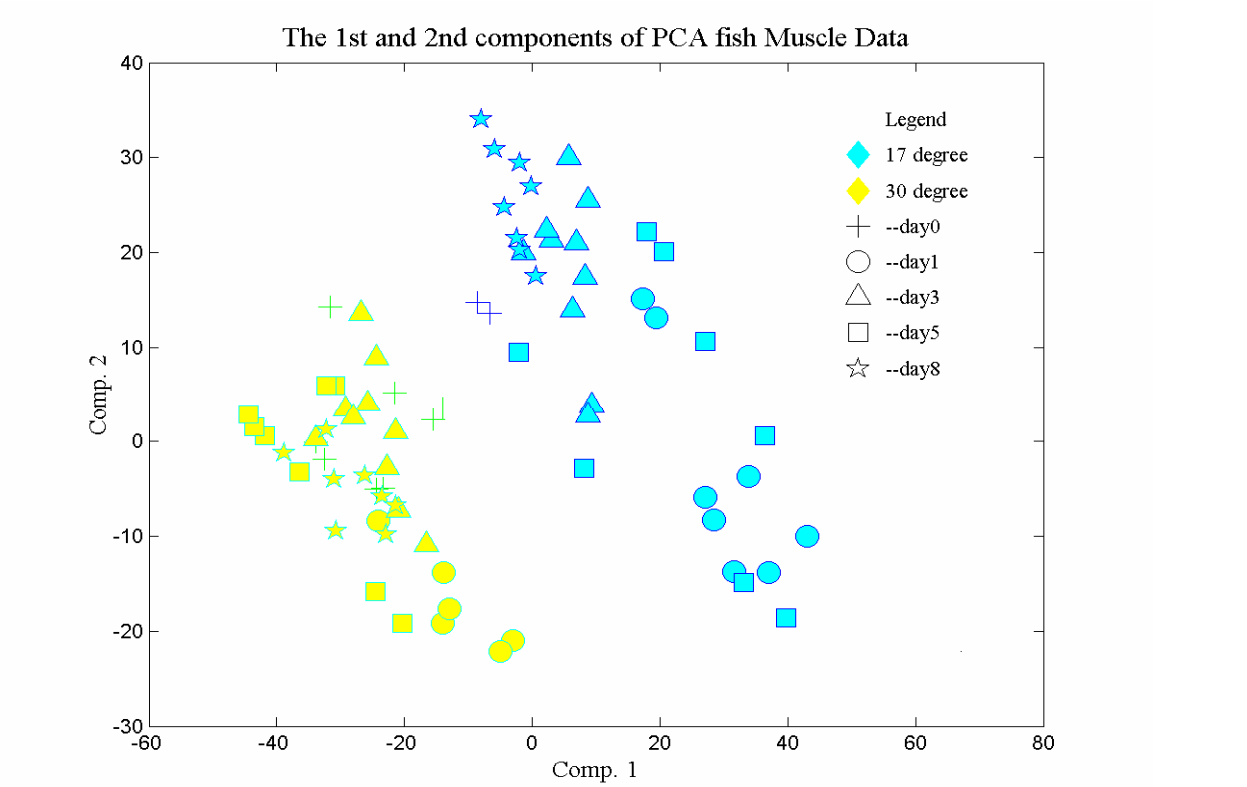


Figure 1. Principal component analysis of expression profiles from epaxial skeletal muscle of the common carp. Data is shown for the experiments conducted at 17 and 30°C, and for 1, 3, 5, and 8 days of hypoxia exposure. We show the first two components only accounting for 80% of the variance in the entire dataset.

A second method of analysing the large-scale data was to group the expression profiles using a hierarchical clustering technique. This generates groups of genes that share patterns of response across treatment space, each of which might constitute a regulated response to the treatment. These clusters generate lists of genes that can be characterised by profiling the gene ontologies. Alternatively, data relating to a specific biological process can be selected from the full dataset and analysed separately. Figure 2 shows the profiles of genes for all of the enzymes involved in the glycolytic pathway, which is an important anaerobic metabolism pathway with liver glycogen the principal energy reserve for fish under hypoxic conditions (Zhou *et al*. 2000). The mean data for the 5 replicate specimens was expressed for each day relative to day 0 with red panels indicating increased expression relative to day 0 and green indicating reduced expression.

The expression profiles were similar for both the 17°C and 30°C fish, with the strongest induction noted for fructose bisphosphate aldolase A & B. Discrepancies between the two temperatures are seen with hexokinase, pyruvate kinase and glucose phosphate isomerase where a decrease in expression is seen at 17°C for the first two, whilst an increase is seen at 30°C. For glucose phosphate isomerase the reverse is seen. Earlier studies have demonstrated that alterations in enzyme kinetics may be an adaptation to hypoxia (Zhou *et al*. 2000). Although we are not examining enzyme activity in this study the lack of increased expression of phosphfructokinase (a major regulatory

enzyme for glycolysis) in the liver support work in the muscle of the common carp where a reduction in phoshpofructokinase activity was found, indicating a decrease in responsiveness to allosteric control (Zhou *et al*. 2000).

It should be noted that for certain genes there are multiple probes included on the array. This provides a specific problem with regard to data analysis and visualisation. One option is to average the information across copies and display each gene as a single expression profile. However, it is clear from Figure 2 that there are differences in the expression profiles between some replicate probes. These differences may simply reflect noise or experimental error, however, there is always the possibility that they represent functionally distinct isoforms of the gene. The common carp is a tetraploid species and is widely thought to have undergone genome duplication, which has resulted in many gene isoforms. This information would be lost if the data were to be averaged.

DISCUSSION

Vertebrate genomes contain in excess of 20,000 genes with many more transcripts, but the microarray technique allows a large fraction of these to be quantified simultaneously. We have shown that even for non-sequenced organisms it is possible for single laboratories to provide the cDNA clone resources and sequence annotation to furnish a high-density microarray composed of many thousands of gene probes. Generating such large datasets produces downstream problems of analysis, visualisation and interpretation, though techniques for this are rapidly maturing. We show here that the PCA technique can give a broad overview of the relationship between samples, treatments taking the entire expression profile into account, and this reveals important conclusions about the main sources of variation in profiles. We also show that highly detailed information can be mined using selected lists of gene probes. In Figure 2, which is a relatively small cluster of only 60 genes from a well-characterised pathway, a complicated pattern emerges with peak expression levels differing over time and contradictory patterns between the two temperatures. These issues are being addressed with constant improvement in pattern finding algorithms and the public accessibility of all microarray data.

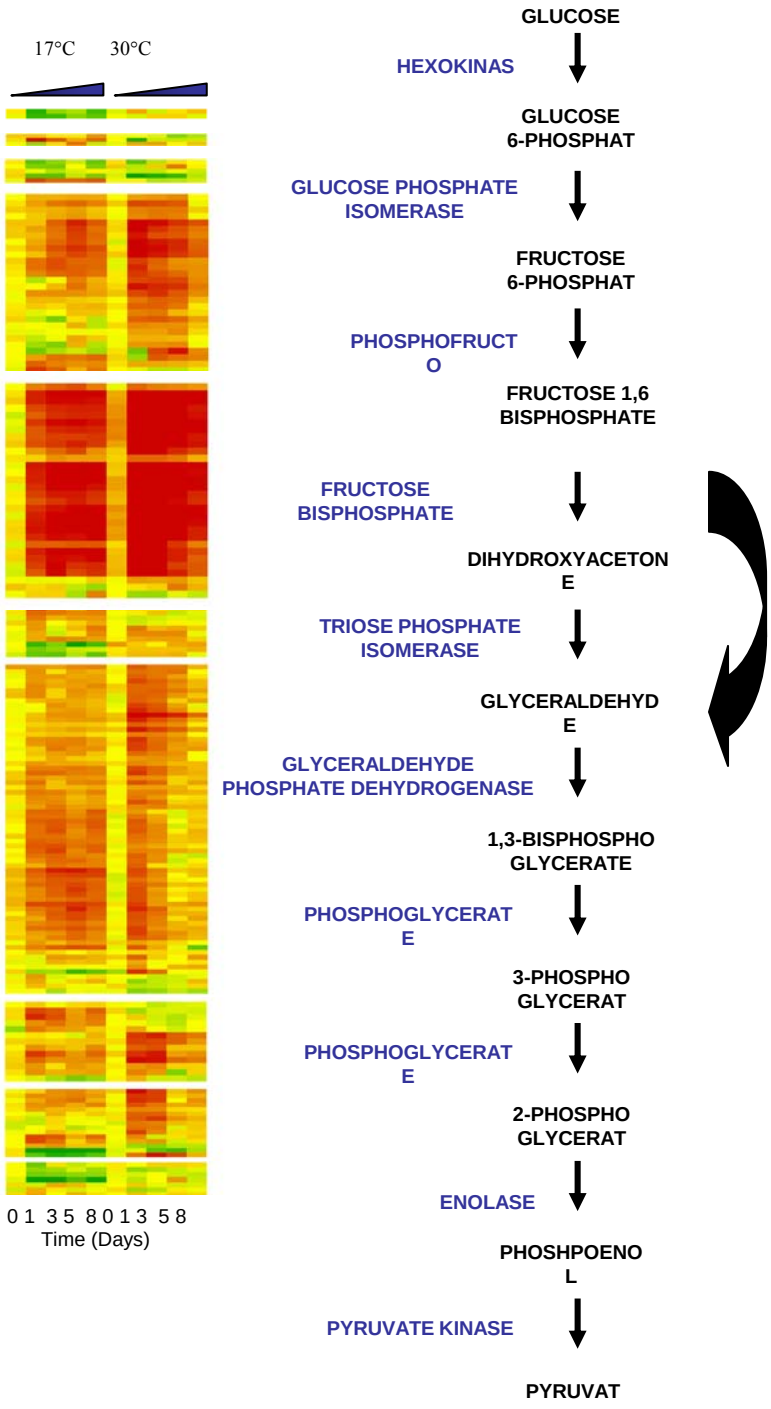


Figure 2. Expression profiles for the genes comprising the glycolytic pathway in liver tissue. The pathway is indicated to the right with chemical intermediates and corresponding genes located next to panels representing expression of replicate probes, each being identified by BLAST as that gene. Expression values were calculated relative to the time zero value and increased levels are indicated in red and decreasing values in green. Yellow indicates no change.

It is important to recognise that microarray data relates to amount of transcript for a particular gene, relative to a specified standard. Whilst it reveals patterns of response in transcript amounts this does not necessarily correlate to changes in protein level or activity, since regulation can be achieved

by adjustment at many hierarchical levels of organisation. Confirming transcript response needs analysis using an alternative technique, such as RT-PCR, but demonstrating that transcript regulation is biologically meaningful requires analysis at the level of protein amounts and enzyme function.

Despite these hurdles the power of microarray technology is evident. The ability to examine thousands of genes across many tissues simultaneously helps to build an idea of whole animal responses to specific stimuli. As well as investigating specific genes of interest the opportunity for novel roles for known genes is always present. Finally, microarray allows scientist working on non-model species to start investigating changes at the transcript level.

ACKNOWLEDGEMENTS

This project was funded by the Natural Environment Research Council (UK). We thank Gregor Govan, Margaret Hughes and Weizhong Li for assistance in the aquarium, genomics and bioinformatics labs, respectively.

REFERENCES

Basilevsky, A. 1994. Statistical Factor Analysis and Related Methods, Theory and Applications. John Wiley & Sons, New York, NY.

Carcinci, P., Y. Shibatat, N.K. Hayatsu, Y. Sugahara and K. Shibata. 2000. Normalization and subtraction of cap-trapper-selected cDNAs to prepare full-length cDNA libraries for rapid discovery of new genes. Genome Research 10: 1617-1630.

Diatchenko, L., Y.F.C. Lau, A.P. Campbell, A. Chenchik, F. Moqadam, B. Huang, S. Lukyanov, K. Lukyanov, N. Gurskaya, E.D. Sverdlov and P.D. Siebert. 1996. Suppression subtractive hybridization: A method for generating differentially regulated of tissue-specific cDAN probes and libraries. Proceedings of the National Academy of Sciences of the United States of America 93. 6025-6030.

Fang, Y., A. Brass, D.C. Hoyle, A. Hayes, A. Bashein, S.G. Oliver, D. Waddington and M. Rattray. 2003. A model-based analysis of microarray experimental error and normalisation. Nucleic Acids Research 31: 96

Gracey, A.Y., E.J. Fraser, W. Li, Y. Fang, R.R. Taylor, J. Rogers, A. Brass and A.R. Cossins. 2004. Coping with cold: An integrative, multitissue analysis of the transcriptome of a poikilothermic vertebrate. Proceedings of the National Academy of Sciences of the United States of America 101: 16970-16975.

Hochachka, P. W., L.T. Buck, C.J. Doll, and S. C. Land. 1996. Unifying theory of hypoxia tolerance: Molecular/metabolic defense and rescue mechanisms for surviving oxygen lack. Proceedings of the National Academy of Sciences of the United States of America. 93: 9493-9498.

Michiels, C. 2004. Physiological and Pathological Responses to Hypoxia. American Journal of Pathology 164: 1875-1882.

Nilsson, G.E., P. Rosen, and D. Johansson. 1993. Anoxic depression of spontaneous locomotor activity in crucian carp quantified by a computerized imaging technique. Journal of Experimental Biology 180: 153-157

Smith, R.W., D.F. Houlihan, G.E. Nilsson and J.G. Brechin. 1996. Tissue-specific changes in protein synthesis rates in vivo during anoxia in crucian carp. American Journal of Physiology 271:897-904.

Stecyk, J.A.W and A.P. Farrell. 2002. Cardiorespiratory responses of the common carp (Cyprinus carpio) to severe hypoxia at three acclimation temperatures. Journal of Experimental Biology 205: 759-768.

Weber, R.E. and G. Lykkeboe. 1978. Respiratory adaptations in carp blood influences of hypoxia, red cell organic phosphates, divalent cations and CO2 on haemoglobin-oxygen affinity. Journal of Comparative Physiology 128: 127-137.

Zhou, B.S., R.S.S. Wu, D.J. Randall, P.K.S. Lam, Y.K. Ip, and S.F. Chew. 2000. Metabolic adjustments in the common carp during prolonged hypoxia. Journal of Fish Biology 57: 1160-1171.

ISOLATION AND CHARACTERIZATION OF THE VEGF-A GENE OF GRASS CARP

Gallant K.L. Chan, Helen O.L. Mok, Patrick K. S. Ng,
Minnie M. L. Wong and Richard Y. C. Kong[3]

ABSTRACT

Vertebrates respond to reduced oxygen concentrations (hypoxia) in many different ways at the systemic, local and cellular levels. For example, adaptations at the systemic level include an increase in ventilation and oxygen-transport capacity by increased erythropoiesis; and at the cellular level, survival of the individual cell under hypoxia is mediated by induction of glycolytic enzymes to facilitate anaerobic ATP production. Local adaptation to hypoxia in tissues is facilitated by the induction of vascular endothelial growth factor (VEGF) which stimulates angiogenesis (or neovascularization) to improve tissue oxygenation. The VEGF family consists of 5 members: VEGF-A, VEGF-B, VEGF-C, VEGF-D, and placental growth factor (PlGF). VEGF-A is the predominant mitogen and plays a critical physiological role both in normal vascular development and response to hypoxic stress. In this report, we describe the isolation and characterization of the *gcVEGF-A* gene from grass carp. In addition, we also demonstrate by RT-PCR the presence of four distinct *gcVEGF-A* splice variants – gcVEGF-121; gcVEGF-145; gcVEGF-189 and gcVEGF-206 – which are differentially expressed in different fish tissues response to hypoxia.

INTRODUCTION

Vascular endothelial growth factor (VEGF-A) is a key regulator of angiogenesis and vascular permeability, and upregulated expression of the *VEGF-A* gene by hypoxia is due to both transcriptional activation by HIF-1 and stabilization of the normally labile VEGF mRNA by various RNA-binding proteins (Levy *et al*. 1996). *In vivo*, VEGF expression is associated with embryonic, physiological, and pathological blood vessel growth (Breier *et al*. 1992; Shweiki *et al*. 1993; Miller *et al*. 1994). In the adult, VEGF is an important angiogenic/mitogenic factor in various estrogen target tissues such as the placenta (Pepe and Albrecht 1999), pituitary (Ochoa *et al*. 2000), testis (Rudolfsson *et al*. 2004), ovary and uterus (Charnock-Jones *et al*. 2002) and normal breast tissue (Nakamura *et al*. 1999). In vertebrates, several different isoforms of the VEGF protein – VEGF-121; VEGF-145; VEGF-165; VEGF-189; and VEGF-206 – are generated via alternative splicing of the *VEGF-A* gene (Tischer *et al*. 1991), and which differ in angiogenic properties (Küsters *et al*. 2003). Numerous studies have demonstrated that estrogens increase VEGF expression in rodent and human uterine tissues (Hyder *et al*. 2000), rat mammary tumors (Nakamura *et al*. 1996) and human breast cancer cells (Ruohola *et al*. 1999) evidently via the binding of estrogen receptors (ERs) α and β to the estrogen-responsive elements (EREs) of the *VEGF-A* gene (Hyder *et al*. 2000; Mueller *et al*. 2000). Moreover, several studies have shown that hypoxia induces proteasome-dependent degradation of the ERα protein (Stoner *et al*. 2002) in a manner similar to the arylhydrocarbon receptor (AhR)-mediated degradation

[3] Department of Biology and Chemistry, City University of Hong Kong, Tat Chee Avenue, Kowloon Tong, Hong Kong Special Administrative Region, China.

of ERα that is known to be associated with the anti-estrogenic activities of various AhR agonists (Wormke *et al*. 2003), whereby proteasome-dependent degradation of ERα were accompanied by downregulated expression of estrogen-responsive genes. Recent studies have shown that hypoxia not only causes reproductive impairment in the common carp via disruptive changes in endocrine function, gonadal development, gamete quality, fertilization success and larval survivorship (Wu *et al*. 2003); but sublethal levels of hypoxia can also produce teratogenic effects as well as delay embryonic development in the zebrafish (Shang and Wu 2004).

To date, the molecular mechanisms by which VEGF proteins regulate reproductive functions in vertebrates are still poorly understood. Based on information in mammalian systems on the effects of hypoxia on VEGF-A and the possible regulatory roles of the different VEGF-A isoforms in reproductive tissues, it is conceivable that the suppressive effects of hypoxia on reproductive fitness in fish may be mediated by changes in the regulation and expression pattern of the VEGF-A mRNA and/or proteins in gonadal tissues of fish. Although various VEGF-A spliced variants have been cloned and characterized in zebrafish (Liang *et al*. 2001; Gong *et al*. 2004), VEGF expression in reproductive tissues of fish under normal physiological conditions or pathological expression of VEGF in response to sublethal levels of hypoxia have not been described. Due to the diverse biological activities of VEGF isoforms and the capacity of VEGF to recruit pleiotropic signaling pathways in various cellular systems (Ferrara 2004), it is conceivable that a specific VEGF isoform or combinations of different isoforms may be enrolled in distinct physiological and pathological processes. On this premise, it is suggested that changes in VEGF expression (and splicing) patterns may play a role in hypoxia-induced endocrine disruption leading to reproductive impairment in fish. As a first step to better understand the role of VEGF-A as a possible mediator of these functions in fish, we have isolated and characterized the *gcVEGF-A* genomic gene and several gcVEGF-A spliced variants from the hypoxia-tolerant grass carp.

MATERIALS AND METHODS

Treatment of samples

Grass carp, *Ctenopharyngodon idellus*, weighing around 500g, were obtained from a commercial hatchery and acclimated in 300 L fiberglass tanks with circulating, filtered and well-aerated tap water at 20 ºC for 1 week prior to experimentation. Fish were fed daily with lettuce that amounted to ≈ 1 % of body weight. Fish were then divided into two groups, one group was reared under normoxia (7.0 ± 0.2 mg O_2/L) and the other under hypoxia (0.5 ± 0.3 mg O_2/L) in a continuous flow system described by Zhang *et al*. (2003). The levels of dissolved oxygen were monitored continuously using an YSI Model 580 dissolved oxygen meter. After the exposure period, fish were anaesthetized by immersion in 2-phenoxyethanol (0.05% v/v) for 5 min, and killed by a blow to the head. Tissues were then dissected out and snap-frozen in liquid nitrogen, and stored at –80 ºC.

Reverse transcription–polymerase chain reaction

Total RNA was isolated using the TRIzol Reagent (Invitrogen, USA) according to the manufacturer's instructions. One microgram of total RNA was annealed to Oligo $(dT)_{12–18}$ primer at 65ºC for 15 min and immediately chilled on ice. First-strand complementary DNA was synthesized using a reverse transcription (RT) mixture (20 μl) containing 200 units of Superscript reverse transcriptase (Invitrogen, USA), 0.1 M dithiothreitol, 0.5 mM deoxynucleotide triphosphates (dNTPs), 10 units of ribonuclease inhibitor (Invitrogen, USA) and 1x first-strand cDNA synthesis buffer and incubated for 1 h at 42ºC. Polymerase chain reaction (PCR) was performed in a PTC 200 Thermal

Cycler (MJ Research, USA) using a PCR reaction mixture (50 µl) containing 5 µl of cDNA, 1 µl of each primer, 1x PCR reaction buffer, 1 µl dNTPs, and 0.3 µl Supertherm *Taq* polymerase. The protocol included a 10-min preincubation at 94ºC, followed by 40 cycles of 1 min at 94ºC (denaturation), 1 min at 60ºC (primer annealing), and 1 min at 72ºC (extension). PCR products (5 µl) were separated on 1% agarose gels using 100-bp DNA ladder as standard (Invitrogen, USA). Gels were stained in 0.1% ethidium bromide, and the DNA visualized using the GelDoc 1000 system (BioRad, USA).

Total RNA (40µg) from fish tissues was reverse-transcribed using Supercript II RNase H^- reverse transcriptase (Invitrogen). 450 ng of the total RT product (from each organ) were used as templates for PCR amplification, and 28S-F and 28S-R primers were used to amplify the 28S rDNA (115 bp) from the same template for control. The 30-cycle amplification profile consisted of: 95°C for 30 s; 63°C for 30 s; and 63°C for 2 min.

DNA sequencing and BLAST analysis

PCR products were cloned into pGEM-T (Promega) or pUC18 (Amersham Biosciences) vectors for DNA sequencing. Plasmid DNA was purified using the Miniprep Concert kit (Invitrogen) and nucleotide sequences were determined in an automated DNA Sequencer (ABI 377, Applied Biosystems) using the BigDye cycle sequencing kit (Applied Biosystems). Homology searches were performed using the online BLAST programs at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih. gov/BLAST/).

Genome Walking

Genomic DNA of grass carp was extracted and digested separately with four different restriction enzymes (*Dra*I, *EcoR*V, *Pvu*II and *Stu*I). Fully-digested DNA was purified by phenol chloroform and ligated to GenomeWalker adaptors (provided in the universal GenomeWalker kit; Clontech). The amplification profile consisted of: 7 cycles of 94°C for 25 s and 72°C for 3 min; 32 cycles of 94°C for 25 s and 67°C for 3 min; followed by a 10-min incubation at 67°C. DNA products were cloned into pGEM-T Easy vector (Promega Corp., USA) for DNA sequencing.

Random Amplification of cDNA Ends (RACE) by PCR

The start and termination sites of gene transcripts were determined by 5′-RACE (Roche) and 3′-RACE PCR (3′-RACE System; Invitrogen), respectively, according to the instructions provided in the kits. Total RNA from the kidney of grass carp exposed to 4 h hypoxia was used as template for first-strand cDNA synthesis. The amplification profile consisted of: 94°C for 2 mins; 35 cycles at 94°C for 30 s, 62°C for 30 s and 72°C for 2 min followed by a 10-min incubation at 72°C. DNA products were cloned into pGEM-T Easy vector (Promega) for sequencing.

RESULTS AND DISCUSSION

In our laboratory, a 355-bp *VEGF-A* cDNA fragment was previously obtained by Zhang (2002) from grass carp by reverse-transcription-PCR (RT-PCR) using VEGF-specific primers directed at conserved sequences derived from multiple alignment of *VEGF-A* genes from different sources. Blast search showed that the DNA fragment shares high sequence identity with the VEGF-A gene of zebrafish (93%), quail (84%), chicken (84%) and human (80%). In an attempt to obtain the full length *gcVEGF-A* cDNA from grass carp, Rapid Amplification of cDNA Ends (RACE)-PCR was performed to obtain additional sequences at the 5′- and 3′-ends of the 355-bp *gcVEGF-A* cDNA fragment (which is designated here as GV-1). However, only 3′-RACE successfully extended the sequence information at the 3′-end of GV-1. Therefore, nucleotide sequences at the 5'-end of GV-1 were obtained by means of genome-walking. Several primers were designed based on the GV-1 sequence for this purpose and were tested on *DraI*, *EcoRV*, *PvuII* and *StuI* "restriction" genomic libraries prepared from grass carp genomic DNA according to instructions in the Genome-Walker kit (Clontech). Several PCR-amplified bands were obtained from the libraries but only the 1081-bp genomic fragment from the *DraI* library was found to contain VEGF-like sequences. Computer analysis showed that a 197-bp exon that shares high sequence identity (85%) with the exon 3 sequence of the mouse *VEGF-A* gene (accession number U41383) is contained in the genomic fragment. To date, all alternatively-spliced variants of the *VEGF-A* genes in vertebrates are known to contain sequences for exons 1-5 (Jin et al., 2000; Mueller et al., 2000; Gong et al., 2004). On this premise, primers based on the 197-bp "exon 3" sequence of the *gcVEGF-A* gene fragment were synthesized for 5′-RACE.

5′- and 3′-RACE of the gcVEGF-A cDNA

Two DNA fragments of 866 bp (VA2) and 1254 bp (VA1) were obtained by means of 3' RACE (Figure 1). DNA sequencing and computer analysis showed that VA1 and VA2 each contains a polyadenylation site as well as 3'-untranslated sequences that overlap with each other indicating that they encode for different gcVEGF-A transcripts with different polyadenylation sites. Comparison of the VA1 and VA2 sequences from grass carp with the human *VEGF-A* isoforms indicated that VA1 and VA2 which lack both exon 6 and exon 7 sequences are likely derived from alternatively-spliced gcVEGF- since these exonic regions are known to be absent in mammalian VEGF-A_{121} isoforms (Jin *et al*. 2000). However, both VA1 and VA2 contain exon 8 sequences and the TGA stop codon. In addition, sequence analysis showed that a 675-bp and a 1081-bp 3′-UTR is present in VA2 and VA1, respectively. We next proceeded to carry out 5' RACE and isolated a 362-bp 5′-RACE product using gene-specific primers (data not shown), DNA sequencing of which confirmed the 5′-RACE fragment contain exon 1-2 sequences of the *gcVEGF-A* gene and the ATG start codon. Based on the collective sequence information of VA1, VA2 and the 5- and 3'-RACE PCR products, exon 1-8 sequences of the grass carp VEGF-A cDNA were assembled which corresponds to the gcVEGF-A_{206} isoform, and the deduced amino acid sequence of which is shown in Figure 2.

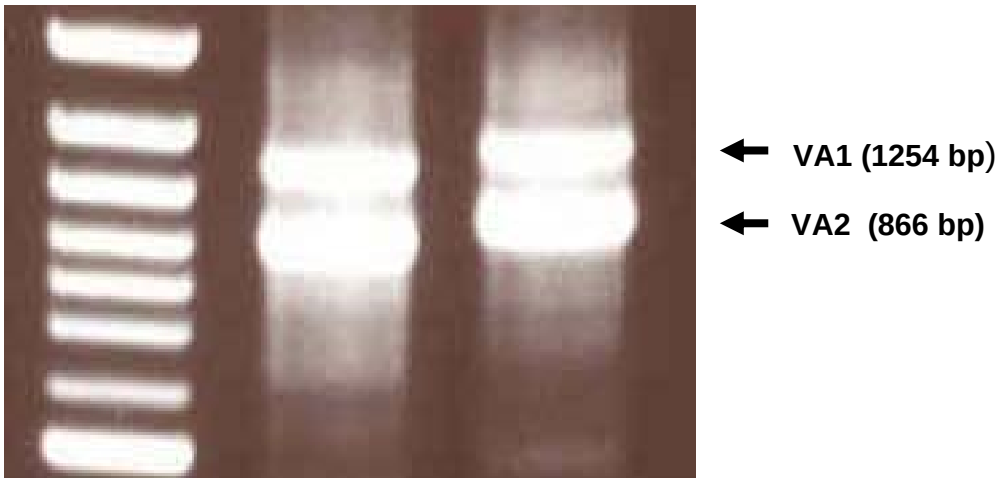


Figure 1. 3′-RACE-PCR of grass carp VEGF-A.
M, 100 bp ladder plus (Fermentus); l**ane 1,** secondary RACE product; and l**ane 2,** primary RACE product. Total RNA from kidney of grass carp exposed to 4 h hypoxia was reverse transcribed into cDNA for RACE reactions. After the ligation of an oligo d(T) adaptor, primers 3'VArace1 (**1**) and VAL1 (**2**) were used for PCR. After 35 cycles of 94°C (30 s), 62°C (30 s) and 72°C (2 min), products were analyzed on 1% agarose.

Deduced amino acid sequence of the gcVEGF-A_{206} isoform

The open reading frame of gcVEGF-A_{206} is 789 bp long and encodes a protein of 263 amino acids, with a predicted molecular mass of 30.5 kDa and *p*I of 9.23. Similar to the mouse VEGF-A protein, gcVEGF-A_{206} contains 8 highly-conserved cysteine residues, six of these – C (49), C (91); C (80), C (124), C (84) and C (126) – are involved in intramolecular disulphide bonding while two – C (74) and C (83) – are involved in intermolecular disulphide bonding to facilitate VEGF-A dimerization. All 8 cysteine residues are located in the peptide region encoded by exons 1 to 5 which are conserved in all vertebrate VEGF-A isoforms analyzed so far (Tischer *et al*. 1991; Shima *et al*. 1996).

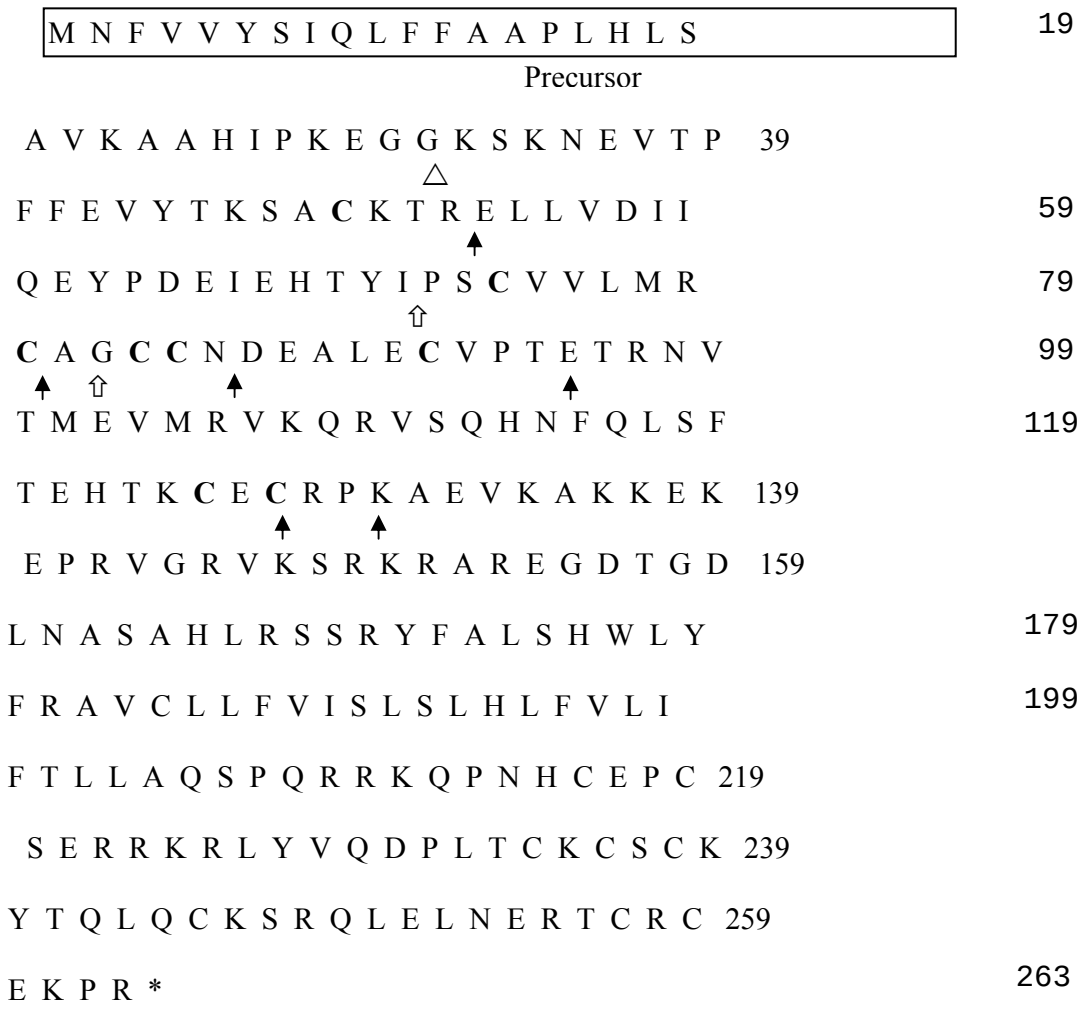


Figure 2. Deduced amino acid sequence of the grass carp VEGF-A_{206} isoform.
The grass carp VEGF-A_{206} deduced amino acid sequences are shown below. The amino acid positions are indicated on the right. The one-letter amino acid code is used and is as follows: A, alanine; R, arginine; N, asparagines; D, asparate; C, cysteine; E, glutamate; Q, glutamine; G, glycine; H, histidine; I, isoleucine; L, leucine; K, lysine; M, methionine; F, phenylalanine; P, Proline; S serine; T, threonine; W, tryptophan; Y, tyrosine; V, valine; *, stop codon. Disulphide bridge sites (cysteine residues) are indicated by arrows. Black arrows (↑) indicate sites for intra-chain disulphide linkages within each monomer, open arrows (⇧) indicate sites for inter-chain disulphide linkages between monomers. The glycosylation (asparagine, N) site is indicated by an upright triangle (△). The precursor protein of gcVEGF is boxed and labelled.

The C-terminus of each isoform, which is associated with VEGF-receptor binding may be different but the core protein structure including the conformation and dimerization domains are maintained.

Detection and isolation of different *gcVEGF-A* isoforms by RT-PCR

In 1998, Liang *et al*. (2001) reported two VEGF-A isoforms (VEGF-A_{121} and VEGF-A_{165}) in zebrafish. More recently, several additional VEGF-A isoforms that show differential expression and distinct spatial distribution patterns were reported in the zebrafish (Gong *et al*. 2004). To determine the number of VEGF-A isoforms that may potentially be expressed in grass carp, total RNA from heart tissue of grass carp exposed to hypoxia for 4 h was used for reverse-transcription PCR (RT-PCR) with primers RTF1 and RTR1. These primers are directed at exon 3 and the 3′-untranslated region (exon 8) of the gcVEGF-A_{206} cDNA, respectively. Four RT-PCR products (697 bp, 829 bp, 922 bp and 1055 bp in size) were obtained and were confirmed by DNA sequencing (and by sequence comparison to the different human VEGF-A isoforms) to encode four distinct gcVEGF-A isoforms which include: gcVEGF-A_{121}, gcVEGF-A_{145}, gcVEGF-A_{189} and gcVEGF-A_{206} (Figure 3).

Genomic organization and promoter structure of the *gcVEGF-A* gene

Based on sequence information of the gcVEGF-A_{206} cDNA, the number and size of introns in the *gcVEGF-A* gene were determined by PCR amplification of grass carp genomic DNA using primers directed at adjacent exons. Numerous primer pairs were designed for this purpose and the genomic PCR products obtained from the different reactions (which ranged between ~100 bp to ~3.5 kb in size) were sequenced and assembled. Computer analysis indicated that the *gcVEGF-A* gene spans ~ 13 kb of nucleotide sequence and consists of 8 exons and 7 introns (Figure 3). Thus it has a very similar structure to the *VEGF-A* genes of human, mouse and zebrafish (Tischer *et al*. 1991; Shima *et al*. 1996; Gong *et al*. 2004). By means of genome walking, ca. 2-kb of 5′-flanking sequence upstream of the transcription start site was determined and analyzed by computer for putative transcription factor-binding sites. Seven AP1 and HBS (HIF-binding sites) are located at various positions in the 2-kb stretch of 5′-flanking sequence (data not shown) which may be involved in molecular responses to hypoxia as in the human *VEGF-A* gene (Salnikow *et al*. 2002). Interestingly, four estrogen-responsive elements (EREs) are detected at several sites in the *gcVEGF-A* gene − two in the 5′-flanking sequence, one in the 5′-UTR and one in the 3′-UTR; a feature very similar to those reported in the human and mouse *VEGF-A* genes (Hyder *et al*. 2000; Mueller *et al*. 2000). This observation suggests that *gcVEGF-A* expression in grass carp is possibly also regulated by estrogen or estrogen-like compounds in a manner similar to those in mammals (Ochoa *et al*. 2000; Long *et al*. 2001).

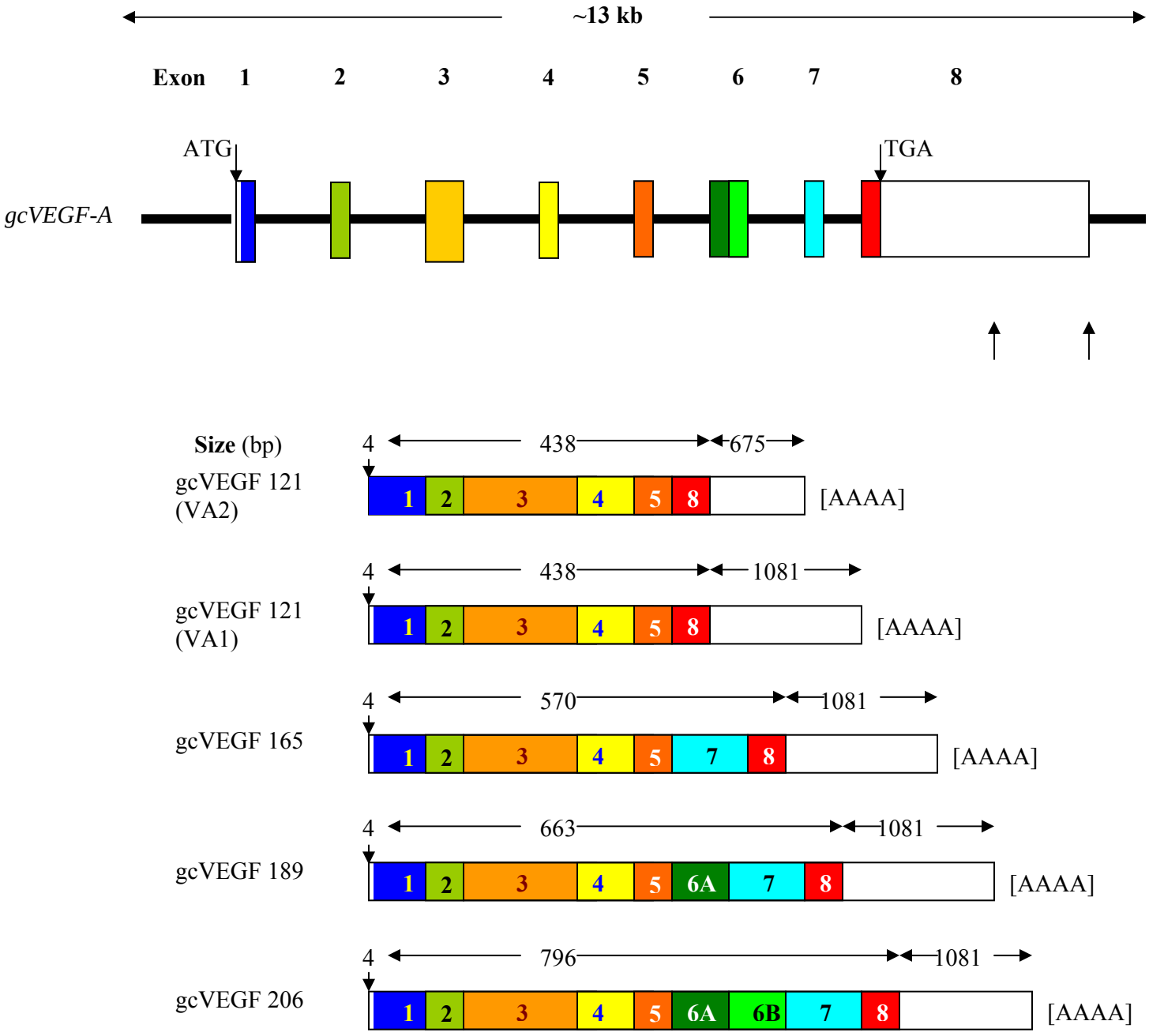


Figure 3. Organization of grass carp VEGF-A gene.
The eight exons of the gcVEGF-A gene are shown as boxes. Filled and open boxes indicate translated and untranslated regions, respectively. The position of the start (ATG) and stop (TAA) codons are indicated by inverted arrows (↓). The two putative polyadenylation sites (ATTAAA) are marked by upright arrows (↑). The 5′-untranslated region, coding sequence and 3′-untranslated region of each transcript are indicated.

Concluding Remarks

VEGF-A is an important angiogenic molecule that not only stimulates new blood vessel formation but is also a potent regulator of vascular permeability (Dvorak *et al*. 1995). In mammals, numerous studies have demonstrated that steroid hormones control angiogenesis and vascular permeability both *in vivo* (Hyder *et al*. 1996) and in primary cultures derived from the reproductive tract (Charnock-Jones *et al*. 2002). These observations strongly suggest that VEGF-A may mediate certain actions of sex steroids, and is likely to play a number of physiological and pathological roles in the regulation of reproductive functions. While comparatively more information is available in mammalian systems on the potential regulatory roles of different VEGF-A isoforms in reproductive tissues, similar information about the functions of VEGF-A in fish remains limited. The isolation and characterization of different VEGF-A isoforms from the grass carp in this study will allow further investigations on the mechanisms by which sex steroids and their agonists/antagonists regulate VEGF-A expression and reproductive functions in fish.

REFERENCES

Breier, G., U. Albrecht, S. Sterrer, and W. Risau. 1992. Expression of vascular endothelial growth factor during embryonic angiogenesis and endothelial cell differentiation. Development 114: 521-531.

Charnock-Jones, D.S., Y. He, and S.K. Smith. 2002. Angiogenesis and vascular endothelial growth factor (VEGF) in reproduction. New Angiotherapy: 115-128.

Dvorak, H.F., L.F. Brown, M. Detmar, and A.M. Dvorak. 1995. Vascular permeability factor/vascular endothelial growth factor, microvascular hyperperbility, and angiogenesis. American Journal of Pathology 146: 1029-1039.

Dvorak, H.F. 2002. Vascular permeability factor/vascular endothelial growth factor: a critical cytokine in tumor angiogenesis and a potential target for diagnosis and therapy. Journal of Clinical Oncology 20: 4368-4380.

Ferrara, N. 2004. Vascular endothelial growth factor: Basic science and clinical progress. Endocrine Reviews 25: 581-611.

Gong, B., D. Liang, T.G. Chew, and R. Ge. 2004. Characterization of the zebrafish vascular endothelial growth factor A gene: comparison with *vegf-A* genes in mammals and Fugu. Biochimica et Biophysica Acta 1676: 33-40.

Hyder, S. M., Z. Nawaz, C. Chiappetta, and G.M. Stancel. 2000. Detection of classical and atypical estrogen response elements in the untranslated regions of the rat VEGF gene. Cancer Research 60: 3183-3190.

Hyder, S.M., G.M. Stancel, C. Chiappetta, L. Murthy, H.L. Boettger-Tong, and S. Makela. 1996. Uterine expression of vascular endothelial growth factor is increased by estradiol and tamoxifen. Cancer Research 56: 3954-3960.

Jin, K. L, X.O. Mao, T. Nagayama, P.C. Goldsmith, and D.A. Greenberg. 2000. Induction of vascular endothelial growth factor receptors and phosphatidylinositol 3'-kinase/Akt signaling by global cerebral ischemia in the rat. Neuroscience 99: 577-585.

Küsters, B., M.W. Robert, P.W. de Waal, V. Kiek, C. Maass, A. Heerschap, J.O. Barentsz, F. Sweep, D.J. Ruiter, and W.P.J. Leenders. 2003. Differential effects of vascular endothelial growth factor A isoforms in a mouse brain metastasis model of human melanoma Cancer Research 63: 5408-5413.

Levy, A.P., N.S. Levy, and M.A. Goldberg. 1996. Post-transcriptional regulation of vascular endothelial growth factor by hypoxia. Journal of Biological Chemistry 271: 2746-2753.

Liang D., J.R. Chang, A.J. Chin, A. Smith, C. Kelly, E.S. Weinberg, and R.W. Ge. 2001. The role of vascular endothelial growth factor (VEGF) in vasculogenesis, angiogenesis, and hematopoiesis in zebrafish development. Mechanisms of Development 108: 29-43.

Long, X-H., K.A. Burke, R.M. Bigsby, and K.P. Nephew. 2001. Effects of the xenoestrogen bisphenol A on expression of vascular endothelial growth factor in the rat. Experimental Biology and Medicine 226: 477-483.

Miller, J. W., A.P. Adamis, D.T. Shima, P.A. D'Amore, R.S. Moulton, M.S. O'Reilly, J. Folkman, H.F. Dvorak, L.F. Brown, B. Berse, T.K. Yeo, and K.T. Yeo. 1994. Vascular endothelial growth factor/vascular permeability factor is temporally and spatially correlated with ocular angiogenesis in a primate model. American Journal of Pathology 145: 574-584.

Mueller, M. D. et al. 2000. Regulation of vascular endothelial growth factor (VEGF) gene transcription by estrogen receptors α and β. Proceedings of the National Academy of Sciences, USA 97: 10972-10977.

Pepe, G. and E. Albrecht. 1999. Regulation of functional differentiation of the placental villous syncytiotrophoblast by estrogen during primate pregnancy. Steroids 64: 624-627.

Nakamura, J., A. Savinvov, Q. Lu, and A. Brodie. 1996. Estrogen regulates vascular endothelial growth/permeability factor expression in 7,12-dimethylbenz(a)anthracene-induced rat mammary tumors. Endocrinology 137: 5589-5596.

Nakamura, J., Q. Lu, G. Aberdeen, E. Albrecht, and A. Brodie. 1999. The effect of estrogen on aromatase and vascular endothelial growth factor messenger ribonucleic acid in the normal non-human primate mammary gland. Journal of Clinical Endocrinology and Metabolism 84: 1432-1437.

Ochoa, A. L. et al. 2000. Vascular endothelial growth factor in the rat pituitary: differential distribution and regulation by estrogen. Journal of Endocrinology 165: 483-492.

Rudolfsson, S, H., P. Wikstroem, A. Jonsson, O. Collin, and A. Bergh. 2004. Hormonal regulation and functional role of vascular endothelial growth factor A in the rat testis. Biology of Reproduction 70: 340-347.

Ruohola, J.K. et al. 1999. Vascular endothelial growth factors are differentially regulated by steroid hormones and antiestrogens in breast cancer cells. Molecular and Cellular Endocrinology 149: 29-40.

Salnikow, K., T. Kluz, M. Costa, D. Piquemal, Z.N. Demidenko, K.P. Xie, and M.V. Blagosklonny. 2002. The regulation of hypoxic genes by calcium involves c-Jun/AP-1, which cooperates with hypoxia-inducible factor 1 in response to hypoxia. Molecular and Cellular Biology 22: 1734-1741.

Shang, E.H.H. and R.S.S. Wu. 2004. Aquatic hypoxia is a teratogen and affects embryonic development. Environmental Science and Technology 38: 4763-4767.

Shima, D.T., M. Kuroki, U. Deutsch, Y.S. Ng, A.P. Adamis, and P. D'Amore. 1996. The mouse gene for vascular endothelial growth factor. Journal of Biological Chemistry 271: 3877-3883.

Shweiki, D., A. Itin, G. Neufeld, H. Gitay-Goren, and E. Keshet. 1993. Patterns of expression of vascular endothelial growth factor (VEGF) and VEGF receptors in mice suggest a role in hormonally regulated angiogenesis. Journal of Clinical Investigation 91: 2235-2243.

Stoner, M., B. Saville, M. Wormke, D. Dean, R. Burghardt, and S. Safe. 2002. Hypoxia induces proteasome-dependent degradation of estrogen receptor α in ZR-75 breast cancer cells. Molecular Endocrinology 16: 2231-2242.

Tischer, E., R. Mitchell, T. Hartman, M. Silva, D. Gospodarowicz, J.C. Fidddes, and J.A. Abraham. 1991. The human gene for vascular endothelial growth factor. Multiple protein forms are encoded through alternative exon splicing. Journal of Biological Chemistry 266: 11947-11954.

Wormke, M., M. Stoner, B. Saville, K. Walker, M. Abdelrahim, R. Burghardt, and S. Safe. 2003. The aryl hydrocarbon receptor mediates degradation of estrogen receptor α through activation of proteasomes. Molecular and Cellular Biology 23: 1843-1855.

Wu, R.S.S., B.S. Zhou, D.J. Randall, Y.S. Norman, and P.K.S. Lam. 2003. Aquatic hypoxia is an endocrine disruptor and impairs fish reproduction. Environmental Science and Technology 37: 1137-1141.

Zhang, Z. P. 2002. Molecular responses to hypoxia in the grass carp, *Ctenopharyngodon idellus*. PhD thesis, City University of Hong Kong, Hong Kong.

Zhang, Z.P., R.S.S. Wu, H.O.L. Mok, Y.L. Wang, W.W.KL. Poon, S.H. Cheng, and R.Y.C. Kong. 2003. Isolation, characterization and expression analysis of a hypoxia-responsive glucose transporter gene from the grass carp, *Ctenopharyngodon idellus*. European Journal of Biochemistry 270: 3010-3017.

MOLECULAR STUDIES OF THE HIF TRANSCRIPTION FACTORS OF GRASS CARP

H.W. Law[4], Richard Y.C. Kong, and Rudolf S.S. Wu

ABSTRACT

Widespread occurrence of aquatic hypoxia as a result of eutrophication and increasing organic pollution is a pressing global problem because of its adverse impact on aquatic life. The hypoxia-inducible factor 1 (HIF-1) in vertebrates senses low oxygen levels and responds by switching on a number of genes such as EPO, VEGF and GLUT to help the organism to adapt and survive. So far, three different HIF-α isoforms have been reported and implicated in diverse regulatory functions such as in oxygen/energy homeostasis, apoptosis and reproduction. Unlike mammals, fish often have to contend with varying levels and periods of hypoxic stress in the aquatic environment and invariably respond by invoking a variety of protective responses (molecular, physiological and behavioural) at sublethal levels of hypoxia. However, the type of responses that are initiated at various levels of hypoxia, and how they are related to each other is not clear. There is a paucity of information at the molecular level on HIF factors and their functional roles in fish. In this report, we describe the isolation of full-length cDNAs encoding three different HIF-α proteins – gcHIF-1α, gcHIF-2α and gcHIF-4α – form the hypoxia-tolerant grass carp. The basic features found in the gcHIF-αs and the pairwise comparison of the gcHIF-αs with other HIF-αs is also explained.

INTRODUCTION

Aquatic hypoxia has been a pressing problem in recent decades because of its widespread occurrence and adverse impact on the aquatic environment. Large scale hypoxia/anoxia events affecting thousands of km^2 have been reported worldwide, including North and South America, Africa, European seas, Pacific and Indian Oceans and many coastal areas (Diaz and Rosenberg 1995; Wu 1999). Aquatic hypoxia is defined as dissolved oxygen (DO) levels between 0 mg/l (anoxia) and 2.8 mg/l (Diaz
and Rosenberg 1995). Aquatic hypoxia is a natural phenomenon and is a consequence of anthropogenic activities, for example, eutrophication, overstocking of fish and shellfish (Gundersen and Jørgensen 1990). Since oxygen availability in aquatic environments fluctuates greatly daily, seasonally and spatially, hypoxia has become one of the major environmental stresses that fish have to contend with in order to survive, and they have evolved a variety of behavioral, physiological and biochemical mechanisms to aid survival under hypoxia (Randall 1982; Storey 1988; Petersen and Petersen 1990). Hypoxia-inducible factor 1 (HIF-1) has been reported to be a master regulator of cellular and systemic oxygen homeostasis in mammals (Semenza 1999) and plays a crucial role in upregulating dozens of hypoxia-responsive genes at the transcriptional level to facilitate oxygen uptake or delivery and anaerobic energy production (Semenza 2000; Bracken *et al*. 2003). More recently, HIF-2 has been shown to play a more dominant role in erythropoiesis (Fine and Norman 2002) and angiogenesis (Takeda *et al*. 2004) while HIF-3 appears to act as a negative regulator of HIF-mediated responses to hypoxia (Hara *et al*. 2001).

To date, there is a paucity of information at the molecular level on HIF factors and their functional

[4] Department of Biology and Chemistry, City University of Hong Kong, Tat Chee Avenue, Kowloon, Hong Kong

roles in fish. Although HIF-1α and HIF-2α have been reported, respectively, in rainbow trout (Soitamo *et al*. 2001) and Atlantic killifish (Powell and Hahn 2002), the molecular mechanisms by which these factors regulate cellular and physiological responses to hypoxia and/or other environmental stresses in fish have not been elucidated. Unlike mammals, fish often have to contend with varying levels and periods of hypoxic stress in the aquatic environment and already it is known that there is a wide range of chronic and protective responses to sublethal levels of hypoxia amongst different fish species. Based on current knowledge about the HIF family of transcription factors in mammals, it is conceivable that many of the hypoxic responses in fish are also likely to be mediated and regulated by HIF proteins. It is of great interest to investigate the HIFs in fish to gain insights on the relationship between HIF functions and hypoxia tolerance in fish. As a first step to investigate the mechanisms by which HIF proteins mediate different biological processes in the hypoxia-tolerant grass carp, full-length cDNAs encoding different gcHIF-α and gcHIF-β (ARNT) proteins have been isolated in this study. Here we report briefly the isolation and characterization of these basic helix loop helix (bHLH) proteins.

MATERIALS AND METHODS

Experimental fish

Animal care and experiments were performed in accordance with the City University of Hong Kong animal care guidelines. Grass carp, *Ctenopharyngodon idella*, with a weight of around 500 g, were obtained from a commercial hatchery in Panyu, Guangdong, PRC, and acclimatized in 400-L freshwater in fiberglass tanks with circulating, filtered and well-aerated tap water at 20 °C for 1 week prior to experimentation.

Hypoxic system for fish

A continuous flow system (modified from Zhou *et al.* 2000) was set up for constant dissolved oxygen for hypoxia exposure of fish (Figure 1). The system consisted of a 400-L fiberglass holding tank, an oxygen stripping column, a filter unit, a cylinder of compressed N_2 gas and a dissolved oxygen meter. Water in fish tank was pumped to filter unit composing of filtering wool and activated charcoal. N_2 was bubbled into the stripping column at the bottom while filtered water was applied at the top. Deoxygenated water flew back to holding tank. Dissolved oxygen was continuously monitored by an YSI Model 580 dissolved oxygen meter at DO 0.5±0.3 mg/L by controlling N_2 flow rate to oxygen stripping column. Control group was maintained at DO 7.0±0.2 mg/L in fiberglass holding tank with continuously filtered water. Water was kept at 20±1°C and the holding tanks were subjected to 12 L : 12 D cycle.

Cloning of HIF-α and ARNT cDNAs in grass carp

Grass carps were exposed to normoxia (DO 7.0±0.2 mg/L) and hypoxia (DO 0.5±0.3 mg/L) for 4 h. After the exposure period, fish were anesthetized, and tissues were dissected out and snap-frozen in liquid nitrogen, stored at −80°C until ready to be processed. Total RNA was isolated from the tissues by Trizol reagent (Invitrogen) according to the manufacturer's instructions. RNA concentration was determined by spectrophotometry using the GeneQuant II RNA/DNA Calculator (Amersham Biosciences) at λ_{260}.

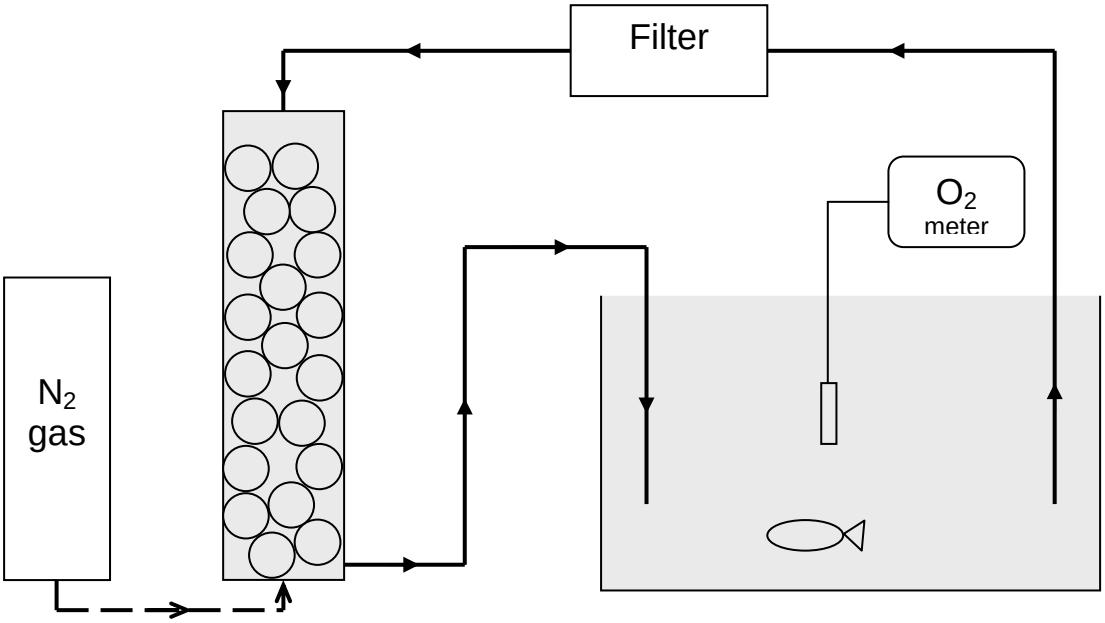


Figure 1. Schematic diagram showing a continuous flow system for hypoxia exposure of grass carp. Water in the fish holding tank is pumped and filtered through filtering wool and a bed of activated charcoal and gravel for removal of solid particles and ammonia waste. Filtered water then passes through an oxygen stripping column where N_2 gas is bubbled at the bottom through a gas stone, in which efficiency of stripping is increased by opposite flow direction of N_2 gas to water, as well as enlarged contact surface area of the gas bubble and water on the engraved bio-balls. Holding tank is replenished with the filtered and deoxygenated water in which a gentle current is created, and dissolved oxygen is monitored at 0.5±0.3 mg/L by an YSI Model 580 dissolved oxygen meter and manual control of the N_2 gas flow rate to the oxygen stripping column. Water flow and gas flow are represented by solid line and broken line, respectively, while direction of flow is denoted by arrows.

DNase I-treated total RNA from kidney of 4 h hypoxically stressed fish was used for reverse transcription. First-strand cDNA was synthesized from 1 μg total RNA with Superscript II RNase H^- Reverse Transcriptase (Gibco/BRL) using Oligo $(dT)_{12\text{-}18}$ primer. PCR amplifications were performed with 10 different combinations of degenerate forward and reverse primers. Amplification products were analysed by agarose gel electrophoresis, purified from gel and cloned into plasmid vectors for DNA sequencing. To obtain the 5′- and 3′-ends of the grass carp HIF-α and ARNT cDNAs, 5′- and 3′-RACE were performed using the Marathon cDNA Amplification Kit (Clontech) with modifications. 5′- and 3′-RACE PCR products were cloned into plasmid vectors for DNA sequencing.

Multiple alignment of nucleotide and deduced amino acid sequences of HIF-1α genes from selected organisms (Table 1) were performed by using Clustal W. Conserved regions in the multiple sequence alignments were identified both manually and by the use of the BOXSHADE program. PCR primers with a degeneracy of less than 100 were designed based on the conserved regions.

Table 1. HIF-1α nucleotide and deduced protein sequences from selected organisms that were used for multiple sequence alignment.

Species	**GenBank accession number**
Oncorhynchus mykiss	AF304864
Danio rerio	(unpublished sequence kindly provided by Dr S.H. Cheng, City University of Hong Kong)
Homo sapiens	NM_001530
Mus musculus	NM_010431
Rattus norvegicus	Y09507
Bos taurus	AB018398
Gallus gallus	AB013746
Xenopus laevis	AJ277829

RESULTS AND DISCUSSION

Degenerate primers directed at highly- conserved regions of the HIF-1αs from different vertebrate species were used in reverse transcription-PCR on total RNA from hypoxic grass carp kidney. Based on DNA sequence analysis, partial cDNA clones encoding three distinct grass carp HIF-αs – gcHIF-1α, gcHIF-2α and gcHIF-4α – were isolated. Using primers directed at two conserved regions of the zebrafish ARNT2b cDNA (GenBank Accession number AF219988), partial gcARNT2b and gcARNT2c cDNAs were also cloned. Since the three partial gcHIF-α cDNAs and the two partial gcARNT cDNAs are incomplete at the 5′- and 3′-ends, gene-specific nested primers (GSP) were designed for 5′- and 3′-RACE PCR in order to obtain the full-length cDNAs. The nucleotide sequence information derived by 5′- and 3′-RACE was assembled to produce the full-length cDNA sequences for gcHIF-1α, gcHIF-2α, gcHIF-4α, gcARNT2b and gcARNT2c. The authenticity of the individual cDNAs was confirmed by full-length reverse transcription-PCR.

Characterization of the gcHIF-α cDNAs

Computer analysis showed that gcHIF-1α (3,848 bp in length) contains a 5′-untranslated (UT) region of 257 bp, a 3′-UT region of 1,238 bp and an open reading frame (ORF) of 2,325 bp which encodes for a protein of 774 amino acids. The gcHIF-2α cDNA (3,195 bp in length) contains 5′- and 3′-UT regions of 263 bp and 424 bp, respectively, and an ORF of 2,508 bp which encodes for a protein of 835 amino acids. The gcHIF-4α (2,092 bp in size) contains a 5′-UT region of 102 bp, a 3′-UT region of 29 bp and an ORF of 1,932 bp which encodes for a protein of 643 amino acids.

Pairwise comparison of gcHIF-1α with selected HIF-α cDNAs from the GenBank database (Table 2) revealed that gcHIF-1α shares high sequence identity at the protein and nucleotide levels with the HIF-1αs of rainbow trout (68%; 72%), human (64%; 63%) and cow (65%; 62%); while only moderate sequence identity (52%–57% at the nucleotide level) with the HIF-2α and HIF-3α paralogs was observed. Homology search showed that gcHIF-2α shares high sequence identity with the killifish (69% at DNA, 62% at amino acid), mammalian (64%–65% at DNA, 57% at amino acid) and avian (63% at DNA, 58% at amino acid) HIF-2α cDNAs.

Table 2. Percentage sequence identities of the ORFs of gcHIF-αs to known HIF-α isoforms in the GenBank database. Identity scores were derived using the GAP program of GCG softwares. "aa" represents identity at amino acid level; "nt" represents identity at nucleotide level.

	GenBank Accession No.	Species	Size (amino acid)	gcHIF-1α		gcHIF-2α		gcHIF-4α	
				aa	nt	aa	nt	aa	nt
HIF-1α	AF208487	*Homo sapiens*	826	64	63	44	51	41	51
	NM_010431	*Mus musculus*	836	64	63	40	52	41	49
	Y09507	*Rattus norvegicus*	825	64	64	43	53	42	52
	AB018398	*Bos taurus*	823	65	62	44	65	42	53
	AB013746	*Gallus gallus*	811	65	65	45	52	47	51
	AJ277829	*Xenopus laevis*	805	57	62	40	53	43	54
	AF304864	*Oncorhynchus mykiss*	766	68	72	44	59	44	56
	AY450269	*Ctenopharyngodon idella*	774	100	100	53	57	47	55
HIF-2α	BC051338	*Homo sapiens*	870	53	57	57	64	44	56
	NM_010137	*Mus musculus*	874	52	57	56	64	45	55
	AJ277828	*Rattus norvegicus*	874	52	57	56	64	43	51
	AB018399	*Bos taurus*	870	53	57	57	65	45	57
	AF129813	*Gallus gallus*	868	50	57	59	64	46	53
	AF212989	*Coturnix coturnix*	870	50	56	58	63	46	53
	AF402782	*Fundulus heteroclitus*	873	52	56	62	69	46	53
	AY577524	*Ctenopharyngodon idella*	835	53	57	100	100	46	55
HIF-3α	AB054067	*Homo sapiens*	667	45	52	43	54	44	54
	AF060194	*Mus musculus*	662	44	52	43	54	47	53
	AJ277827	*Rattus norvegicus*	662	44	52	42	55	46	53
HIF-4α	AY450270	*Ctenopharyngodon idella*	643	47	55	46	55	100	100

Interestingly, exceptionally high sequence identity (93%) was observed in a 32-bp nucleotide stretch (nucleotide 71–102) in the 5′-UT region of gcHIF-2α which is highly conserved in the corresponding positions of the quail and human HIF-2α cDNAs. In contrast to gcHIF-1α and gcHIF-2α, gcHIF-4α shares almost equal sequence identity at the protein level with the HIF-1α, -2α and -3α isoforms from various animal species. For example, a 40–44% identity to the HIF-1αs, 45% identity to the HIF-2αs and 45% identity to the HIF-3αs were observed which strongly suggests that gcHIF-4α encodes for a novel HIF-α isoform in grass carp. The full-length gcHIF-1α, gcHIF-2α and gcHIF-4α cDNA sequences have been deposited in GenBank under accession numbers AY450269,

AY577524 and AY450270, respectively.

With reference to the deduced amino acid sequence of human HIF-1α, the characteristic domains typical of HIF-α proteins are present in all three gcHIF-αs proteins which include: the basic helix-loop-helix (bHLH) domain; Per-Sim-ARNT (PAS) -A and -B domains; PAS-associated C-terminal domain (PAC); oxygen dependent degradation (ODD) domain; and the N- and C-terminal transactivation domains (N-TAD and C-TAD) (Figure 2). These domains are known to have a specialized function, for example bHLH, PAS-A and -B and PAC are required for dimerization with ARNT molecules and DNA binding to target genes (Jiang et al., 1996); ODD senses cellular oxygen tension and controls HIF-α protein stability (Jiang *et al*. 1997) and; the N-TAD and C-TAD regions are for transcriptional activation of HIF target genes (Jiang *et al*. 1997).

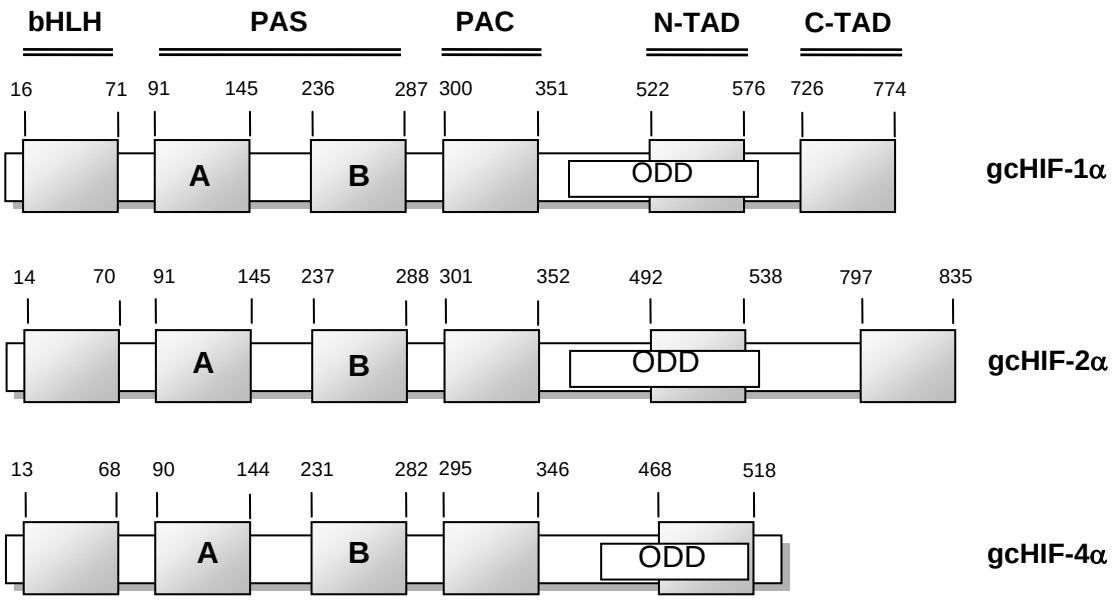


Figure 2. Comparison of homologous domains between the gcHIF-1α, -2α and -4α proteins. Each horizontal bar represents a gcHIF-α protein and the amino acid positions demarcating each structural domain are shown accordingly in the diagram. The structural domains are shaded and drawn to scale.

As depicted in Figure 2, both gcHIF-1α and gcHIF-2α contain all of the above structural domains typical of HIF-α while gcHIF-4α lacks a discernible C-TAD region – no significant similarity between the C-terminal region of the putative gcHIF-4α protein and the C-TAD region from the known HIF-1α and -2α proteins could be detected. The structural region of gcHIF-4α that mediates transcriptional activation therefore remains to be elucidated.

Characterization of the gcARNT cDNAs

Computer analysis revealed that the gcARNT2b cDNA contains a 5′-UT region of 128 bp and an ORF of 2214 bp which encodes for a 737-amino acid protein while the gcARNT2c cDNA is an alternatively-spliced variant differing from gcARNT2b in that the former has a 45-bp deletion in the ORF (nucleotide 318–362 of gcARNT2b). The 45-bp deleted region encodes for a 15-amino acid peptide, YDDDQIPGDKERYAR, in the N-terminal half of the protein. The putative gcARNT2b and gcARNT2c proteins were compared with the ARNT sequences from different species (available in the GenBank) and amongst them, the zebrafish ARNT2b (AF219988) and zebrafish ARNT2c (AF219989) proteins share the highest amino acid sequence identity with gcARNT2b (94%) and gcARNT2c (96%), respectively. The gcARNT2b and gcARNT2c sequences have been deposited in GenBank under accession numbers AY596921 and AY596922, respectively.

CONCLUSIONS

In this study, the full-length cDNAs of gcHIF-1α, gcHIF-2α and the novel isoform gcHIF-4α have been cloned. The sequence similarity of the grass carp proteins to mammalian and avian HIF-αs was examined by computer and gcHIF-4α was found to be a novel isoform sharing almost equal sequence identity with the three types of HIF-α proteins (HIF-1α, HIF-2α and HIF-3α) that have been reported thus far in mammals. Our studies also indicated that gcARNT2b and the alternatively spliced variant gcARNT2c are present in grass carp. It will be interesting to investigate the dimerization affinities between the different gcHIF-αs and gcARNTs and the transactivation capacity and biological functions of the different HIF proteins in fish.

ACKNOWLEDGEMENT

Funding for this study was supported partially by a Strategic Grant (Project No. 7001415) and a Central Earmarked Research Grant (Project No. CityU1094/01M) from the Research Grants Council of Hong Kong Special Administrative Region, People's Republic of China. SHWL was supported by a City University of Hong Kong studentship.

REFERENCES

Bracken, C.P., M.L. Whitelaw, and D.L. Peet. 2003. The hypoxia-inducible factors: key transcriptional regulators of hypoxia responses. Cellular and Molecular Life Science 60: 1376–1393.

Diaz, R.J., and R. Rosenberg. 1995. Marine benthic hypoxia: a review of its ecological effects and the behavioral responses of benthic macrofauna. In: Oceanography and Marine Biology: an Annual Review. Volume 33. Ansell, A.D., Gibson, R.N., and Barnes, M. (eds) UCL Press, London. 245–303.

Fine, L.G., and J.T. Norman. 2002. The breathing kidney. Journal of the American Society of Nephrology 13: 1974–1976.

Gundersen, J.K., and B.B. Jørgensen. 1990. Microstructure of diffusive boundary layers and the oxygen uptake of the sea floor. Nature 345: 604–607.

Hara, S., J. Hamada, C. Kobayashi, Y. Kondo, and N. Imura. 2001. Expression and characterization of hypoxia-inducible factor (HIF)-3α in human kidney: suppression of HIF-mediated gene expression by HIF-3α. Biochemical and Biophysical Research Communications 287: 808–813.

Jiang, B., E. Rue, G.L. Wang, R. Roe, and G.L. Semenza. 1996. Dimerization, DNA binding, and transactivation properties of hypoxia-inducible factor 1. Journal of Biological Chemistry 271: 17771–17778.

Jiang, B., J.Z. Zheng, S.W. Leung, R. Roe, and G.L. Semenza. 1997. Transactivation and inhibitory domains of hypoxia-inducible factor 1α. Journal of Biological Chemistry 272: 19253–19260.

Petersen, J.K., and G.I. Petersen. 1990. Tolerance, behavior and oxygen consumption in the sand goby, *Pomatoschistus minutus* (Pallas), exposed to hypoxia. Journal of Fish Biology 37: 921–933.

Powell, W.H., and M.E. Hahn. 2000. The evolution of aryl hydrocarbon signaling proteins: diversity of ARNT isoforms among fish species. Marine and Environmental Research 50: 39–44.

Randall, D.J. 1982. The control of respiration and circulation in fish during exercise and hypoxia. Journal of Experimental Biology 100: 275–288.

Semenza, G.L. 1999. Regulation of mammalian O_2 homeostasis by Hypoxia-inducible Factor 1. Annual Review Cell and Developmental Biology 15: 551–578.

Soitamo, A.J., C.M.I. Råbergh, M. Gassmann, L. Sistonen, and M. Nikinmaa. 2001. Characterization of a hypoxia-inducible factor (HIF-1α) from rainbow trout. Accumulation of protein occurs at normal venous oxygen tension. Journal of Biological Chemistry 276: 19699–19705.

Storey, K.B. 1988. Suspended animation: the molecular basis of metabolic depression. Canadian Journal of Zoology 66: 124–132.

Takeda, N., K. Maemura, Y. Imai, T. Harada, D. Kawanami, T. Nojiri, I. Manabe, and R. Nagai. 2004. Endothelial PAS domin protein 1 gene promotes angiogenesis through the transactivation of both vascular endothelial growth factor and its receptor, fetal liver tyrosine kinase 1. Circulation Research 95: 146–153.

Wu, R.S.S. 1999. Eutrophication, water borne pathogens and xenobiotic compounds: environmental risks and challenges. Marine Pollution Bulletin 39: 11–22.

Zhou, B.S., R.S.S. Wu, D.J. Randall, P.K.S. Lam, Y.K. Ip, and S.F. Chew. 2000. Metabolic adjustments in the common carp during prolonged hypoxia. Journal of Fish Biology 57: 1160–1171.

GENE EXPRESSION PROFILING OF RAW264.7 CELL SURVIVAL AND APOPTOSIS UNDER OXIDATIVE STRESS

C. C. Fong[5], Y. Zhang, M.S. Wong, C.H. Tzang, W.P. Lai and M. Yang

ABSTRACT

Organisms living in an aerobic environment are continuously exposed to reactive oxygen species (ROS). ROS can induce apoptosis of cells and cells also develop negative feedback mechanisms to limit ROS induced cell death. In this study, we used cDNA microarray to investigate the molecular mechanism of survival and apoptosis of RAW 264.7 cells under ROS. The cells were treated with H_2O_2 and cDNA microarray assay was carried out to produce gene expression profiles. We found that H_2O_2 treatment up-regulated stress, survival and apoptosis related genes, and down regulated growth and cell cycle promoting genes. Metabolism related genes showed special expression pattern under oxidative stress: glycolysis and lipid synthesis related gene were down-regulated whereas the genes of lipid catabolism and protein synthesis were up-regulated. We also identified several signaling pathways as ROS sensitive pathways, including p53, Akt, NF-κB, ERK, JNK, p38, PKC and INF-r. They played important roles in the process of apoptosis or cell survival.

INTRODUCTION

Organisms living in an aerobic environment are continuously exposed to reactive oxygen species (ROS). ROS, including superoxide anion, hydrogen peroxide, singlet oxygen and hydroxy radical, can raise from normal metabolism and pathological processes such as stress, heat, irradiation, hyperoxia, and any increases in metabolism including exercise, injury, and even repair processes, and in wide variety of human disease including tumor, AIDs, Parkinson's, Huntington's (Finkle *et al.* 2000; Chandra *et al*. 2000; Fehrenbach and Northoff 2001). The roles of ROS in living cells have been studied for many years. Direct treatment of cells with oxidants such as hydrogen peroxide (H_2O_2) was thought to exclusively cause necrosis, but more recent studies have shown that under certain situations, ROS can act as signaling molecules to trigger apoptosis, and apoptosis not the only responses of living cells to ROS. To different levels of ROS, the spectrum of response ranges from proliferation to growth arrest, to senescence, and to cell death. In addition, cells also develop negative feedback mechanisms that involve the regulated expression of survival genes to limit the ability of ROS in inducing cell death, in order to survive under oxidative stress (Carmody and Cotter 2001; Nishikawa *et al.* 2000).

To understand how ROS act as signal transduction molecules, people have investigated the regulation of gene expression and signal transduction pathways under ROS exposure. Many genes and signal transduction pathways have been identified as ROS sensitive genes and pathways (Allen and Tresini 2000; Flohe *et al.* 1997). However, previous studies have been limited in that only a small number of genes can be examined for their response to ROS in each experiment by using traditional molecular and cell biological methods. If people want to address ROS sensitive signal pathways,

[5] Department of Biology and Chemistry, City University of Hong Kong, Tat Chee Avenue, Kowloon, Hong Kong

especially the relationships among these pathways, they have to use various small pieces of data collecting from different experiments even in different cell models to make the big pictures. However, this may cause some biases.

In current study, we used cDNA microarray combined with traditional molecular and cell biological methods to investigate the molecular mechanism of survival and apoptosis in H_2O_2 treated RAW 264.7 cells, a mouse monocytic-macrophage like cell line. In the present study, a number of ROS sensitivity genes and signaling pathways related to apoptosis and cell survival were identified. A partial cellular signaling web on how ROS trigger apoptosis in H_2O_2 treated RAW 264.7 cells and how the cells survive under oxidative stress has been culminated through analysis of the data from current study and other people's reports. The signaling web provided insight into the molecular mechanism of apoptosis induced by ROS and feedback mechanisms that involve in cell survival.

MATERIAL AND METHODS

Cell culture and cell viability assay

RAW 264.7 cells (ATCC, VA) were cultured in Dulbecco's modified Eagle's medium (DMEM) (GIBCO BRL, Caithersburg, MD) supplement with 10% fetal bovine. For cell viability assay, 1×10^4 RAW 264.7 cells seeded in a 96 well cell culture plates were treated with H_2O_2 in DMEM at various concentrations from 1.25×10^{-5} to 1.20×10^{-3} mol/L for 30 or 60 min, then H_2O_2 medium was replaced with fresh medium and the cells were cultured for 5hr followed by SRB assay.

Cell cycle analysis and assessment of apoptosis by flow cytometry

Flow cytometry was used to analyze cell cycle and apoptosis. Cells were treated with H_2O_2 in DMEM at dose of 0, 0.5, 0.75 and 1 pmol/cell for 1 hr. After removing the H_2O_2 medium, the cells were cultured in fresh medium for overnight. The cells were then harvested and stained by hypotonic propidium iodide (Sigma, St. Louis, MO) solution. Stained cells were analyzed by EPICS Elite *ESP* flow cytometry (Coulter Electronic, USA) at excitation 488/emmision 600 nm. Data were analyzed by cycle distribution software ModFit LT version 2.0 (Verity Software House, USA).

cDNA microarray analysis

RAW264.7 cells were treated with H_2O_2 in DMEM at dose 1 pmol/cell for 1 hr, then, continuously cultured in fresh medium for 24 hr, meanwhile, untreated cells as control. They were harvested at 1, 4, 8, 12 and 24 hours after H_2O_2 treatment. TRIZOL reagent (Life Technologies, Gaithersburg, MD) was added to extract total RNA from the cultures according to the manufacturer's instruction.

40 μg of total RNA from control or treated cells was reverse-transcripted to cDNA in the presence of SuperScript II reverse transcriptase (Life Technologies, CA). During the reaction, two distinct fluorescent dyes, Cy3-dUTP and Cy5-dUTP (Amersham Pharmacia Biotech, NJ), were incorporated into the cDNA for the control and treated sample, respectively. The purified cDNA sample was mixed with a hybridization solution and applied to a home-made microarray which

presented 1000 genes selected from a mouse clone set provided from NIA, including cell proliferation or cycle, apoptosis, metabolism, transcription and signal transduction related genes. To ensure the reproducibility of the microarray results, each experiment was repeated with newly extracted total RNA samples.

Microarray images were scanned with a confocal laser scanner (ScanArray 4000, GSI Lumonics, MA) and the results were analyzed using ScanAnalyze program (Michael Eisen, Standford University). Genes showing a greater than 2-fold induction or repression after normalization (Cy5/Cy3 ratios above 2 or below 0.5) were selected for further analysis. The cutoff value of 2-fold is conventionally used by other investigators (Quackenbush 2001). Clustering analysis was carried out by using Cluster software offered by Elsein' lab (Standford University, California, USA).

Western blot analysis of phosphorylated p53, Akt and JNK

The cells were lysed in Nonidet P-40 buffer supplemented with NaF, Na_3VO_4 and protease inhibitors (Roche/Boehringer Mannheim, Indianapolis, USA) and subjected to western blot analysis with primary antibodies, p-p53, p-Akt or p-JNK(Santa Cruz Biotechnology Inc.)

Electrophoretic mobility shift assay (EMSA)

Nuclear proteins were isolated using method provided by CellDeath.de. And gel shift assay system (Promega) was used to perform EMSA according to manufacturer's instruction. Briefly, nuclear extracts were incubated with a [γ- ^{32}P] labeled oligonucleotide probe, which contains the specific recognition sequence for NF-κB. After binding, the samples were separated on a non-denaturating PAGE gel and the bands were detected by autoradiography.

RESULTS

The analysis of cellular viability, cell cycle and apoptosis in H_2O_2 treated RAW264.7 cells

The toxic dose of H_2O_2 varies with both the density of the cells and the cell types studied, therefore, we first evaluated the effects of cells density, dose and time exposure of H_2O_2 on our current used RAW264.7 cells. Figure1 summarized the RAW 264.7 cells viability treated with H_2O_2 for 30 or 60 min ranging from 1.25×10^{-5} to 1.20×10^{-3} mol/L. After 30 and 60 minutes exposure, maximum ratio of cell death was 25% and 50%, respectively. The purpose of our experiments is to study the mechanism of apoptosis and cell survival in RAW 264.7 cells treated with H_2O_2. 30% of cell death and 70% of cell survive are optimal ratios and was chosen for studying gene expression profiles. The result of cell viability showed that 30% cell death was induced when the 1000 cells in 0.1 ml of DMEM were treated with H_2O_2 for 1 hr at concentration 100 μM, which is equal to 1 pmol/cell H_2O_2.

Furthermore, RAW 264.7 cells treated with various H_2O_2 concentrations were analyzed by flow cytometer and result of cell cycle distribution analysis were summarized in Table 1. It was found that sub-G1 cells that were destined to enter apoptosis increased proportionally with increasing H_2O_2

concentrations and the percentage of G_0/G_1 phase increased with the increasing H_2O_2 concentration and reached peak at 1.0 pmol/cell; oppositely, S phase reduced significantly. The concentration of 1 pmol/cell H_2O_2 also induced G_2/M arrest slightly.

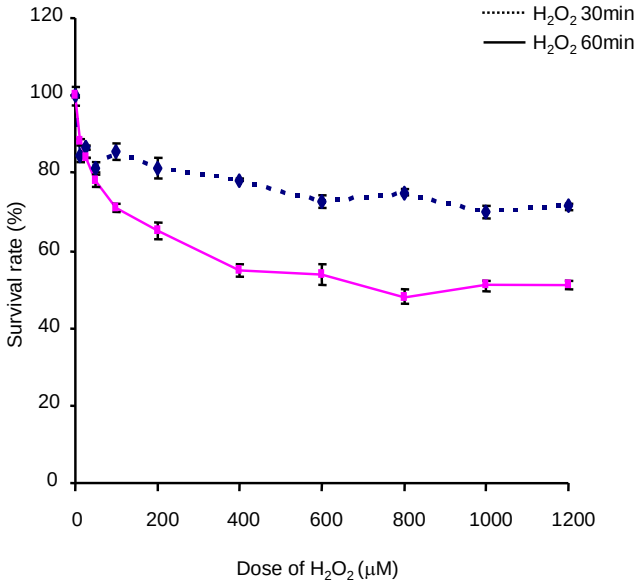


Fig. 1. Cell viability assay. RAW264.7 cells were exposed to H_2O_2 in various concentrations for 30 and 60 minutes. Cell viability was determined using SRB assay. The toxic effect of 60 min H_2O_2 treatment is stronger than 30 min and the percentage of death cells caused by H_2O_2 treatment was time- and dose-dependent. Results were obtained from three independent experiments and expressed as mean±s.d.

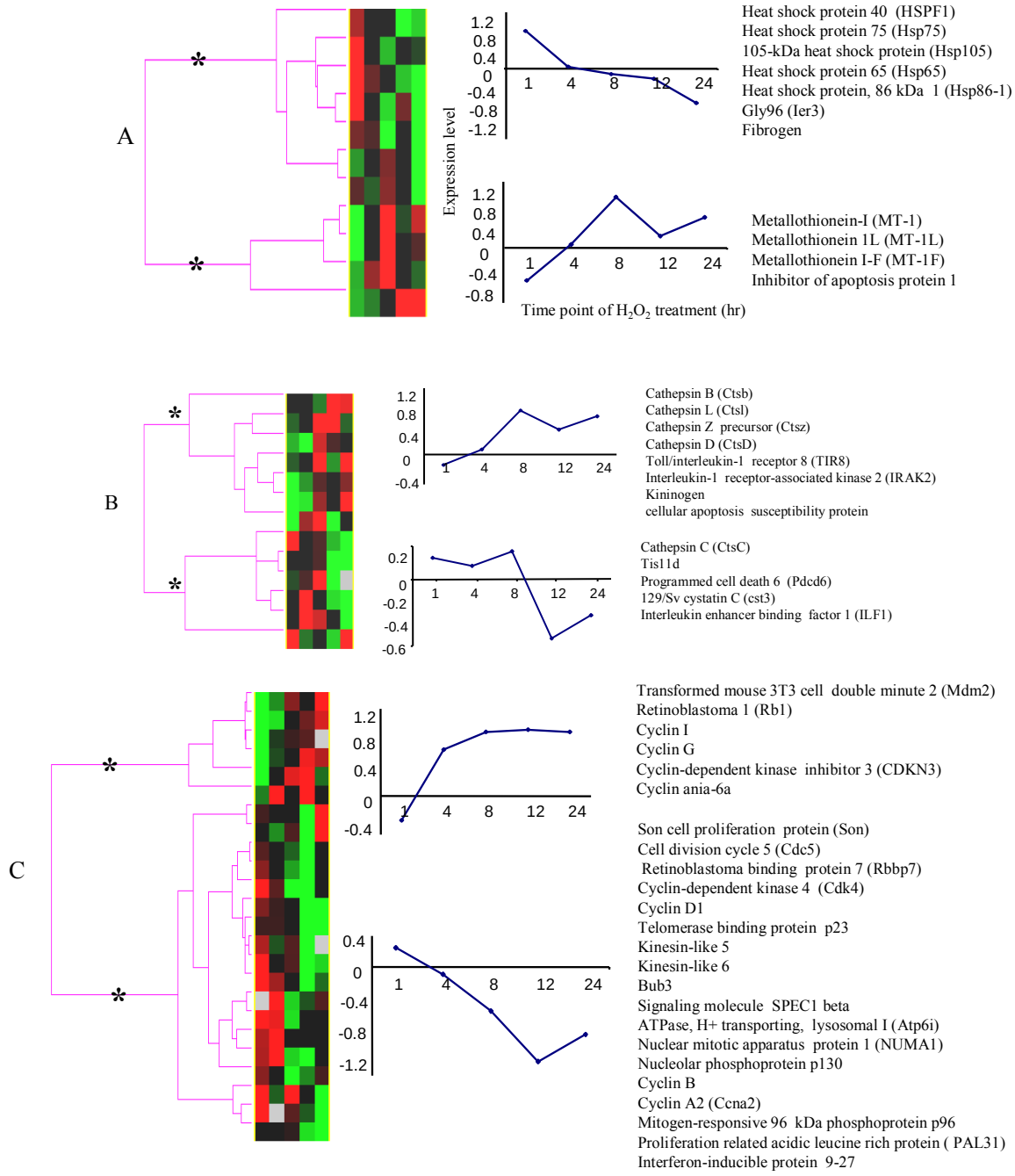
H_2O_2 concentration (pmol/cell)	Distribution of cell cycle (%) (mean + s.d.)			
	G0/G1	S	G2/M	Sub-G1
0.00	51.11±2.12	36.50 ± 3.85	12.39±1.02	0.91±0.00
0.50	67.45±0.23	21.57±2.12	10.98±0.11	6.67±0.85
0.75	78.79±1.58	9.02+1.34	12.18±0.23	19.62±3.85
1.00	82.51±2.36	0.86+0.06	16.63±1.55	28.45±2.69

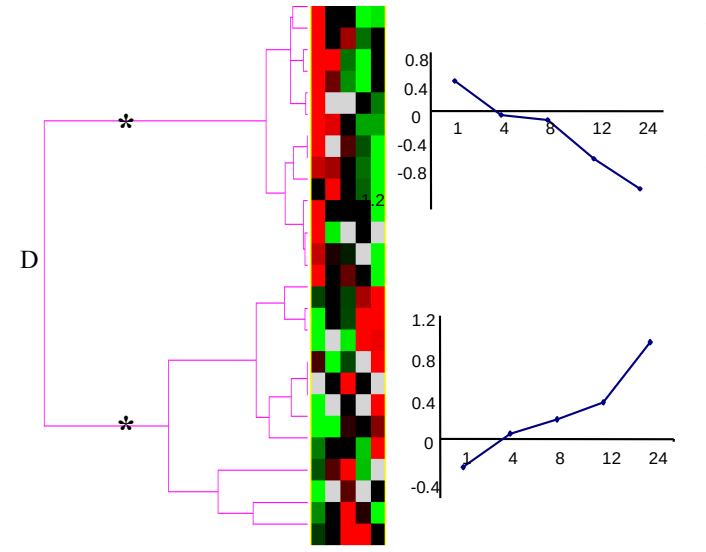
Table 1. Flow cytometry analysis of H_2O_2 treated RAW264.7 cells
(Results were obtained from three independent experiments and expressed as mean ± s.d.)

Gene expression profiles of RAW264.7 cells treated with H_2O_2 and functional cluster analysis

After critical data analysis, genes with a ratio of Cy5/Cy3 >2.0 or <0.5 at least at one time point among five were selected as genes that can be regulated by H_2O_2. After critical data analysis, 113 genes were identified as redox-sensitivity genes. They were divided into eight clusters, including cell survival, apoptosis, cell cycle and proliferation, metabolism, signaling, transcription, proteasomes and cell structure based on functional analysis. A hierarchical clustering algorithm was used to determine

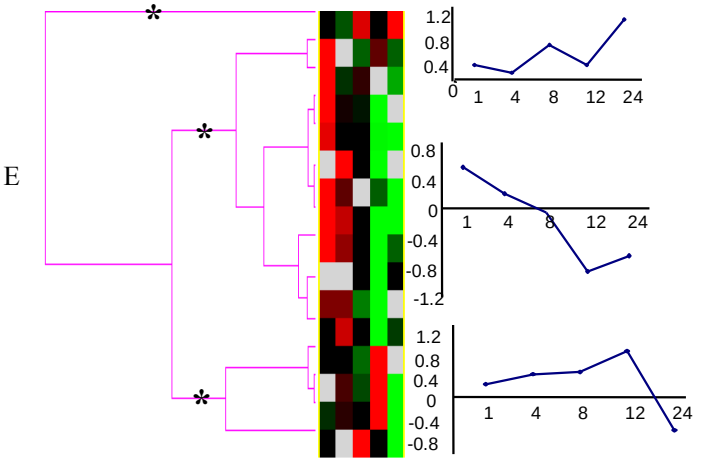
the numbers of genes expression pattern in each functional group, and then K means analysis was applied to produce trend lines that represent the gene expression patterns in each group as summarized in Figure 2A to 2H, respectively. The genes that show coordinated fluctuations in gene expression across 5 time points might have same regulatory mechanism.





Aldolase 1(Aldo1)
Phospholipase c neighboring (Png)
Eno1
High density lipoprotein binding protein (HDLBP)
Lactate dehydrogenase 1, A (Ldh1)
Disulfide isomerase (ERp59
Fatty acid desaturase 1 (FADS1)
Fatty acid synthase (Fasn)
Triosephosphate isomerase (Tpi)
Transketolase (Tkt)
Methylenetetrahydrofolate dehydrogenase (Mthfd1)
Dkc1
Stearoyl-Coenzyme A desaturase 2 (Scd2)

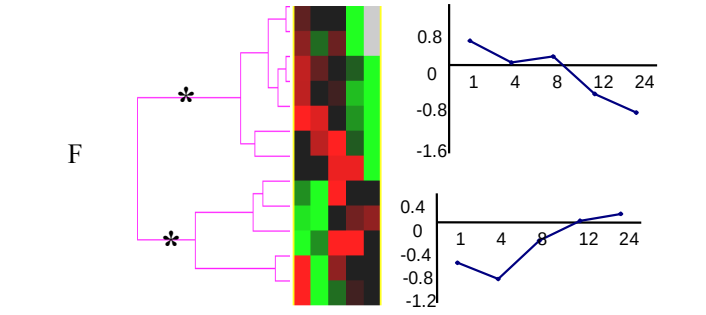
Lipoprotein lipase (Lpl)
Malate dehydrogenase, soluble (Mor2
Elongation factor 1-alpha (EF 1-alpha)
Medium-chain acyl-CoA dehydrogenase (Acadm)
Ca2+-independent phospholipase A2
Small intestine apolipoprotein B (Apobec 1)
Low density lipoprotein receptor related protein (Lrp)
Adipose differentiation-related protein (Adrp)
RPL31
TLS-associated protein TASR-2
Glycyl-tRNA synthetase (Gars)
Paired basic amino acid cleaving enzyme (Furin)



Intracellular signaling protein (Trip6)

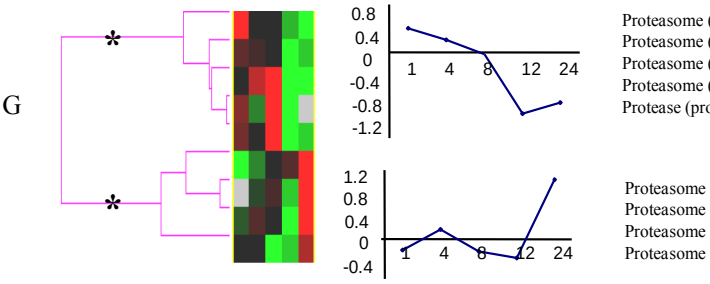
Mitogen-activated protein kinase 6 (Mapk6)
Mitogen inducible gene mig-2 (Mig-2)
Ras 21 activator
p97 ATPase pseudogene (Vcp)
MyD88 mRNA induced by interleukin-6 (Myd88)
Thymoma viral proto-oncogene (Akt)
MAP kinase/Erk kinase (Mek2)
Partial Prkar1a gene for cAMP-dependent protein kinase
COP9 (constitutive photomorphogenic), subunit 7a (Cops7a)
Clathrin adaptor protein, large, beta 1
Clathrin adaptor protein, medium 1

JNK-binding protein 1 (Jnkbp1-pending)
Interferon (alpha and beta) receptor (Ifnar)
Interferon gamma receptor 2 (Ifngr2)
Protein kinase C substrate 80K-H (Prkcsh)



p53 cellular tumor antigen
Transcription factor Sp3 (Sp3)
Interleukin enhancer binding factor 3 (Ilf3)
Adaptor protein complex AP-2, mu1 (Ap2m1)
CCAAT/enhancer binding protein (C/ebp)
Ctbp1
Synaptotagmin 11 (Syt11)

C-fos
E74-like factor 1 (Elf1)
synaptotagmin 4 (Syt4)
Histone deacetylase 1 (Hdac1)
Nuclear receptor co-repressor 1 (Ncor1)



Proteasome (prosome, macropain) 26S subunit, non-ATPase, 1 (Psmd1)
Proteasome (prosome, macropain) 26S subunit, non-ATPase, 8 (Psmd8)
Proteasome (prosome, macropain) 26S subunit, ATPase 3 (Psmc3)
Proteasome (prosome, macropain) subunit, beta type, 2 (Psmb2)
Protease (prosome, macropain) 28 subunit, alpha (Psme1)

Proteasome (prosome, macropain) subunit, alpha type 4 (Psma4)
Proteasome (prosome, macropain) subunit, alpha type 2 (Psma2)
Proteasome beta-subunit C5 (Psmb1)
Proteasome (prosome, macropain) activator subunit 2 (PA28 beta)

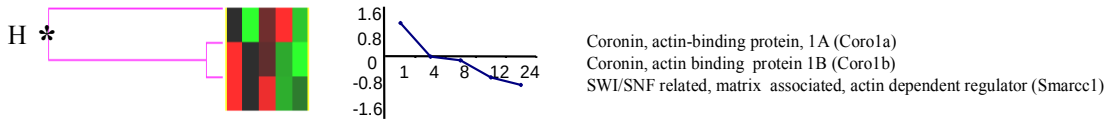


Fig. 2. Clustering analysis. 113 of ROS sensitive genes were divided into 8 groups according to their functions. Hierarchical clustering and K means were used to analyze expression patterns of these genes. (A) Cell survival related genes. (B) Apoptosis related genes. (C) Cell cycle related genes. (D) Metabolism related genes. (E) Signaling molecules. (F) Transcription related genes. (G) Proteasomses. (H) Structural proteins

The change of molecular activity in certain early response molecules in H_2O_2 treated RAW264.7 cell

From gene expression profiles, it was found that the expression of some signaling pathway related genes, such as Akt, p53 and others was regulated by ROS in H_2O_2 treated RAW264.7 cells. In order to get more information about signaling pathways which might be regulated by ROS, the molecular activity of some important signaling molecules in H_2O_2 treated RAW264.7 cell were further investigated.

H_2O_2 up regulates the nuclear NF-κB activity in RAW264.7 cell

Electrophoretic mobility shift assays were used to assess activation of NF-κB in RAW264.7 cells as described before. Our studies revealed an increase in NF-κB activation in RAW264.7 cells at 30 and 60min after H_2O_2 treatment. NF-κB activation had attenuated to the level observed in controls after 2h (Figure 3).

H_2O_2 induces the phosphorylation of p53, Akt and JNK

p53 is a transcriptional factor and its transcriptional activation function requires post-translational modification of the p53 protein via phosphorylation, acetylation, or glycosylation. In this study, the level of the phosphorylated p53 protein was examined using an antibody that specifically recognizes the phosphorylated serine 15 residue of p53. Western blot analysis of cell lysates prepared from H_2O_2-treated RAW264.7 cells demonstrated the phosphorylation of p53 was induced at about 15 min after H_2O_2 treatment (Figure 4a). Like p53, the activation function of Akt and JNK requires phosphorylation. Figure 5b and c shows Akt and JNK activation by H_2O_2. After RAW264.7 cells were treated by H_2O_2 at concentration 1 pmol/cell, phosphorylation of Akt was detected at 15min (Figure 4b), whereas JNK at 30 min, peaked at 1h and attenuated after 2h (Figure 4c).

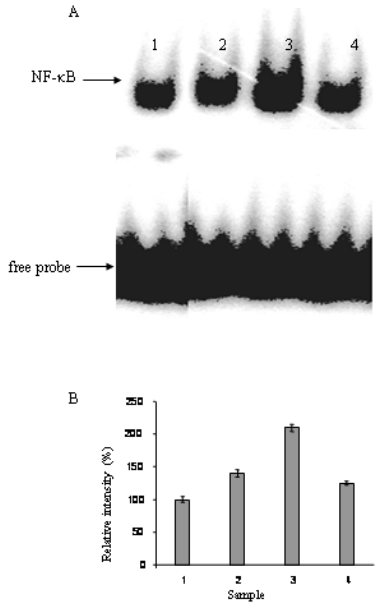


Fig. 3. Typical image for EMSA binding assays of nuclear NF-κB in H_2O_2 treated RAW264.7 cells (1 pmol/cell) (A). Lane1: H_2O_2 treatment for 0 hr; lane 2: H_2O_2 treatment for 0.5 hr; lane 3: H_2O_2 treatment for 1hr, lane 4: H_2O_2 treatment for 2hr. The activity of NF-κB peaked at 1hr and attenuated at 2hr. (B): The bar chart represents the relative intensity of the samples. Results were obtained from three independent experiments and expressed as mean±s.d.

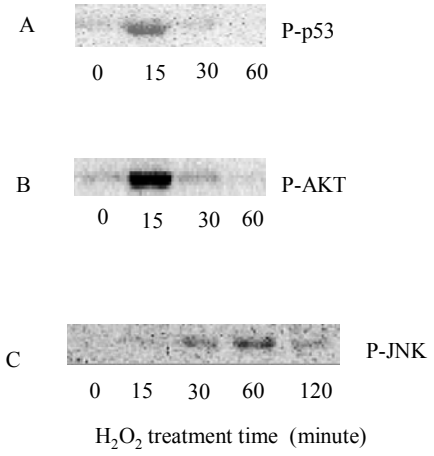


Fig. 4. Western blot analysis of phosphorylated p53, Akt and JNK. Phosphorylation of p53 (A) and Akt (B) was detected in RAW264.7 cells after 15 min of H_2O_2 treatment. Phosphorylated JNK peaked at 1hr then, attenuated at 2hr in H_2O_2 treated RAW264.7 cells (C).

DISCUSSION

The responses of 264.7 under oxidative stress and the roles of ROS sensitive protein families and signal pathways in these responses

In our study, the major responses of RAW 264.7 cells under oxidative stress (1 pmol/cell of H_2O_2) are arresting growth, processing apoptosis and fighting back for cell survival. The change of metabolism is involved in all of the responses. According to our investigation and other people's reports, several ROS sensitive protein families and signal pathways plays played important roles in the responses of cells to oxidative stress. They are heat shock proteins, metallothioneins, cathepsins, cytokine and proteasomes families, and p53, Akt, NF-κB, ERK, JNK, p38, PKC and INF-r pathways.

ROS sensitive protein families

The heat shock protein family involve in regulating the folding, transport and degradation of other cellular proteins. The general function of heat-shock proteins is protecting cells from various cellular stressors such as hypoxia, oxygen radicals, endotoxin, infections, and fever [Morimoto and Santoro 1998; Beere and Green 2001]. Under oxidative stress, HSPs protect cell from apoptosis through stabilizing intracellular protein structures and suppressing caspase activation [Finkel *et al.* 2000].

Metallothioneins (MTs) are intracellular, low molecular and cysteine-rich proteins. They have unique structural characteristics to give potent metal-binding and redox capabilities. A primary role has not been identified. Different types of stress including heavy toxic metals, reactive oxygen species can cause a rapid and robust induction of metallothioneins (MT)-I and II. In vitro and in vivo evidence shows that MTs have an antioxidant function. MTs may suppress apoptosis through inhibiting p38 MAPK activation (Kang *et al.* 2000).

In eukaryotes, the ubiquitin/proteasomes play a pivotal role in non-lysosomal degradation of intracellular protein. In the periods of oxidative stress, protein oxidation is significantly increased and become a threat to cell survival. Oxidative damaged proteins appear to be removed, by proteasomes through selective proteolysis (Hershko and Ciechanover 1998; Grune *et al.* 1997). The ROS sensitive sub-units of proteasomes identified in our research might play major roles in degradation of oxidized proteins.

In current study, it was found that a group of actin related genes were regulated by ROS. Actin is the major constituent of the cytoskeleton of almost all the eukaryotic cells. Evidences have indicated that oxidative stress can cause non-muscle mammalian cells to undergo remarkable changes in their morphology and in the structure of the actin cytoskeleton (Dalle-Donne *et al.* 2001). In this study, ROS did not change the gene expression of actin, but regulated the gene expression pattern in a group of actin-related molecules, such as coronin, an actin-binding protein and play a role in regulating the organization of actin cytoskeletal network that mediates cell morphology and proliferation. (Asano *et al.* 2001).

Cellular signaling pathways regulated by ROS

In our investigation, it was found that the activity and gene expression of p53 quickly increased after H_2O_2 treatment. ROS are potent activators of p53 [Martindale and Holbrook 2002]. p53 is activated through signaling pathways that induce phosphorylation of p53 protein in response to stress

(Sakaguchi *et al.* 1998) and selectively activates a different subset of target genes to regulate different cellular processes, including apoptosis, growth arrest, DNA repair, or cell differentiation (Hupp *et al.* 1992). In our investigation, active p53 mediated efficient G_1 arrest through down-regulation of cyclin D1 and Cdk4, because Cdk4 and associated cyclin D control the G 1 to S phase transition (Ohtsubo and Roberts 1993; Ekholm and Reed 2000). p53 may cause slight G2/M arrest by the decrease in gene expression of cyclin A2 and cyclin B1 because progression through G2 and M is regulated by different CDKs and their associated cyclin A and B (Lewin 1990). Active p53 also triggers the activation of genes related to apoptosis and leads to apoptosis (El-Deiry 1998). However, p53 was tightly regulated by other molecules such as Mdm2, and cyclin G1 [Chen 2002], Cyclin I might be also included. Mdm2 is a cellular proto-oncogene and negative cellular regulator of p53, which can abrogate p53-mediated G1 arrest and apoptosis and also accelerate cell cycle progression by independent way of p53 degradation (Argentini *et al.* 2000). Our results indicated the possibility that p53, Mdm2 and cyclin G1 form an auto-regulatory feedback loop in which p53 positively regulates Mdm2 and cyclin G1 levels whereas Mdm2 and cyclin G1 negatively regulates p53 levels and activity (Figure 5). The accumulation of Mdm2 and cyclin G1 could be critical for cell survival.

The second ROS sensitive pathway is NF-κB pathway. In our study, the activity of NF-κB increased after RAW 264.7 cells were treated by H_2O_2 (Figure 3). A wide range of signals, including ROS, can induce activation of NF-κB. NF-κB has been shown to exert protective effects under various conditions. However, NF-κB activity has also been correlated with apoptosis and the activation of apoptosis-associated genes such as Fas ligand and p53. Investigations examining the influence of NF-κB on cell survival following oxidant injury have likewise produced mixed findings (Flohe *et al.* 1997).

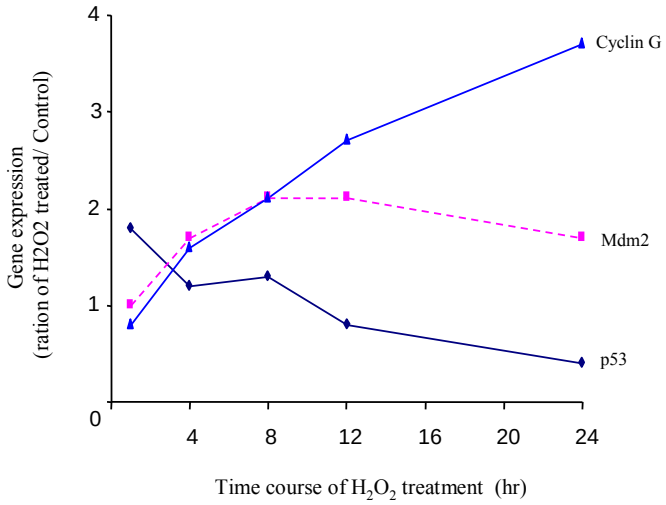


Fig. 5. The Gene expression patterns of p53, Mdm2 and cyclin G1 under H_2O_2 treatment.

In our investigation, Akt was activated and its expression was up-regulated by ROS. Recent studies have demonstrated that Akt regulates cell survival at multiple sites, including preventing release of cytochrome *c* from mitochondria and inactivating forkhead transcription factors, which was known to induce expression of pro-apoptotic factors, such as Fas ligand, phosphorylated and inactivated Bad and caspase9, and activated IκB kinase, which is a positive regulator of NF-κB and results in transcription of anti-apoptotic genes (Testa *et al.* 2001).

Evidence shows that PKC pathway is involved in both cell survival and apoptotic functions during oxidative stress (Martindale 2002). In our study, we found that the expression of PKC substrate 80K-H (Prkcsh) was up-regulated, which may suggest that PKC signaling pathway was also involved in the cellular response to ROS but it is not clear what roles PKC signaling pathway plays.

The change of metabolism in H_2O_2 treated RAW 264.7 cells

Under oxidative stress, regulation of metabolism patterns is important not only for apoptosis but also for cell survival. In this study, we found the genes related to glycolysis and lipid synthesis were gradually down-regulated whereas the genes related to lipid catabolism and protein synthesis were gradually up-regulated in H_2O_2 treated RAW 264.7 cells (Figure 2D). It was reported that oxidative stress can block glycolysis and the stress-induced block of glycolysis is not the result of a passive oxidative damage, but rather an active cell reaction programmed for cell self-defense (Colussi *et al.* 2000). According to our results, the block of glycolysis could be the consequence of the depletion of intracellular NAD pools or of the inactivation of the glycolytic enzymes or the down-regulation of the glycolysis related genes. However, both apoptosis and cell survival are energy spending processes. If intracellular ATP concentration is too low, cells will switch from apoptosis to necrosis (Leist *et al.* 1997). Our result suggests that when glycolysis was blocked, H_2O_2 treated RAW 264.7 cells may continuously process apoptosis or promote survival by using fatty acid as main source of ATP through up-regulation of fatty acid catabolism related genes to stabilizing the levels of intracellular ATP. Consistent with the report by others, activation of phospholipases, particularly phospholipase A2 (PLA2), plays important role by destabilizing lysosomal and mitochondrial membranes in H_2O_2 treated cells (Zhao *et al.* 2001).

In conclusion, this study might help to understand the molecular mechanism of apoptosis induced by oxidative stress and the feedback mechanisms that involve cell survival.

ACKNOWLEDGEMENTS

The work described in this paper was supported by the Areas of Excellent Scheme established under the University Grant Committee of the Hong Kong SAR Government (Project No: AOE/P-04/2004).

REFERENCES

Allen, R.G. and M. Tresini. 2000. Oxidative stress and gene regulation. Free Radic. Biol. Med. 28: 463–499.

Argentini, M., N. Barboule, and B. Wasylyk. 2000. The contribution of the RING finger domain of Mdm2 to cell cycle progression. Oncogene 19: 3849-3857.

Asano, S., M. Mishima, and E. Nishida. 2001. Coronin forms a stable dimer through its C-terminal coiled coil region: an implicated role in its localization to cell periphery. Genes to Cells 6: 225-235.

Beere, H.M. and D.R. Green. 2001. Stress management - heat shock protein-70 and the regulation of apoptosis. Trends Cell Biol. 11: 6-10.

Carmody, R.J. and T.G. Cotter. 2001. Signalling apoptosis: a radical approach. Redox Rep. 6: 77-90.

Chandra, J., A. Samali, and S. Orrenius. 2000. Triggering and modulation of apoptosis by oxidative stress. Free Radic. Biol. Med. 29: 323-333.

Chen, X. 2002. Cyclin G: a regulator of the p53-Mdm2 network. Dev. Cell 2: 518-519.

Colussi, C., M.C. Albertini, S. Coppola, S. Rovidati, F. Galli, and L. Ghibelli. 2000. H_2O_2-induced block of glycolysis as an active ADP-ribosylation reaction protecting cells from apoptosis FASEB J. 14: 2266-2276.

Dalle-Donne, I., R. Rossi, A. Milzani, P. Di Simplicio, and R. Colombo. 2001. The actin cytoskeleton response to oxidants: from small heat shock protein phosphorylation to changes in the redox state of actin itself. Free Radic. Biol. Med. 31: 1624-1632.

Ekholm, S.V. and S.I. Reed. 2001. Regulation of G (1) cyclin-dependent kinases in the mammalian cell cycle. Curr.Opin. Cell. Biol.12: 676-684.

El-Deiry, W. 1998. Regulation of p53 downstream genes. Semin. Cancer Biol. 8: 345–357.

Fehrenbach, E., H. Northoff. 2001. Free radicals, exercise, apoptosis, and heat shock proteins. Exerc Immunol. Rev. 7: 66-89.

Finkel, T. and N.J. Holbrook. 2000. Oxidants, oxidative stress and the biology of ageing. Nature 408: 239-247.

Flohe, L., R. Brigelius-Flohe, C. Saliou, M.G. Traber, L. Packer. 1997. Redox regulation of NF-kappa B activation. Free Radic. Biol. Med. 22: 1115–1118.

Grune, T., T. Reinheckel, and K.J. Davies. 1997. Degradation of oxidized proteins in mammalian cells. FASEB J. 11: 526-534.

Hershko, A. and A. Ciechanover. 1998. The ubiquitin system. Annu. Rev. Biochem. 67: 425-479.

Hupp, T.R., D.W. Meek, C.A. Midgley, and D.P. Lane. 1992. Regulation of the specific DNA binding function of p53. Cell 71: 875–886.

Kang, Y.J, Z.X. Zhou, G.W. Wang, A. Buridi, and J.B. Klein. 2000. Suppression by metallothionein of doxorubicin-induced cardiomyocyte apoptosis through inhibition of p38 mitogen-activated protein kinases. J. Biol. Chem. 275: 13690-13698.

Leist, M., B. Single, A.F. Castoldi, S. Kuhnle, and P. Nicotera. 1997. Intracellular adenosine triphosphate (ATP) concentration: a switch in the decision between apoptosis and necrosis. J. Exp. Med. 185: 1481-1486.

Lewin, B. 1990. Driving the cell cycle: M phase kinase, its partners, and substrates. Cell 61: 743-752.

Martindale, J.L. and N.J. Holbrook. 2002. Cellular response to oxidative stress: signaling for suicide and survival. J. Cell Physiol. 192: 1-15.

Morimoto, R.I. and M.G. Santoro. 1998. Stress-inducible responses and heat shock proteins: new pharmacologic targets for cytoprotection. Nat. Biotechnol. 16: 833-838.

Nishikawa, T., D. Edelstein, X.L. Du, S. Yamagishi, T. Matsumura, Y. Kaneda, M.A. Yorek, D. Beebe, P.J. Oates, H.P. Hammes, I. Giardino, and M. Brownlee. 2000. Normalizing mitochondrial superoxide production blocks three pathways of hyperglycaemic damage. Nature 404: 787-790.

Ohtsubo, M. and J.M. Roberts. 1993. Cyclin-dependent regulation of G1 in mammalian fibroblasts. Science 259: 1908-1912.

Quackenbush, J. 2001. Computational analysis of microarray data. Nature 2: 418-427.

Sakaguchi, K., J.E. Herrera, S. Saito, T. Miki, M. Bustin, A. Vassilev, C.W. Anderson, and E. Appella. 1998. DNA damage activates p53 through a phosphorylation–acetylation cascade. Genes Dev. 12: 2831–2841.

Testa, J.R. and A. Bellacosa. 2001. AKT plays a central role in tumorigenesis. Proc. Natl. Acad. Sci. U S A 98: 10983-10985.

Torres, M. and H.J. Forman. 2003. Redox signaling and the MAP kinase pathways. Biofactors 17: 287-296.

Zhao, M., U.T. Brunk, J.W. Eaton. 2001. Delayed oxidant-induced cell death involves activation of phospholipase A2. FEBS Lett. 509: 399-404.

EXTREME ADAPTATIONS TO HYPOXIA AND ANOXIA IN CRUCIAN CARP

Göran E. Nilsson[6]

ABSTRACT

The crucian carp has the capacity to survive anoxia for days at room temperature and several months at temperatures close to 0°C. The extreme respiratory, cardiovascular and neurobiological adaptations employed by the crucian carp to tolerate anoxia are discussed.

INTRODUCTION

The rule among vertebrates is that anoxia is synonymous with rapid death. To study animals that deviate from a rule has again and again proven very useful not only for the understanding of how such animals work, but also for shedding light on the mechanisms behind the rule - in this case the mechanisms behind anoxic damage. Anoxia related diseases are the most common causes of death in the industrialized world.

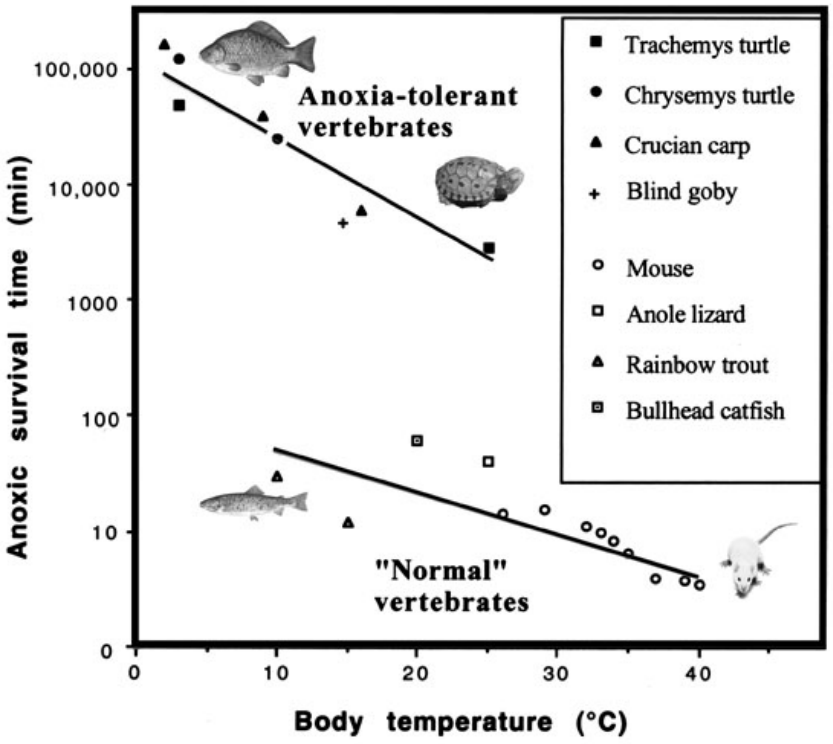


Figure 1. Anoxic survival time in "normal" vertebrates and in anoxia tolerant vertebrates. Note that cold blooded vertebrates in general are as sensitive to anoxia as mammals, if temperature is taken into account, and that the anoxia-tolerant vertebrates survive anoxia about 1000 times longer than other

[6] Department of Molecular Biosciences, University of Oslo, POBox 1041, NO-0316 Oslo, Norway

vertebrates. In general, metabolic rate falls sharply with body temperature and a main reason why anoxia tolerant vertebrates survive anoxia longer at cold temperatures is that their glycogen stores last longer. For anoxia-intolerant vertebrates, a main benefit of low temperature is that it slows down the loss of ATP and the onset of degenerative processes. (from Lutz and Nilsson, 2003)

Few vertebrates have the ability to live without oxygen (anoxia). The crucian carp (No.: karuss, Lat.: *Carassius carassius*), a common fish in Northern Europe, and some North American freshwater turtles (genera *Trachemys* and *Chrysemys*) are the best studied examples of anoxia tolerant vertebrates (Lutz *et al*. 2003; Nilsson and Lutz 2004) (Figure 1). Both the crucian carp and the turtles have evolved their anoxia tolerance to allow overwintering in ice-covered lakes, streams and ponds. However, there are some striking differences in their responses to anoxia, reflected by the fact that the crucian carp survives anoxia in an active state, still swimming around, while anoxic turtles are virtually comatose (Nilsson 2001).

Respiratory adaptations

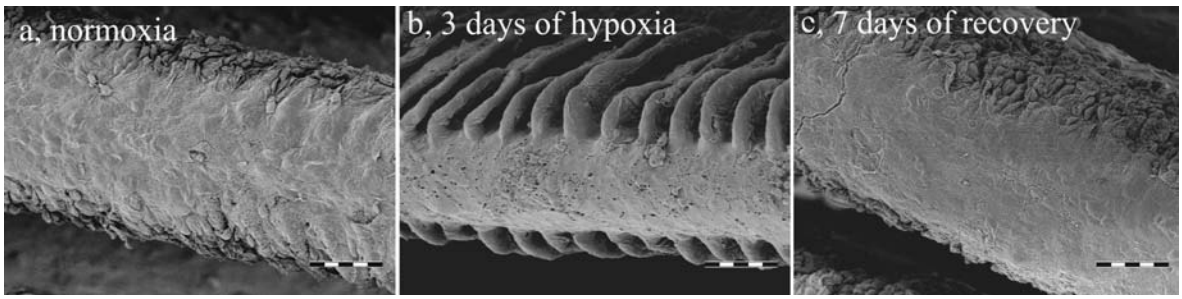


Figure 2. Scanning electron micrographs of crucian carp gill filaments before, during and after exposure to hypoxia. Note the appearance of lamellae during hypoxia. Scale bar = 50 µm. From Sollid et al. (2003).

The crucian carp is, so far, the only adult vertebrate known to be able to reversibly adjust the morphology of the respiratory organ to match its oxygen needs (Sollid *et al*. 2003) (Figure 2). When the crucian carp is exposed to hypoxia, it starts remodeling its gills within days, resulting in a 7-8 fold increase the respiratory surface area and, thereby, boosting the ability to take up oxygen. In normoxic crucian carp, Sollid *et al*. (2003) found that a cell mass, situated outside the gill epithelium, is filling up the space between the gill lamellae (the respiratory units of fish). The mitotic and apoptotic activity in this interlamellar cell mass (ILCM) was found to vary with ambient oxygen levels. Thus, in normoxic water, mitosis was dominating, causing ILCM to fill up the interlamellar space, while in hypoxic water, apoptosis prevailed and the ILCM nearly disappeared, exposing the respiratory epithelium to the water.

Even if the crucian carp can survive without any oxygen, this is costly as it involves releasing an energy rich hydrocarbon (ethanol - the exotic anaerobic end product of this genus) to the water, resulting in a depletion of the glycogen stores. Thus, getting access to oxygen is highly desirable from an energetic point of view, and this is obviously what it strives to do in hypoxia by remodeling its gills. On the other hand, in well oxygenated waters, it can apparently get all the oxygen it needs without having protruding lamellae, so here the ILCM may function to reduce water and ion fluxes between blood and water, thereby reducing osmoregulatory costs.

Circulatory adaptations

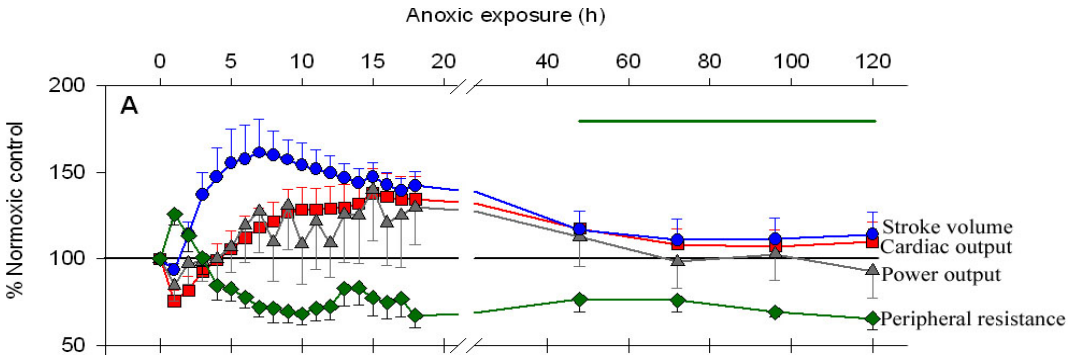


Figure 3. The unique performance of the crucian carp heart and circulatory system during 5 days of anoxia in vivo (from Stecyk et al, 2004).

Although we knew that crucian carp remains active in anoxia (Nilsson *et al*. 1993b), it was surprising to find that it actually maintain heart work (cardiac output, cardiac power output, and heart rate) in anoxia (Stecyk *et al*. 2004) (Figure 3). Thus, crucian carp kept anoxic for 5 days show no signs of reduced cardiac work, autonomic nervous control is maintained, and systemic vascular resistance is untypically reduced. No other vertebrates even come close to such a cardiac performance in anoxia. Their hearts either stop working almost immediately, or, in a few cases, cope with anoxia by slowing down to a few beats a minute (like in anoxia tolerant turtles) in combination with widespread systemic vasoconstriction and blunted autonomic control. This finding immediately poses some very basic questions that need experimental attention, the most important being: why and how? Moreover, it obviously makes the crucian carp heart an attractive and unique model for studying adaptive anoxia-defense mechanisms in cardiac tissue, bearing in mind that anoxic or ischemic heart conditions are major killers in humans. Our present hypothesis is that crucian carp need to maintain cardiac output to shuttle its main glycolytic substrates and products: glucose, lactate and ethanol. It may for example be that the crucian carp has to maintain heart work in anoxia to avoid ethanol intoxication, as ethanol is the major anaerobic end product in this species.

The brain

Because of its high energy demands, the brain is generally the most anoxia sensitive organ. During anoxia, the oxidative phosphorylation by the respiratory chain stops, and glycolysis becomes the only available route for ATP production. Since every molecule of glucose yields less than 1/10th of the ATP produced by oxidative phosphorylation, the energy (ATP) demand of the mammalian brain can not be met by glycolysis alone and there is a rapid fall in cellular ATP levels causing a slow down, and eventually a stop in the Na/K pump activity. Ion-homeostasis is therefore lost and ions start running down their concentration gradients. After one or a few minutes, a general depolarization of the brain occurs and Ca^{2+} starts flooding the cells. At about the same time, there is a release of excitatory neurotransmitters, particularly glutamate, which speeds up the Ca^{2+} inflow. The increased levels of intracellular Ca^{2+} triggers an array of degenerative processes that soon leads to neuronal damage and death. These degenerative processes involves the degradation of DNA, proteins and the cell membrane itself, and besides glutamate and Ca^{2+}, the key players in these events include lytic enzymes, free radicals and nitric oxide (NO).

There is a vast literature on the mechanisms involved in anoxic brain damage in mammals, but we have found that the important events of this anoxic catastrophe are probably common to most vertebrate

brains, as judged from our experiments with rainbow trout. Thus, during anoxia the rainbow trout brain looses its ion-homeostasis and depolarises virtually as fast as the mammalian brain (when temperature differences are taken into account) (Nilsson *et al*. 1993a). Moreover, there is a release of glutamate as well as other amino acids in the anoxic rainbow trout brain (Hylland *et al*. 1995). Due to the extended time frame during which these events occur at the low body temperature (15°C) of the rainbow trout, we could in fact show that the release of glutamate proceeds rather than precedes the loss of ion homeostasis - a matter that has been difficult to resolve in experiments with mammals.

So how can crucian carp and freshwater turtles avoid brain damage during anoxia? From the result we have obtained so far, we can conclude that a key to anoxic survival is a continued ability to match ATP consumption with ATP production in the absence of oxygen, thereby avoiding energy failure and consequent neuronal depolarization.

The glycolytic ATP production is supported by extra large glucose (glycogen) stores. Indeed, the only factor that appears to limit the crucian carp's survival time is anoxia is a complete depletion of its main glycogen store in the liver (Nilsson 1990). It is possible that the unique ability of the genus *Carassius* to produce ethanol as the major end product of anaerobic metabolism (Shoubridge and Hochachka 1980) is the key adaptation allowing the crucian carp to maintain physical activity during anoxia (Nilsson *et al*. 1993b; Lutz and Nilsson 1997; Nilsson 2001; Nilsson and Lutz 2004). The turtles lack the ability to produce ethanol in anoxia and have to cope with very high lactate levels. In both crucian carp and turtles there is a redistribution of blood flow to direct the glucose available to anoxia sensitive organs like the brain, changes in which adenosine appears to play another key role (Nilsson *et al*. 1994; Hylland *et al*. 1994).This is accomplished by a rapid temporary increase in the rate of anaerobic glycolysis followed by a depression of energy needs termed metabolic depression. Using microcalorimetry, we have been able to show that isolated brain slices of crucian carp depress their metabolic rate and maintains their ATP levels during anoxia (Johansson *et al*. 1995).

One mechanism employed by the crucian carp and turtles for depressing energy use in brain is the suppression of neurotransmission caused by release inhibitory neuromodulators like adenosine (Nilsson 1991; Nilsson and Lutz 1992) and inhibitory neurotransmitters such as gamma-amino butyric acid (GABA) (Nilsson 1990, 1992; Nilsson *et al*. 1990, 1991; Nilsson and Lutz 1991). In 1986, Peter Hochachka suggested in a review article in Science (Hochachka 1986) that anoxia tolerance could be accomplished by a down regulation of the ion channel density and/or activity of the cell membrane, and termed this hypothetical phenomenon "channel arrest". The rationale for the hypothesis is that the cost for ion pumping will go down drastically if the membrane becomes less permeable to ions - ion pumping being the main energy consumer in the brain (Ericinska and Silver 1989). There is now substantial evidence for “channel arrest” in turtles (Doll *et al*. 1993; Nilsson and Lutz 2004). For example, Bickler (1992) used iodoacetate to block glycolysis in turtle cortical brain slices and found that the rate of Ca^{2+} inflow into the cells decreased dramatically if the slices were pre incubated in anoxia, clearly suggesting an anoxia induced down regulation of Ca^{2+} permeability of the neuronal membrane. However, similar experiments on the crucian carp indicate that there is now down regulation of ion permeability in the neuronal membranes (Johansson and Nilsson 1995; Nilsson 2001). In light of the continuous physical activity displayed by the crucian carp in anoxia, it is tempting to suggest that a down regulation of neuronal ion channels is a much too drastic measure for saving energy. The anoxic crucian carp is probably relying more on a fine tuned down regulation of brain activity initiated by inhibitory neurotransmitters and modulators such as GABA and adenosine (Nilsson 1991, 1992; Hylland and Nilsson 1999).

REFERENCES

Bickler, P.E. 1992. Cerebral anoxia tolerance in turtles: regulation of intracellular calcium and pH. American Journal of Physiology 263: 1298-1302.

Doll, C.J., P.W. Hochachka and P.B. Reiner. 1993. Reduced ionic conductance in turtle brain. American Journal of Physiology 265: 929-933.

Erecinska, M., and I.A. Silver. 1989. ATP and brain function. Journal of Cerebral Blood Flow and Metabolism 9: 2-19.

Hochachka, P.W. 1986. Defense strategies against hypoxia and hypothermia. Science 231: 234-241.

Hylland, P., G.E. Nilsson, and P.L. Lutz. 1994. Time course of anoxia induced increase in cerebral blood flow rate in turtles: evidence for a role of adenosine. Journal of Cerebral Blood Flow and Metabolism 14: 877-881.

Hylland, P., G.E. Nilsson, and D. Johansson. 1995. Anoxic brain failure in an ectothermic vertebrate: release of amino acids and K^+ in rainbow trout thalamus. American Journal of Physiology 269: 1077-1084.

Hylland, P., and G.E. Nilsson. 1999. Extracellular levels of amino acid neurotransmitters during anoxia and forced energy deficiency in crucian carp brain. Brain Research 823: 49-58

Johansson, D., and G.E. Nilsson. 1995. Roles of energy status, K_{ATP} channels, and channel arrest in fish brain K^+ gradient dissipation during anoxia. Journal of Experimental Biology 198: 2575-2580.

Johansson, D., G.E. Nilsson, and E. Törnblom. 1995. Effects of anoxia on energy metabolism in crucian carp brain slices studied with microcalorimetry. Journal of Experimental Biology 198: 853-859.

Lutz, P.L. and G.E. Nilsson. 1997. Contrasting strategies for anoxic brain survival - glycolysis up or down. Journal of Experimental Biology 200: 411-419.

Lutz, P.L., G.E. Nilsson, and H. Prentice. 2003. *In:* The Brain Without Oxygen. 3rd Ed. Kluwer Academic Publishers.

Nilsson, G.E. 1990. Long term anoxia in crucian carp: changes in the levels of amino acid and monoamine neurotransmitters in the brain, catecholamines in chromaffin tissue, and liver glycogen. Journal of Experimental Biology 150: 295-320.

Nilsson, G.E. 1991. The adenosine receptor blocker aminophylline increases anoxic ethanol production in crucian carp. American Journal of Physiology 261: 1057-1060.

Nilsson, G.E. 1992. Evidence for a role of GABA in metabolic depression during anoxia in crucian carp (*Carassius carassius* L.). Journal of Experimental Biology 164: 243-259.

Nilsson, G.E., A.A. Alfaro, and P.L. Lutz. 1990. Changes in turtle brain neurotransmitters and related substances during anoxia. American Journal of Physiology 259: 376-384.

Nilsson, G.E., P.L. Lutz, and T.L. Jackson. 1991. Neurotransmitters and anoxic survival of the brain: a comparison between anoxia-tolerant and anoxia-intolerant vertebrates. Physiological Zoology 64: 638-652.

Nilsson, G.E., and P.L. Lutz. 1991. Release of inhibitory neurotransmitters in response to anoxia in turtle brain. American Journal of Physiology 261: 32-37.

Nilsson, G.E., and P.L. Lutz. 1992. Adenosine release in the anoxic turtle brain: a possible mechanism for anoxic survival. Journal of Experimental Biology 162: 345-351.

Nilsson, G.E., M. Pérez-Pinzón, K. Dimberg, and S. Winberg. 1993a. Brain sensitivity to anoxia in fish as reflected by changes in extracellular potassium-ion activity. American Journal of Physiology 264: 250-253.

Nilsson, G.E., P. Rosén, and D. Johansson. 1993b. Anoxic depression of spontaneous locomotor activity in crucian carp quantified by a computerized imaging technique. Journal of Experimental Biology 180: 153-162.

Nilsson, G.E., P. Hylland, and C.O. Löfman. 1994. Anoxia and adenosine induce increased cerebral blood flow rate in crucian carp. American Journal of Physiology 267: 590-595.

Nilsson, G.E. 2001. Surviving anoxia with the brain turned on. News in Physiology Science 16: 218-221.

Nilsson, G.E., and P.L. Lutz. 2004. Anoxia tolerant brains. Journal of Cerebral Blood Flow and Metabolism 24: 475-486.

Shoubridge, E.A., and P.W. Hochachka. 1980. Ethanol: novel end product in vertebrate anaerobic metabolism. Science 209: 308-309.

Sollid, J., P. De Angelis, K. Gundersen, and G.E. Nilsson. 2003. Hypoxia induces adaptive and reversible gross-morphological changes in crucian carp gills. Journal of Experimental Biology 206: 3667-3673.

Stecyk, J.A.W., K-O, Stensløkken, A.P. Farrell, and G.E. Nilsson. 2004. Maintained cardiac pumping in anoxic crucian carp. Science 306: 77-77.

Suppressive Effects of Hypoxia on Male Rat Reproductive Function

Weigong Liao, Yuqi Gao[7], Mingchun Cai, Jian Chen, Yi Wu

ABSTRACT

Our objectives were to explore the effects of hypobaric hypoxia on the male rat reproductive function. Adult male Wistar rats were randomly divided into four groups: control group raised at sea level; 5d, 15d and 30d hypoxic groups raised in a hypobaric chamber stimulating 5000 m altitude for 5 days, 15 days and 30 days respectively. The spermatozoa density was found to decrease in 15d and 30d hypoxic groups, compared with the control group ($P<0.01$). Motilities of epididymal caudal spermatozoa decreased approximately by 50% in all of the three hypoxic groups ($P<0.01$, compared with control group). Morphologically abnormal spermatozoa increased by more than 50% in all three hypoxic groups ($P<0.01$, compared with control group). The LPC-induced AR was suppressed by about 60% in all three hypoxic groups ($P<0.01$, compared with control group). Acrosin protease activity decreased significantly after exposure to hypoxia for 5, 15, and 30 days ($P<0.01$, compared with control group). Exposure to hypobaric hypoxia significantly reduced the tetraploid cell populations (approximately 28%) compared with the control group ($P<0.01$). After exposure to hypoxia for 30d, the ratio of haploid and diploid cell populations was reduced by 25% when compared with control group ($P<0.05$). Proliferation index of 15d and 30d hypoxic groups was significantly lower than that of the control group ($P<0.05$). The apoptosis of spermatocytes and spermatogonia increased after exposure to hypoxia for 5d, 15d and 30d. Hypoxia caused testicular interstitial edema, occasional necrosis and degenerative changes in testicular germ cells**.** Hypobaric hypoxia suppresses the spermatozoa quantity and quality, as well as inhibiting the acrosome reaction and acrosin protease activity of rat spermatozoa. Hypobaric hypoxia inhibits the spermatogenesis in testis, by decreasing the number of tetraploid spermatogenic cells (primary spermatocytes). The apoptosis of primary spermatocytes and spermatogonia, occurring under hypoxic conditions may contribute to the loss of tetraploid cell populations.

INTRODUCTION

A large number of studies have revealed that hypoxia causes changes of respiration and circulation and the composition of the blood. Studies on the changes in the reproductive system caused by hypoxia are limited. High altitude resident fertility rate is not lower than populations at sea level. However newcomers from low areas seem to have reduced fertility at high altitude. Cattle and other animals from sea level fail to reproduce (Eckes 1976). Previous studies indicated that hypoxia decreased the production of seminal sperm and caused morphologic changes of the germinal epithelium in rat (Gosney 1984), monkey (Saxena 1995), mouse, sheep and man (Donald 1981). Little is known about which stages of spermatogenesis are affected by hypoxia and how does hypoxia affect them. These questions should be answered. Further, in the previous studies, spermatogenesis was evaluated only by carrying out histological analyses of testicular tissues. In contrast, the use of flow cytometry facilitates rapid and accurate quantitation of large numbers of testicular germ cells and it is widely accepted as an effective method of assessing spermatogenesis (Spano and Evenson 1993).

The acrosome reaction (AR) was believed to be a prerequisite that enables the spermatozoa to penetrate the zona pellucida (ZP) and fertilize the egg. Acrosin probably aids spermatozoa penetration

[7] Department of Pathophysiology and High Altitude Physiology, Institute of High Altitude, Third Military Medical University, ChongQing 400038, PR China

by limited proteolysis of ZP glycoproteins. Acrosin protease activity has also been involved in dissolution of the acrosomal matrix, and in a regulated release of acrosomal contents at the time of the acrosome reaction (Hardy *et al.* 1991). But no work has addressed the effect of hypoxia on spermatozoa acrosome reaction and acrosin protease activity.

The present investigation was undertaken, therefore, to evaluate the effects of hypoxia on the spermatogenesis in rat testis by flow cytometry, histological examination and TUNEL immunohistochemistry. Acrosome reaction and acrosin protease activity of sperm in rat were also studied.

MATERIALS AND METHODS

Animals

Adult male Wistar rats were randomly divided into four groups: control group, 5d, 15d, and 30d hypoxic group. Hypoxic groups were maintained in a hypobaric chamber stimulating altitude 5000m. The animals had free access to food and water, with a controlled light / dark cycle (12 and 12 h of light and dark respectively).

Determination of motility and viability of spermatozoa

Right cauda epididymises were excised from each animal and minced in 5 ml prewarmed saline. Epididymal caudal spermatozoa were obtained by filtering through a mesh. Then a 0.5 ml aliquot was removed from the epididymal caudal spermatozoa suspension and mixed with 1 ml saline prewarmed at 37°C. A drop of the mixture was loaded on a slide and examined with a phase contrast microscope for the number of spermatozoa with progressive motility. The number of progressively motile spermatozoa and motile spermatozoa was determined by counting spermatozoa exhibiting forward motion and any motion in a population of 100 cells respectively. The procedure must be completed within 10 minutes. Viability of spermatozoa was expressed as percentage of motile spermatozoa.

Determination of the density of spermatozoa

A 0.5 ml aliquot was removed from the filtered epididymal caudal spermatozoa suspension and diluted with 2 ml saline. Subsequently, each diluted spermatozoa sample was loaded onto a hemocytometer and allowed to equilibrate for a few minutes prior to counting. Four squares per hemocytometer chamber were counted in duplicate using phase contrast microscopy at a magnification of 200×. Spermatozoa density was calculated by the following formula: total number of spermatozoa in the epididymal caudal extract/ right epididymal caudal weight. Duplicate values were averaged.

Determination of abnormal spermatozoa

To assess the percentage of morphologically abnormal spermatozoa, spermatozoa smears were prepared on clean glass slides and dried. Smears were fixed by formaldehyde for 5 minutes and stained by 2% eosin for 2 hours. One thousand spermatozoa per animal were screened and classified into normal and abnormal types. Abnormal types included blunt hook-head, banana-head, amorphous-head, two-tail, coiled-tail, and folded-tail (Wyrobek and Bruce 1975; Briz *et al.* 1996).

Acrosome reaction examination (Hsu *et al.* 1999)

Spermatozoa suspension preparation: after sacrificing animals, the right epididymal cauda was dissected from each rat, and slashed in 2 ml human tubule fluid (HTF)-albumin buffer (4.0 g/L bovine serum albumin) prewarmed at 37°C. Spermatozoa suspension was obtained by filtering through mesh.

A 1:10 dilution of spermatozoa was prepared, and an epididymal spermatozoa count was done by hemocytometer. The concentration of spermatozoa was adjusted to 1×10^6 cells/ml for the assay of CTC fluorescence.

Induction of acrosome reaction: After 3 h preincubation at 37°C, 10 µl stock LPC (Sigma) solution (4 mM in DMSO) was added to 200 µl spermatozoa suspension so that the final concentrations of LPC were 200 µM. After incubation for 2 h, the spermatozoa were examined by the chlortetracycline fluorescence assay.

CTC fluorescence assay: After 5 h of incubation at 37°C, aliquots of cauda epididymal spermatozoa were taken for the CTC fluorescence assay. The CTC fluorescence assay was used to study the status of the acrosome reaction in rat spermatozoa. CTC (Sigma) was prepared freshly at a concentration of 1.5 mM in 20 mM Tris buffer containing 130 mM NaCl and 5 mM L-cysteine. The pH was adjusted to 7.8 and the solution was shielded from light at room temperature. Of the spermatozoa suspension, 50 µl were mixed with 50 µl CTC solution in an Eppendorf tube. After 30 s, 8 µl of 12.5% (w/v) paraforamaldehyde in 0.5 mM Tris buffer (pH 7.4) were added. After mixing by gentle eddying, 50 µl of 0.22 mM 1,4-diazabicyclo[2.2.2]octane in glycerol was introduced and mixed with the spermatozoa suspension to retard fading of fluorescence. Fluorescence patterns of spermatozoa were analyzed on an Olympus BX-60 microscope (Tokyo, Japan) equipped with phase-contrast and fluorescence optics. The excitation beam was passed through a band-pass filter of 400–410 nm. A volume of 5 µl spermatozoa suspension was placed on a slide and covered with a 22 mm ×22 mm cover slip. In each sample, a total of 200 cells was assessed and classified as ‘acrosome-reacted spermatozoa’ with dark heads except for the tip, which retained some fluorescence, and ‘incapacitated spermatozoa’ with uniform bright fluorescence over the entire head.

Determination of acrosin Proteolytic activity

Acrosin proteolytic activity of spermatozoa was determined using a gelatin substrate film assay. Gelatin substrate film slides were prepared on one side of glass slides with a 3.4% gelatin solution. Film slides were fixed in 0.05% glutaraldehyde for 2 minutes, washed by acetic acid-sodium barbiturate buffer twice, and dried in air for at least 1 hour. Subsequently, film was stained with 0.5% Trypan Blue.

After centrifugation at 1000 rpm for 5 minutes and resuspended in PBS, epididymal spermatozoa were spread on the surface of gelatin-coated slides and incubated at 37°C for 7 hours to allow proteolysis of the gelatin by acrosin. Positive spermatozoa showing a clear halo around the spermatozoa head were counted in a population of 100 cells.

Histopathological examination

Light microscopy

Testes were fixed in 10% formalin, dehydrated and embedded in paraffin. Sections were cut at 5 µm thicknesses and stained with hematoxylin and eosin for light microscope observation.

Electron microscopy

Testes were sliced and fixed in 3% glutaraldehyde. Ultra-thin sections were stained in uranyl acetate and lead citrate and observed in a Phillips TECNAI 10 transmission electron microscope (TEM).

Flow cytometry

The germ cells were released from seminiferous tubules in phosphate buffered saline (PBS), by mincing the testes with fine scissors and digesting the testes with Collagenase□ (Sigma) at 37°C for 30 minutes. The suspension was filtered through a nylon mesh twice and fixed in 70% chilled ethanol. The samples were stored at 4°C. The germ cells were stained with ethidium bromide (Sigma). The tubes were kept in the dark at room temperature for 30 minutes before flow cytometry analysis.

TUNEL immunohistochemistry

Immunohistochemical apoptotic detection (TUNEL assay) was performed on 10% formalin-fixed 5μm paraffin sections using In Situ Cell Death Detection Kit, POD (Roche) according to the manufacturer's instructions. Germ cells were restained with hematoxylin. The percentage of seminiferous tubules with TUNEL staining was determined (Yamamoto *et al.* 2002).

Statistical analysis

Results were presented as mean ± SD. All data were analyzed by one-way ANOVA. $P<0.05$ was considered statistically significant.

RESULTS

Sperm density, motility, viability and abnormality

As shown in Table 1, spermatozoa density in cauda epididymis decreased significantly in hypoxic 15d group and 30d group (P<0.01, compared with control group, shown in Figure 1). Spermatozoa motility and viability in cauda epididymis were severely impaired in all of three hypoxic groups (P<0.01, compared with control group, shown in Figure 2 and 3). The number of morphologically abnormal spermatozoa increased significantly in hypoxic condition (P<0.01, compared with control group, shown in Figure 4). The main types of spermatozoa abnormality were coiled and folded tail.

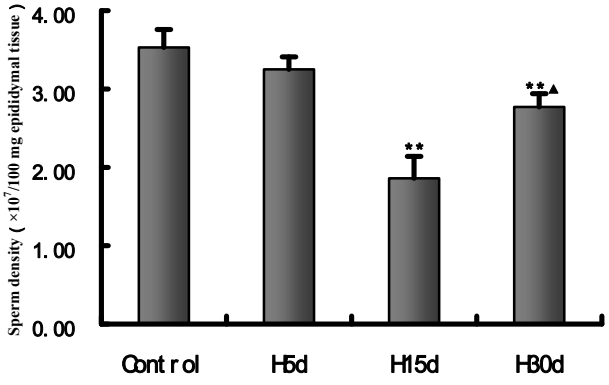


Figure 1. Effect of hypoxia on sperm density. (**: P<0.01 vs. Control group ▲: P<0.05vs Hypoxic 15d group

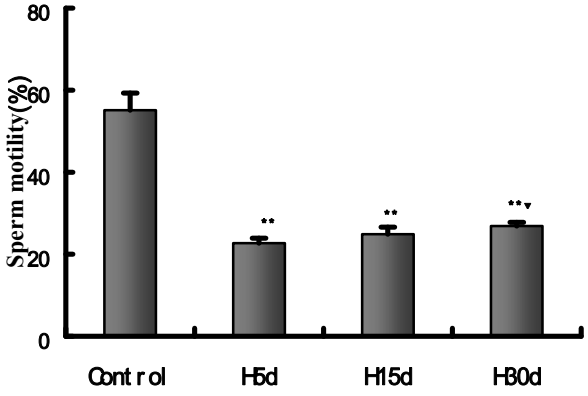


Figure 2. Effect of hypoxia on sperm motility. (**: P<0.01 vs Control group ▾ : P<0.05 vs Hypoxic 5d group)

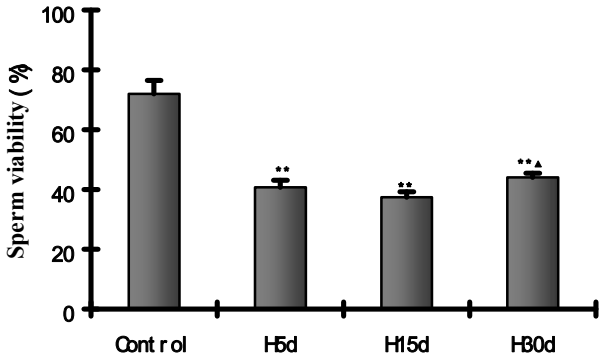


Figure 3. Effect of hypoxia on sperm viability. (**: P<0.01 *vs.* Control group)

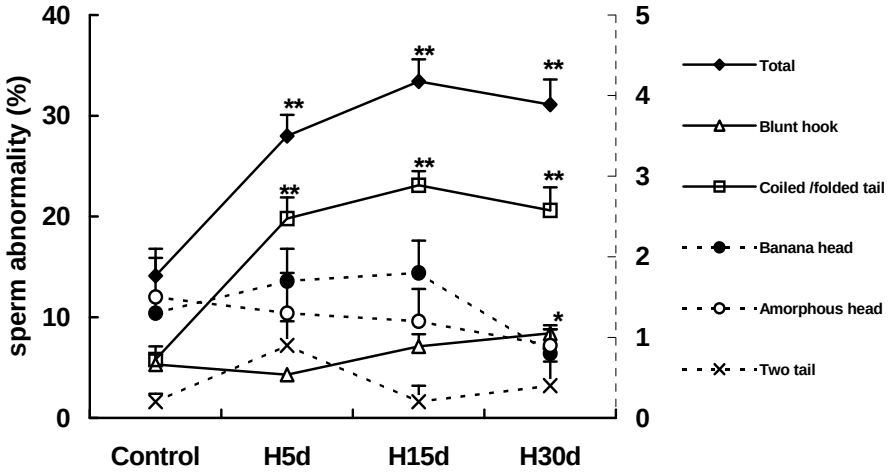


Figure 4. Effect of hypoxia on sperm abnormality. (*:P<0.05 **:P<0.01 vs Control group)

The acrosome reaction (AR)

LPC-induced AR was suppressed by about 60% in all three hypoxic groups (P<0.01, compared with control group, shown in Figure 5).

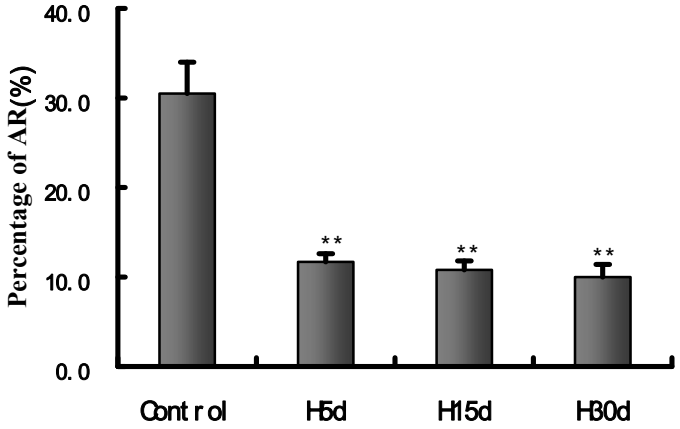


Figure 5. Effect of hypoxia on the percentage of acrosome reaction in rats. (**: P<0.01 *vs.* Control group)

Acrosin activity

The percentage of acrosin positive reaction spermatozoa decreased significantly in hypoxic groups (P<0.01, compared with control group, shown in Figure 6).

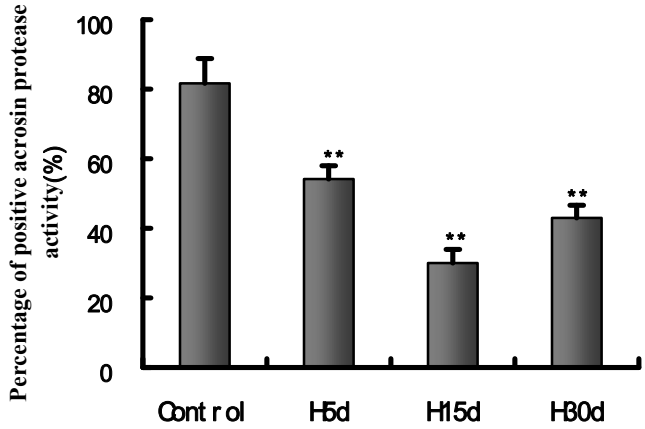


Figure 6. Effect of hypoxia on the percentage of positive acrosin protease activity spermatozoa in rats. (**:P<0.01 vs Control group)

Histopathological examination

LM: Hypoxia caused testicular interstitial congestion and edema. Occasionally, seminiferous tubule atrophy and germ cell depletion were observed after exposure to hypoxia for 30d. (Shown in Figure 7)

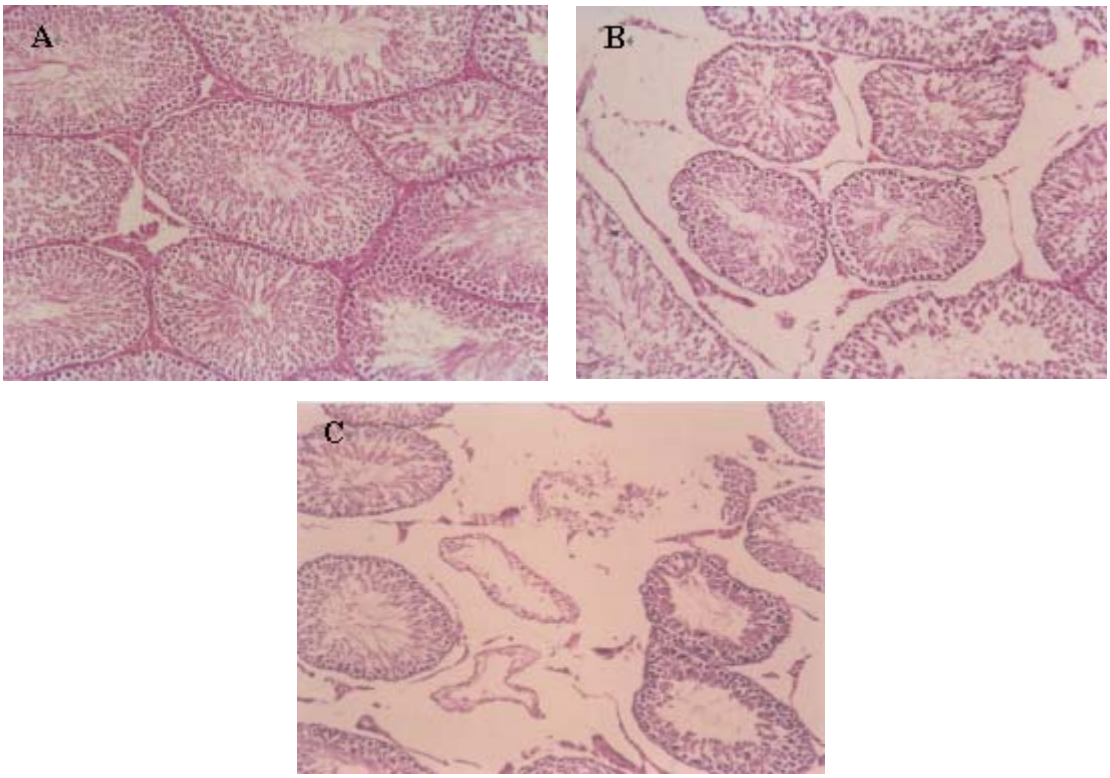


Figure 7. Representative photomicrographs of testes from control and hypoxic rats (original magnification: 100X). (A) Control rat (B) 5d hypoxic cat (C)30d hypoxic rat

Lipid droplets were found in the Sertoli cells in hypoxic rats. Degenerating spermatogonia with slight chromatin margination were observed in 15d and 30d hypoxic rats. (Shown in Figure 8).

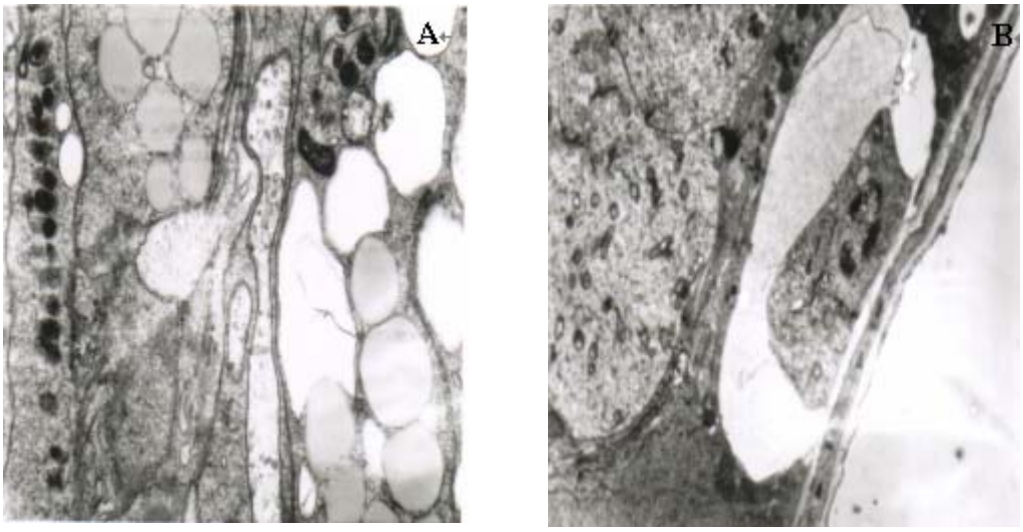


Figure 8. Electron micrograph of seminiferous tubules. (A) 5d hypoxic rat, showing lipid droplets in Sertoli cells. 10000×, (B) 15d hypoxic rat, showing degenerating spermatogonia. 4200×

Flow cytometry analysis

5d, 15d, and 30d hypobaric hypoxia exposure significantly reduced the tetraploid cell populations (approximately 28%) compared with the control group. (Shown in Figure 9)

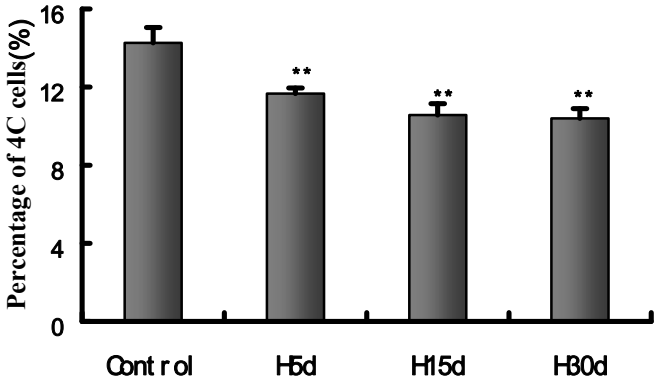


Figure 9. Effect of hypoxia on the tetraploid (4C) cell population (**:P<0.01 vs. Control group)

Diploid cell populations in hypoxia 30d group rats were significantly more than that in the control group rats. (Shown in Figure 10)

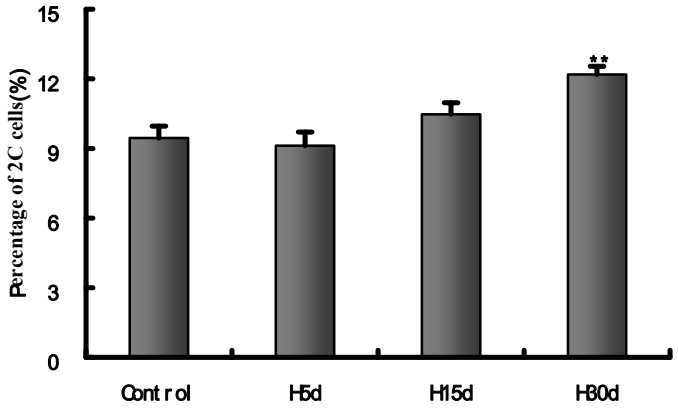


Figure 10. Effect of hypoxia on diploid (2C) cell population (**:P<0.01 vs. Control group)

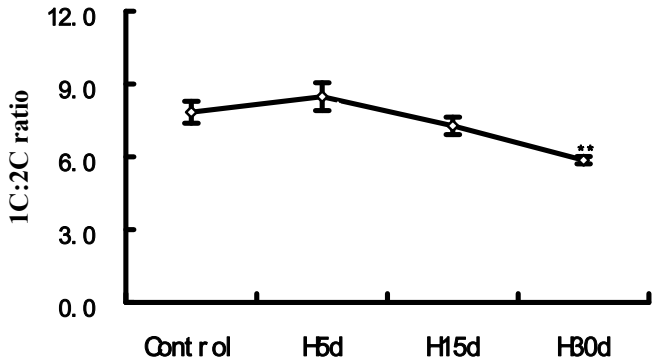


Figure 11. Effect of hypoxia on the ratio of haploid (1C) to diploid (2C) cells in rat tastes (**:P<0.01 vs. Control group)

After exposed to hypoxia for 30d, the ration of haploid and diploid cell population was reduced by 25% when compared with control group. (Shown in Figure 11)

The ratio of tetraploid cells to diploid cells decreased significantly in rats exposed to hypoxia for 15d and 30d. (Shown in Figure 12)

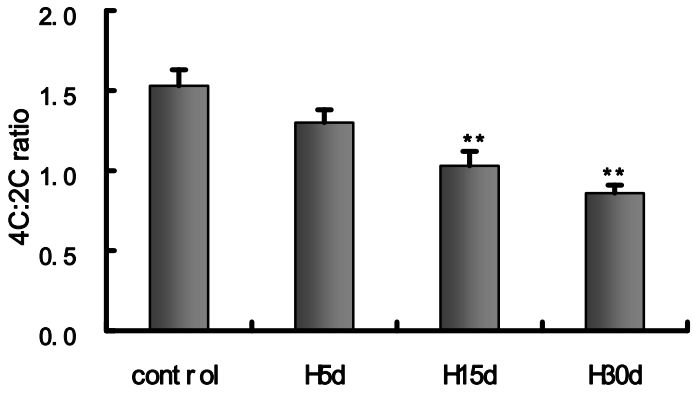


Figure 12. Effect of hypoxia on the ratio of 4C:2C in testes (**:P<0.01 vs Control group)

Proliferation index of 15d and 30d hypoxic groups was significantly lower than that of the control group (Shown in Figure 13). Relative percent of G0/G1 cells in hypoxic 15d group and 30d group was more than control group (P<0.01, shown in Figure 14)

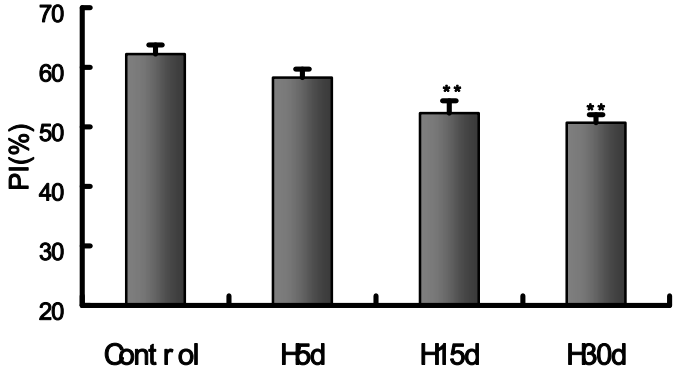


Figure 13. Effect of hypoxia on proliferation index of germ cells in testes (**:P<0.01 vs. Control group)

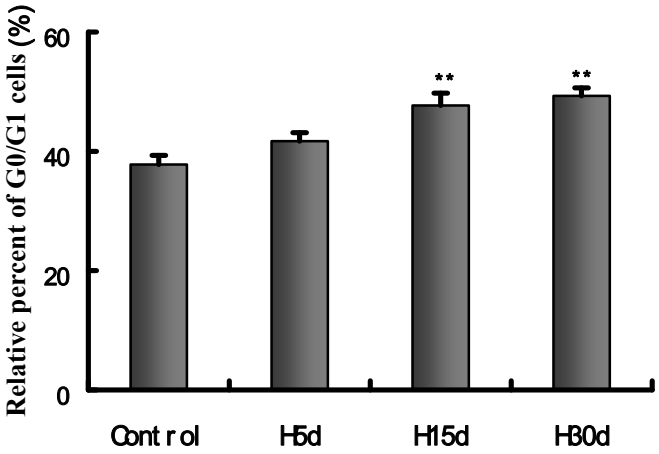


Figure 14. Effect of hypoxia on relative percent of G0/G1 cells in tests (**:P<0.01 vs Control group)

TUNEL

The apoptosis of spermatogonia and spermatocytes increased after exposure to hypoxia for 5d, 15d and 30d. (Shown in Figure 15, 16)

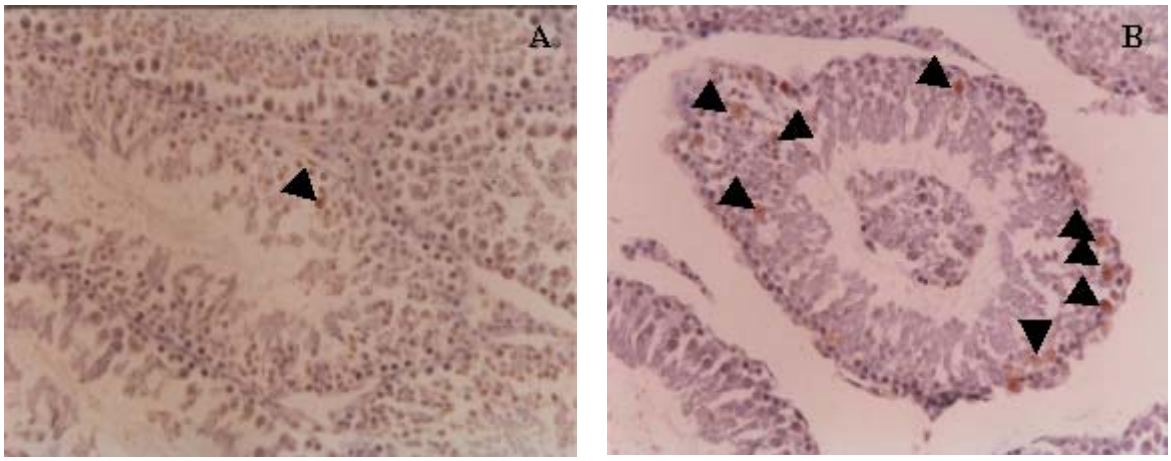


Figure 15. Immunohistochemical demonstration of apoptotic cells by the TUNEL method (magnification: 200×). (A) Control rat (B) 5d hypoxic rat

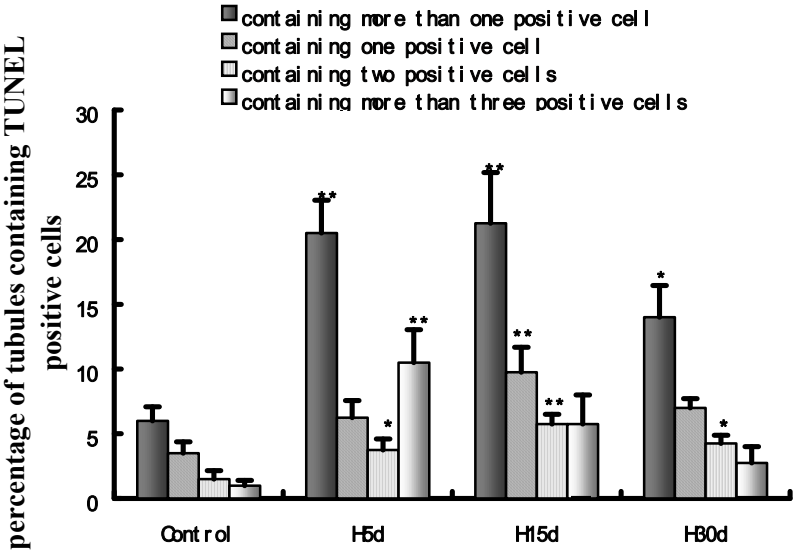


Figure 16. Effect of hypoxia on apoptosis of seminiferous germ cells (*:P<0.05 **:P<0.01 vs. Control group)

DISCUSSION

The density of spermatozoa in the cauda epididymis decreased in hypoxic 15d group and 30d group (Table 1), suggesting adverse effects of hypoxia on the spermatogenic process. This was coincident with the results of previous researches, which indicated that hypoxia changed the seminiferous epithelium and inhibited spermatogenesis in rats (Gosney 1984; Astakhov 1990). Previous studies have revealed that spermatozoa acquire motility and form tail abnormalities mainly in the epididymis (Axner *et al.* 1999). Our study found motility and viability of spermatozoa in hypoxic rats was reduced significantly, and spermatozoa with abnormal tails increased significantly after exposure to hypoxia for 5 days. These results indicate that hypoxia suppressed the maturation of spermatozoa in the epididymis. Since low spermatozoa number and motility and high rates of abnormal spermatozoa have negative correlations with fertility (Bostofte *et al*. 1984), our findings indicate that hypoxia will decrease the fertility of male rats.

Mammalian spermatozoa must undergo a preparation period known as capacitation to become capable of fertilizing oocytes. This process was associated with the removal of cholesterol, leading to an increase in membrane fluidity, calcium influx, and cAMP levels and changes in some enzymatic activities. These biochemical modifications lead to a transient change in the pattern of spermatozoa motility called hyperactivation. The preparation process ends with an exocytotic event called the acrosome reaction, a prerequisite step for oocyte fertilization. The membrane-perturbing properties of LPC have been useful to the study of capacitation. LPC induces the acrosome reaction in capacitated but not in noncapacitated spermatozoa (Byrd and Wolf 1986). The effect of hypoxia on the acrosome reaction was not explored in previous studies. Our study found acrosome reaction rate was suppressed by approximately 60% when exposed to hypoxia. This indicates hypoxia inhibits acrosome reaction of spermatozoa (Figure 1).

Acrosin has function in spermatozoa penetration by limited proteolysis of ZP glycoproteins. Acrosin protease activity has also been involved in dissolution of the acrosomal matrix, and in a regulated release of acrosomal contents at the time of the acrosome reaction (Hardy *et al.* 1991). Previous researches have not revealed the effect of hypoxia on acrosin protease activity. In the present study, we found that acrosin protease reaction rate decreased significantly when exposed to hypoxia (Figure 2), suggesting hypoxia suppressed the acrosin protease activity of spermatozoa. Low acrosome reaction rate and acrosin protease activity in hypoxic conditions will decrease the opportunity for oocyte fertilization.

In this study, we found hypoxia caused testicular interstitial edema, occasional atrophy of seminiferous tubules and germ cell depletion (Figure 3). We also found hypoxia caused more lipid droplets to be deposited in Sertoli cells and spermatogonia to degenerate with slight chromatin margination under electron microscopy (Figure 4). These changes of morphology in testes indicate hypoxia affects spermatogenesis in rats.

Flow cytometry can provide a rapid way to investigate germinal epithelium integrity and to perform a quantitative analysis of spermatogenesis, and has been used in many laboratories to examine spermatogenesis in men and animals (Spano and Evenson 1993; Dong *et al.* 1999). The effect of spermatogenesis was evaluated only by morphological methods in previous studies (Astakhov 1990;

Saxena 1995). In the present studies, quantitative examination was done by flow cytometry. Using this method we demonstrated that ratio of G0/G1 phase cells in hypoxic 15d and 30d group significantly increased (Figure 10). It suggests that hypoxia is capable of inducing cell cycle arrest in G0/G1 phase. Proliferation index of 15d and 30d hypoxic rats decreased significantly (Figure 9) and the total spermatogenic transformation (1C:2C cell ratio) dropped in 30d hypoxic rats (Figure 7), indicating hypoxia restrains spermatogenesis in rats. Flow cytometry also provided convincing evidence that the suppressive effects of hypoxia on spermatogenesis were mainly caused by decreasing tetraploid spermatogenic cells. This was supported by the observation that the ratio of tetraploid to diploid cells was lower in hypoxic rats and the proportions of tetraploid cells were decreased under hypoxic condition (Figure 5, 8), which indicates the direct cytotoxic effect of hypoxia on the pachytene spermatocytes. A similar early reduction in 4C population was observed in mice after administration of 2-methoxyacetic acid (Spano *et al.* 1991) and teniposide (Jyothi *et al*. 2001). Consequently, after exposure to hypoxia 30d, there was a compensatory increase in the relative percentage of 2C cells (Figure 6). Such an increase in relative percentage of 2C cells is due to the accumulation of surviving spermatogonia (Krishnamurthy *et al.* 1998).

The TUNEL assay is a method for quantifying apoptosis in germ cells of rat testes. Percentage of seminiferous tubules containing apoptotic germ cells increased in hypoxic conditions (Figure 12). The apoptotic germ cells were mainly primary spermatocytes and spermatogonia (Figure 11). The apoptosis of spermatogonia and primary spermatocytes may contribute to the loss of tetraploid cells.

CONCLUSIONS

Hypobaric hypoxia suppresses spermatozoa density, motility, viability, and increases the number of morphologically abnormal spermatozoa in rat. Hypoxia also inhibits the acrosome reaction and acrosin activity of rat spermatozoa. Hypobaric hypoxia inhibits spermatogenesis in rats by decreasing the tetraploid spermatogenic cells (primary spermatocytes). Apoptosis of primary spermatocytes and spermatogonia under hypoxic condition may contribute to the decrease in the tetraploid cell population.

REFERENCES

Astakhov, O. B. 1990. Dinamika morfometricheskikh pokazatelei semennikov u krys v khode adaptatsii k vysokogor'iu Antarktidy i posleduiushchei deadaptatsii. [Dynamics of morphometric indicators of rat testes during adaptation to high-altitude in the Antarctic regions with subsequent dysadaptation]. Arkh Anat Gistol Embriol 99: 17-9.

Axner, E., C. Linde Forsberg, and S. Einarsson. 1999. Morphology and motility of spermatozoa from different regions of the epididymal duct in the domestic cat. Theriogenology 52: 767-78.

Bostofte, E., J. Serup and H. Rebbe. 1984. Interrelations among the characteristics of human semen, and a new system for classification of male infertility. Fertil Steril 41: 95-102.

Briz, M., S. Bonet, B. Pinart and R. Camps. 1996. Sperm malformations throughout the boar epididymal duct." Animal Reprod Sci 43: 221-39.

Byrd, W. and D. P. Wolf. 1986. Acrosomal status in fresh and capacitated human ejaculated sperm. Biol Reprod 34: 859-69.

Donald, H. 1981. Man at high altitude. Churchill Livingstone.

Dong, Q., H. Li, Y. Yang, M. Huang and S. Lei. 1999. The effect of vasectomy on spermatogenesis and proliferation of rat spermatogenic cell. Hua Xi Yi Ke Da Xue Xue Bao 30: 417-9.

Eckes, L. 1976. Hohenadaptation. Teil IV: Fertilitat und Fortpflanzung in grossen Hohen [Altitude adaptation. IV. Fertility and reproduction at high altitudes. Gegenbaurs Morphol Jahrb 122: 761-70.

Gosney, J. R. 1984. Effects of hypobaric hypoxia on the Leydig cell population of the testis of the rat. J Endocrinol 103: 59-62.

Hardy, D. M., M. N. Oda, D. S. Friend and T. T. Huang, Jr. 1991. A mechanism for differential release of acrosomal enzymes during the acrosome reaction. Biochem J 275: 759-66.

Hsu, P. C., C. C. Hsu and Y. L. Guo. 1999. Hydrogen peroxide induces premature acrosome reaction in rat sperm and reduces their penetration of the zona pellucida. Toxicology 139: 93-101.

Jyothi, P., G. C. Jagetia and H. Krishnamurthy. 2001. Evaluation of teniposide (VM-26)-induced toxicity on mouse spermatogenesis by flow cytometry. Toxicology 163: 163-74.

Krishnamurthy, H., G. C. Jagetia and P. Jyothi. 1998. Radioprotective effect of zinc aspartate on mouse spermatogenesis: a flow cytometric evaluation. Mutat Res 401: 111-20.

Saxena, D. K. 1995. Effect of hypoxia by intermittent altitude exposure on semen characteristics and testicular morphology of male rhesus monkeys. Int J Biometeorol 38: 137-40.

Spano, M., R. Amendola, C. Bartoleschi, S. Emiliani, E. Cordelli, J. M. Petit, R. Julien and M. H. Ratinaud. 1991. Evaluation of 2-methoxyacetic acid toxicity on mouse germ cells by flow cytometry. J Toxicol Environ Health 34: 157-76.

Spano, M. and D. P. Evenson. 1993. Flow cytometric analysis for reproductive biology. Biol Cell 78: 53-62.

Wyrobek, A. J. and W. R. Bruce. 1975. Chemical induction of sperm abnormalities in mice. Proc Natl Acad Sci U S A 72: 4425-9.

ENDOCRINE DISRUPTING AND TERATOGENIC EFFECTS OF HYPOXIA ON FISH, AND THEIR ECOLOGICAL IMPLICATIONS

Rudolf Wu[8], Eva Shang and B.S. Zhou

ABSTRACT

Hypoxia occurs over thousands of km^2 and poses a major threat to aquatic ecosystems. Elimination of sensitive species, population decline and changes in community have been reported worldwide. We demonstrated that hypoxia is an endocrine disruptor, and can lower the levels of testosterone and estradiol in adult carps (by 77-91%). Disturbances of sex hormones retarded gonad development in both male and female carps, leading to a decline in sperm motility, decrease in fertilization success (from 99.4% to 55.5%), hatching rate (from 98.8% to 17.2%) and larval survival (from 93.7% to 46.4%). Overall, the survival of eggs to larvae decreased from 92.3% in the normoxic group to only 4.4% in the hypoxic group. Experiment on zebrafish further demonstrated that hypoxia is a teratogen, and can significantly increase % malformation (by 77.4%) during their embryonic development. Disruption of apoptotic pattern was clearly evident at 24 hpf, which may be a major cause of malformation. Hypoxia can also delay embryonic development, and upset the balance of testosterone and estradiol at very early developmental stages, implicating that subsequent sexual development may also be affected. Taken together, our results imply that hypoxia may reduce species fitness and threaten the sustainability of natural fish populations over large areas on a global scale.

INTRODUCTION

Hypoxia affects thousands of km^2 of freshwater and marine waters worldwide, and some coastal areas (e.g. Black Sea) have become permanently hypoxic / anoxic (Jin 1994; Diaz and Rosenberg 1995; Smith *et al*. 1999; Levin 2003). Helly and Levin (2004) estimated that the global area of permanent hypoxic shelf and bathyal sea floor are over one million km^2. The severity, frequency occurrence and spatial scale of aquatic hypoxia have increased in the last few decades, and due to rapid human population growth and global warming, the situation is likely to become worse in coming years (Wu 2002). Indeed, hypoxia is now regarded as one of the most serious threats to aquatic populations and genetic diversity (Goldberg 1995; McIntyre 1995; Wu 1999; Gray *et al*. 2002), and there is a pressing need to understand the functional consequences of oxygen depletion in aquatic ecosystems (Levin 2003).

In the course of evolution, fish has developed various strategies to survive hypoxic conditions. These include: (1) enhanced capability to obtain oxygen from water by increasing the binding affinity of oxygen to haemoglobin, increasing water flow over the gills, a reduction of blood flow, promotion of gill diffusing capacity or increasing the perfused number of gill lamellae (Randall 1982); (2) promoting anaerobic metabolism to compensate for a lack of oxygen (Dunn and Hochachka 1986); (3) change in enzyme activities and invoking metabolic depression (Hochachka 1997). Despite the numerous studies on the biochemical and physiological responses of fish to hypoxia, the effects of hypoxia on fish reproduction and development remain very poorly known, albeit reproductive success

[8] Department of Biology and Chemistry, City University of Hong Kong, Kowloon, Hong Kong, SAR, China

is one of the key Darwinian fitness traits in determining species survival.

Reproductive success in fish depends on a number of inter-related factors, namely gonad development, reproductive behavior, fecundity, gamete quality, fertilization success, as well as larval hatchability and larval survival. In fish, all these important processes are regulated by sex steroid hormones (Fostier *et al*. 1983; Tyler and Sumpter 1996; Kime 1998; McCormick 1998; 1999). Thus, disruption of any of these hormones may affect the reproductive process as a whole (Kime 1998). For example, disturbance of the gonadal steroid hormone production will impair gonadal development and hence reproductive success (Kime 1998; Arcand-Hoy and Benson 1998). Effects of hypoxia on sex hormones, gametogenesis, fertilization success and reproductive hormones are virtually unknown.

It is generally accepted that embryonic and larval development are much more sensitive to stress than adult stages (Rand 1995; Rolland *et al*. 1995; Connell *et al*. 1999) since normal histogenesis and organogenesis depend on a series of programmed, highly intricate processes. Disturbance of any of these processes is likely to result in malformation and birth defects, which may ultimately affect the survival and fitness of adults. Surprisingly, effects of hypoxia on embryonic and larval development of fish remain virtually unknown.

This paper presents scientific evidence to show that hypoxia is an endocrine disruptor, and can disrupt the balance of sex hormones, leading to severe reproductive impairments. We also provide data to demonstrate that hypoxia is a teratogen, and fish exposed to sub-lethal levels of hypoxia during their embryonic and larval development will result in a significantly higher percentage of malformation, which may adversely affect species survival.

Hypoxia is an endocrine disruptor

We compared serum levels of testosterone, 17β-estradiol (using ELISA), gonadosomatic index, gonadal histology, spawning success, sperm motility (using a CRISMAS image analysis system), fertilization success and larval hatchability of adult carp (*Cyprinus carpio)* reared under hypoxia (1.0±0.2 mg O_2 l^{-1}) and normoxia (7.0±0.2 mg O_2 l^{-1}) for 8 weeks and 12 weeks (Wu *et al*. 2003).

Changes of sex hormomes

After 8 weeks of exposure to hypoxia, serum testosterone levels were significantly reduced (-77%), but estradiol (E_2) levels increased significantly (+90%) in male carps ($p<0.001$). Serum testosterone concentrations showed a significant correlation with degrees of maturation of the testis ($r=0.69$). Serum estradiol and testosterone levels in female carp exposed to hypoxia for 8 weeks were reduced by 80% to 91% respectively ($p<0.01$) (Table 1).

Table 1. GSI and serum hormone levels (ng ml^{-1}) of carp after exposure to normoxia (7 mg O_2 l^{-1}) hypoxia (1 mg O_2 l^{-1}) for 8 weeks

	Male		Female	
	7.0 mg O_2 l^{-1}	1.0 mg O_2 l^{-1}	7.0 mg O_2 l^{-1}	1.0 mg O_2 l^{-1}
GSI	10.23±0.88	4.26±0.61***	18.51±2.18	6.09±2.81**
Testosterone	9.83±0.95	2.22±0.72***	8.23±1.15	0.73±0.31***
Estradiol	0.033±0.004	0.063±0.06***	0.99±0.35	0.19±0.14**

Mean ± SE; n=7-11. Asterisk(s) indicate values significantly different from the normoxic control. (t-test: *, P<0.05; **, P<0.01; ***, P<0.001).

GSI and gonadal histology

A significant decrease in the gonadosomatic index (GSI) was found in both male and female carps after 8 weeks of exposure to hypoxia (Table 1). Histological examination further showed a reduced number of spermatocysts (SPC) cells and smaller lobules, but a larger number of spermatogonia (SPG) cells in hypoxic fish, indicating gametogenesis was impaired by hypoxia. (P<0.001; Figure1). Retardation of gonad development in female carp was exemplified by smaller gonads and less yolk in each egg (P<0.001; Figure 1).

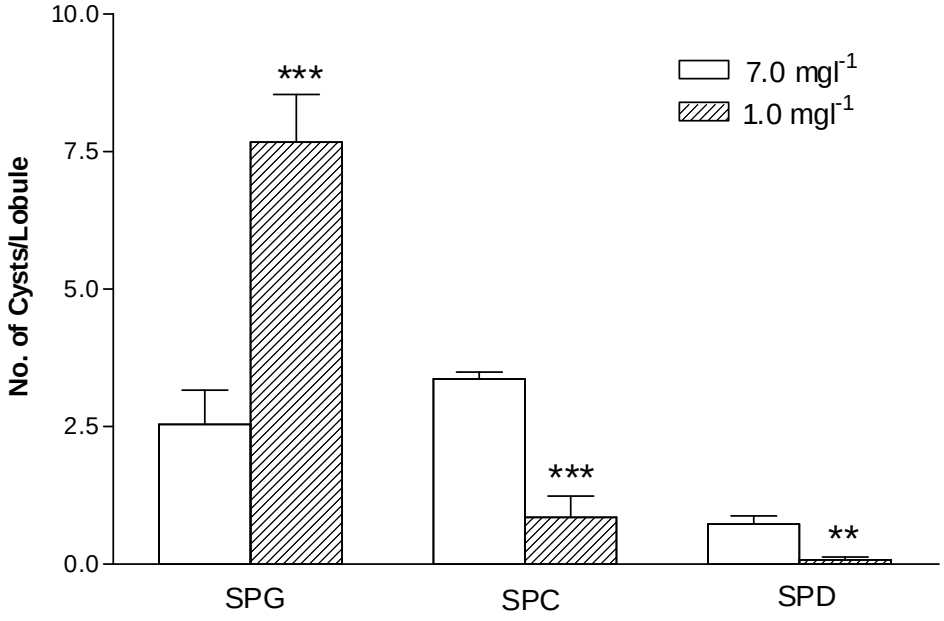


Figure 1. Number of spermatogonia (SPG), spermatocytes (SPC), and spermatid (SPD) of the testes of *C. carpio* upon exposure to 7.0 and 1.0 mg O_2 l^{-1} for 8 weeks. Values significantly different from the control are indicated by asterisks. (N=7-11, Mean ± SD). (**, P<0.01; ***, P<0.001).

Sperm motility, fertilization success, hatching success and larval survivorship

A significant decrease in sperm motility was found in fish after exposure to hypoxia for 12 weeks (Table 2), indicating that sperm quality was impaired. Percentage fertilization success was reduced from 99.4% in the normoxic group to 55.5% in the hypoxic group (P<0.001; Figure 2b). 98.8% fertilized eggs produced by the normoxic group hatched to fry, while only 17.2% of fertilized egg produced by the hypoxic group hatched (P<0.01; Figure 2b). 93.7% hatched larvae survived in the normoxic group, while larval survival decreased to 46.4% in the hypoxic group 24h post-hatching (P<0.001; Figure 2b). Overall, survival from eggs to larvae was 92.3% in the normoxic group but only 4.4% in the hypoxic group (P<0.001; Figure 2A).

Table 2. S perm motility of carp after exposure to normoxia (7.0 mg O_2 l^{-1}) and hypoxia (1.0 mg O_2 l^{-1}) for 12 weeks.

	7.0 mg O_2 l^{-1}	**1.0 mg O_2 l^{-1}**
VCL	77.42±13.03	46.25±4.84*
VSL	38.83±9.39	10.65±1.74*
VAP	47.96±2.69	21.12±4.49*

Mean ± SE, N=6. The velocity is expressed as μm S^{-1}. Asterisk indicates values significantly different from the control. (t-test; *: P<0.05). (VCL: mean curvilinear velocity; VSL: mean straight-line velocity; VAP: angular path velocity)

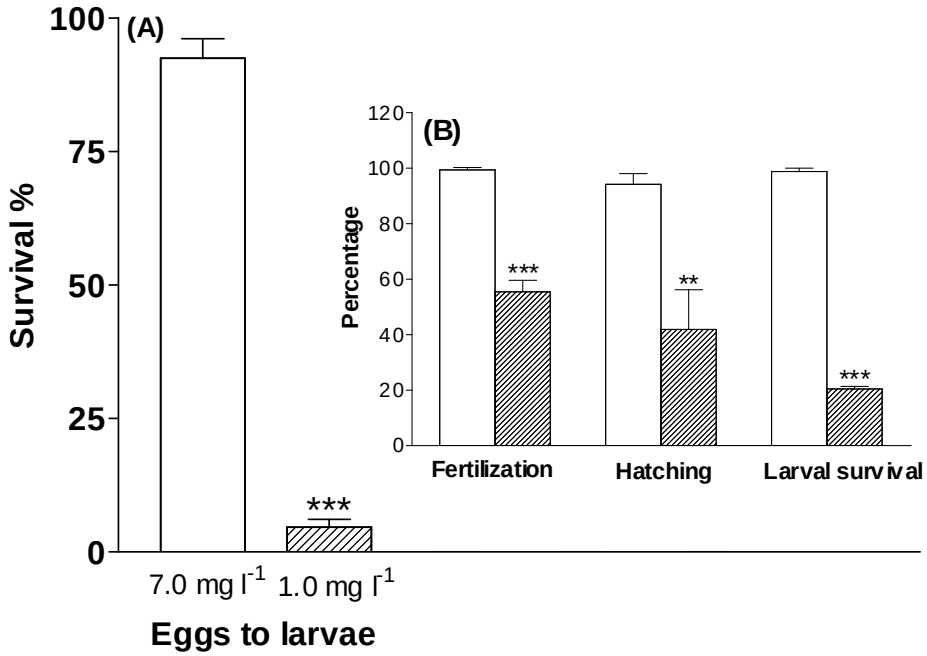


Figure 2. (A) Percentage survival of eggs to larvae; (B) Percentage of fertilization, hatching rate and larval survivorship of *C. carpio* upon exposure to 7.0 and 1 mg O_2 l^{-1} for 12 weeks. Values significantly different from the control are indicated by asterisks. (N=6, Mean±SD). (**: P<0.01; ***: P<0.001)

The above results provide clear evidence that hypoxia disrupts endocrine function, reduces GSI, gametogenesis and maturation of gonads, quality of gametes, fertilization, hatching, and larval survival.

In male fish, testosterone plays an important role in stimulating and maintaining spermatogenesis (Kime 1998; Fostier 1983) Estradiol normally occurs only in trace amounts in male fish, albeit ineffective in regulating spermatogenesis (Kime 1998), higher levels of estradiol can inhibit spermatogenesis (Billard 1981). Thus both low levels of testosterone and high levels of estradiol may have contributed to the reduction in spermatogenesis in male hypoxic fish.

Sperm motility is indicative of sperm quality and also a major factor determining successful fertilization and quality of fish larvae (Evans 1998; Kime 1998, 1999). Time to spawning, egg size, egg viability, fertilization success, time to hatching, and larval survivorship have all been regarded as important attributes leading to reproductive success (Fahræus-Van Ree 1997; Arcand-Hoy 1998). Fertilization success and the proportion of normally developing embryos and larvae depend upon the quality of the eggs and sperm (Moodie 1989). Factors influencing the maternal levels of testosterone can have a major impact on larval survivorship (Moodie 1989). It is clear from our results that fish exposed to hypoxia produce inferior quality gametes and offspring, which in turn, probably accounted for the observed low hatching success and high larval mortality.

The above evidence showed that hypoxia reduces the overall reproductive success of fish by disturbing endocrine functions, which in turn affect gametogenesis, sexual maturity, gamete quality, fecundity, fertilization success, hatching, development as well as viability of larvae. The impairment of fish reproduction by hypoxic stress may be an important mechanism accounting for the population decline and changes of fish species composition observed in hypoxic environments.

Hypoxia is a teratogen

We compared development of zebrafish embryos exposed to normoxia (5.8 mg/L) and hypoxia (0.8 mg/L) at time interval (24, 48, 72, 96, 120 and 168hpf) (Shang and Wu, 2004). Developmental stages were classified according to Kimmel *et al*. (1995), and embryos displaying noticeable external abnormalities such as spinal deformity and cardiac deformities were considered as malformed embryos. Embryos were sampled from the hypoxic and normoxic group at 24hpf when significant extent of apoptosis occurs and stained by Acridine Orange (Cole and Ross 2001). Total number of apoptotic cells was counted and compared.

Development was clearly delayed when zebrafish embryos were exposed to hypoxia. Embryos exposed to 0.5 mg O_2 L^{-1} required twice as long for them to develop to the same developmental stages as their normoxic counterpart (Figure 3). Besides, embryos exposed to hypoxia developed lost synchronization in their development, with their tails developed much faster than the head.

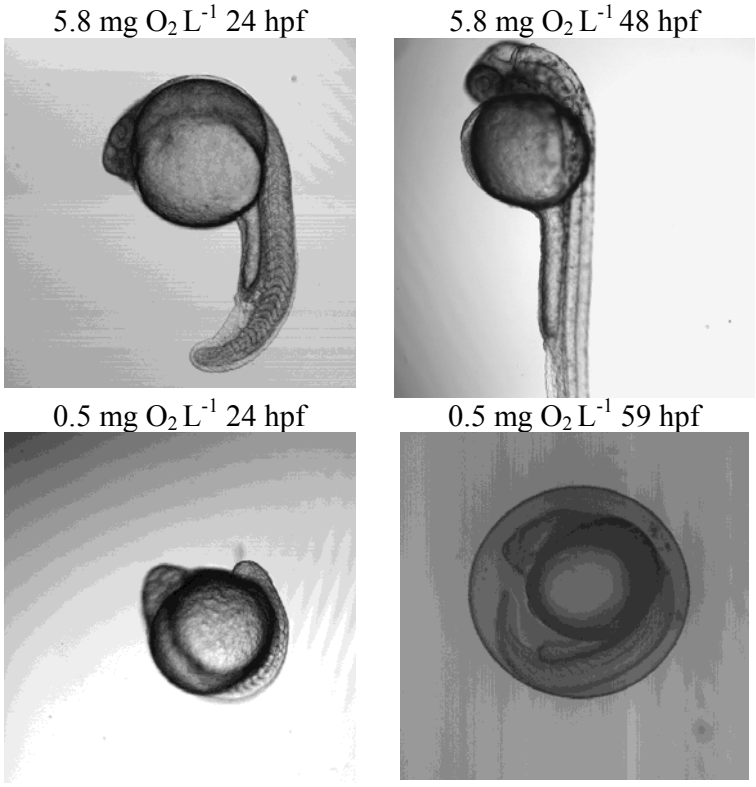


Figure 3. A comparison of zebrafish development under hypoxia (0.5mg O_2 L^{-1}) and normoxia (5.8 mg O_2 L^{-1})

After 168 hpf, percentage of malformation (i.e. spinal deformity and altered axial curvature) in the hypoxic group (18.3 ± 2.31%) was significantly higher than that of the control (10.3 ± 1.15%, Student's t-test, $P<0.01$) (Figure 4). Under hypoxia, many embryos failed to develop their vascular systems after several days and died.

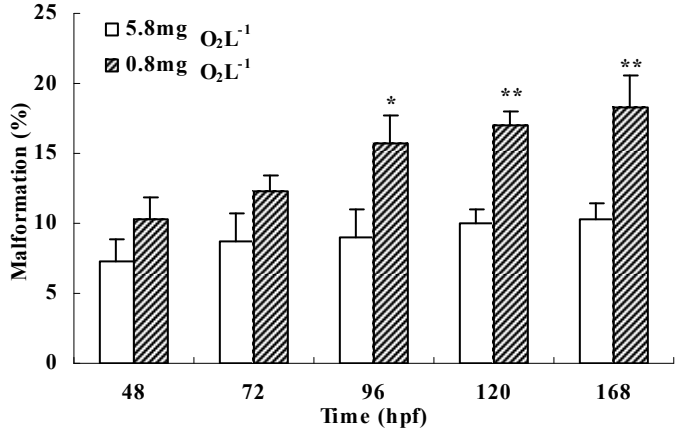


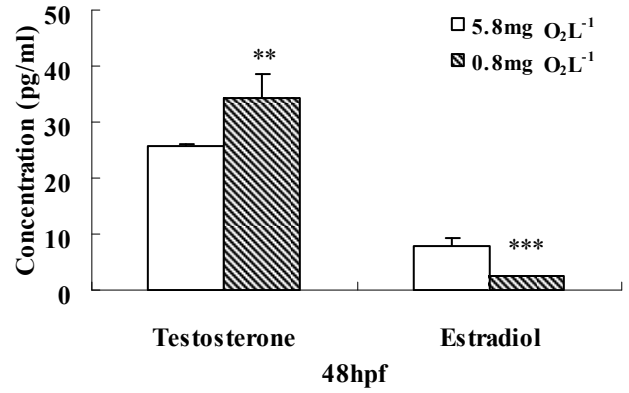
Figure 4. Percentage malformation in zebrafish embryos during different developmental stages (48, 72, 96, 120, 168 hpf) upon exposure to 5.8 mg O_2 L^{-1} (Normoxia) and 0.8 mg O_2 L^{-1} (Hypoxia). Values significantly different from the normoxic control are indicated by asterisks (N=5, Mean ± SD) (t-test: *, $p<0.05$, **, $p<0.01$).

Sex Hormones

At 48hpf, testosterone levels in hypoxic embryos showed a significant increase from 25.6 ± 0.56 to 34.4 ± 4.2 pg mL^{-1} ($P<0.01$), while estradiol was significantly reduced from 8.0 ± 1.4 to 2.4 ± 0.1 pg mL^{-1} ($P<0.001$) (Figure 5A).

A reversed pattern was observed at 120hpf: testosterone in hypoxic embryos was significantly reduced from 36.1 ± 1.43 to 27.3 ± 2.75 pg mL^{-1} ($P<0.01$), while estradiol was significantly increased from 4.61 ± 1.28 to 12.5 ± 4.10 pg mL^{-1} ($P<0.05$) (Figure 5B).

A:



B:

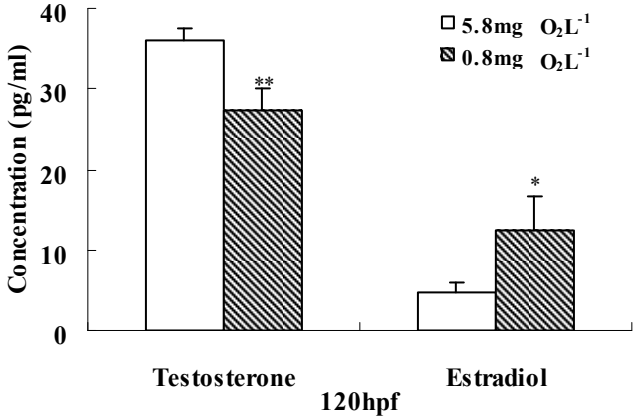


Figure 5. Testosterone and estradiol (pg/mL) in zebrafish embryos at 48 hpf (A) and 120 hpf (B) upon exposure to 5.8 mg O_2/l (Normoxia) and 0.8mg O_2/l (Hypoxia). Values significantly different from the normoxic control are indicated by asterisks (N=4, Mean ± SD) (t-test: *, $p<0.05$, **, $p<0.01$, ***, $p<0.001$).

Apoptosis

Compared with the normoxic control, number of apoptotic cells in the tail of hypoxic embryos were significantly reduced (-63.7%, $p<0.01$). In contrast, a significantly higher percentage (+116%, $p<0.05$) of apoptotic cells was found in the brain of hypoxic embryos as compared with control embryos (Figure 6). The above observations clearly indicated that the apoptotic pattern in zebrafish embryos was altered by hypoxia.

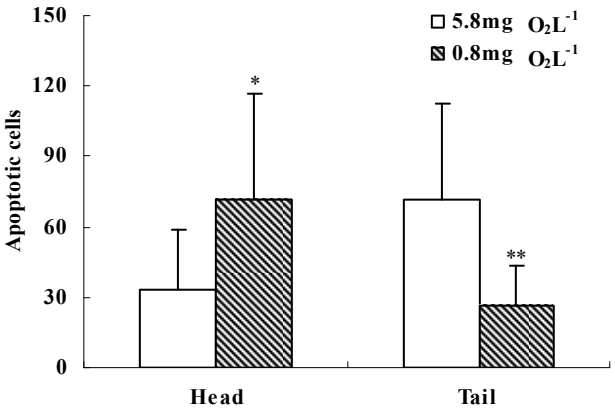


Figure 6. Number of apoptotic cells at 24 hpf in zebrafish embryos upon exposure to 5.8 mg O_2 L^{-1} (Normoxia) and 0.8 mg O_2 L^{-1} (Hypoxia). Values significantly different from the control are indicated by asterisks (N=10, Mean ± SD) (t-test: *, $p<0.05$, **, $p<0.01$).

Ecological implications and significance

Endocrine disruption by chemical pollutants has been related to the decline in many fish, reptile, bird and wildlife populations (Colborn 1993; Tyler *et al*. 1998), and has therefore become a major environmental concern in the last decade. For example, tributyl tin has caused imposex in female whelks, leading to a male biased sex ratio and hence extinction of natural populations worldwide, ranging from the North Sea to New Zealand (Ten 1994). The present study documents, for the first time, that hypoxia can also cause endocrine disruption in fish. The facts that (a) hypoxia occurs over one million km^2 globally (Levin 2003) imply that endocrine disruption caused by hypoxia may be a wide spread phenomenon, and the problem of endocrine disruption caused by hypoxia is potentially much more serious than that caused by any known, anthropogenic chemicals. Since the modulating sex hormones are highly conservative, hypoxia induced endocrine disruption observed in carp and zebrafish may possibly also occur in other fish species or animal groups.

Our results also provided, for the first time, clear evidence that hypoxia does not only affect adults but also embryonic and larval development of fish. Indeed, the fact that a higher percentage of malformed fish is often found in polluted water (Au 2004) where hypoxia frequently occur lend further support to our laboratory findings. These developmental impairments and malformation at the earlier stages of life cycle may subsequently reduce the fitness and chance of survival of individuals in natural populations.

In conclusion, hypoxia could significantly impair fish reproduction, through a significant decrease in GSI, retardation of gonad development; disruption in reproductive hormones; lowering of sperm quality and fertilization ability. These impairments at various stages culminated in a reduction in hatching rate and survivorship of the larvae. Hypoxia also affects embryonic development of fish, leading to malformation. Taken together, these findings may form a scientific basis for interpreting and/or predicting population decline and changes of fish species composition in hypoxic environments.

ACKNOWLEDGEMENT

The work described in this paper was supported by the Central Allocation Grant Scheme of the Research Grants Council of the Hong Kong Special Administrative Region, China (CityU 2/03C) and a grant from the Area of Excellence Scheme (Project No. AoE/P-04/04).

REFERENCES

Arcand-Hoy, L.D., and W.H. Benson. 1998. Fish reproduction: An ecologically relevant indicator of endocrine disruption. Environmental Toxicology and Chemistry (Annual Review) 17: 49-57.

Au, D.W.T. 2004. The application of histo-cytopathological biomarkers in marine pollution monitoring: A review. Marine Pollution Bulletin 48: 817-834.

Billard, R., B. Breton, and M. Richard. 1981. On the inhibitory effect of some steroids on spermatogenesis in adult rainbow trout *Salmo gairdneri*. Canadian Journal of Zoology 59: 1479-1487.

Colborn T., F. S. Vom Saal, and A. M. Soto. 1993. Developmental effects of endocrine disrupting chemicals in wildlife and humans. Environmental Health Perspectives. 101: 378-384.

Cole L.K., and L.S. Ross. 2001. Apoptosis in the developing zebrafish. Developmental Biology 240: 123-153.

Connell, D.W., P.K.S. Lam, B.R. Richardson, and R.S.S. Wu. 1999. *In:* Ecotoxicology. Blackwell Science, UK, pp50-77.

Diaz, R. J., and R. Rosenberg. 1995. Marine benthic hypoxia: a review its ecological effects and the behavioural responses of benthic marcofauna. Oceanography and Marine Biology Annulal Review) 33: 245-303.

Dunn, J.F., and P.W. Hochachka. 1986. Metabolic responses of the trout (*Salmo gairdneri*) to acute environmental hypoxia. Journal of Experimental Biology 123: 229-242.

Evans, J. P., and A.J. Geffen. 1998. Male characteristics, sperm traits, and reproductive success in winter-spawning Celtic Sea Atlantic herring, Clupea harengus. Marine Biology 132: 179-186.

Fahræus-Van Ree, and G.E., J.F. Payne. 1997. Effects of toxaphene on reproduction of fish. Chemosphere 34: 855-867.

Fostier, A., B. Jalabert, B. Billard, B. Breton, and Y. Zohar. 1983. *In:* Fish Physiology, W.S. Hoar, D.J. Randall, E.M. Donaldson, Eds.; Academic Press: New York, pp. 277-346.

Goldberg, E.D. 1995. Emerging problems in the coastal zone for the twenty-first century. Marine Pollution Bulletin 31: 152-158.

Gray J.S. 2002. The species diversity of marine soft sediments. Marine Ecology-Progress Series. 244: 285-297.

Helly J.J., and L.A. Levin. 2004. Global distribution of naturally occurring marine hypoxia on continental margins. Deep-Sea Research I 51: 1159-1168.

Hochachka, P.W. 1997. Oxygen-A key regulatory metabolite in metabolic defense against hypoxia. American Zoology 37: 595-603.

Jin X.C. 1994. An analysis of lake eutrophication in China. *In:* Inland Waters of Tropical Asia and Australia: Conservation and Management, Dudgeon D. and P.K.S Lam (eds.). pp. 207-211

Kime, D.E. 1998. *In:* Endocrine disruption in fish, Kluwer-Acad. Press.

Kime, D.E., and J.P. Nash. 1999. Gamete viability as an indicator of reproductive endocrine disruption in fish. Science of the Total Environment 233: 123-129.

Kimmel, C.B., W.W. Ballard, S.R. Kimmel, B. Ullmann, and T.F. Schilling. 1995. Stages of embryonic development of zebrafish. Developmental Dynamics 203: 253-310.

Levin L.A. 2003. Oxygen minimum zone benthos: adaptation and community response to hypoxia. Oceanography and Marine Biology: an Annual Review 41: 1-45.

McCormick, M.I. 1998. Behaviorally induced maternal stress in a fish influences progeny quality by a hormonal mechanism. Ecology 79: 1873-1883.

McCormick, M.I. 1999. Experimental test of the effect of maternal hormones on Larval quality of a coral reef fish. Oecologia 118: 412-422.

McIntyre, A.D. 1995. Human impact on the oceans: The 1990s and beyond. Marine Pollution Bulletin 31: 147-151

Moodie, G.E.E., N.L. Loadman, M.D. Wiegand, and J.A. Mathias. 1989. Influence of egg characteristics on survival, growth and feeding in larval walleye (*Stizostedion vitreum*). Canadian Journal of Fisheries and Aquatic Sciences 46: 516-521.

Rand, G.M. 1995. Fundamentals of aquatic toxicology. Effects, environmental fate and risk assessment, 2nd edition. Taylor and Francis, Washington, D.C., USA.

Randall, D.J. 1982. The control of respiration and circulation in fish during exercise and hypoxia. Journal of Experimental Biology 100: 275-288

Rolland, R.M., M. Gilbertson, and R.E. Peterson. (Eds.) 1995. Chemically induced alterations in functional development and reproduction of fishes. Proceedings from a session at the Wingspread Conference Center; 21-23 July 1995; Racine WL, SETAC Press, Pensacola, FL, USA

Shing, E.H.H., and R.S.S. Wu. 2004. Aquatic hypoxia in a teratogen and affects fish embryonic development. Environmental Science and Technology 38: 4763-4767.

Smith V.H., G.D. Tilman, and J.C. Nekola. 1999. Eutrophication: impacts of excess nutrient inputs on freshwater, marine, and terrestrial ecosystems. Environmental Pollution 100: 179-196

Ten Hallers-Tjabbes, C.C., J.F. Kemp, and J.P. Boon. 1994. Imposex in whelks (*Buccinum undatum*) from the open North Sea: relation to shipping traffic intensities. Marine Pollution Bulletin 28: 311–313

Tyler C.R. and J.P. Sumpter. 1996. Oocyte growth and development in teleosts. Reviews in Fish Biology and Fisheries 6: 287-318.

Tyler C.R., S. Jobling, and J.P. Sumpter. 1998. Endocrine disruption in wildlife: a critical review of the evidence. Critical Review Toxicology 28:319-361.

Wu, R.S.S. 1999. Eutrophication, trace organics and water-borne pathogens: pressing problems and challenge. Marine Pollution Bulletin 39: 11-22.

Wu, R.S.S. 2002. Hypoxia: from molecular responses to ecosystem responses. Marine Pollution Bulletin 45: 35-45

Wu, R. S. S., B.S. Zhou, D.J. Randall, N.Y.S. Woo, and P.K.S. Lam. 2003. Aquatic hypoxia is an endocrine disruptor and impairs fish reproduction. Environmental Science and Technology 37: 1137-1141

EFFECTS OF HYPOXIA ON LIPID METABOLISM IN TELEOSTS

Guido van den Thillart[9]

ABSTRACT

Hypoxia is a naturally occurring phenomenon for fishes. So, a protection mechanism can be expected particularly with respect to lipid catabolism. By measuring the lactate response it is possible to determine whether a teleost is able to survive and even to adapt to a certain oxygen level. It is hypothesized that recovery will occur at oxygen levels above the critical oxygen level (PO_2)crit reflected by a transient lactate increase. In contrast, continuous lactate accumulation occurs at oxygen levels below the (PO_2)crit, which will be lethal in case of prolonged exposure. Results from cannulated common carp and rainbow trout exposed to stepwise decreasing oxygen levels show that both individual and inter-specific differences in lactate response occur during exposure to hypoxia. Both in carp and trout a strong correlation exists between changes in lactate and both catecholamine levels.

Hypoxia increases the levels of catecholamines, which in mammals activate lipolysis. The oxidation of the released fatty acids, however, is impaired at low oxygen levels. Thus plasma and tissue free fatty acids accumulate in (mammalian) tissues under hypoxia which ultimately leads to death. There are no indications, however, that this type of tissue damage develops also in fishes. We observed a strong negative correlation between the level of noradrenaline and the level of plasma free fatty acids in fish during chronic hypoxia exposure. The fact, that noradrenaline levels remain elevated when the hypoxic condition persists, suggests that this hormone has a controlling function. Infusion studies with specific α- and β- agonists and antagonists demonstrate that there are β_1-adrenoceptors that inhibit lipolysis. In-vitro studies with adipocytes, isolated from several fish species, confirmed these results. There is also evidence that this is mediated by the inhibition of hormone sensitive lipase. Thus we can conclude that in fishes the down-regulation of plasma FFA levels under hypoxia is controlled by catecholamines. As fatty acid cannot be oxidized under hypoxia, this downregulation may be a protection mechanism in fish against fatty acid intoxication.

INTRODUCTION

Over the last two decades, the hormonal regulation of carbohydrate metabolism in fish, especially in liver, has received increasing attention. It has been demonstrated that the mobilization of glucose from the liver is stimulated by hormones such as catecholamines, cortisol and glucagon (Janssens and Waterman 1988; Danulat and Mommsen 1990; Puviani *et al*. 1990; Brighenti *et al*. 1991) in particular during stress conditions such as hypoxia (Wright *et al*. 1989). In contrast, little information is available on the regulation of lipid metabolism in teleost fish. Several reviews on fish lipid metabolism have been published showing a restricted knowledge compared to the mammalian situation (Greene and Selivonchick 1987, Hendersen and Tocher 1987, Sheridan 1988). In mammals two types of stress responses are described i.e. coping and non-coping (Bohus *et al*. 1987). These responses are related to the prevelance of cortisol vs catecholamine release in the circulation. More recently Van Raaij *et al*. (1996a) have demonstrated these two responses in hypoxic trout. The non-coping group showed a

[9] Institute of Biology, section Integrative Zoology, University of Leiden, Van der Klaauw Laboratories, Kaiserstraat 63, PO Box 9516, 2300 RA Leiden, The Netherlands

surge of catecholamines and high lactic acid levels. This group did not survive the hypoxic conditions, whereas the coping group with higher cortisol levels and lower catecholamine levels did. This suggests that animals under stress may show opposing responses with respect to behaviour, hormones, and metabolites. In mammals epinephrine levels increase in response to a psycho-physiological disturbance, while norepinephrine increase is related to exercise stress (Scheurink *et al*. 1989, Bohus *et al*. 1987). Increased norepinephrine levels are often due to sympathetic overflow from postganglionic adrenergic nerve terminals. In mammals catecholamines have a strong lipolytic action (Lafontan and Berlan 1993) which is connected to an increased energy requirement. Hypoxia causes a surge of catecholamines in all vertebrates. Under such conditions a consistent activation of lipolysis is observed in mammals, due to the high release of catecholamines (Prinzen *et al*. 1984; Prasad *et al*. 1991). The effect of hypoxia on lipolysis in fish is, however, not clear and the available results are conflicting (see review Van den Thillart *et al*. 2002). Considering the strong lipolytic character of catecholamines in mammals, a reduction of lipolysis during hypoxia is a puzzling phenomenon and strongly suggests a different regulatory mechanism in fish. The role of catecholamines in fish is summarized in a several recent reviews (Randall and Perry 1992, Wendelaar Bonga 1997, Reid *et al*. 1988), describing the control of catecholamines release, the effects of catecholamines on the cardiovascular system, and on the ion transport mechanisms. The effect of catecholamines on metabolism has been reviewed recently by Fabbri *et al* (1998). This review is mainly focused on glucose metabolism and signal transduction mechanisms. With respect to lipid metabolism in fish reviews have been published by Sheridan (1988, 1994) and Van den Thillart *et al*. 2002).

Hypoxia can be a regularly occurring phenomenon for many teleost fishes under natural circumstances. These fish species have developed adaptations, which enable them to survive different hypoxic situations (Van den Thillart 1982). Adaptations may include adjustments in the oxygen-carrying capacity/affinity (Perry and Wood 1989; Boutilier *et al*. 1988), a diminution of the oxygen consumption rate by a reduction in locomotor activity (Lomholt and Johansen 1979; Van den Thillart *et al*. 1994) or even a depression of the overall energy metabolism (Van Ginneken *et al*. 1997). However, generally these adaptations occur with a certain delay before reaching the required levels and therefore, mostly they are not sufficient to meet the energy demands at the onset of the hypoxia exposure, which is then connected with a certain level of stress. To compensate energy imbalance all activate anaerobic glycolysis resulting in accumulation of lactic acid (Jørgensen and Mustafa 1980; Dunn and Hochachka 1986, 1987; Dalla Via *et al*. 1994) and consequently in metabolic acidosis (Boutilier *et al*. 1988; Van Raaij *et al*. 1996a). Since low pH inhibits physiological processes and can even cause cell damage and/or cell death (Van den Thillart and Van Waarde 1985) anaerobic metabolism can only be maintained for a short period. For survival of long-term hypoxia it is important for the animal to obtain a new balance in oxygen extraction and oxygen consumption, which must allow aerobic recovery from earlier anaerobic stimulation. In this case the lactate level may rise first and drop later due to recovery. Below a certain critical oxygen level ((PO_2)crit) no recovery will be possible resulting in a continuous accumulation of lactic acid. Therefore, monitoring plasma lactate during hypoxia will tell us whether the animal is able to recover at that level. Recently, it has been demonstrated (Van Raaij *et al*. 1996b) that with respect to behaviour in rainbow trout two opposite coping strategies can be distinguished during deep hypoxia. A passive coping strategy of "wait and see" reflected by a quiet behaviour and an active coping strategy characterized by a large amount of burst type activity. Plasma lactate levels were 4-5 fold higher in the active animals suggesting that the anaerobic threshold was reached almost immediately in the active fish, while it could be postponed in quiet animals. In general, oxygen restriction is experienced as a stressful condition resulting in the release of adrenaline, noradrenaline (Boutilier *et al*. 1988; Randall and Perry 1992; Van Raaij *et al*. 1996c) and cortisol (White and Fletcher 1989; Van Raaij *et al*. 1996c).

MATERIAL AND METHODS

Experimental protocol for cannulation experiments

The experiments were performed in a recirculation system with a total volume of 96.5 L. The water of this system was kept at a temperature of 20.0 ± 0.5 °C for carp and 15.0 ± 0.5 °C for trout. It was air saturated at a normoxic level of 80-90% air saturation (AS; 15.5-17.5 kPa PO_2) and pumped through flow-chambers at a rate of 0.8-1.0 l min-1. Before the start of the experiment three fish were captured and placed individually into the flow-chambers with dimensions 55x10x18cm (carp) or 55x8x14cm (trout). Each experimental chamber was closed with a gas-tight perspex lid, which was partially darkened over the head of the fish in order to block visual perception. During the experimental period the fish were deprived of food.

After three days of acclimation the dorsal aorta was cannulated, according to a modification of the method of Soivio *et al*. (1975) as described by Van Dijk *et al*. (1993), with a poly-ethylene (PE 50) catheter (Rubber, Hilversum, The Netherlands) filled with saline containing 200 IU ml-1 heparin (Sigma, St. Louis, U.S.A.). After surgery the fish was placed into the flow-chamber and allowed to recover for two days before the experiments were carried out. This careful 5-day pre-experimental protocol has been shown to minimize the amount of stress associated with handling, anesthesia and surgery (Van Raaij *et al*. 1996c). Each day the cannulae were flushed with heparinized saline (200 IU ml-1) and filled with a viscose solution of poly-vinyl-pyrrolidon (Merck, F.R.G., 1 g PVP ml-1 saline) containing 500 IU ml-1 heparin. Using this procedure, the diffusion of heparin into the circulation is negligible.

Before the experiments were started two control blood samples were taken within 30 minutes (between 08:00 and 09:00 a.m.) for measurement of the initial values of metabolites and stress hormones. Directly after the second blood sample carp were exposed to a controlled linear PO_2 decline from 80% AS to 30% AS, which was reached in 2 hours. Thereafter, the PO_2 was stepwise decreased further depending on the experiment. Trout experienced a PO_2 decline from 80% AS to 40% AS (over 2 hours), after which the PO2 was stepwise decreased further depending on the experiment. After the hypoxia exposure, the water was rapidly re-aerated again and the cannulae were removed. Blood samples and analyses were carried out as described earlier (Van Raaij *et al*. 1996c).

Isolation and incubation protocol for adipocytes

Tilapias were killed by a sharp blow on the head followed by spinal transection at the cervical level. Samples of abdominal adipose tissue were removed rapidly from the gastro-intestinal tract without connective tissue obtaining portions of 8-10 g ($fish^{-1}$) and placed immediately into a Petri dish with Krebs-Henseleit buffer (in mmol l^{-1}: 117.5 NaCl, 5.6 KCl, 1.18 $MgSO_4$, 2.52 $CaCl_2$, 1.28 NaH_2PO_4, 25.0 $NaHCO_3$ and 5.5 D-glucose) pregassed with 5% CO_2 in O_2, pH 7.4. The portions of adipose tissue were chopped obtaining slices of 1 mm^2. Cells were isolated by incubation in a water shaking bath in a Teflon vessel containing 20 ml of Krebs-Henseleit buffer per portion with the addition of 1% bovine serum albumin, BSA (Fraction V, Sigma, St. Louis, U.S.A.) and collagenase 130 U ml^{-1} (type II, Sigma, St. Louis, U.S.A.), at 25°C under an atmosphere of 5% CO_2 in O_2, pH 7.4. After 1.5 hour, the suspensions of adipocytes were filtered through a nylon cloth and washed three times with Krebs-Henseleit buffer (1% BSA). In a water shaking bath equilibrated at 5% CO_2 in O_2 portions of 100 µl adipocyte-suspension (approximately $3x10^5$ cells) were incubated in Teflon vessels for 5 h at 25°C in a total volume of 3 ml Krebs-Henseleit buffer with or without (controls) the addition of an adrenoceptor (ant)agonist and

containing 2% bovine serum albumin. Control measurements were performed in triplicate and experimental measurements in duplicate. When both an agonist and antagonist were used, the adipocytes were pre-incubated with the antagonist 15 minutes before the agonist was added. The incubation period was terminated by adding the content of each vessel to 3 ml of extraction medium (1-propanol / n-heptane / 1N H_2SO_4 = 40:20:1). The tubes were mixed for 60 seconds and centrifuged for 5 minutes at 2000 rpm in a Hettich Rotixa/KS centrifuge. From the upper layer 400 µl was used for FFA-determination. (Vianen *et al*. 2002).

RESULTS AND DISCUSSION

Response of cannulated carp and trout to stepwise decreasing oxygen levels

Cannulated common carp (Cyprinus carpio) and rainbow trout (Oncorhynchus mykiss) were exposed to stepwise decreasing oxygen levels and during each oxygen level plasma lactate and stress hormones were measured repeatedly. It was hypothesized that at oxygen values (just) above the (PO_2)crit activation of the anaerobic metabolism will be followed by aerobic recovery. In contrast, continuous accumulation of lactate will be observed during oxygen levels below the (PO_2)crit associated with strong increases in circulating stress hormone levels.

Changes in lactate levels in cannualted carp and trout is shown in figure 1. Exposure of carp to 30% AS (5.8 kPa PO_2) caused activation of the anaerobic glycolysis in 2 out of 6 animals. However, after 1-2 hours at 30% AS the lactate levels declined in these animals indicating that the anaerobic activation was temporal and that 30% AS is above the (PO_2)crit. During 15% AS (2.9 kPa PO_2) all animals showed an increase in plasma lactate. In two fish (fish II and III) lactate levels stabilized during this period indicating an oxygen level just above (PO_2)crit. In contrast, there was a continuous accumulation of plasma lactate in the other fish indicating that more lactate was produced than oxidized. For these fish 15% AS is below (PO_2)crit and will be lethal in case of prolonged exposure. Exposure to 8% AS resulted in all carp in continuous lactate accumulation revealing an oxygen level below the (PO_2)crit. So, the results demonstrate that for carp 15% AS (2.9 kPa PO_2) is close to the critical oxygen level. Exposure of trout to 40% AS (7.9 kPa PO_2), two out of five animals showed a moderate but persistent increase of blood [lactate], while there was no change in plasma [lactate] in the other fish. From this observation it can be concluded that for the latter group 40% AS is above the (PO_2)crit, whereas for the animals showing the permanent increase in lactate levels 40% AS is already too severe. During 35% AS (6.9 kPa O_2) the individual variation in lactate response became more pronounced between surviving (fish 1 and 4) and non-surviving trout (fish 2, 3 and 5). Nevertheless, the lactate accumulation was continuous in all trout indicating that 35% AS is below (PO_2)crit. It is obvious that the switch to continuous lactate accumulation occurs in carp at lower oxygen levels than in trout. One of the main reasons explaining this difference is the fact that carp hemoglobin has a higher affinity for oxygen than trout hemoglobin (Jones *et al*. 1970). While in trout half-maximal Hb-O_2 saturation (P50) values range from 15-25 mmHg (Boutilier *et al*. 1988; Perry and Reid 1994), P50 values in carp range from 1-8 mmHg (Itazawa and Takeda 1978; Wurm and Albers 1989; Glass *et al*. 1990), which means that at reduced oxygen levels carp is able to extract more oxygen from the environment than trout.

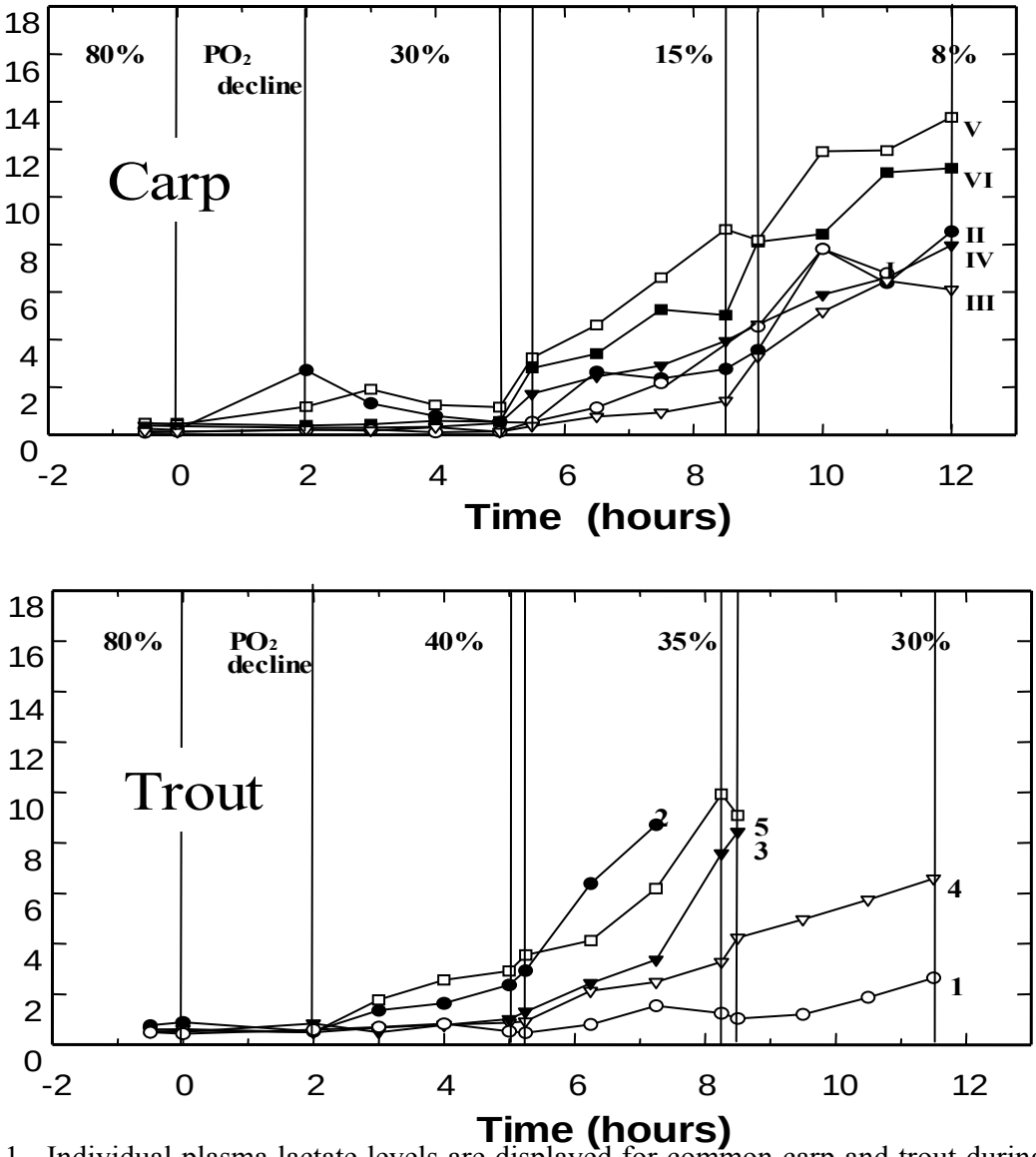


Figure 1. Individual plasma lactate levels are displayed for common carp and trout during stepwise hypoxia. The oxygen concentration in the water was reduced over a period of two hours from 80% AS (15.5 kPa PO_2) to subsequently 30%, 15% and 8% AS, each level for three hours. The PO_2 decline for trout was from 80%AS to subsequently 40%, 35% and 30% AS. All values at $t \geq 5$ ½ h for carp and at $t \geq 2$ ½ h for trout were significantly different ($P<0.05$) from those at normoxia (t= -0.5 and 0 h). Data from Vianen 1999.

To minimize the amount of stress associated with the experimental conditions carp and trout were acclimated to the experimental well-aerated flow-chambers for three days before they were cannulated. After surgery the fish were allowed to recover for 36-48 hours. The fish displayed a quiet behaviour before the stepwise hypoxia was induced, which is reflected by low initial levels of catecholamines both in carp and trout (about 0.04 ng/ml adrenaline and about 0.3 ng/ml noradrenaline). These levels are similar to values observed by Van Raaij *et al*. (1996c) in carp and trout, which were obtained under identical experimental conditions. In fish the resting values for plasma adrenaline are usually between 0.05 and 1.5 ng/ml and for noradrenaline between 0.04 and 2.5 ng/ml (Randall and Perry 1992; Thomas and Perry 1992) and thus the concentrations observed in this study are among the lowest reported in

fish. The initial values of plasma cortisol are comparable with resting concentrations in cannulated fish observed by other investigators (Eros and Milligan 1996; Van der Boon *et al*. 1991; Van Raaij *et al*. 1996c). Changes in noradrenaline levels in cannulated carp and trout is shown in figure 2. Changes in adrenaline levels (not shown) were less dramatic, significant changes occurred later at the lower PO_2 levels. ADR and NA remained at low levels (< 5 ng/ml) in quiet reacting trout, while in the active non-surviving trout higher values were measured for both ADR (5-15 fold) and NA (2-10 fold). These results support the idea that the neuroendocrine activition in fish resembles the stress coping strategies observed in higher vertebrates (Van Raaij *et al*. 1996b).

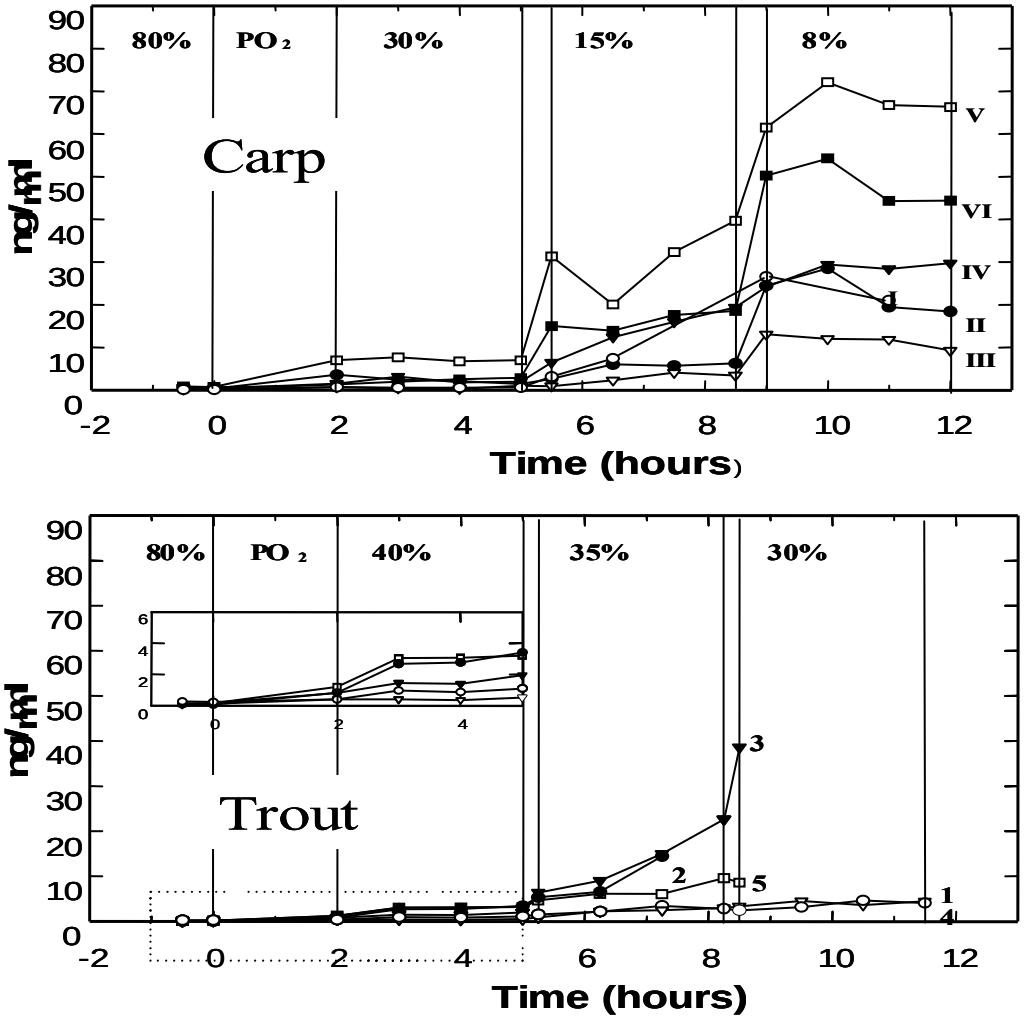


Figure 2. Individual concentrations of noradrenaline levels are shown for common carp and trout during stepwise hypoxia. Details as in figure 1. Data from Vianen 1999.

Comparing figure 1 with figure 2, it follows that the individuals with the highest plasma lactate levels are associated with the highest plasma NA levels and vice versa. This positive coupling was tested for significance with a nonparametric ranking test and resulted both in case of a two-tailed and a one-tailed test in a significant ($P<0.05$) correlation between these compounds. Similarly, in trout especially the marked increase in plasma lactate in the non-surviving trout is accompanied by strong elevations in both ADR and NA. In this fish species a significant correlation was found between the lactate and ADR levels. The correlation between the lactate concentration and catecholamine levels provides strong indications that these stress hormones play in fish an important regulatory function in the activation of anaerobic metabolism during hypoxia.

Response of cannulated carp and trout to chronic hypoxia

For chronic hypoxia the animals were exposed to oxygen levels above the critical PO_2. From the stepwise hypoxia exposure data on lactate and noradrenaline it is obvious that PO_2 for carp and trout of respectively 30 and 40% should be above lethal. This is because in most fish under these conditions recovery could be observed. The 40%AS levels proved however lethal for some trout. This is likely due to the fact that trout can be extremely active during escape reactions, after which recovery becomes impossible. In figure 3 plasma FFA levels of carp and trout are shown over a hypoxia period of 48h. Both in carp and trout exposure to chronic hypoxia resulted in markedly decreased plasma FFA levels stabilizing around new steady state levels during the whole hypoxia period (Figure 3). With respect to carp a reduction in plasma [FFA] from circa 0.5 mmol l^{-1} to 0.1-0.4 mmol l^{-1} has been earlier observed during an exposure of several hours to deep hypoxia (Farkas 1967 a/b; Mazeaud 1973; Van Raaij *et al*. 1996a). These data, however, show that FFA levels in carp can fall to much lower levels (about 0.05 mmol l^{-1}) when exposed to a longer period (48 hours). In contrast to previous publications (Mazeaud 1973; Plisetskaya 1980; Van Raaij *et al*. 1996a) we observed with rainbow trout also a marked reduction of plasma [FFA] during hypoxia.

The continuously reduced FFA levels were accompanied with significant amounts of both adrenaline and noradrenaline in carp and trout during prolonged hypoxia (Figure 4). With respect to adrenaline there was a transient increase (factor 12) in carp at the beginning of the hypoxia period, while in HT-trout a similar transient increase was observed after 24 hours. In contrast, both in carp and trout NE levels remarkably increased (by about factor 35) remaining at these elevated levels during the whole hypoxia period. The permanently increased NE levels are in contrast to the transient changes observed with catecholamines so far (Perry and Reid 1994; Van Raaij *et al*. 1996a) and suggest an important regulatory function for NE during prolonged hypoxia. Van Raaij *et al*. (1995) have demonstrated that stress-free infusion of NE in carp resulted in a reduction of FFA levels providing strong indications that NE is responsible for the FFA reduction during hypoxia. It was hypothesized by the same authors that the NE-induced reduction of plasma [FFA] is due to a reduction of lipolytic rates most probable in the adipose tissue. Indeed, recent experiments with isolated adipocytes showed that the release of FFA decreases dose-dependently during exposure to increasing concentrations of NE, which surprisingly is mediated through stimulation of β-adrenoceptors (Vianen *et al*. 2002). This is in sharp contrast to the mammalian situation, where both noradrenaline and adrenaline are known to be potent activators of lipolysis via β-stimulation (Scheurink *et al*. 1989; Connacher *et al*. 1991; Germack *et al*. 1997; Mills 2000). Thus, the chronically increased NE levels were correlated with the chronically reduced FFA levels in both carp and trout. This suggests that noradrenaline has an important function in controlling lipid catabolism in fish.

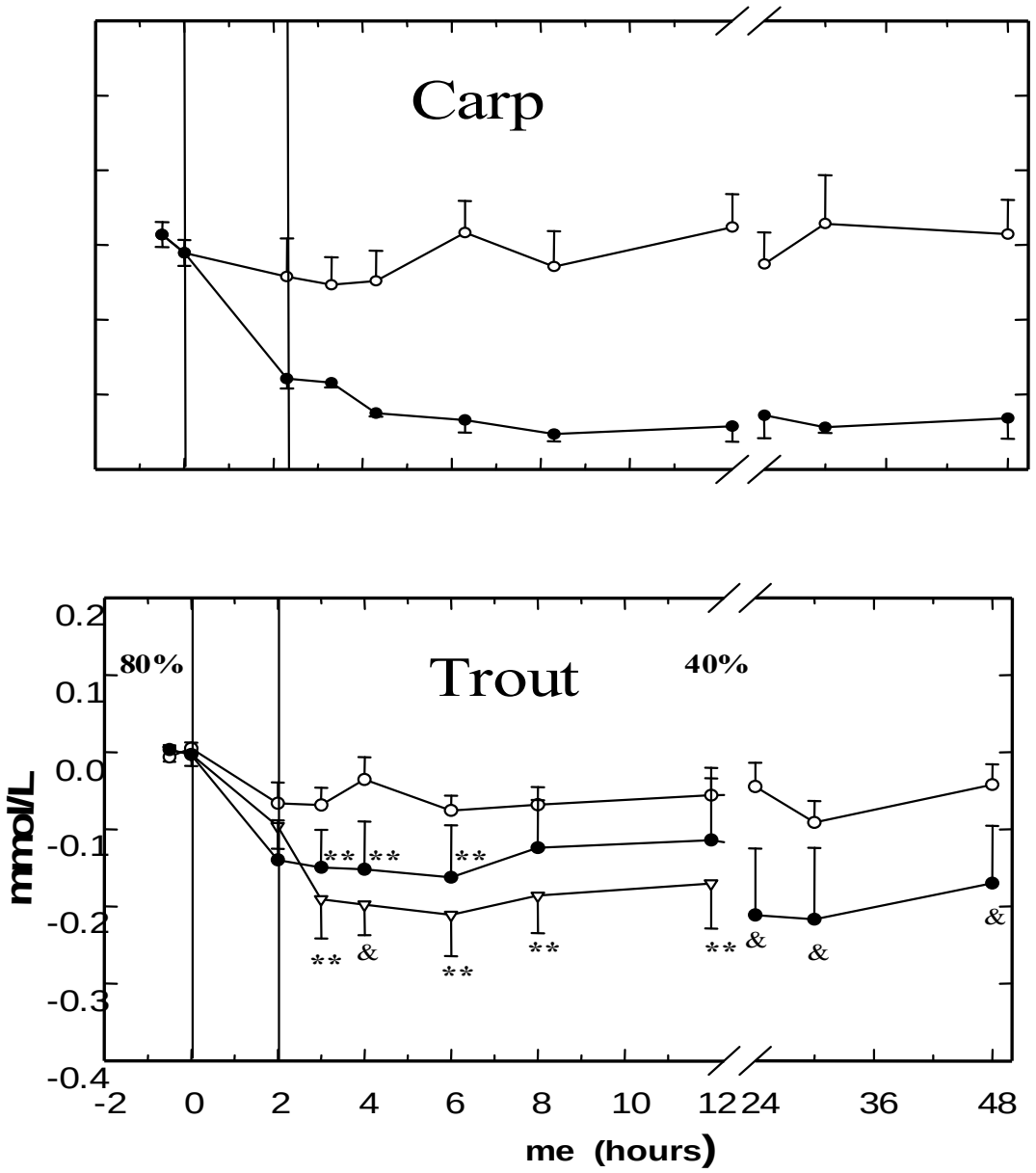


Figure 3. Plasma concentrations of free fatty acids (FFA) in common carp and trout during normoxia (n=7; open circles) and prolonged hypoxia (n=8; closed circles). For carp the oxygen concentration in the water decreased over a period of 2:20 h from 80% air saturation to 20% AS, for trout it was reduced to 40% AS. Values are expressed as mean ± SEM; significant differences ($P<0.05$) with normoxic carp are indicated by a single asterisk (*), with the initial value (t= 0 h) by a double asterisk (**), and both by the "&" sign. Data from Vianen 1999.

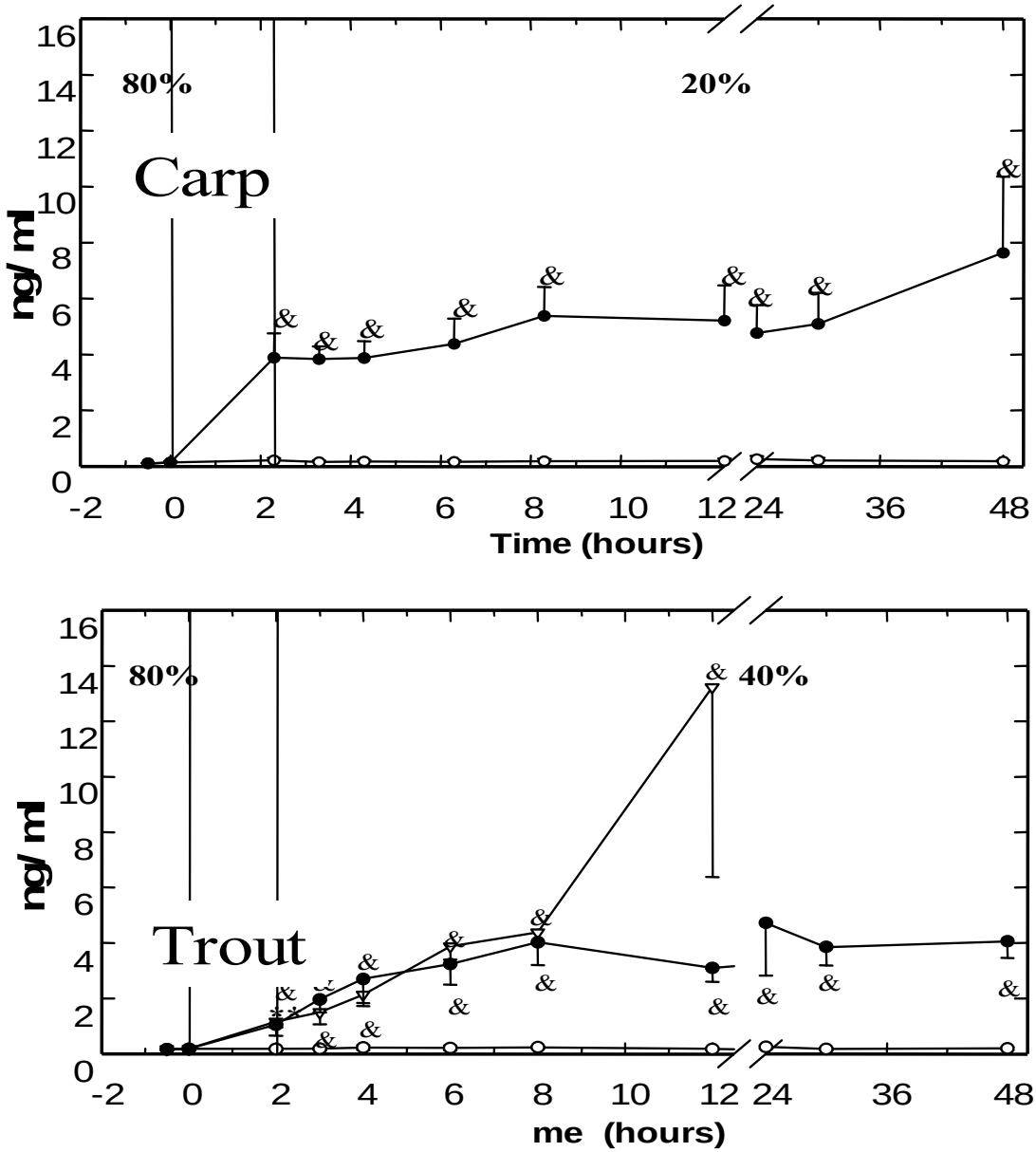


Figure 4. Plasma concentrations of noradrenaline in carp and rainbow trout during normoxia (n=6; open circles) and prolonged hypoxia (n=9). Four trout were hypoxia sensitive (open triangles). Values are expressed as means ± SEM. Details as in Figure 3. Data from Vianen 1999.

Adrenergic control of lipid metabolism; in-vivo studies

To determine the receptor type we carried out infusion experiments with cannulated carp using different salines (Van den Thillart *et al*. 2001). In the first series we tested the involvement of α_2-adrenoceptors. The animals were infused for 1.5h with noradrenaline, noradrenaline + yohimbine (α_2-antagonist), and clonidine (α_2- agonist). Upon infusion with noradrenaline plasma glucose levels began to rise and FFA levels fell, reaching +60% for glucose and -50% for FFA at about 2h after the onset. When the animals were pretreated with yohimbine the glucose response did not differ, but the decline of FFA was retarded. Thus, during infusion with noradrenaline FFA only decreased by about 10%. The same inhibition as without yohimbine was reached after five hours, suggesting that the effect of yohimbine diminishes in a few hours. Infusion with the α_2-agonist clonidine, however, resulted in a transient 10% decline of plasma FFA independent of the applied concentration. This would suggest that α_2-adrenoceptors are indirectly involved, possibly via an effect on tissue perfusion. Infusion of isoproterenol (non-selective β-agonist) according to the same protocol as with noradrenaline (Van den

Thillart *et al*. 2001), showed a similar stimulation of glucose release but inhibition of FFA release of the same magnitude and speed as with noradrenaline. This unexpected result suggests that the direct inhibitory action of noradrenaline on lipolysis is mediated by β-adrenoceptors and not via α_2-adrenoceptors. To determine the type of β-adrenoceptor involved we applied ICI-118.551 (β_2-antagonist) or atenolol (β_1-antagonist) in combination with isoproterenol. The strong increase of plasma glucose by isoproterenol appeared to be attenuated by both antagonists in the same way: the peak value was about 10% lower, and the control levels were reached three hours earlier, six instead of nine hours after the onset of infusion. This suggests that β-receptor stimulation of glycogenolysis (in the liver) is mediated by both β_1- and β_2-adrenoceptors. A different picture was observed with plasma FFA levels: the decline of plasma FFA by isoproterenol was more pronounced in the presence of ICI-118.551, while the administration of atenolol even caused an increase of plasma FFA levels (Figure 2). This result suggests that there are two opposite actions on FFA release. The stronger decline after pretreatment with ICI-118.551 reveals an inhibitory action of β_1-adrenoceptors, presumably located on cell membranes of adipose tissues. The opposite effect of atenolol, showing an increase of plasma FFA by isoproterenol, suggests that β_2-adrenoceptors (possibly located on liver cells) may stimulate lipolysis.

Adrenergic control of lipid metabolism; in-vitro studies

The difficulty with using whole fish is that catecholamines may alter the levels of other circulating hormones, and that catecholamine turnover in the circulation is rapid. Furthermore, the responses of the different fat storing tissues including the liver may be different. This problem is prevented when using isolated fat or liver tissue, or isolated adipocytes and hepatocytes. Migliorini *et al*. (1992) investigated lipolysis in adipose tissue slices of fish, toad, and snake. Remarkably, basal rates were much lower than those of mammals. Catecholamines had, however, no effect on lipolysis in fish and toad adipose tissue. Adrenaline decreased lipolysis in snake adipose tissue, while glucagon had a clear lipolytic effect on snake, both in-vivo and in-vitro. The release of FFA from all adipose preparations was stimulated by cAMP, xanthine derivatives and phosphodiesterase-inhibitors. Lipolysis was also stimulated by fluoride and forskolin, which also increased cAMP levels. These results, suggesting that lipolysis is mediated via phosphorylation of a lipase, did not support Farkas' idea that lipase is not regulated by cAMP (Farkas 1969b).

The advantage of working with isolated cells instead of chopped adipose tissue is that the influence of damaged cells is negligible, and that reproducible incubations from the same source can be made to study concentration dependent effects of different agonists and antagonists. To verify the hypothesis that β-adrenoceptor stimulation leads to inhibition of lipolysis in adipose tissue, we carried out experiments with isolated adipocytes from tilapia (*Oreochromis* mossambicus) mesenteric fat tissue (Vianen *et al*. 2002). The paper of Vianen *et al*. (2002) was the first to show in-vitro lipolysis in isolated fish adipocytes. Zhou *et al*. (1996) described the histology and size range of Atlantic salmon adipocytes. Christiansen *et al*. (1985) describing glucose uptake studies pointed to the fragility of adipocytes isolated from trout adipose tissue. Their adipocytes could not be incubated much longer than 4 minutes. The procedure of Vianen *et al*., however, allows incubations for 5 hours, in some cases overnight incubations were even performed. A general draw-back of working with lipolysis in fish adipose tissue appears the large variability in FFA release. The basal lipolytic rates in teleost fish range from 2-1500 nmoles/h/10^6 cells. In catfish (Clarias (*Clarias gariepinus*), trout and seabream the basal lipolytic rates were about 650, 130 and 2 nmol/h/10^6 cells, respectively (Van Heeswijk, Van den Thillart and Vianen, unpublished results), whereas in tilapia values of about 1500 nmoles/h/10^6 cells were found (Vianen et al 2002).. The values are dependent on age, season, nutritional condition, and

also on the level of maturation. Basal lipolytic rates observed in mammals range between 50-150 nmol/h/10^6 cells. Published rates are: 65-100 nmol/h/10^6 cells for rat (Hollenga *et al*. 1991, Germack *et al*. 1997), 150 nmol/h/10^6 cells for sheep (Cochrane and Rogers 1990), 150 nmol/h/10^6 cells for pig (Mills 2000), and 65 nmol/h/10^6 cells for human (Hollenga *et al*.1991). These rates are relatively low compared with the values obtained from fish adipocytes especially in view of the higher incubation temperature (37°C) with mammalian adipocytes. On the other hand, β-receptor stimulation in mammals leads to a strong increase of lipolysis reaching maximum rates of 2.000-40.000 nmol/h/10^6 cells (Hollenga *et al*.1991, Mills 2000, Germack *et al*. 1997), which are 10-100 fold higher than those observed with teleosts.

The paper of Vianen *et al*. (2002) confirmed that noradrenaline reduces the FFA release of adipocytes dose-dependently, which became significant at concentrations of ≥1μM. Co-incubation of noradrenaline with phentolamine ($\alpha_{1,2}$-antagonist) showed no effect, indicating that no α-adrenoceptor is involved. On the other hand co-incubation with timolol ($\beta_{1,2}$-antagonist) showed that the anti-lipolytic effect of noradrenaline was caused by β-adrenoceptor stimulation in sharp contrast to the situation in mammals (Galitzky *et al*. 1995, Mills 2000). We also found evidence for inhibitory β_3-adrenoceptors; which are activated at concentrations of isoproterenol ≥10 μM and by the selective β_3-adrenoceptor agonist BRL 35135 (Vianen *et al*. 2002). A possible function for those receptors is a safety valve: at high catecholamine concentrations that are found during hypoxia lipolyic activity becomes arrested. This finding was the first report of a functional β_3-adrenoceptors in fish. Thus far no reports are known of an inhibitory β_3-adrenoceptor. Nickerson *et al*. (2003) cloned β-adrenoceptor genes of trout, and found that two were homologous with mammalian β_3-adrenoceptors. In a detailed study they showed two subtypes in trout, a β_3-a and a β_3-b form. The first expressed predominantly in gill and heart, and the latter exclusively in erythrocytes. Binding studies suggested that the β_3-adrenoceptor in erythrocytes is involved in sodium/proton exchange, the function of this adrenoceptor in the other tissues remains to be resolved. With respect to the β-adrenoceptors on adipocyte membranes, we could confirm with trout, catfish, and sea bream the inhibitory effect on lipolysis (van Heeswijk, Van den Thillart, Vianen, unpublished results). This suggests a general mechanism in teleost fish.

Contrary to the observations by Migliorini *et al*. (1992) with fat tissue from the adult male tigerfish (*Hoplias malabaricus*), exposure of tilapia adipocytes to increasing concentrations of isobutyl-methylxanthine (IBMX) and forskolin resulted in a marked reduction of FFA-release (Vianen and Van den Thillart, unpublished results). These results suggest a cAMP-dependent inhibition of TG lipase activity, which is the opposite from the transduction mechanism in mammals. The observation that Migliorini *et al*. (1992) found an increase in lipolytic activity may be due to the (very) high concentrations of IBMX and forskolin, which were applied, 10 and 0,1 mM, respectively. So, fish adipocytes have β-adrenoceptors which upon activation reduce lipolysis via a cAMP mediated mechanism. Liver slices of coho salmon incubated in vitro (Sheridan 1987) release fatty acids upon stimulation by 1 μM noradrenaline but not by adrenaline. Also isoproterenol (β-receptor agonist) stimulated FFA release. The lipolysis occurred at a 3 to 1 ratio of FFA and glycerol respectively. The effects of noradrenaline and isoproterenol could be blocked by propranolol (β-receptor antagonist) but not by phentolamine (α-receptor antagonist), prazosin (α_1- receptor antagonist) and yohimbine (α_1-receptor antagonist). This shows that lipolysis in the salmon liver is activated through β-adrenoceptors. In a recent study with trout hepatocytes (van Heeswijk et al unpublished) we could demonstrate that lipolysis is activated by β_2-adrenoceptors.

CONCLUSIONS

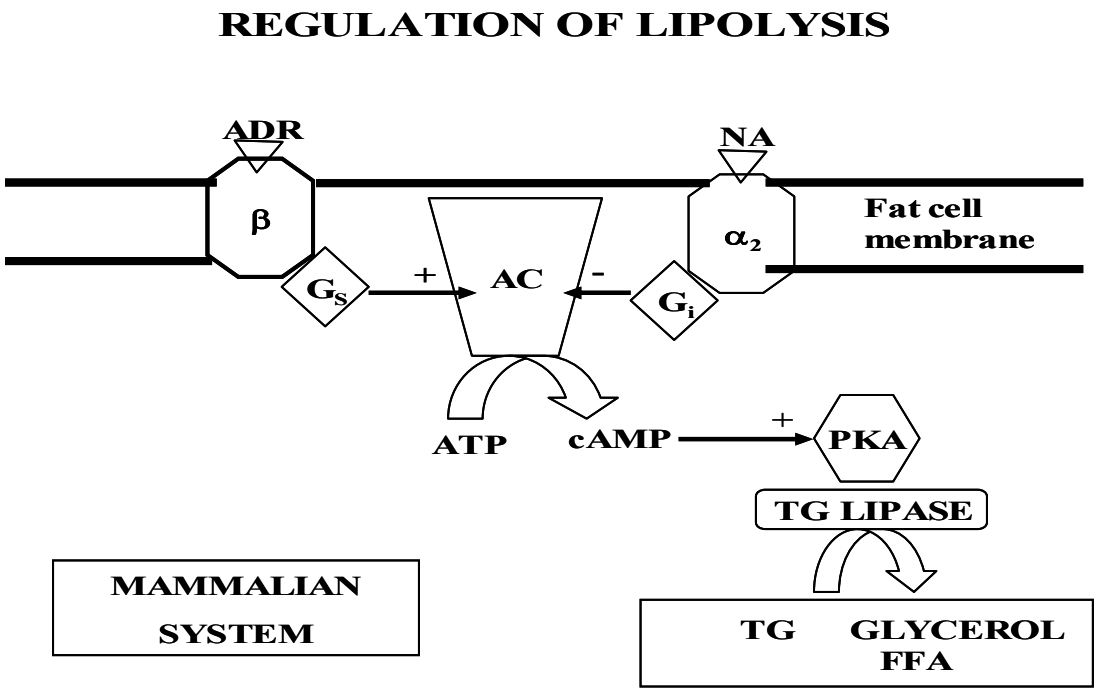
There is overwhelming evidence that lipid mobilization is differently organized in fishes compared to mammals. In general it appears that lipolysis in mammals is strongly activated by catecholamines via β-adrenoceptors (figure 5), while in fishes lipolysis is mostly inhibited. In mammals some inhibition may occur via α2-adrenoceptors. The basal lipolytic rates in fish are sometimes barely measurable, which may compromise the effect of controlling hormones. This pattern is related to the changing needs for storage and mobilization in fishes; lipids are stored in different locations and mobilized differentially for maturation, migration, and long periods of starvation. The fat storage near and in red muscle forms a very short transport route to the muscle. This obscures FFA turnover measurements. The FFA concentrations per se do not provide an indication about lipolysis. However, assuming a fast response on catecholamine release, the fall in oxidation capacity due to oxygen shortage will be slower than the inhibition on HSL. Thus this will in general lead to a lowering of the plasma FFA levels.

The oxygen content of water is some 30 times lower than that of air, in addition oxygen diffusion rates in water are much lower than those in air (800 times), and this makes water-breathers very sensitive to changes in oxygen content. Actually water can become hypoxic or even anoxic in a few hours under certain conditions. Since hypoxia is a generally occurring phenomenon, it may be expected that fish are better adapted to hypoxia than mammals. Between fish species the tolerance to hypoxia varies greatly, and some species have developed "unorthodox" metabolic pathways and behaviour in order to survive extreme hypoxic/anoxic conditions (Van den Thillart and van Waarde 1985). During normoxic conditions, lipids and amino acids are the major fuels for the energy metabolism in teleost fish whereas carbohydrates become important during anaerobic conditions such as burst activity and hypoxia (Van den Thillart and van Raaij 1995). In mammals ischemia and severe hypoxia results in membrane damage. This damage is mainly caused by the accumulation of amphiphiles such as fatty acids and the intermediates of β-oxidation. Fatty acids are amphipatic molecules and are known to destabilize biomembranes (Hoekstra and Golovina 2002, Katz and Messineo 1981, Hütter and Sobol 1992). The flux through β-oxidation is high, particularly in mammalian heart muscle during normoxic conditions (Van der Vusse *et al*. 1983). Due to inhibition of the lipid oxidation fatty acids and their metabolites will accumulate rapidly (Moore 1985). In addition, hypoxia gives always rise to a massive release of catecholamines. In mammals these hormones cause a strong activation of lipolysis. Thus the combination of catecholamine-induced lipolysis and strongly reduced capacity to oxidize fatty acids results in a fast and major accumulation of fatty acids and intermediates of the β-oxidation. Severe ischemia and hypoxia may lead to a marked depletion of the phosphorylation potential resulting in ion rearrangements, in particular in Ca^{++}-influx (Weiss *et al*. 1992, Kidooka *et al*. 1987). Increased Ca^{++}-influx results in turn in activation of phospholipases and consequently in membrane leakage (Hochachka 1985, Kidooka *et al*. 1987, Prasad *et al*. 1991). The above processes ultimately lead to cell damage and to leakage of intracellular enzymes (such as LDH and CK) into the circulation. While hypoxia in mammals is considered as a pathological condition, this is not the case for (water-breathing) fishes. Actually hypoxia/anoxia induced membrane damage does not occur in fish even after near lethal exposures (Van Raaij *et al*. 1994a). So obviously fish, including salmonids, are better protected against hypoxia than most mammals. Since hypoxic conditions are common for water-breathers, it is assumed that catecholamine induced inhibition of lipolysis is a strategy by which fishes are able to prevent accumulation of amphipatic molecules and hence membrane damage.

In fish we observed low lipolytic rates combined with inhibiting effect of catecholamines on lipolysis in adipose tissue. The transduction mechanism of inhibition appears to be cAMP dependent and mediated via activation of β_1- and possibly also β_3- adrenoceptors. From in-vivo measurements in carp it appears that β_2- adrenoceptor activation results in activation of lipolysis in the liver (Van den

Thillart *et al*. 2001). Activation of lipolyis in trout liver (Sheridan 1987) and inhibition of lipolysis in tilapia adipocytes (Vianen *et al*. 2002) via β-adrenoceptors has been described. These opposite effects may explain the variable results obtained in the past. Further research focused on the expression of HSL in the different fat depots and the hormone sensitivity of these depots for lipolysis should be the next step in resolving the problems related to the regulation of lipolysis in fish.

Figure 5. Model showing the action of α_2- and β-adrenoceptors on the membrane bound adenylate cyclase. The α_2-adrenoceptors bind to inhibiting G-proteins and the β-adrenoceptors to stimulating G-proteins. The interaction between the two adrenoceptors determines the activity of TG-lipase.



REFERENCES

Bohus, B., R.F. Benus, D.S. Fokkema, J.M. Koolhaas, C. Nayakas, G.A. van Oortmerssen, A.J.A. Prins, A.J.H. de Ruiter, A.J.W. Scheurink, and A.B. Steffens. 1987. Neuroendocrine states and behavioral and physiological stress responses. Progress in Brain Research 72: 57-70.

Boutilier, R.G., G. Dobson, U. Hoeger, and D.J. Randall. 1988. Acute exposure to graded levels of hypoxia in rainbow trout (*Salmo gairdneri*): metabolic and respiratory adaptations. Respiratory Physiology 71: 69-82.

Brighenti, L., A.C. Puviani, M.C. Gaviolo, E. Fabbri, and C. Ottolenghi. 1991. Interaction of salmon glucagon, glucagon-like peptide, and epinephrine in the stimulation of phosphorylase a activity in fish isolated hepatocytes. General and Comparative Endocrinology 82: 131-139.

Christiansen, D.C., L. Skarstein, and L. Klungsøyr. 1985. Uptake studies in adipocytes isolated from rainbow trout (*Salmo gairdnerii*). A comparison with adipocytes from rat and cat. Comparative Biochemistry and Physiology 82A: 201-205.

Cochrane, W. and M.P. Rogers. 1990. Sensitivity of freshly isolated ovine adipocytes to inhibition of lipolysis by insulin. Comparative Biochemistry and Physiology 96B: 331-333.

Connacher, A.A., W.M. Bennet, R.T. Jung, D.M. Bier, C.C.T. Smith, C.M. Scrimgeour, M.J. Rennie. 1991. Effect of epinephrine infusion on fatty acid and glucose turnover in lean and obese human subjects in the post-absorptive and fed states. Clinical Science 81: 635-644.

Dalla Via, J., G. Van den Thillart, O. Cattani, and A. De Zwaan. 1994. Influence of long-term hypoxia exposure on the energy metabolism of Solea solea. II. Intermediary metabolism in blood, liver and muscle. Marine Ecology Progress Series 111: 17-27.

Danulat, E. and T.P. Mommsen. 1990. Norepinephrine: A potent activator of glycogenolysis and gluconeogenesis in rockfish hepatocytes. General and Comparative Endocrinology 78: 12-22.

Dunn, J.F. and P.W. Hochachka. 1986. Metabolic responses of trout (*Salmo gairdneri*) to acute environmental hypoxia. Journal of Experimental Biology 123: 229-242.

Dunn, J.F. and P.W. Hochachka. 1987. Turnover rates of glucose and lactate in rainbow trout during acute hypoxia. Canadian Journal of Zoology 65: 1144-1148.

Eros, S.K. and C.L. Milligan. 1996. The effect of cortisol on recovery from exhaustive exercise in rainbow trout (*Oncorhynchus mykiss*): Potential mechanisms of action. Physiology Zoology 69: 1196-1214.

Fabbri, E., A. Capuzzo, and T.W. Moon. 1998. The role of circulating catecholamines in the regulation of fish metabolism: An overview. Comparative Biochemistry and Physiology C 120: 177-192

Farkas, T. 1967a. The effect of catecholamines and adrenocorticotropic hormone on blood and adipocyte tissue FFA levels in the fish *Cyprinus carpio* L. Progress in Biochemistry Pharmacology 3: 314-319.

Farkas, T. 1967b. Examinations of fat metabolism in freshwater fishes. The sympathetic nervous system and mobilisation of fatty acids. Annual Inst. Biology Hungarian Academy of Science 34: 129-138.

Farkas, T. 1969b. Effects of agents modifying the level of 3'-5'-adenosine monophosphate in adipose tissue on mobilization of fats in fish and frogs. Annual Inst. Biology Hungarian Academy of Science 36: 163-171.

Galitzky, J., C. Carpene, A. Bousquet-Melou, M. Berlan, and M. Lafontan. 1995. Differential activation of beta(1)-, beta(2)- and beta(3)-adrenoceptors by catecholamines in white and brown adipocytes. Fundamental Clinical Pharmacology 9: 324-331.

Germack, R., A.B. Starzec, R. Vassy, and G.Y. Perret. 1997. β-Adrenoceptor subtype expression and function in rat white adipocytes. Br. Journal of Pharmacology 120: 201-210.

Glass, M.L., N.A. Andersen, M. Kruhoffer, E.M. Williams, and N. Heisler. 1990. Combined effects of environmental PO_2 and temperature on ventilation and blood gases in the carp (*Cyprinus carpio* L.). Journal of Experimental Biology 148: 1-17.

Greene, D.H.S. and D.P. Selivonchick. 1987. Lipid metabolism in fish. Progress in Lipid Research 26: 53-85.

Henderson, R.J. and D.R. Tocher. 1987. The lipid composition and biochemistry of freshwater fish. Progress in Lipid Research 26: 281-347.

Hochachka, P.W. 1985. Assessing metabolic strategies for surviving O_2-lack: role of metabolic arrest coupled with channel arrest. Molecular Physiology 8: 331-350

Hoekstra, F.A. and E.A. Golovina. 2002. The role of amphiphiles. Comparative Biochemistry and Physiology A 131: 527-533.

Hollenga, Ch., M. Haas, J.T. Deinum, and J. Zaagsma. 1991. Differences in functional cyclic AMP compartments mediating lipolysis by isoprenaline and BRL 37344 in four adipocyte types. European Journal of Pharmacology 200: 325-330

Hütter, J.F. and S. Sobol. 1992. Role of fatty acid metabolites in the development of myocardial ischemic damage. International Journal of Biochemistry 24: 399-403.

Itazawa, Y. and T. Takeda. 1978. Gas exchange in the carp gills in normoxic and hypoxic conditions. Respiratory Physiology 35: 263-269.

Janssens, P.A. and J. Waterman. 1988. Hormonal regulation of gluconeogenesis and glycogenolysis in carp (*Cyprinus carpio*) liver slices cultured in vitro. Comparative Biochemistry and Physiology 91A: 451-455.

Jones, D.R., D.J. Randall, and G.M. Jarman. 1970. A graphical analysis of oxygen transfer in fish. Respiratory Physiology 10: 285-298.

Jørgensen, J.B. and T. Mustafa. 1980. The effect of hypoxia on carbohydrate metabolism in flounder (*Platichthys flesus* L.). I. Utilization of glycogen and accumulation of glycolytic end products in various tissues. Comparative Biochemistry and Physiology 67B: 243-248.

Katz, A.M. and F.C. Messineo. 1981. Lipid-membrane interactions and the pathogenesis of ischemic damage in the myocardium. Circulation Research 48: 1-12.

Kidooka, M., M. Matsuda, and J. Handa. 1987. Effect of calcium antagonist and agonist on free fatty acid liberation in the ischemic brain of rats. Surgical Neurology 28: 41-45.

Lafontan, M. and M. Berlan. 1993. Fat cell adrenergic receptors and the control of white and brown cell function. Journal of Lipid Research 34: 1057-1083.

Lomholt, J.P. and K. Johansen. 1979. Hypoxia acclimation in carp: How it affects O_2 uptake, ventilation, and O_2 extraction from water. Physiological Zoology 52: 38-50.

Mazeaud, F. 1973. Recherches sur la regulation des acides gras libres plasmatiques et de la glycemie chez les poissons. Thesis, Paris.

Migliorini, R.J., Lima-Verde, J.S., Machado, C.R., Cardona, G.M.P., Garofalo, M.A.R. and Kettelhut, I.C. 1992. Control of adipose tissue lipolysis in ectotherm vertebrates. American Journal of Physiology 263: R857-862.

Mills, S. 2000. Beta-adrenergic receptor subtypes mediating lipolysis in porcine adipocytes. Studies with BRL-37344, a putative β_3-adrenergic agonist. Comparative Biochemistry and Physiology 126C: 11-20.

Moore, K. 1985. Fatty acid oxidation in ischaemic heart. Molecular Physiology 8: 549-565.

Nickerson, J.G., S.G. Dugan, G. Drouin, S.F. Perry, T.W. Moon. 2003. Activity of the unique beta-adrenergic Na+/H+ exchanger in trout erythrocytes is controlled by a novel beta3-AR subtype. American Journal of Physiology 285: R526-535

Perry, S.F. and C.M. Wood. 1989. Control and coordination of gas transfer in fishes. Canadian Journal of Zoology 67: 2961-2970.

Perry, S.F. and S. G. Reid. 1994. The effects of acclimation temperature on the dynamics of catecholamine release during acute hypoxia in the rainbow trout, *Oncorhynchus mykiss*. Journal of Experimental Biology 186: 289-307.

Plisetskaya, E. 1980. Fatty acid levels in the blood of cyclostomes and fish. Environmental Biology of Fishery 5: 273-290.

Prasad, M.R., L.M. Popescu, I.I. Moraru, X. Liu, S. Maity, R.M. Engelman, and D.K. Das. 1991. Role of phospholipases A_2 and C in myocardial ischemic reperfusion injury. American Journal of Physiology 29: H877-883.

Prinzen, F.W., G.J. Van der Vusse, T. Arts, T.H.M. Roemen, W. Coumans, and R.S. Reneman. 1984. Accumulation of non-esterified fatty acids in ischemic myocardium. American Journal of Physiology 247: H264-272.

Puviani, A.C., C. Ottolenghi, M.E. Gaviolli, E. Fabbri, and L. Brighenti. 1990. Action of glucagon and glucagon-like peptide on glycogen metabolism of trout isolated hepatocytes. Comparative Biochemistry and Physiology 96B: 387-391.

Randall, D.J. and S.F. Perry. 1992. Catecholamines. *In:* Fish Physiology, vol XIIB (ed. D.J. Randall and W.S. Hoar), pp. 255-300.

Reid, S.G., N.J. Bernier, and S.F. Perry. 1988. The adrenergic stress response in fish: control of catecholamine storage and release. Comparative Biochemistry and Physiology C 120: 1-27

Scheurink, A.J.W, A.B. Steffens, H. Bouritius, G.H. Dreteler, R. Bruntink, R. Remie, and J. Zaagsma. 1989. Sympathoadrenal influence on glucose, FFA, and insulin levels in exercising rats. American Journal of Physiology 256: R161-168.

Sheridan, M.A. 1987. Effects of epinephrine and norepinephrine on lipid mobilization from coho salmon liver incubated in vitro. Endocrinology 120: 2234-2239.

Sheridan, M.A. 1988. Lipid dynamics in fish: aspects of absorption, transportation, deposition, and mobilization. Comparative Biochemistry and Physiology 90B: 697-690.

Sheridan, M.A. 1994. Mini Review. Regulation of lipid metabolism in poikilothermic vertebrates. Comparative Biochemistry and Physiology 107B: 495-508.

Sheridan, M.A. and N.A. Muir. 1988. Effects of epinephrine and norepinephrine on glucose release from chinook salmon (*Oncorhynchus tshawytscha*) liver incubated in vitro. Journal of Experimental Zoology 248: 155-159.

Soivio, A., K. Nyholm, and K. Westman. 1975. A technique for repeated sampling of blood of individual resting fish. Journal of Experimental Biology 63: 207-217.

Thomas, S. and S.F. Perry. 1992. Control and consequences of adrenergic activation of red blood cell Na+/H+ exchange on blood oxygen and carbon dioxide transport in fish. Journal of Experimental Zoology 263: 160-175.

Van den Thillart, G. 1982. Adaptations of fish energy metabolism to hypoxia and anoxia. Molecular Physiology 2:L 49-61.

Van den Thillart, G. and A. van Waarde. 1985. Teleosts in hypoxia: aspects of anaerobic metabolism. Molecular Physiology 8: 393-411.

Van den Thillart, G., J. Dalla Via, G. Vitali, and P. Cortesi. 1994. Influence of long-term hypoxia exposure on the energy metabolism of Solea solea. I. Critical O_2 levels for aerobic and anaerobic metabolism. Marine Ecology Progress Series 104: 109-117.

Van den Thillart, G. and M. van Raaij. 1995. Endogenous fuels: non-invasive versus invasive approaches. *In:* Biochemistry and molecular biology of fishes. Vol 4, pp 33-63. Edited by Hochachka & Mommsen, Academic Press, New York, NY.

Van den Thillart, G.E.E.J.M., G.J. Vianen, M. Campos-Ponze, H. Lelieveld, M. Nieveen, M. van Raaij, A. Steffens, and J. Zaagsma. 2001. Differential role of adrenoceptors in the control of plasma glucose and fatty acids in carp, *Cyprinus carpio* (L). American Journal of Physiology 281: R615-624.

Van den Thillart, G., G.J. Vianen, and J. Zaagsma. 2002. Adrenergic regulation of lipid mobilization in fishes; a possible role in hypoxia survival. Fish Physiology and Biochemistry 27: 189-204

Van der Boon, J., G.E.E.J.M. Van den Thillart, and A.D.F. Addink. 1991. The effects of cortisol administration on intermediary metabolism in teleost fish. Comparative Biochemistry and Physiology 100A: 47-53.

Van der Vusse, G.J., T.H.M. Roemen, W. Flameng, and R.S. Reneman. 1983. Serum myocardium gradients of non-esterified fatty acids in dog, rat, and man. Biochimica et Biophysica Acta 752: 362-370.

Van Dijk, P.L.M., G.E.E.J.M. Van den Thillart, P.H.M. Balm, and S.E. Wendelaar Bonga. 1993. The influence of gradual water acidification on the acid/base status and plasma hormone levels in carp. Journal of Fish Biology 42: 661-671.

Van Ginneken, V.J.T., A.D.F. Addink, G.E.E.J.M. Van den Thillart, F. KÖrner, L. Noldus, and M. Buma. 1997. Metabolic rate and level of activity determined in tilapia (Oreochromis mossambicus Peters) by direct and indirect calorimetry and videomonitoring. Thermochimica Acta 291: 1-13.

Van Raaij, M.T.M., E. Bakker, M.C. Nieveen, H. Zirkzee, and G.E.E.J.M. van den Thillart. 1994a. Energy status and free fatty acid patterns in tissues of common carp (Cyprinus carpio L.) and rainbow trout (*Oncorhynchus mykiss* L.) during severe oxygen restriction. Comparative Biochemistry and Physiology 109A: 755-767.

Van Raaij, M.T.M., G.E.E.J.M. van den Thillart, M. Hallemeesch, P.H.M. Balm, and A.B. Steffens. 1995. The effect of arterially infused adrenaline, noradrenaline and insulin on plasma hormones and the levels of glucose and free fatty acids in common carp *(Cyprinus carpio* L). American Journal of Physiology 268: R1163-1170.

Van Raaij, M.T.M., D.S.S. Pit, P.H.M. Balm, A.B. Steffens, and G.E.E.J.M. van den Thillart. 1996a. Behavioral strategy and the physiological stress response in rainbow trout exposed to severe hypoxia. Hormones and Behavior 30: 85-92.

Van Raaij, M.T.M., G.J. Vianen, and G.E.E.J.M. Van den Thillart. 1996b. Blood gas parameters and the responses of erythrocytes in carp exposed to deep hypoxia and subsequent recovery. Journal of Comparative Physiology 166B: 453-460.

Van Raaij, M.T.M., G.E.E.J.M. Van den Thillart, G.J. Vianen, D.S.S. Pit, P.H.M. Balm, and A.B. Steffens. 1996c. Substrate mobilization and hormonal changes in rainbow trout (*Oncorhynchus mykiss*, L.) and common carp (*Cyprinus carpio*, L.) during deep hypoxia and subsequent recovery. Journal of Comparative Physiology 166B: 443-452.

Vianen, G.J. 1999. Hormonal control of energy metabolism in teleost fish during hypoxia. Thesis, Leiden.

Vianen, G.J., P.P. Obels, G.E.E.J.M. van den Thillart, and J. Zaagsma. 2002. β-Adrenoceptors mediate inhibition of lipolysis in adipocytes of tilapia (*Oreochromis mossambicus*). American Journal of Physiology 282: E318-325.

Weiss, J.N., N. Venkatesh, and S.T. Lamp. 1992. ATP sensitive K^+-channels and cellular K^+ loss in hypoxic and ischemic mammalian ventricle. Journal of Physiology 447: 649-673.

Wendelaar Bonga, S.E. 1997 The stress response in fish. Physiological Reviews 77: 591-625

White, A. and T.C.Fletcher. 1989. The effect of physical disturbance, hypoxia and stress hormones on serum components of the plaice, *Pleuronectus platessa*. Comparative Biochemistry and Physiology 93A: 455-461.

Wright, P.A., S.F. Perry, and T.W. Moon. 1989. Regulation of hepatic gluconeogenesis and glycogenolysis by catecholamines in rainbow trout during environmental hypoxia. Journal of Experimental Biology 147: 169-188.

Wurm, T.H. and C. Albers. 1989. Interaction of allosteric effectors (ATP, CO_2, H^+) modulating oxygen affinity of the hemoglobin in the carp (*Cyprinus carpio*) in vitro. Journal of Comparative Physiology 159B: 255-261.

Zhou, S., R.G. Ackman, and C. Morrison. 1996. Adipocyte and lipid distribution in the muscle tissue of Atlantic salmon (Salmo salar). Canadian Journal of Fisheries and Aquatic Science 53: 326-332.

THE INFLUENCE OF OXYGEN SHORTAGE ON THE DEVELOPMENT OF THE CARDIOVASCULAR SYSTEM IN ZEBRAFISH

Bernd Pelster[10]

ABSTRACT

Oxygen shortage typically elicits characteristic adaptations in the cardio - respiratory systems and in cellular metabolism, as well documented in adult vertebrates. Even embryonic and larval stages of fish, for example, respond to hypoxia. Oxygen shortage can be the result of a reduced oxygen availability in the environment (= general, environmental hypoxia), but it can also be the consequence of an increased oxygen demand in certain tissues, exceeding the capacity of oxygen supply to the particular tissue (= functional, locally restricted hypoxia).

In early embryonic stages of the zebrafish *Danio rerio* severe environmental hypoxia may cause developmental arrest. Chronic exposure to mild hypoxia (P_{O_2} = 10 kPa), however, provoked an increase in heart rate as well as cardiac output at about hatching time or shortly thereafter. A reduced hemoglobin oxygen-carrying capacity in the blood of zebrafish, however, did not induce any change in cardiac activity until 12-14 dpf. Beyond 7 dpf animals raised under chronic hypoxia showed a significantly elevated red cell concentration in their blood, and a redistribution of blood away from the gut to the segmental muscle tissue in the trunk region was observed. A comparison of the vascular bed of various organs revealed no obvious differences in hypoxic animals compared to normoxic animals.

Functional hypoxia, achieved by chronic exposure of zebrafish larvae to a constant water current inducing an increase in swimming activity, did not cause any adaptations in cardiac activity until 32 dpf. Nevertheless, critical P_{O_2} in trained animals was reduced, indicating an improvement of the oxygen transport system in these larvae. In the muscle tissue an increase in mitochondrial density especially in red and intermediate muscle cells was observed. At 32 dpf an increase in capillary density of the segmental muscle tissue as well as of the tail fin was detected in trained larvae, and this correlated with an enhanced expression of VEGF in muscle tissue at this stage. In gills and in the head region a stimulation of VEGF expression was already detectable at 15 dpf in trained animals.

Taken together these results indicate that at about hatching time receptors are present which sense hypoxic conditions, and this information can be used to stimulate convective oxygen transport in order to compensate for a reduction in bulk oxygen diffusion in the face of a reduced oxygen gradient between environmental water and tissues. Erythropoiesis can be stimulated a few days after hatching. The first modification of angiogenesis was detected at 32 dpf in trained fish. VEGF expression suggests, however, that gill development might respond even earlier to the lack of oxygen.

INTRODUCTION

In vertebrate embryos, the heart is the first functional organ, and with the onset of cardiac activity blood flow is observed very early in development. Given the notion that the unfailing beat of the heart is necessary for life this observation may at first glance not be surprising. The convective flow of blood through the circulation provides the ceaseless transport of the respiratory gases and nutrients

[10] Institute for Zoology and Limnology and Center for Molecular Biosciences, University of Innsbruck, Austria

required by metabolism, the waste products produced by metabolism, and the hormonal cues that regulate metabolism and many other physiological functions. This role of the circulation clearly is indisputable in adult vertebrates. Within recent years many studies provided striking examples of the inaccuracy of this perspective with respect to the embryonic cardiovascular system. Compelling evidence on many fronts revealed that the convective flow of blood generated by the early embryonic vertebrate heart is simply not required for transport of oxygen, nutrients, metabolic wastes, or hormones, all of which can be achieved entirely by diffusion (Burggren 2004; Pelster 2002; Territo and Altimiras 2001). In fact, fish, amphibian, and even bird embryos lacking a functional heart can survive and develop for several days after fertilization.

Thus, the often expressed view that embryonic physiology merely is the physiology of adult animals but conducted on a smaller physical scale must be rejected. The fact that in early developmental stages blood flow is not required for nutrient and respiratory gas transport implies that the coupling between metabolic activity of tissues and cardiac activity is not yet established during early development (Pelster 2002). This may be related to the observation that the autonomic nervous system gains control over the cardiovascular system only quite late during development in spite of the fact that histochemistry and neurophysiological experiments revealed that adrenergic and cholinergic receptors are present in cardiac muscle cells and in the central vascular system much earlier than the sympathetic or parasympathetic innervation is completed (Holeton 1971; Pappano 1977; Protas and Leontieva 1992). In these early stages cardiac activity and peripheral resistance obviously can be adjusted by various hormones (Fritsche 1997; Fritsche *et al*. 2000; Kloberg and Fritsche 2002).

If in early developmental stages of fish and amphibians the cardiovascular system is not required for gas and nutrient supply to the tissues and is controlled in a different way as in adult animals the question arises how the embryos and larvae respond to changes in the environmental oxygen availability. Do they notice environmental hypoxia? Under hypoxic conditions, when a reduced oxygen partial pressure gradient across the skin impairs cutaneous oxygen uptake, convective oxygen transport maybe required in order to supply the tissues sufficiently.

Conditions with a reduced oxygen availability can be achieved in several ways. First of all, environmental PO_2 can drop, resulting in a decrease in the PO_2 gradient between the environment and the internal blood and tissues. Second, if the blood contributes to the oxygen supply to tissues, a reduced oxygen supply can be achieved by reducing the oxygen carrying capacity of the blood, i.e. by reducing the oxygen carrying capacity of the hemoglobin (hypoxemia). Thirdly, the lack of oxygen can be achieved by increasing the oxygen demand of the tissues. By stimulating muscular activity, for example, an increase in ATP turn over is induced, which requires an additional supply with oxygen in order to generate ATP via the aerobic metabolism. Chronic muscular activity thus may bring the oxygen transport pathway to its limits and cause oxygen shortage (= functional hypoxia).

Within recent years the effect of all three conditions has been tested in a couple of larval stages, and in this study I would like to summarize our current knowledge about the influence of oxygen shortage on the development of the cardiovascular system in lower vertebrates, especially in fish.

THE EFFECT OF ENVIRONMENTAL HYPOXIA

Cardiac Activity

One possibility to test the capability of the cardiovascular system to cope with the lack of oxygen during development is to expose animals to varied environmental PO_2 values (Fritsche and Burggren 1996; Orlando and Pinder 1995). Soon after fertilization fish eggs are surprisingly resistant towards a lack of oxygen. Embryos of the Arctic charr (*Salvelinus alpinus*) exposed to anoxia for 8 hours at about 80% of embryonic development show a remarkable metabolic depression. Metabolic activity assessed by measuring oxygen consumption as well as heat production is reduced to a few percent of control values, and heart rate decreased by about 90% (Pelster 1999). Zebrafish embryos survive 24 hours of anoxia in a status of suspended animation, in which cardiac activity ceases and mitotic activity of the blastomeres is arrested (Padilla and Roth 2001). The ability to survive anoxic conditions is restricted to very early stages, however. If the zebrafish embryos are exposed to anoxic conditions at about 30 h after fertilization, less than 10% of the embryos were able to survive. A significant decrease in heart rate and in cardiac output has been reported for very early stages of amphibians during periods of oxygen deficiency (Fritsche and Burggren 1996; Orlando and Pinder 1995; Pelster 1999). This decrease in activity has been attributed to a direct effect of the lack of oxygen on the metabolism of cardiomyocytes, and it does not appear to be a controlled response, which involves hormonal or neuronal activity (Orlando and Pinder 1995).

In *Xenopus* larvae a coordinated stimulation of cardiac activity in order to enhance convective oxygen transport during periods of hypoxia was only observed in later developmental stages (Orlando and Pinder 1995). In contrast to anoxia hypoxic conditions provoked a minor but significant tachycardia in salmonid larvae (Holeton 1971; McDonald and McMahon 1977). If the direct effect of hypoxia on cardiac muscle cells is a reduction in activity, an increase in heart rate under these conditions must be due to an external stimulation, which can only be explained as a coordinated response.

In order to identify the point in development when cardiac activity becomes responsive to hypoxia and in order to question the importance of diffusional oxygen supply through the skin versus convective oxygen transport in the blood, we raised zebrafish under normoxic and hypoxic conditions (P_{O_2} = 10kPa, prepared by equilibrating water with a mixture of air and N_2; T = 28^{o}C). We used zebrafish larvae because the small body size should facilitate diffusional oxygen transport and render convectional oxygen transport less important even in later developmental stages. The results revealed that even in the small zebrafish larvae cardiac activity becomes responsive to environmental hypoxia already at about the time of hatching (Jacob *et al*. 2002). In hypoxic incubated animals heart rate started to increase significantly compared to control animals at 4 dpf (Figure 1A), and cardiac output was significantly elevated at 3,4, and 5 dpf (Figure 1B). The comparison of systolic and end-diastolic ventricular volume revealed a significantly increased diastolic volume at 3 and 4 dpf, but this increase did not persist on 5dpf. The increase in cardiac output can only be explained as a coordinated response. The reduction in water PO_2 is detected by the animal, and this information is used to stimulate cardiac activity.

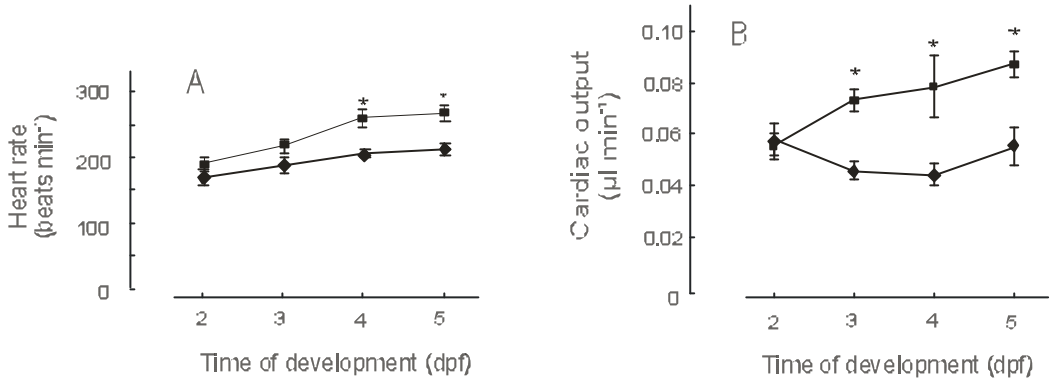


Figure 1. Heart rate (A) and cardiac output (B) in zebrafish larvae raised under normoxia (PO_2 = 20 kPa) and under chronic hypoxia (PO_2 = 10 kPa) at a temperature of 28°C; n = 12 for 2 and 5 dpf; 3 dpf, n = 18; 4 dpf, n = 16. *, significantly different from controls ($p < 0.05$). (Modified after (Jacob *et al*. 2002))

The positive response to hypoxia in zebrafish is observed even earlier than in *Xenopus* larvae, which is significantly larger than zebrafish. *Xenopus* raised under normoxia (P_{O_2} = 21 kPa), hypoxia (P_{O_2} = 11 kPa) or under hyperoxia (P_{O_2} = 35 kPa) until Nieuwkoop-Faber stage 51 showed no difference in oxygen uptake and in cardiac activity (Territo and Altimiras 1998; Territo and Burggren 1998). Thus, fish larvae may respond to environmental hypoxia at an earlier stage than amphibian larvae. Alternatively, a P_{O_2} of 11 kPa may not have been low enough to elicit a coordinated response in *Xenopus* larvae. This explanation does not appear very likely, however, because for our experiments we used a similar P_{O_2} of 10 kPa, and this was very close to the lower threshold which permitted a complete development of zebrafish larvae without significantly increasing the rate of mortality.

Blood Distribution

A redistribution of blood in response to different metabolic demands represents a well established physiological adaptation in many species. While in zebrafish larvae raised under chronic hypoxia (10 kPa P_{O_2}) until 7 dpf no changes in blood distribution could be detected, at 12 and 15 dpf blood perfusion was significantly higher in the muscle tissue of hypoxic animals than in control animals (Figure 2). In turn, perfusion of the gut was significantly lower in hypoxic 12 dpf animals. Brain perfusion was not affected by hypoxia (Schwerte *et al*. 2003). Thus, at 12 dpf under hypoxic conditions blood was shifted from the gut to the muscles in the tail region. The so-called red-layer of muscle has been implicated in the uptake of oxygen in early larvae and at about the time of hatching (El-Fiky and Wieser 1988). Possibly, blood is redistributed towards the muscle tissue in order to enhance oxygen uptake through the body surface under hypoxic conditions.

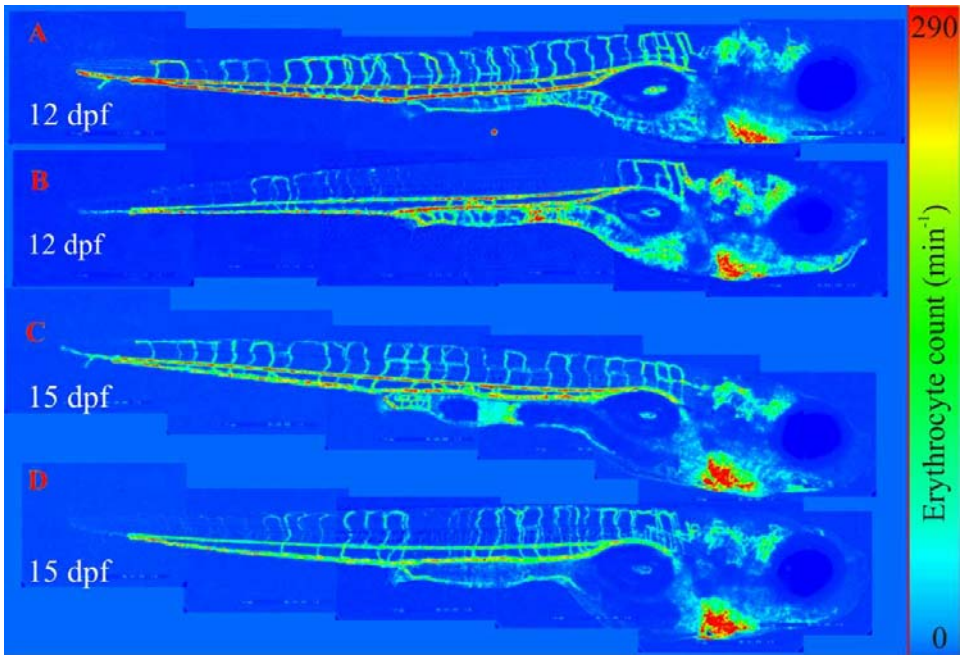


Figure 2. Typical changes in erythrocyte cell count in zebrafish raised under control conditions (B, D) and under chronic hypoxia (P_{O_2} = 10 kPa; A,C). Red cell count per minute is given in false colors according to the calibration bat on the right side. (From (Schwerte *et al*. 2003) with permission).

Tissue Vascularization

In zebrafish larvae exposed to hypoxia no significant changes in the vascular bed were observed (Schwerte *et al*. 2003). There appeared to be quite a high interindividual variation in the expression of small blood vessels like the parachordal vessels, running longitudinally along the myoseptum, or the caudal vascular tree, but the overall morphometric analysis did not show significant differences between control and hypoxic animals. Compared with the development of amphibians such as *Xenopus* or *Rana* or the development of salmonid larvae, zebrafish development is very rapid, and it could be that a rearrangement of the vascular bed is only possible in later developmental stages. In a number of other vertebrates a stimulation of angiogenesis by hypoxic incubation has been demonstrated. (Yue and Tomanek 1999) demonstrated that coronary vessels from cultured 6 day old quail embryo grow faster under hypoxic conditions, while hyperoxia induced a delayed angiogenesis. Chorioallantoic membrane capillarization of chicken embryos has been shown to increase during hypoxia (Dusseau and Hutchins 1988; Hudlicka *et al*. 1992). In larval amphibians, aquatic hypoxia stimulates growth of respiratory surfaces and enhances the transition from gill respiration to lung respiration (Burggren and Mwalukoma 1983). The lack in the angiogenetic response in hypoxic zebrafish is in line with the current concept of blood vessel formation in this species. Weinstein and coworkers proposed that the initial wiring of blood vessels follows a fixed pattern and thus is largely based on the genetic information, leaving little or no room for adaptations to environmental influences (Isogai *et al*. 2003; Weinstein 1999). On the other hand, molecular signals involved in the formation of blood vessels have been identified in early embryonic stages. The molecular mechanisms which lead to the extremely regular pattern in the zebrafish trunk have been shown by (Childs *et al*. 2002), and VEGF up- and down regulation seems to be involved in this process. Whole mount in situ hybridization of zebrafish embryos indicated strong expression of VEGF already at 18 hours post fertilization (Gong *et al*. 2004; Liang *et al*. 1998; Liang *et al*. 2001; Tan *et al*. 2001; Weinstein 1999).

Red Cell Concentration

In mammals hypoxia usually causes an increase in oxygen transport capacity of the blood, i.e. an increase in the number of circulating erythrocytes and in hemoglobin concentration. Similar observations have been reported for embryos of the turtle *Pseudemys nelsoni* (Kam 1993), while in chicken embryos during early development a stimulation of red cell production was not observed (Baumann and Meuer 1992). In zebrafish larvae hypoxia increased red blood cell concentration larvae during the second week after fertilization, while no effect was observed until 7dpf (Schwerte *et al*. 2003). Until 7 dpf the concentration of red cells remained fairly constant in normoxic as well as in hypoxic animals. The first presumptive proerythroblast-like cells can be detected in the zebrafish by the end of the first day (20 hpf), and this primitive cohort of cells originates in the intermediate cell mass (IMC) (Weinstein *et al*. 1996). By tracing transfused fluorescence labeled blood cells into 1.5 day old host embryos these authors concluded that this first cohort of primitive red cells provides the embryo with all, or nearly all, red blood cells for at least 4 days. A population of new, larger and more adult appearing erythrocytes becomes predominant by 10 dpf. The constant concentration of red cells observed in zebrafish until 7 dpf is in line with these results. The elevated red cell concentration in 12 dpf larvae appears to be due to a hypoxic stimulation of erythropoiesis in zebrafish larvae, as observed in fetal mammals (Richardson and Bocking 1998), for example, or in embryos of the turtle *Pseudemys nelsoni* (Kam 1993). In this case the production of the second population of cells, primarily observed at 10 dpf, would have been stimulated by hypoxia.

INFLUENCE OF A REDUCTION IN THE OXYGEN CARRYING CAPACITY (HYPOXEMIA)

A reduction in the oxygen carrying capacity of the blood can be achieved by impairing the oxygen binding of the hemoglobin. Zebrafish have been incubated in an atmosphere containing CO, and also in the presence of phenylhydrazine, which oxidizes hemoglobin and thus reduces its oxygen binding capacity. Zebrafish raised until 4 or 5 dpf under these conditions have a similar oxygen consumption compared to control animals (Pelster and Burggren 1996). Measurements of whole body lactate content did not provide any indication for a switch to anaerobic metabolism in the animals with a reduced oxygen carrying capacity until 12 dpf (Jacob *et al*. 2002). Similar results have been reported for *Xenopus* larvae raised under chronic CO exposure until Nieuwkoop Faber stage 54 (Territo and Altimiras 1998; Territo and Burggren 1998).

A comparison of heart rate in zebrafish raised with a reduced oxygen carrying capacity with animals raised under control conditions overall revealed no significant differences until 15 dpf (Jacob *et al*. 2002). At 15 dpf animals raised in the presence of phenylhydrazine had a reduced heart rate, while heart rate of animals raised in the presence of CO was not different from controls. Stroke volume was also hardly affected and, accordingly, cardiac output was hardly influenced, except for the phenylhydrazin group at 15 dpf, where deleterious effects of the drug increased the mortality of the larvae. Thus, hypoxemia did not stimulate cardiac activity until about two weeks after fertilization.

HYPOXIA VERSUS HYPOXEMIA

Comparing the effect of hypoxemia on cardiac activity of zebrafish larvae with the effect of hypoxia it is apparent that hypoxemia does not stimulate cardiac activity, but hypoxia does. Under hypoxemia the P_{O_2} gradient between the environmental water and the tissues is not different from normoxic conditions, and thus bulk oxygen diffusion is sufficient to meet the oxygen requirements of the tissues. Under hypoxia, however, the diffusion gradient of oxygen is reduced, and even in the small zebrafish larvae (body weight less than 1 mg) this induces an increase in convective oxygen transport in order to compensate for the reduction in oxygen diffusion. This clearly demonstrates that in zebrafish larvae at hatching time the afferent nervous system is able to sense hypoxic conditions. Furthermore, central control units are active and bring about a coordinated response to environmental perturbations (like oxygen deficiency), either by direct stimulation of the heart, or by stimulation of hormone secreting cells like the chromaffin cells. The secreted hormone in turn could then activate cardiac activity.

An obvious conclusion from these considerations is that convective oxygen transport is not necessary in zebrafish larvae until about two weeks after fertilization, if the P_{O_2} gradient through the skin is normal. If this gradient is reduced due to a decrease in environmental P_{O_2}, cardiac activity can be stimulated at about half a day or a day after hatching. This appears to be even earlier than in the larger salmonid or *Xenopus* larvae, where the hypoxic stimulation was not observed before 1 day after hatching or even later. Thus, the time in development at which a coordinated response of the ventricle becomes possible is not only governed by body mass. Interesting and not yet completely explained is the observation of Territo and Altimiras (Territo and Altimiras 1998) that stroke volume and cardiac output are significantly enhanced in *Xenopus laevis* under chronic exposure to CO, but not under hypoxia. This is in clear contrast to the results reported for zebrafish larvae, and the increase in cardiac output was only observed in very early stages of *Xenopus*, not in later stages with a significantly larger body mass. Territo and Altimiras concluded that the increase in cardiac output observed in embryonic stages was intrinsic in nature and did not significantly improve oxygen uptake. In fact, aerobic metabolism was similar in all experimental groups.

THE INFLUENCE OF CHRONIC SWIMMING ACTIVITY

Hypoxic conditions can also result from an increased metabolic activity. This is possible for example during periods of exercise, where the increased activity of myosin ATPase significantly stimulates oxygen consumption of the muscle cells. Swim training leads to a modification of growth rate and of food conversion efficiency in adult fish (Christiansen and Jobling 1990; Wieser *et al*. 1988). To assess possible influences of chronic swimming activity on metabolism and growth of zebrafish larvae were trained in a swim tunnel system for about 15h every day at a water velocity of 5 bl/sec (Bagatto *et al*. 2001). The training protocol had no influence on growth of the larvae, although yolk absorption was significantly faster in animals exposed to a constant water current.

An exciting observation was that compared to control animals free swimming larvae after 11 days of training between 21 dpf and 32 dpf had a higher M_{O_2} at rest, and a significantly lower rate of oxygen consumption in the swim tunnel at any given water velocity. The higher rate of oxygen consumption at rest may be attributable to an increased activity level at rest. The reduction in M_{O_2} during activity, however, clearly shows that the efficiency of muscular contraction and/or the efficiency of energy conversion have been improved in trained animals. Furthermore, training enhanced survival of larvae during exposure to low oxygen partial pressures (Bagatto *et al*. 2001).

Exposure of early larval stages of zebrafish to a constant water current therefore induced physiological adaptations resulting in an enhanced swimming efficiency, and in an increased tolerance towards hypoxia.

A typical adaptation to endurance training is the so-called sports heart. Cardiac activity of trained zebrafish larvae, however, showed no difference compared to untrained control animals in terms of heart rate, stroke volume and cardiac output (Pelster *et al*. 2003). Erythropoiesis was also not stimulated in trained animals. Significant adaptations have been found at the tissue level. In animals trained between 9 and 15 dpf and also in animals trained between 21 and 32 dpf mitochondrial density of red and intermediate muscle tissue was increased. In adult fish like rainbow trout and Danube bleak, sustained swim training can induce an increase in the aerobic capacity even in white muscle fibres (Davie *et al*. 1986; Sänger and Pötscher 2001). In zebrafish mitochondrial density of white muscle fibres of endurance and interval trained animals appeared to be higher as in untrained animals, but this difference was not significant (Pelster *et al*. 2003). It could well be that during this early stage of zebrafish development it is mainly the red and intermediate muscle portion recruited for the endurance swimming activity. In mammals endurance exercise may even induce a fibre type transition in white muscle cells (Pette and Staron 2001). Such an adaptational change in white muscle fibres would enable the fish to use the more profitable aerobic pathways for energy production, prevent local fatigue and enhance the removal of metabolic end products like lactate.

In the older larvae total length of the tail fin blood vessels as well as the vascularization index of the axial tail muscle increased significantly after 11 days of continuous or interval swim training, suggesting an improvement in the blood supply to the muscle cells (Figure 3). In larvae trained between 9 and 15 dpf angiogenesis was not enhanced (Pelster *et al*. 2003). As in the older zebrafish larvae, in adult rainbow trout (Davie *et al*. 1986) and also in some cyprinid species like *Chalcalburnus chalcoides mento, Chondrostoma nasus* and *Leuciscus cephalus* endurance exercise training has been shown to induce a significant increase in muscle capillarity (Sänger 1992; Sänger and Pötscher 2001). These results indicate that angiogenesis is stimulated in fish during exercise training, but this effect is not observed in very early developmental stages, i.e. within the first two weeks of development in the zebrafish. This is again in line with the idea that in the earliest developmental stages the wiring of blood vessels a fixed pattern, and leaves little or no room for adaptations to environmental factors (Isogai *et al*. 2003; Weinstein 1999). On the other hand, if in zebrafish convective oxygen transport is not required to supply oxygen to the tissues until about two weeks after hatching, it could also be that at this stage of development a modification of the vascular bed is not yet necessary.

Perspectives: The observation that the circulatory system of embryonic and early larval stages is not working in order to supply the tissue with oxygen and nutrients can be attributed to the small size of the larvae. This implies, however, that the early larval circulatory system is not controlled the same way as the adult circulatory system. Nevertheless, recent data indicate that early developmental stages can sense their environment, and via hormonal control cardiovascular activity can modified in response to environmental information. In addition, the patterning of the cardiovascular system can be influenced by environmental parameters. Accordingly, functional development is not only controlled by genetic information, but it appears to be responsive to environmental input. Thus, studying development in the light of genetic and environmental constituents appears to be a promising task.

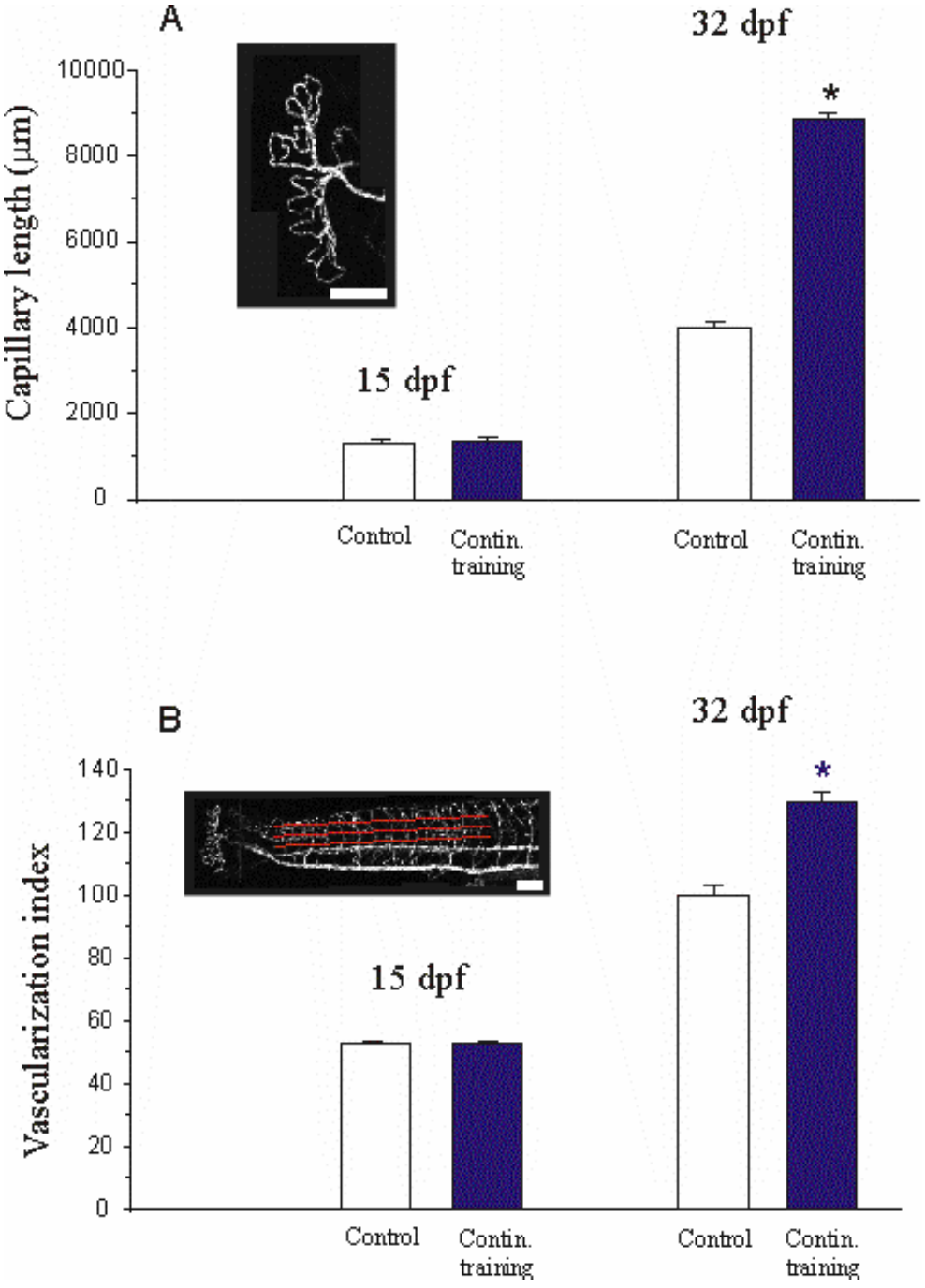


Figure 3. A. Capillary length measured in the tail fin in control animals and in interval or continuously trained animals. N = 9. B. Vascularization index determined in the axial muscle tissue caudal from the anus in control and in interval or continuously trained animals. N = 10. Scale bars, 250 □m; * indicates significant differences between trained and control animals ($p<0.05$); (modified after Pelster *et al.* 2003).

ACKNOWLEDGEMENT

The study was financially supported by the Fonds zur Förderung der wissenschaftlichen Forschung (FWF, P12571-BIO; P14976-BIO).

REFERENCES

Bagatto, B., B. Pelster, and W.W. Burggren. 2001. Growth and metabolism of larval zebrafish: effects of swim training. Journal of Experimental Biology 204: 4335-4343.

Baumann, R. and H.J. Meuer. 1992. Blood oxygen transport in early avian embryo. Physiological Reviews 72: 941-965.

Burggren, W.W. 2004. What is the purpose of the embryonic heart beat? or How facts can ultimately prevail over physiological dogma. Physiological and Biochemical Zoology 77: 333-345.

Burggren, W.W. and A. Mwalukoma. 1983. Respiration during chronic hypoxia and hyperoxia in larval and adult bullfrogs (Rana catesbeiana) I. Morphological responses of lungs, skin and gills. Journal of Experimental Biology 105: 191-203.

Childs, S., J.N. Chen, D.M. Garrity, and M.C. Fishman. 2002. Patterning of angiogenesis in the zebrafish embryo. Development 129: 973-982.

Christiansen, J.S. and M. Jobling. 1990. The behaviour and the relationship between food intake and growth of juvenile Arctic charr, Salvelinus alpinus L., subjected to sustained exercise. Canadian Journal of Zoology 68: 2185-2191.

Davie, P.S., R.M.G. Wells, and V. Tetens. 1986. Effects of sustained swimming on rainbow trout muscle structure blood oxygen transport, and lactate dehydrogenase isozymes: evidence for increased aerobic capacity of white muscle. Journal of Experimental Zoology 237: 159-171.

Dusseau, J.W. and P.M. Hutchins. 1988. Hypoxia-induced angiogenesis in chick chorioallantoic membranes: a role for adenosine. Respiration Physiology 71: 33-44.

El-Fiky, N. and W. Wieser. 1988. Life styles and patterns of development of gills and muscles in larval cyprinids (Cyprinidae; Teleostei). Journal of Fish Biology 33: 135-145.

Fritsche, R. 1997. Ontogeny of cardiovascular control in amphibians. American Zoologist 37: 23-30.

Fritsche, R. and W.W. Burggren. 1996. Development of cardiovascular responses to hypoxia in larvae of the frog Xenopus laevis. American Journal of Physiology 271: R912-917.

Fritsche, R., T. Schwerte, and B. Pelster. 2000. Nitric oxide and vascular reactivity in developing zebrafish, Danio rerio. American Journal of Physiology 279: R2200-R2207.

Gong, B., D. Liang, T.G. Chew, and R. Ge. 2004. Characterization of the zebrafish vascular endothelial growth factor A gene: comparison with vegf-A genes in mammals and Fugu. Biochimica et Biophysica Acta 1676: 33-40.

Holeton, G.F. 1971. Respiratory and circulatory responses of rainbow trout larvae to carbon monoxide and to hypoxia. Journal of Experimental Biology 55: 683-694.

Hudlicka, O., M. Brown, and S. Egginton. 1992. Angiogenesis in skeletal and cardiac muscle. Physiological Reviews 72: 369-417.

Isogi, S., N.D. Lawson, S. Torrealday, M. Horiguchi, and B.M. Weinstein. 2003. Angiogenic network formation in the developing vertebrate trunk. Development 130:5281-5290.

Jacob, E., M. Drexel, T. Schwerte, and B. Pelster. 2002. The influence of hypoxia and of hypoxemia on the development of cardiac activity in zebrafish larvae. American Journal of Physiology 283: R911-R917.

Kam, Y.C. 1993. Physiological effects of hypoxia on metabolism and growth of turtle embryos. Respiration Physiology 92: 127-138.

Kloberg, A.J. and R. Fritsche. 2002. Catecholamines are present in larval Xenopus laevis: A potential source for cardiac control. Journal of Experimental Zoology 292: 293-303.

Liang, D., J.R. Chang, A.J. Chin, A. Smith, C. Kelly, E.S. Weinberg, and R. Ge. 2001. The role of vascular endothelial growth factor (VEGF) in vasculogenesis, angiogenesis, and hematopoiesis in zebrafish development. Mechanisms of Development 108: 29-43.

Liang, D., X. Xu, A.J. Chin, N.V. Balasubramaniyan, M.A. Teo, T.J. Lam, E.S. Weinberg, and R. Ge. 1998. Cloning and characterization of vascular endothelial growth factor (VEFG) from zebrafish, Danio rerio. Biochemica Biophysica Acta 1397:14-20.

McDonald, D.G. and B.R. McMahon. 1977. Respiratory development in Arctic char Salvelinus alpinus under conditions of normoxia and chronic hypoxia. Canadian Journal of Zoology 55: 1461-1467.

Orlando, K. and A.W. Pinder. 1995. Larval cardiorespiratory ontogeny and allometry in Xenopus laevis. Physiological Zoology 68: 63-75.

Padilla, P.A. and M.B. Roth. 2001. Oxygen deprivation causes suspended animation in the zebrafish embryo. Proceedings of the National Academy of Science USA 98:7331-7335.

Pappano, A.J. 1977. Ontogenetic development of autonomic neuroeffector transmission and transmitter reactivity in embryonic and fetal hearts. Pharmacological Reviews 29: 3-33.

Pelster, B. 1999. Environmental influences on the development of the cardiac system in fish and amphibians. Comparative Biochemistry and Physiology Pt.A 124: 407-412.

Pelster, B. 2002. Developmental plasticity in the cardiovascular system of fish, with special reference to the zebra fish. Comparative Biochemistry and Physiology 133A: 547-553.

Pelster, B. and W.W. Burggren. 1996. Disruption of haemoglobin oxygen transport does not impact oxygen- dependent physiological processes in developing embryos of zebrafish (*Danio rerio*). Circulation Research 79: 358-362.

Pelster, B., A.M. Sänger, M. Siegele, and T. Schwerte. 2003. Influence of swim training on cardiac activity, tissue capillarization, and mitochondrial density in muscle tissue of zebrafish larvae. American Journal of Physiology 285: R339-R347.

Pette, D. and R.S. Staron. 2001. Transitions of muscle fiber phenotypic plasticity. Histochemistry and Cell Biology 115: 359-372.

Protas, L.L. and G.R. Leontieva. 1992. Ontogeny of cholinergic and adrenergic mechanisms in the frog (Rana temporaria) heartr. American Journal of Physiology 262: R150-R161.

Richardson, B.S. and A.D. Bocking. 1998. Metabolic and Circulatory Adaptations to Chronic Hypoxia in the Fetus. Comparative Biochemistry and Physiology 119A: 717-723.

Sänger, A.M. 1992. Effects of training on axial muscle of two cyprinid species: Chondrostoma nasus (L.) and Leuciscus cephalus (L.). Journal of Fish Biology 40: 637-646.

Sänger, A.M. and U. Pötscher. 2001. Endurance exercise training affects fast white axial muscle in the cyprinid species Chalcalburnus chalcoides mento (Agassiz, 1832), Cyprinidae, Teleostei. Basic and Applied Myology 10: 297-300.

Schwerte, T., D. Überbacher, and B. Pelster. 2003. Non-invasive imaging of blood cell concentration and blood distribution in hypoxic incubated zebrafish in vivo (Danio rerio). Journal of Experimental Biology 206: 1299-1307.

Tan, D.C., R.M. Kini, S.D. Jois, D.K. Lim, L. Xin, and R. Ge. 2001. A small peptide derived from Flt-1 (VEGFR-1) functions as an angiogenic inhibitor. FEBS Letters 494: 150-156.

Territo, P.R. and J. Altimiras. 1998. The ontogeny of cardio-respiratory function under chronically altered gas compositions in Xenopus laevis. Respiration Physiology 111: 311-323.

Territo, P.R. and J. Altimiras. 2001. Morphometry and estimated bulk oxygen diffusion in larvae of Xenopus laevis under chronic carbon monoxide exposure. Journal of Comparative Physiology B 171: 145-153.

Territo, P.R. and W.W. Burggren. 1998. Cardio-respiratory ontogeny during chronic carbon monoxide exposure in the clawed frog Xenopus laevis. Journal of Experimental Biology 201: 1461-1472.

Weinstein, B.M. 1999. What guides early embryonic blood vessel formation? Developmental Dynamics 215: 2-11.

Weinstein, B.M., A.F. Schier, S. Abdelilah, J. Malicki, L. Solnica-Krezel, D.L. Stemple, D.Y. Stainier, F. Zwartkruis, W. Driever, and M.C. Fishman. 1996. Hematopoietic mutations in the zebrafish. Development 123: 303-309.

Wieser, W., H. Forstner, N. Medgyesy, and S. Hinterleitner. 1988. To switch or not to switch: partioning of energy between growth and activity in larval cyprinids (Cyprinidae: Teleostei). Functional Ecology 2: 499-507.

Yue, X. and R. J. Tomanek. 1999. Stimulation of coronary vasculogenesis/angiogenesis by hypoxia in cultured embryonic hearts. Developmental Dynamics 216: 28-36.

A BIOCOMPUTATIONAL TOOL TO MEASURE CARDIAC RHYTHMS IN ZEBRAFISH

Shuk Han Cheng[11], Eric Po Kwok Chan and Alex Chun Chi Lin

ABSTRACT

The zebrafish embryo has become one of the popular vertebrate model organisms for developmental toxicology research. The transparent embryo becomes a viable model for providing assessment of cardiovascular functions. However, the most commonly used measurement tool for heart beat rate is simple visual examination. In our laboratory, we have developed a prototypical biocomputational tool to quantify the cardiac function of heart beat. Heart beat pattern was digitally recorded and the images were assessed by digital motion analysis in the caudal blood vessels whereby the flow of blood cells was measured. The software gave an indication of heart beat rates which were correlated positively with the heart beat rates measured by visual observation. Using the insecticide carbaryl, we demonstrated that this toxicant elicited irregular heart beat. This perturbation of cardiovascular function is previously unreported as carbaryl is thought to be a rather safe insecticide for humans and animals. Our biocomputational technique can be readily adapted to screen for toxic effects of other physical and chemical toxicants in this test system.

INTRODUCTION

The developing fish embryos and larvae are often used in toxicity testing due to their high sensitivity towards toxicants. Increasingly, developmental toxicity has been incorporated in risk assessment procedures. Detailed understanding on how toxicants affect physiological and developmental processes in embryonic fish serves as a foundation for assessment of fitness and survival. Notwithstanding, there is still a paucity of data on anatomical malformations and physiological impairment of internal organs caused by exposure to toxicants.

The transparent zebrafish embryo is an ideal model for studying cardiovascular toxicity of chemicals (reviewed in Schwerte and Fristsche 2003). For example, polycyclic aromatic hydrocarbons induced bradycardia and arrhythmias characteristic of atrioventricular conduction block in zebrafish embryos (Incardona JP *et al*. 2004). Apart from morphological description of the cardiovascular system and measurements of heart rates, the assessment of heartbeat rhythm is also of paramount importance in cardiovascular physiology. Irregular heartbeat rhythm, caused by side-effects of non-cardiac drugs, is often related with sudden death in human. However, assessment of the regularity of heartbeat rhythm is not readily available in these zebrafish embryos.

We report here the prototype of a non-invasive method to measure regularity of heartbeat. The method involves two separate steps, namely, image analysis using digital motion analysis of caudal blood flow and, the power spectral analysis of image data. Digital motion analysis was developed by Schwerte and Pelster (2000), in which movement of blood cells was analyzed by image subtraction between 2 video frames (figure 1A). In this image subtraction, pixels in each frame were separated in

[11] Department of Biology and Chemistry, City University of Hong Kong, Tat Chee Avenue, Kowloon, Hong Kong

2 categories. The first category included static pixels which were located in the non-moving area where no intensity change occurred between 2 video frames. The second category included dynamic pixels which were located in the moving area where intensity change occurred between 2 video frames. Since the time interval between 2 video frames was constant, the amount of dynamic pixels was larger if the object moved faster (figure 1B and figure 1C).

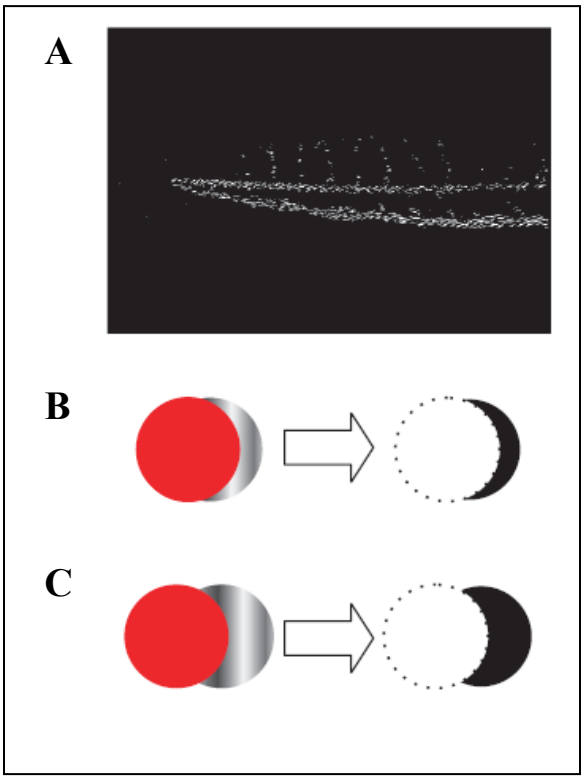


Figure 1. (A) Image subtraction of 2 video frames. (B & C) Schematic diagram illustrates the amount of dynamic pixels obtained if object is moved slowly (B) or quickly (C).

Based on this idea, a pilot program was developed to perform image subtraction in video sequence recording caudal blood flow in 48-hpf zebrafish embryos. Instead of summing up the dynamic pixels as described in the original methodology (Schwerte and Pelster 2000), the amount of dynamic pixels of each video frame was treated as a sequence of data in a time series. Plotting of the time series data of dynamic pixels from control embryo showed a waveform correlation (figure 2A). This effect was similar to the direct observation of pulsatile blood cell movements under a microscope. We performed power spectral analysis on the waveform pattern of dynamic pixels in order to extract frequency specific characteristics from the data set comprising the dynamic pixels. The power spectrum of waveform represented in figure 2A actually consisted of 3 peaks (figure 2B), suggesting the existence of 3 basic frequency components in this time series data of dynamic pixels. Since the second and the third frequency component were the higher integer multiples of the first frequency component, they were considered as the harmonics of the first frequency component (Stefanovska and Bracic 1999). In this preliminary study, power spectra of control embryos (n=6) were analyzed (figure 3A). It consisted of 3 frequency components and the second and the third component were the exact multiples of the first frequency (figure 3A). Conversion of the units of major frequency values from per second to per minute resulted in values correlating directly to measurements of heart rates being counted visually from video recordings of heart chamber beating (figure 3B). This pilot experiment showed that the

power spectral analysis gave us an direct indication of the heart beat rate.

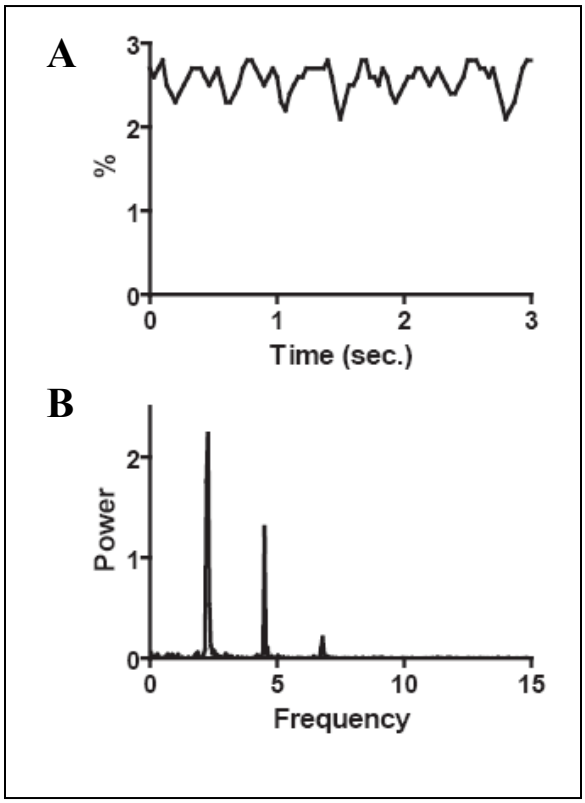


Figure 2. Percentage of dynamic pixel (A) and its corresponding power spectrum (B) of a control embryo.

We then perturbed the experimental system with the addition of carbaryl, a carbamate insecticide, to the fish embryo culture. Carbaryl was considered a low toxicity insecticide for both human and other vertebrates. However, previous studies showed that carbaryl induced ultrastructural changes in the heart (Tos-Luty *et al*. 2001) and caused reduced heart rate (Kossakowski 1987). In a preliminary experiment to demonstrate the application of our method, an embryo exposed to 10 ppm of carbaryl for 48 hours was analyzed. The waveform of dynamic pixels appeared to be similar to those obtained from control embryos (figure 4A). However, using power spectrum of the carbaryl-treated embryo, we could not identify any peak corresponding to a specific frequency component (figure 4B). Therefore, this preliminary experiment suggested that carbaryl disturbed the regularity of heartbeat resulting in the heart beating in a random manner in this carbaryl-treated embryo.

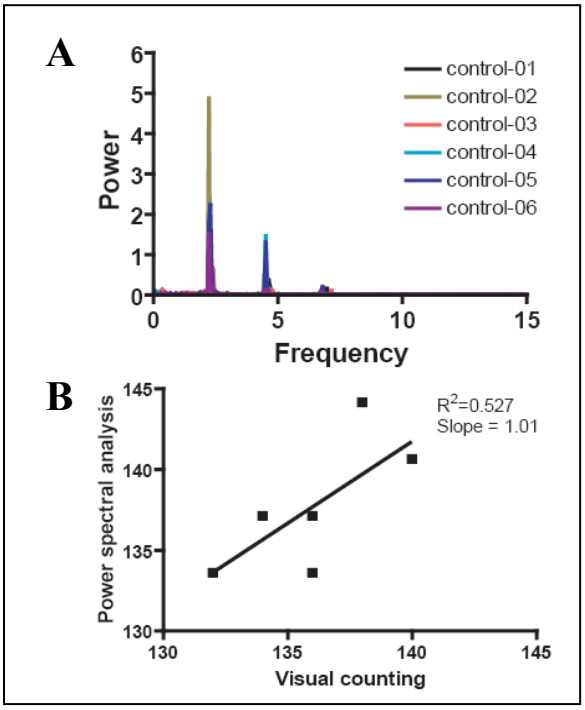


Figure 3. (A) Power spectra of six control embryos. (B) Correlation of heart rate determined by power spectral analysis and direct visual counting in heart.

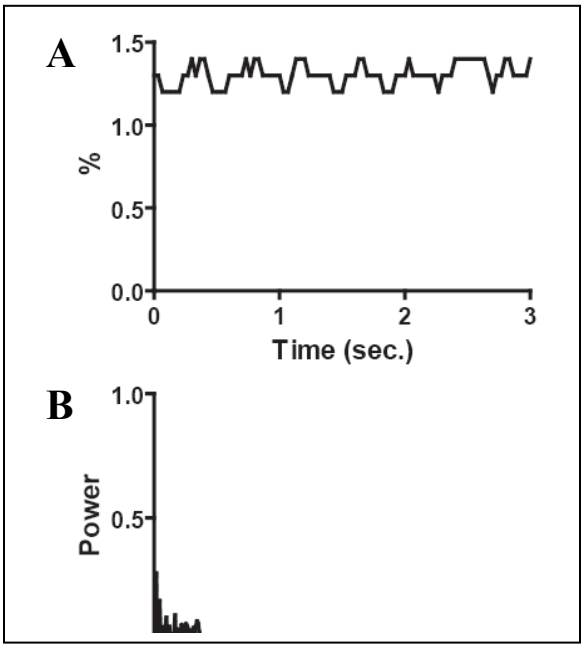


Figure 4. Percentage of dynamic pixels (A) and its corresponding power spectrum (B) of a carbaryl-treated embryo.

In summary, our preliminary results showed that our method was able to measure heart rate digitally as compared to the conventional visual method. In addition, this method can demonstrate the regularity of heartbeat and identify irregular beatings in zebrafish embryos. However, it still requires

a large sample size in order to investigate the correlation between irregular heartbeats and absence of frequency component peak in power spectrum after toxicant treatments. Furthermore, we need to identify parameters of the power spectrum which are necessary for the quantification of heartbeat rhythms.

REFERENCES

Incardona, J.P., T.K. Collier, and N.L. Scholz. 2004. Defects in cardiac function precede morphological abnormalities in fish embryos exposed to polycyclic aromatic hydrocarbons. Toxicol Appl Pharmacol. 196:191-205.

Kossakowski, S. 1987. Electrocardiogram of rabbits experimentally intoxicated with carbaryl. Polskie Archiwum Weterynaryjne (Warszawa). 27:15-28.

Schwerte, T. and R. Fritsche. 2003. Understanding cardiovascular physiology in zebrafish and Xenopus larvae: the use of microtechniques. Comparative Biochemistry and Physiology A: Molecular Integrated Physiology 135:131-145.

Schwerte, T. and B. Pelster. 2000. Digital motion analysis as a tool for analyzing the shape and performance of the circulatory system in transparent animals. Journal of Experimental Biology 203:1659-1669.

Stefanovska, A. and M. Bracic. 1999. Physics of the human cardiovascular system. Contemporary Physics 40: 31-55.

Tos-Luty, S., D. Przebirowska, J. Latuszynska, and M. Tokarska-Rodak. 2001. Histological and ultrastructural studies of rats exposed to carbaryl. Annual Agric Environ Med. 8:137-44.

HYPOXIA IN REPTILES

Tobias Wang[12] and Nini Skovgaard

ABSTRACT

Reptiles are amongst the most hypoxia and anoxia-tolerant vertebrates, and some species such as turtles are therefore favored organisms for studies on metabolic adaptations to oxygen lack. Although reptiles are unlikely to experience hypoxic environments, they are often hypoxic at the tissue level due to large cardiac shunts and prolonged periods of breath-hold. Here we briefly review some of the physiological responses to hypoxia, with particular emphasis on local responses in the pulmonary circulation. Hypoxic pulmonary vasoconstriction is more prevalent in species with anatomically complex lungs and is particularly expressed in species that also have a functionally divided heart where increased pulmonary vascular resistance does not affect the cardiac shunt flow. We also review the metabolic responses to hypoxia and argue that the hypoxic conditions created by intracardiac shunts and breath-hold may serve to downregulate metabolism thorough direct effects of low oxygen levels on cellular metabolism.

INTRODUCTION

Reptiles and other air-breathing animals are, at least in comparison with aquatic animals, unlikely to experience severe hypoxic environments unless when inhabiting or entering high altitude. High altitude is normally associated with low environmental temperatures, which, rather than low oxygen levels, is likely to be a limiting factor for the distribution of reptiles. However, reptiles exhibit a number of anatomical, physiological and behavioural traits that results in low oxygen levels of lung gases and arterial blood. In particular, they have undivided hearts, so arterial and venous blood is mixed within the ventricle leading to low oxygen levels in the blood. Also, many species exhibit prolonged periods of breath hold where lung PO_2 and arterial oxygen levels can decline to very low values. Many reptiles, therefore, routinely experience hypoxia at the tissue level. Furthermore, reptiles are rather tolerant to oxygen lack and species such as freshwater turtles are amongst the most anoxia-tolerant vertebrates. Many studies, therefore, have been performed on the anoxia and hypoxia-tolerance of reptiles with an aim to understand basic mechanisms common to all vertebrates.

Reptiles are ectohermic vertebrates with a relatively low metabolism compared to endothermic vertebrates. Because of the low metabolism, reptiles are characterized by much lower rates of pulmonary ventilation and many species breathe episodically with long-lasting periods of breath holds. The different taxonomic groups of reptiles vary substantially with regards to heart morphology and lung structure (Farrell *et al*. 1998; Hicks 1998; Perry 1989, 1998) and it is likely that studies on the effects of hypoxia in reptiles with diverse cardiac and pulmonary morphologies can provide insight into the evolution of physiological responses to hypoxia amongst air-breathing vertebrates.

In terms of evolutionary studies, reptiles represent a phylogenetically important group because endothermic birds and mammals evolved independently from reptilian ancestors (Page 2000; Fig. 1).

[12] Department of Zoophysiology, Building 131, University of Aarhus, 8000 Aarhus C, Denmark

The evolution of endothermy was associated with an approximate ten-fold rise in resting and maximal rates of oxygen consumption (*e.g.* Bennett and Ruben 1979). This transition placed great demands on the circulatory and respiratory systems (Taylor and Weibel 1981), requiring higher resting and maximal heart rates to match the increased need for oxygen delivery, and elevated blood pressure to secure adequate perfusion pressure for the higher capillary density (Burggren *et al,* 1997; Lillywhite *et al.* 1999). In the pulmonary circulation, higher structural complexity allowed for smaller gas exchange units and increased surface area which, together with a thinner blood-gas barrier, increased the pulmonary diffusive capacity for O_2 (Perry 1990; West 2003). The very thin blood-gas barrier in endothermic vertebrates required a complete cardiac division and separation of the pulmonary and systemic circulations to allow for low pulmonary pressure to protect the respiratory epithelium from a high vascular pressure (West 2003). The higher complexity, however, also increased the possibilities for ventilation-to-perfusion in homogeneities (Piiper 1992; Powell and Hopkins 2004). These cardio-respiratory changes certainly enabled a substantial rise in oxygen transport from air to the metabolising tissue, which allows for increased exercise stamina, but the higher metabolic rate also leads to an increased reliance on oxidative metabolic pathways and made the endothermic animals more susceptible to hypoxia. This sensitivity to hypoxia is perhaps best illustrated by the intolerance to ischemia and hypoxia, which characterizes the heart and brain of mammals, where few minutes of ischemia can lead to irreversible brain damage and persisting depressions of cardiac contractility.

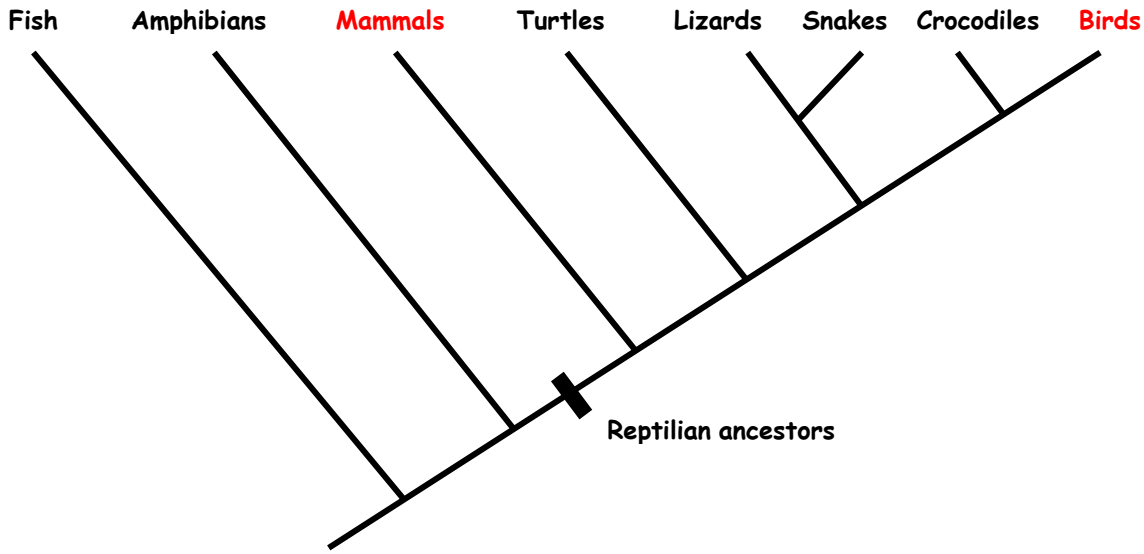


Figure 1. The phylogenetic relations between vertebrates. Endothermic birds and mammals evolved independently from reptilian ancestors and the four groups of extant reptiles (turtles, lizards, snakes and crocodiles), therefore, constitute an important group for comparative studies on the evolution of physiological responses to hypoxia.

Here we review the physiological and metabolic responses to hypoxia in reptiles and compare these responses to the endothermic mammals and birds. Rather than providing a detailed review on all aspects of the hypoxic responses, we will place emphasis on the pulmonary circulation and the possible effects of the internal hypoxia that can be caused by prolonged breath hold and cardiac shunts.

THE REPTILIAN HEART AND ITS EFFECT ON ARTERIAL OXYGEN LEVELS

In most non-crocodilian reptiles, the ventricle is anatomically and functionally undivided and blood pressures are, therefore, equal in systemic and pulmonary circulations (*e.g.* Hicks 1998; Fig. 2B). This allows for cardiac shunting of blood flow where systemic (right-to-left, R-L) or pulmonary (left-to-right, L-R) circulation is by passed. In species where the ventricle is undivided the heart basically functions as a single pressure source and blood flow distribution between pulmonary and systemic circulations is primarily determined by pulmonary and systemic vascular resistances, respectively. Thus, when pulmonary vascular resistance is low, a L-R shunt will prevail, whilst large R-L shunts exist when pulmonary vascular resistance is high. This relationship between vascular resistances and blood flow distribution is illustrated in Figure 2A, which shows that turtles exhibit large R-L shunts (i.e. a low Qpul/Qsys) when pulmonary vascular resistance is high relative to systemic vascular resistance (i.e. a low Rpul/Rsys) and vice versa.

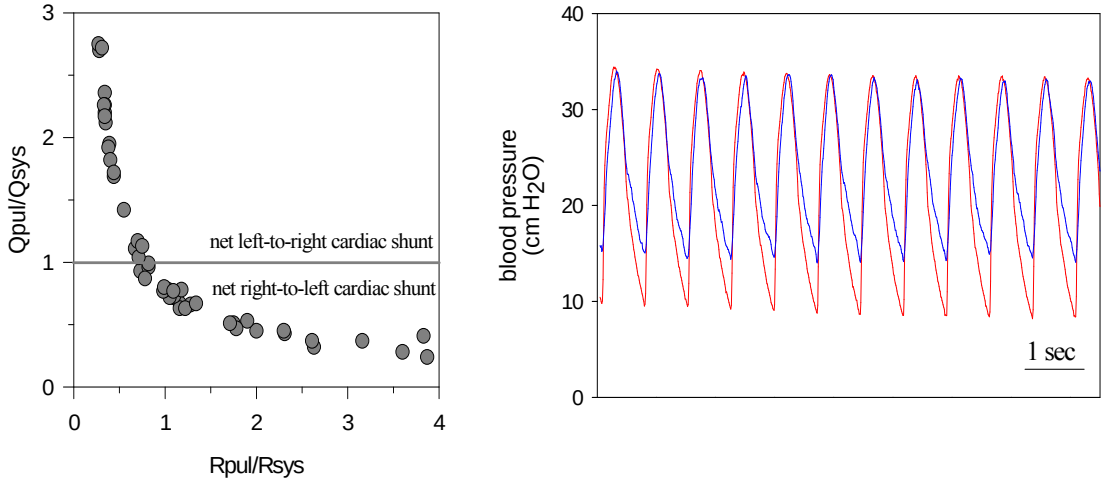


Figure 2. A. The distribution of blood flows between the systemic and pulmonary circulations in anaesthetized turtles where the cardiac shunt pattern is expressed as Qpul/Qsys. Qpul, pulmonary blood flow; Qsys systemic blood flow; Rpul, pulmonary vascular resistance; Rsys, systemic vascular resistance (modified from Crossley *et al.* 1998). B. Simultaneous recording of systemic and pulmonary blood pressures (in blue and red, respectively) in an anaesthetized turtle. Note that systolic pressures are similar in the two circulatory systems, whereas diastolic pressures are lower in the pulmonary circulation due to the lower resistance.

It is well established that virtually all reptiles regulate pulmonary vascular resistance through constriction or dilatation of smooth muscle surrounding the common pulmonary artery close to where it emerges from the ventricle. This smooth muscle is innervated by the vagus nerve and increased vagal activity leads to a constriction of the pulmonary artery, which can be blocked with atropine. Reptiles, therefore, are able to regulate their cardiac shunt pattern and it is now well established that the low heart rate that is characteristic during breath hold and diving is attended by large R-L cardiac shunts where most of the blood recirculates within the systemic circulation without being oxygenated. In contrast, during pulmonary ventilation, pulmonary vascular resistance is greatly reduced and

pulmonary blood flow increases markedly, which in some cases is associated with L-R shunts.

There are a few reptiles where the ventricle is functionally divided, so that pulmonary blood pressures are much lower than systemic blood pressures. This deviation from the normal reptilian pattern of low systemic and pulmonary blood pressures has only been described in varanid lizards and pythons and these two groups have blood pressures that resemble those of mammals (Burggren and Johansen, 1982; Wang *et al.* 2002, 2003). The functional division of the ventricle allows for high systemic blood pressures, whilst protecting the lungs by having low pulmonary blood pressures and has often been linked to the high metabolic rates and exercise stamina of varanid lizards. A similar teleological reasoning is not obvious for pythons, but it is possible that the functional division of their hearts is related to the high metabolic rates that these animals have during incubation of the eggs (Wang *et al.* 2003).

The cardiac right-to-left shunt has pronounced effects on arterial blood gas composition and oxygen transport. During a right-to-left shunt, oxygen poor venous blood bypasses the lungs, and consequently does not become oxygenated and is shunted directly back into the systemic circulation. The oxygen concentration of arterial blood, therefore, becomes the weighed average of the oxygen concentrations in the blood returning from the lungs and that of the blood which is shunted away from the pulmonary circulation. Formally, this can be described by the shunt equation:

$$[O_2]a = (Qpul \times [O_2]pv + Q_{R\text{-}L} \times [O_2])/(Qpul + Q_{R\text{-}L}),$$

where $[O_2]a$, $[O_2]pv$ and $[O_2]sv$ are the oxygen concentration in arterial blood, the pulmonary vein and the systemic venous blood, respectively, while Qpul, Qsys and $Q_{R\text{-}L}$ are pulmonary, systemic and shunt blood flows, respectively. Arterial oxygen concentration, and thereby the arterial partial pressure of oxygen, are reduced according to the magnitude of the shunt flow, but also according to venous oxygen concentrations. The effects of cardiac shunting has been measured in several species (see Burggren *et al.* 1979; White *et al.* 1989; Wang *et al.* 1998).

A number of recent studies show that resting undisturbed reptiles normally exhibit large cardiac right-to-left shunts (*e.g.* White *et al.* 1989; Wang *et al.* 1996a; Wang *et al*. 1998; Krosniunas and Hicks 2003). However, when metabolic rate is elevated due to muscular exercise, increased temperature or digestion, the R-L shunt is reduced (Wang *et al.* 1997; Krosniunas and Hicks 2003). Thus, it seems that reptiles regulate oxygen delivery by altering the cardiac shunt according to metabolic demand (Wang *et al.* 1997; Wang *et al.* 2001).

PHYSIOLOGICAL RESPONSES TO HYPOXIA

When exposed to hypoxia, animals can increase the transport of oxygen through the ventilatory and cardiovascular systems to maintain oxygen delivery at a sufficiently high level that allows metabolism to be maintained at the normoxic value. Reptiles certainly do exhibit both increases in ventilation and heart rate in response to hypoxia, but it is also becoming clear that many reptiles, in common with other vertebrates, respond by decreasing metabolism, which reduces the demand on oxygen transport. The physiological responses to hypoxia are elicited through stimulation of oxygen sensitive chemoreceptors which seem to be located rather diffusively within the systemic arterial arches, but there is also some evidence for receptors on the pulmonary artery. In this case, hypoxic responses may also be elicited through reductions in venous oxygen levels.

EFFECTS OF HYPOXIA ON CARDIAC SHUNT PATTERNS

During exposure to hypoxia, it is clearly advantageous for oxygen transport to reduce or eliminate the cardiac R-L shunt. A reduction in cardiac shunt has indeed been documented in hypoxic turtles (Wang *et al.* 1997). This reduction can, at least in part, be ascribed to the rise in ventilation, which due to the tight interaction between ventilation and cardiac shunt patterns reduces the periods of R-L shunt that are associated with breath hold. Thus, it remains to be established whether the cardiac shunt pattern is directly affected by stimulation of oxygen sensitive chemoreceptors or merely a consequence of increased ventilation. However, when arterial oxygen concentration is reduced through reductions in blood haemoglobin concentration or exposure to carbon monoxide there are large reductions in the cardiac R-L shunt, which certainly is beneficial for oxygen transport. The receptors that are responsible for these responses remain to be characterized (Wang *et al.* 1997).

THE REPTLIAN LUNG AND THE DIRECT EFFECT OF HYPOXIA

The reptilian lung is structurally rather simple compared to that of mammals and birds. Thus, in most snakes and lizards the lungs merely consist of large air sacs with few subdivisions and the area for gas exchange is, therefore, very limited compared to mammals and birds (Luchtel and Kardong 1981; Hlastala *et al.* 1985; Maina 1989). On the other hand, the lungs of turtles, varanid lizards and crocodiles are more complex and compartmentalized increasing the surface area and the capacity for oxygen diffusion (Perry 1983, 1989, 1998).

A great number of studies have shown that reptiles increase ventilation when exposed to hypoxia and the rise in overall ventilation is mainly accomplished through an increased breathing frequency while tidal volume only increases in some cases (Milsom 1991).

Low oxygen levels in the lungs of birds and mammals cause constriction of the pulmonary vasculature elevating resistance to pulmonary blood flow and increasing pulmonary blood pressure (Euler and Liljestrand 1946; Motley *et al.* 1947; Faraci *et al.* 1984). This response, hypoxic pulmonary vasoconstriction (HPV), diverts pulmonary blood flow from poorly ventilated and hypoxic areas of the lung to more well-ventilated parts. HPV is considered important for local matching of ventilation to blood perfusion improving pulmonary gas exchange and maintaining systemic arterial PO_2 (Euler and Liljestrand 1946; Dawson 1984; Brimioulle *et al.* 1996). The primary site of HPV is the precapillary muscular pulmonary arteries (Weir and Archer 1995). HPV is detrimental during exposure to hypoxic gases, where the entire pulmonary circulation becomes hypoxic, and increases pulmonary arterial pressure.

HPV is a locally mediated response that persists in isolated, perfused lungs with no neurohumoral influences and also occurs in isolated rings of pulmonary vascular smooth musculature (Fishman 1976; McMurty *et al.* 1978, Robertson *et al.* 1995). HPV reflects an inherent property of the pulmonary vascular smooth muscle and is likely to be mediated by inhibition of one or several different types of K^+-channels, whilst various endothelium-derived vasoactive compounds act as modulators (Ward and Robertson 1995; Weir and Archer 1995; López-Barneo *et al.* 2004; Ward *et al.* 2004). In contrast, the systemic circulation of most vertebrates responds to hypoxia by vasodilatation (Detar 1980; Crossley *et al.* 1998; Shimizu *et al.* 2000; Smith *et al.* 2001).

The breathing pattern of many reptiles, particularly aquatic species, is characterized by ventilatory episodes of one or several breaths interspersed between non-ventilatory periods of varying

length (Milsom 1991). During non-ventilatory periods, lung and blood PO_2 decrease as the oxygen stores are exhausted (*e.g.* Burggren and Shelton 1979). In species with an undivided ventricle, blood flow distribution between pulmonary and systemic circulations is primarily determined by pulmonary and systemic vascular resistances (Hicks 1998; Crossley *et al.* 1998), so HPV would induce a pulmonary by pass of blood flow (right-to-left cardiac shunt) and reduce the ability to exploit the pulmonary oxygen reserve during breath hold. In animals with a divided heart, such as caimans, HPV during breath hold will, nevertheless, cause pulmonary hypertension that may disturb pulmonary fluid balance (Welling *et al.* 1997).

We have recently shown that hypoxia causes a reversible constriction of the pulmonary vasculature in turtles, varanids and caimans (Crossley *et al.* 1998; Skovgaard *et al.* 2005). These three species possess complex multicameral lungs and it is likely that HPV would aid to secure V/Q homogeneity. There is no response to hypoxia in rattlesnakes (Skovgaard *et al.* 2005), which is consistent with the view that species with structurally simple lungs rely less on local responses for proper V/Q matching. However, tegu lizards that also have simple unicameral lungs do exhibit HPV (Skovgaard *et al.* 2005), albeit at a lower hypoxic threshold than varanids and caimans. Thus, while the data support our general hypothesis that HPV is more pronounced in species with complex lungs and divided hearts, it is also clear that other components are involved. HPV evolved to improve the V/Q matching; however, in species with a poorly divided ventricle, this benefit is counterbalanced by the fact that HPV impairs the ability to increase pulmonary blood flow during non-ventilatory periods. The apparent blunted HPV in turtles compared to caimans may, therefore, ensure that pulmonary blood flow can be increased during for instance submergence (Crossley *et al.* 1998).

REDUCTIONS IN METABOLISM DURING HYPOXIA: a DECREASE IN PREFERRED BODY TEMPERATURE AND/OR REDUCTIONS IN BASAL METABOLIC RATE

Most of the physiological responses during hypoxia, such as the increased ventilation and elevated cardiac output, serve to enhance oxygen transport ensuring an adequate oxygen delivery to maintain normal oxygen consumption. However, reptiles can also utilise an opposite or alternative strategy where they reduce metabolism and, therefore, the need for oxygen during hypoxia (see Hicks and Wang 2004). This metabolic depression reduces energy demand, and therefore lessens the demands on the cardio-respiratory system, limits utilization of substrate reserves and reduces the accumulation of toxic metabolites. A reduction in metabolism during hypoxia is not limited to reptiles and has been extensively documented in newborn mammals and birds (*e.g.* Mortola and Frappell 2000; Mortola 2004). Also, diving birds and mammals appear to reduce metabolic rate when diving (Butler 2004), and it is becoming increasingly clear that metabolic rate is more plastic than previously thought.

There are two strategies to reduce metabolism during periods of limited oxygen availability. Firstly, animals can utilise a behavioural reduction of the preferred body temperature that, through the direct effect of temperature on biochemical processes (the so-called "Q_{10} effect" or the "Arhenius effect") decreases the rate of metabolic processes. Assuming a Q_{10} of ~ 2.5 for oxygen consumption, aerobic demands decrease by approximately 11% per degree C reduction of body temperature. Second, at a constant body temperature, animals can reduce ATP demands, which reduce basal metabolic rate. As a corollary mechanism, reptiles and other animals can reduce physical activity during hypoxia, which obviously reduces oxygen usage.

Ectothermic animals, such as reptiles, generate a limited amount of heat through metabolism and rely on the environment to maintain body temperature, and use behavioural mechanisms such as basking and/or shuttling between sun and shade to achieve the preferred body temperature. By these mechanisms reptiles regulate body temperature within a rather narrow range that is determined by a thermal set-point within the central nervous system (Cabanac 1975; Steiner and Branco 2002). The mechanism by which hypoxia lowers this set-point remains obscure (Wood, 1991; Steiner and Branco, 2002), but a reduction in the preferred body temperature has been documented in teleost fish, amphibians, reptiles, mammals and birds (*e.g.* Bryan *et al.* 1984; Hicks and Wood 1985), and even appears to be a universal response amongst all animals (Wood 1991; Malvin and Wood 1992). Apart from low oxygen levels in the inspired air, anemia can also cause a reduction in body temperature as shown in lizards (Hicks and Wood 1985), and a reduction of the preferred body temperature is also a common response to fasting and starvation where the reduction on metabolism may prolong the period that animals can tolerate being without food.

A reduction in metabolism during hypoxia can be accomplished through an actual depression of cellular metabolism at unchanged temperature. This metabolic depression has been studied extensively during complete lack of oxygen, *i.e*. anoxia, and it is particularly very anoxia-tolerant species, such as freshwater turtles, that can survive for many months without oxygen. Thus, using calorimetric measurements, Jackson and Schmidt-Nielsen (1966) showed that heat production of turtles decreases approximately ten-fold within hours after being exposed to anoxia. A number of subsequent studies on individual organs, such as brain, heart and liver have shown that the metabolic depression during anoxia is a regulated response that involves reductions in active membrane transport and membrane permeability, as well as a down-regulation of various synthetic pathways, such as protein synthesis (Nilsson *et al.* 1991; Buck *et al.* 1993a; Buck *et al.* 1993c; Land *et al.* 1993; Hochachka *et al.* 1996, 1997, 2001; Lutz and Kabler 1997). The depression of metabolism obviously extends the period of survival during anoxia, but does not prevent the accumulation of lactate and anoxia is invariably associated with a progressive development of a metabolic acidosis.

While much attention has been paid to describing and understanding the suppression of metabolism during anoxia, fewer studies have been devoted to understanding how hypoxia affects metabolism. In turtles exposed to progressive levels of hypoxia there was a lowering of their metabolic rate. This response was most pronounced at higher temperatures where metabolism is elevated. It is not easy to interpret these results, because the turtles did increase ventilation in response to hypoxia and the decline in oxygen consumption may reflect an inability to deliver sufficient oxygen to the metabolising tissues. To circumvent some of these uncertainties, Hicks and Wang (1999) investigated the effects of progressive hypoxia on oxygen consumption in anaesthetized turtles, while making simultaneous measurements of blood flows as well as arterial and venous blood gases. The turtles were artificially ventilated, so the levels of hypoxia could be controlled for precise control of inspired gases. Hypoxia and the attending fall in arterial and venous PO_2 and O_2 content was associated with significant reductions in VO_2 (up to 73% of the normoxic value), which was fully reversible upon return to normoxia. As with the previous study on recovered turtles (Glass *et al.* 1983), the reduction in VO_2 could either result from an inability of the cardiopulmonary system to supply sufficient oxygen to the tissues or by an actual down regulation of metabolism, *i.e.* a reduction of ATP demand. To distinguish between these two possibilities, the metabolic uncoupler 2,3-dintrophenol (DNP) was infused during hypoxia to determine whether a higher VO_2 could be stimulated (Hicks and Wang 1999). The infusion of DNP did indeed increase VO_2 of the hypoxic turtles, while arterial and venous blood O_2 levels remained low and without affecting cardiac output. Furthermore, there was no observable rise of plasma lactate or potassium levels. It was concluded, that the reduction of VO_2 during hypoxia reflects

an actual reduction in the rate of ATP turnover. Similar results have been obtained on rats (Saiki *et al.* 1997) and hypoxic hepatocytes from turtles (Buck *et al.* 1993b).

As discussed above, most reptiles normally have a pronounced right-to-left cardiac shunt, which leads to a reduction in systemic arterial PO_2 and oxygen concentration (*e.g.* Shelton and Burggren 1976; Wang *et al.* 1997). Given the rather distinct reduction of oxygen uptake during hypoxia that was observed in the anaesthetized turtles, it seems possible that the low arterial oxygen levels, which are common during breath hold and diving, could trigger fast and reversible metabolic down-regulations (Hicks and Wang 2004). Pulmonary blood flow and the cardiac shunt pattern of most reptiles are regulated by a vagal innervation on smooth muscles within the wall of the pulmonary artery (*e.g.* Milsom *et al.* 1977; Wang *et al.* 2001). To mimic these responses, Platzack *et al.* (2001) electrically stimulated the vagus nerve of anaesthetized turtles and induced a reduction in heart rate and an increase in the R-L cardiac shunt that occurs normally in diving turtles. Under these conditions, the lowering of arterial oxygen levels was attended by a decrease in oxygen uptake, which further strengthens the possibility of cardiac right-to-left shunt being able to affect aerobic metabolism.

Some species experience hypoxia at altitude, and aquatic species experience reductions in lung and blood PO_2 during breath-holds associated with prolonged diving. In contrast to mammals and birds, many reptiles also routinely experience varying levels of hypoxemia resulting from their unique cardiac morphology, in which the undivided ventricle allows for admixture of systemic venous blood (right-to-left or R-L cardiac shunt) with subsequent reductions in arterial PO_2 (Shelton and Burggren 1976; Burggren *et al.* 1979; White *et al.* 1989; Wang *et al.* 1996a; Hicks 1998). Because the amount of systemic venous admixture can be regulated, reptiles have the ability to alter arterial PO_2 independently of lung PO_2. This ability may represent a mechanism of regulating metabolism through modulation of oxygen supply to the tissue (Wang *et al.* 1996b; Wang *et al.* 1997; Wang *et al.* 2001).

ACKNOWLEDGEMENTS

Supported by the Danish Research Council.

REFERENCES

Bennett, A.F. and J.A. Ruben. 1979. Endothermy and activity in vertebrates. Science 206: 649-654.

Brimioulle, S., P. Lejeune and R. Naeije. 1996. Effects of hypoxic pulmonary vasoconstriction on pulmonary gas exchange. Journal of Applied Physiology 81: 1535-1543.

Bryan, J.D., L.G. Hill and W.H. Niell. 1984. Interdependence of acute temperature preference and respiration in the plains minnow. Transactions of the American Fisheries Societies 113: 557-562.

Buck, L.T. and P.W. Hochachka. 1993a. Anoxic suppression of Na(+)-K(+)-ATPase and constant membrane potential in hepatocytes: support for channel arrest. American Journal of Physiology 265: R1020-1025.

Buck, L.T., P.W. Hochachka, A. Schon and E. Gnaiger. 1993b. Microcalorimetric measurement of reversible metabolic suppression induced by anoxia in isolated hepatocytes. American Journal of Physiology 265: R1014-1019.

Buck, L.T., S.C. Land and P.W. Hochachka. 1993c. Anoxia-tolerant hepatocytes: model system for study of reversible metabolic suppression. American Journal of Physiology 265: R49-56.

Burggren, W., A. Farrel and H. Lillywhite. 1997. Vertebrate cardiovascular systems. In: Dantzler, W.H. (Ed.) Handbook of physiology, Sec. 13, Comparative physiology, vol. 1. American Physiological Society. Oxford, New York, pp. 215-308.

Burggren, W.W. and K. Johansen. 1982. Ventricular haemodynamics in the monitor lizard Varanus exanthematicus: pulmonary and systemic pressure separation. Journal of Experimental Biology 96: 343-354.

Burggren, W.W. and G. Shelton. 1979. Gas exchange and transport during intermittent breathing in Chelonian reptiles. Journal of Experimental Biology 82: 75-92.

Butler, P.J. 2004. Metabolic regulation in diving birds and mammals. Respiratory Physiology and Neurobiology 141:297-315.

Cabanac, M., 1975. Temperature regulation. Annual Review of Physiology 37: 415-439.

Crossley, D.A., T. Wang and J. Altimiras. 1998. Hypoxia elicits pulmonary vasoconstriction in anaesthetized turtles. Journal of Experimental Biology 201: 3367-3375.

Dawson, C.A., 1984. Role of pulmonary vasomotion in physiology of the lung. Physiological Review 64: 544-616.

Detar, R., 1980. Mechanisms of physiological hypoxia-induced depression of vascular smooth muscle contraction. American Journal of Physiology 238: H761-H769.

Euler, U.S.V. and G. Liljestrand. 1946. Observations on the pulmonary arterial blood pressure in the cat. Acta Physiologica Scandinavica 12: 301-320.

Faraci, F.M., D.L. Kilgore and M.R. Fedde. 1984. Attenuated pulmonary pressor response to hypoxia in bar-headed geese. American Journal of Physiology 247: R402-R403.

Farrell, A.P., A.K. Gamperl, and E.T.B. Francis. 1998. Comparative aspects of heart morphology. In: Gans, C., Gaunt, A.S. (Eds.), Biology of Reptilia, vol. 19 (Morphology). Society for the study of amphibians and reptiles. Ithaca, New York, pp. 375-424.

Fishman, A.P. 1976. Hypoxia on the pulmonary circulation. How and where it acts. Circulation Research 38: 221-231.

Glass, M. L., R.G. Boutilier and N. Heisler. 1983. Ventilatory control of arterial PO2 in the turtle *Chrysemys picta bellii*: effects of temperature and hypoxia. Journal of Comparative Physiology B 151:145-153.

Hicks, J.W. 1998. Cardiac Shunting in Reptiles: Mechanisms, Regulation and Physiological Function. In: Gans, C., Gaunt, A.S. (Eds.), Biology of the Reptilia, vol. 19 (Morphology G). Society for the Study of Amphibians and Reptiles, Ithaca, N.Y., pp. 425-483

Hicks, J.W. and T. Wang. 1999. Hypoxic hypometabolism in the anesthetized turtle, *Trachemys scripta*. American Journal of Physiology 277: R18-23.

Hicks, J.W. and T. Wang. 1996. Functional role of cardiac shunts in reptiles. Journal of Experimental Zoology 275: 204-216.

Hicks, J.W. and T. Wang. 2004. Hypometabolism in reptiles: behavioural and physiological mechanisms that reduce aerobic demands. Respiratory Physiology and Neurobiology 141: 261-271, 2004.

Hicks, J.W. and S.C. Wood. 1985. Temperature regulation in lizards: effects of hypoxia. American Journal of Physiology 248: R595-R600.

Hlastala, M.P., T.A. Standaert, D.J. Pierson and D.L. Luchtel. 1985. The Matching of Ventilation and Perfusion in the Lung of the Tegu Lizard, *Tupinambis nigropunctatus*. Respiration Physiolology 60: 277-294.

Hochachka, P.W. and P.L. Lutz. 2001. Mechanism, origin, and evolution of anoxia tolerance in animals. Comparative Biochemistry and Physiology 130B: 435-459.

Hochachka, P.W., S.C. Land and L.T. Buck. 1997. Oxygen sensing and signal transduction in metabolic defense against hypoxia: lessons from vertebrate facultative anaerobes. Comparative Biochemistry and Physiology 118A: 23-29.

Hochachka, P.W., L.T. Buck,C.J. Doll, and S.C. Land. 1996. Unifying theory of hypoxia tolerance: molecular/metabolic defense and rescue mechanisms for surviving oxygen lack. Proceedings of the National Academy of Sciences USA 93: 9493-9498.

Jackson, D. C. and K. Schmidt-Nielsen. 1966. Heat production during diving in the fresh water turtle, *Pseudemys scripta*. Journal of Cellular Physiology 67: 225-232.

Krosniunas, E. and J.W. Hicks. 2003. Cardiac Output and Shunt during Voluntary Activity at Different Temperatures in the Turtle, *Trachemys scripta*. Physiological and Biochemical Zoology 76: 679-694.

Land, S.C., L.T. Buck and P.W. Hochachka. 1993. Response of protein synthesis to anoxia and recovery in anoxia-tolerant hepatocytes. American Journal of Physiology 265: R41-48.

Lillywhite, H.B., K.C. Zippel and A.P. Farrell. 1999. Resting and maximal heart rates in ectothermic vertebrates. Comparative Biochemistry and Physiology 124A: 369-382.

López-Barneo, J., R. del Toro, K.L. Levitsky, M.D. Chiara, and P. Ortega-Sáenz. 2004. Regulation of oxygen sensing by ion channels. Journal of Applied Physiology 96: 1187-1195.

Luchtel, D.L. and K.V. Kardong. 1981. Ultrastructure of the lung of the rattlesnake, *Crotalus viridis oreganos*. Journal of Morphology 169: 29-47.

Lutz, P.L. and S. Kabler. 1997. Release of adenosine and ATP in the brain of the freshwater turtle (*Trachemys scripta*) during long-term anoxia. Brain Research 769: 281-286.

Maina, J.N. 1989. The morphology of the lung of the black mamba *Dendroaspis polylepis* (Reptilia: Ophidia: Elapidae). A scanning and transmission electron microscopic study. Journal of Anatomy 167: 31-46.

Malvin, G.M. and S.C. Wood. 1992. Behavioral hypothermia and survival of hypoxic protozoans *Paramecium caudatum*. Science 255: 1423-1425.

McMurtry, I.F., B.W. Hookway and B.W. Roos. 1978. Red blood cells but not platelets prolong vascular reactivity of isolated rat lungs. American Journal of Physiology 234: H186-H191.

Milsom, W.K. 1991. Intermittent breathing in reptiles. Annual Review of Physiology 53: 87-105.

Milsom, W.K., B.L. Langille and D.R. Jones. 1977. Vagal control of pulmonary vascular resistance in the turtle *Chrysemys scripta*. Canadian Journal of Zoology 55: 359-367

Mortola J.P. 2004. Implications of hypoxic hypometabolism during mammalian ontogenesis. Respiratory Physiology and Neurobiology 141: 345-356.

Mortola, J. P. and K. Labbe. 2005. Oxygen consumption of the chicken embryo: interaction between temperature and oxygenation. Respiratory Physiology and Neurobiology 146: 97-106.

Mortola J. P. and P.B. Frappell. 2000. Ventilatory responses to changes in temperature in mammals and other vertebrates. Annual Review of Physiology 62: 847-874.

Motley, H.L., A. Cournand, L. Werko, A. Himmelstein and D. Dresdale. 1947. The influence of short periods of induced acute anoxia upon pulmonary artery pressures in man. American Journal of Physiology 150: 315-320.

Nilsson, G.E. and P.L. Lutz. 1991. Release of inhibitory neurotransmitters in response to anoxia in turtle brain. American Journal of Physiology 261: R32-37.

Page, R.D.M. 2000. Extracting species trees from complex gene trees: Reconciled trees and vertebrate phylogeny. Molecular Phylogenetics and Evolution 14: 89-106.

Perry, S.P. 1998. Lungs: Comparative anatomy, functional morphology and evolution. In: Gans, C., Gaunt, A.S. (Eds.), Biology of Reptilia, vol. 19 (Morphology). Society for the study of amphibians and reptiles. Ithaca, New York, pp. 1-92.

Perry, S.F. 1990. Recent advances and trends in the comparative morphometry of vertebrate gas exchange organs. In: Boutilier, R.G. (Ed.) Advances in Comparative and Environmental Physiology, vol.6. Springer-Verlag, Berlin, pp. 45-71.

Perry, S.F. 1989. Mainstreams in the evolution of vertebrate respiratory structures. In: King, A.S., McMelland, J. (Eds.) Form and function in birds, vol. 4. Academic Press, London, pp. 1-67.

Perry, S.F. 1983. Reptilian lungs. Functional anatomy and evolution. Advances in Anatomy, Embryology and Cellular Biology 79: 1-81.

Piiper, J. 1992. Diffusion-perfusion inhomogeneity and alveolar-arterial O_2 diffusion limitation – theory. Respiration Physiology 87: 349-356.

Platzack, B. and J.W. Hicks. 2001. Reductions in systemic oxygen delivery induce a hypometabolic state in the turtle *Trachemys scripta*. American Journal of Physiology 281: R1295-1301.

Powell, F.L. and S.R. Hopkins. 2004. Comparative physiology of lung complexity: Implications for gas exchange. News in Physiological Sciences 19: 55-60.

Robertson, T.P., P.I. Aaronson and J.P.T. Ward. 1995. Hypoxic intracellular Ca2+ in pulmonary arteries: evidence for PCK-independent Ca^{2+} sentization. American Journal of Physiology 268: H301-307.

Saiki, C. and J.P. Mortola. 1997. Effect of 2,4-dinitrophenol on the hypometabolic response to hypoxia of conscious adult rats. Journal of Applied Physiology 83: 537-542.

Shelton, G. and W.W. Burggren. 1976. Cardiovascular dynamics of the chelonia during apnoea and lung ventilation. Journal of Experimental Biology 64: 323-343.

Shimizu, S., P.S. Bowman, G. Thorne III, and R.J. Paul. 2000. Effects of hypoxia on isometric force, intracellular Ca2+, pH, and energetics in porcine coronary artery. Circulation Research 86: 862-870.

Smith, M.P., M.J. Russell, J.T. Wincko and K.R. Olson. 2001. Effects of hypoxia on isolated vessels and perfused gills of rainbow trout. Comparative Biochemistry and Physiology 130A: 171-181.

Skovgaard, N., A.S. Abe, D.V. Andrade and T. Wang. 2005. Hypoxic pulmonary vasoconstriction in reptiles: A comparative study. American Journal of Physiology (in review)

Steiner, A.A. and L.G. Branco. 2002. Hypoxia-induced anapyrexia: implications and putative mediators. Annual Review of Physiology 64: 263-288.

Taylor, C.R. and E.R. Weibel. 1981. Design of the mammalian respiratory system. I. Problem and strategy. Respiration Physiology 44: 1-10.

Wang, T., J. Altimiras, W. Klein and M. Axelsson. 2003. Ventricular haemodynamics in *Python molurus*: separation of pulmonary and systemic pressures. Journal of Experimental Biology 206: 4241-4245.

Wang, T., J. Altimiras and M. Axelsson. 2002. Intracardiac flow separation in an in situ perfused heart from Burmese python *Python molurus*. Journal of Experimental Biology 205: 2715-2723.

Wang, T., S. Warburton, A. Abe and T. Taylor. 2001. Vagal control of heart rate and cardiac shunts in reptiles: Relation to metabolic state. Experimental Physiology 86: 777-784.

Wang, T., A.S. Abe and M.L. Glass. 1998. Effects of temperature on lung and blood gases in the South American rattlesnake, *Crotalus durissus terrificus*. Comparative Biochemistry and Physiology 121A: 7-11.

Wang, T., E.H. Krosniunas and J.W. Hicks. 1997. The role of cardiac shunts in the regulation of arterial blood gases. American Zoologist 37: 12-22.

Wang, T. and J.W. Hicks. 1996a. Cardiorespiratory synchrony in turtles. Journal of Experimental Biology 199: 1791-1800.

Wang, T. and J.W. Hicks. 1996b. The interaction of pulmonary ventilation and the right-left shunt on arterial oxygen levels. Journal of Experimental Biology 199: 1435-1446.

Ward, J.P.T., V.A. Snetkov and P.I. Aaronson. 2004. Calcium, mitochondria and oxygen sensing in the pulmonary circulation. Cell Calcium 36: 209-220.

Ward, J.P.T. and T.P. Robertson. 1995. The role of endothelium in hypoxic pulmonary vasoconstriction. Experimental Physiology 80, 793-801.

Weir, E.K. and S.L. Archer. 1995. The mechanism of acute hypoxic pulmonary vasoconstriction: the tale of two channels. FASEB Journal 9: 183-189.

Welling, K.-L.K., M. Sander, J.B. Ravn, B. Larsen, U. Abildgaard and O. Amtorp. 1997. Effect of alveolar hypoxia on segmental pulmonary vascular resistance and lung fluid balance in dogs. Acta Physiologica Scandinanavica 161: 177-186.

West, J.B. 2003. Thoughts on the pulmonary blood-gas barrier. American Journal of Physiology 285: L501-L513.

White, F.N., J.W. Hicks and A. Ishimatsu. 1989. Relationship between respiratory state and intracardiac shunts in turtles. American Journal of Physiology 256: R240-R247.

Wood, S.C. 1991. Interactions between hypoxia and hypothermia. Annual Review of Physiology 53: 71-85.

BIOACCUMULATION AND ENANTIOSELECTIVE BIOTRANSFORMATION OF FIPRONIL BY RAINBOW TROUT (*ONCORHYNCHUS MYKISS)*

Brad J. Konwick, Aaron T. Fisk, Jimmy K. Avants,
J. MacArthur Long[13], Arthur W. Garrison

ABSTRACT

Dietary accumulation and enantioselective biotransformation was determined for rainbow trout (*Oncorhynchus mykiss*) exposed to fipronil, a widely used chiral pesticide. Measurement of the fish carcass tissue (whole fish minus GI tract and liver) showed a rapid accumulation of fipronil at microgram per gram concentrations during the 32 day uptake phase. The low biomagnification factor (0.05) and short depuration half-life (0.58 d) determined for fipronil indicated a rapid clearance of this compound by the fish, thus fipronil would not be expected to biomagnify in the food chain. Changes in enantiomer fractions (EFs) over the course of the study indicated that depuration was due to biotransformation, with preferential metabolism of the (+) enantiomer of fipronil. Formation of the metabolite fipronil sulfone, detected in the fish carcass, validated the observation of biotransformation indicated by chiral analysis. Fipronil sulfone was more persistent than fipronil in fish with a depuration half-life of 2.36 d, thus making this compound a possible tracer of fipronil exposure.

INTRODUCTION

Fipronil is a phenylpyrazole-class insecticide first approved in 1996 for use on a number of crops in the U.S. Typical fipronil applications include rice culture, turf grass management, and residential pest control (US EPA 1997, US EPA 1996). Fipronil has been recognized as a disrupter of γ-aminobutyric acid (GABA) gated chloride channels in nerve cells, leading to hyper-excitation and eventual mortality (Gant *et al*. 1998). This toxicity is much greater in invertebrates than in mammalians due to their different GABA receptor binding affinities (Hainzl *et al*. 1998). While fipronil is effective in insect control at comparable potencies to organophosphorous (OP) and carbamate insecticides, its use is expected to increase relative to other classes of pesticides because of developing species resistance to OP insecticides and fipronil's commercial availability (Hosie *et al*. 1995). Fipronil applications can impact aquatic environments at low concentrations (US EPA 2001, US EPA 1997), thus there is potential for adverse effects on non-target species. In addition, fipronil's degradation products, which are similar in potency to fipronil (Schlenk *et al*. 2001) and more environmentally persistent (Walse *et al*. 2004, US EPA 1997), may lead to long-term effects on non-target species.

To assess the potential risk of contaminants, such as current-use pesticides (e.g., fipronil), it is important to understand their environmental accumulation in aquatic biota. Typically, the bioaccumulation potential for most current-use pesticides is considered minimal because of their low octanol-water partition coefficients (log K_{ow}), a good predictor of bioaccumulation. However, fiponil's log K_{ow} (4.01) (US EPA 1996) is in the range of some persistent chemicals (e.g., α-HCH,

[13] U.S. Environmental Protection Agency, National Exposure Research Laboratory, Athens, GA

β-HCH), thus indicating the potential for bioaccumulation in aquatic biota. On the other hand, current-use pesticides are readily metabolized by cytochrome P450 isozymes due to the presence of structural groups that are susceptible to biotransformation reactions. Biotransformation is a key component of a fish's, or any species, elimination pathway for contaminants, making them more water soluble and excretable. Ultimately, elimination rate (e.g., via biotransformation) is what determines the potential for a chemical to bioaccumulate and cause adverse effects in organisms (Borga *et al*. 2004). Unfortunately, methods to assess biotransformation are limited, especially for fish, which are believed to have little biotransformation ability. This is inferred from fish having lower concentrations and activities of cytochrome P450s compared to mammals and birds (Stegeman and Kloeppper-Sams 1987, Kleinow *et al*. 1987). Also, when modeling contaminant movement through food webs, biotransformation in fish is assumed to be negligible (Thomann 1989).

Investigations focusing on chiral analysis have been used recently to study the biotransformation dynamics of contaminants. Approximately 25% of current-use pesticides are chiral (Williams 1996), in addition to many legacy pesticides (e.g., chlordanes, some DDT isomers) and some polychlorinated biphenyls (PCBs) (Kaiser 1974). Chiral compounds exist as two non-superimposable mirror image forms called enantiomers, which are designated as (+) and (-) based on their rotation of plane-polarized light. The manufacture of chiral chemicals results in a mixture designated as racemic (±), which contains 50% of each enantiomer and is the form in which they are typically applied and released into the environment. Enantiomers have identical physical-chemical properties (Garrison *et al*. 1996); however, the relative abundances of enantiomers can change after being subjected to metabolic processes (e.g., enzyme mediated receptors, biochemical reactions) due to the different spatial orientation of the enantiomers (Wong *et al*. 2002). Thus, changes in the enantiomer fractions (EF) of chiral contaminants within species can serve as a tracer for biotransformation. The observation of non-racemic residues, using chiral analysis, has previously shown the ability of fish (Wong *et al*. 2002), cetaceans (Hoekstra *et al*. 2002), and birds (Fisk *et al*. 2001) to bioaccumulate and biotransform chiral organochlorine (OC) contaminants enantioselectively. Determining the biotransformation potential of OC contaminants due to their environmental persistence has been the focus of most chiral studies. However, chiral analysis of current-use pesticides in biota can achieve these same goals, yet has not received much attention.

In this paper, we report the dietary accumulation and biotransformation of racemic fipronil in juvenile rainbow trout (*Oncorhynchus mykiss*). Rainbow trout was chosen as the test species because of its widespread species distribution and extensive knowledge regarding its bioaccumulation and physiology. Dietary exposure was used because it is the major contributor in food chain transfer, especially for hydrophobic contaminants and higher trophic organisms (Fisk *et al*. 1998). To our knowledge, this is the first experiment to determine the toxicokinetics of fipronil in fish via dietary exposure or its chiral biotransformation in any species.

MATERIALS AND METHODS

Chemicals and Food Preparation

Fipronil was obtained from ChemService (West Chester, PA, 98% pure). All solvents (Ultra Resi-Analyzed®) were obtained from J.T. Baker (Phillipsburg, NJ). A stock solution of fipronil in methanol (1000 μg/mL) was diluted in hexane (1:100) and mixed with the commercial trout food (Zeigler, Gardner, PA) to give a nominal concentration of 10 μg/g in the feed; the solvent was then slowly evaporated to dryness in a rotary evaporator. Control food was treated in an identical manner

but without addition of the target compound. The food was air dried for 48 hours and then stored in amber jars at 4°C. The trout food consisted of 38% protein, 14% lipid, and 3% fiber. The concentrations of fipronil were determined in spiked and control food using the same technique described below to determine concentrations in rainbow trout tissue (Table 1).

Table 1. Concentrations (μg/g wet wt) and Enantiomeric Fractions (EFs) of Fipronil (mean ± SE) in Control and Treated Food (n = 3)

Compound	Control food		Treated food	
	C_{food}	EF	C_{food}	EF
Fipronil	ND	-	7.68 ± 0.18	0.497 ± 0.001

ND = Not Detected

Experiments

Juvenile rainbow trout (initial weights 10 ± 0.5 g, mean ± SE) were collected from Lake Burton Fish Hatchery (GA) and randomly assigned to one of two 800 gallon fiberglass aquaria (45 fish per tank) with recirculating carbon-dechlorinated tap water chilled (Frigid Units, Toledo, OH) to 12°C. Fish were maintained with a 12 h light: 12 h dark photoperiod. One tank of fish was exposed to the fipronil treated food whereas the other tank served as a control. Fish were exposed to the spiked food for 32 days (uptake) followed by 96 days of clean food (depuration). The daily rate of feeding was 1.5% of the mean weight of the rainbow trout, corrected for weight gain after each sampling day. All food was consumed within ~ 5 minutes of feeding. Three fish each were randomly sampled from the fipronil treatment and control treatment on days 0, 2, 4, 8, 16, and 32 of the uptake phase, and days 34, 36, 40, 48, 64, and 128 of the depuration phase. Sampled fish were separated into liver, gastrointestinal (GI) tract (including stomach and contents, spleen, pyloric caeca, intestines and adipose tissue associated with these organs), and carcass (whole fish minus GI tract and liver). The GI tract was removed so as to not measure the target analyte that remained in undigested food. Only carcass results were used in calculating bioaccumulation parameters and enantiomer fractions.

Chemical Analysis

Carcass samples were weighed, stored frozen at -8°C , and then freeze-dried. For analysis, samples were homogenized/extracted in dichloromethane (DCM): hexane (1:1) using a polytron (PowerGen 125, Fisher Scientific). The whole fish carcass was homogenized, except for the last sampling day on which only 10-12 g of carcass fillet was extracted due to the large size of the fish. Polychlorinated biphenyl 65 was added to samples as a surrogate recovery standard just before extraction. Samples were extracted twice; the extracts were then combined and evaporated to 10 ml. One ml of the extract was used to determine lipids gravimetrically. The remaining extract was then evaporated to 2 ml and applied to a gel permeation chromatography (GPC) column to remove lipids. The GPC columns (inner diameter 29.5 mm, length 400 mm, reservoir 500 ml) were packed with 60 g (dry weight) of 200-400 mesh Bio-Beads® S-X3 (Bio-Rad Laboratories, Hercules, CA). The column was eluted with 340 ml of DCM: hexane (1:1); the first 140 ml contained the lipids and was discarded. The next 200 ml eluate contained the target analyte and was rotary evaporated to 1 ml before analysis.

Analysis of the extracts for fipronil was by chiral capillary gas chromatography with mass spectrometric detection using a Hewlett-Packard 6890 GC with a 5973 mass selective detector. The

column was a BGB 172 (BGB Analytik AG, Anwil, Switzerland) containing a chiral phase composed of 20% tert-butyldimethylsilylated-β-cyclodextrin. The column was 30 m long, 0.25 mm ID, and of 0.25 µm film thickness. GC conditions were: column temperature, 150 to 220° at a rate of 1.5°/min; injection, splitless at 250°; carrier gas, helium at a flow rate of 1.5 mL/min. Fipronil was detected and measured using selected ion monitoring of ions 367 and 369; electron voltage was 70 eV. This BGB column resolved the enantiomers of fipronil to baseline. Calculation of concentrations of fipronil in the fish extracts was based on the sum of GC areas of both enantiomers compared to the sum of GC areas of both enantiomers of a fipronil standard run on the same day. Typically, 3 fipronil standards were analyzed with a batch of 10 fish samples. Fipronil concentrations were corrected for each extract sample by comparison to the recovery of the surrogate standard (PCB 65), which had been added in known amount to each whole fish carcass before extraction; recovery of the surrogate standard ranged from 35 to 77%.

To assess biotransformation, enantiomeric fractions (EFs) for fipronil were calculated from concentration data using

$$EF = (+) / [(+) + (-)]$$

where (+) and (-) are concentrations of the individual fipronil enantiomers. Using pure fipronil enantiomers, it was observed that the (+)-enantiomer eluted first from the BGB 172 column under these analytical conditions. EFs of the standard sample of fipronil used for spiking the fish food was racemic (0.50 ± 0.01) (n = 4) as was the fipronil in the food residues (Table 1).

Data analysis

Growth rates were determined by fitting all fish weight data to an exponential model (ln fish weight = a + bt ; where a is a constant, b is the growth rate, and t is time in days). As growth dilution can cause differences in concentration between individual fish, all concentrations were corrected for growth by multiplying the fish concentrations by a factor of (1 + bt), where b is the growth rate. Depuration rate (k_d) constants were determined by fitting the depuration data to a first order decay curve (ln concentration = a + k_dt ; where a is a constant and t is time in days). Half-life ($t_{1/2}$) values were calculated using ln 2/k_d. Equilibrium biomagnification factors (BMF_{eq}) were predicted from the equation

$$BMF = C_{fish}/C_{food}$$

where C_{fish} is the average fipronil concentration at steady state in the fish and C_{food} is the average concentration in the food, normalized to lipid content. Steady state was assumed when a significant increase in fish concentrations was not observed over three consecutive time intervals and did not increase thereafter. Because we did not use a replicate for each treatment (only one aquarium), pseudoreplication was addressed by using average concentration values of the three replicate fish taken at each time to determine all rate constants. Statistical differences for tested parameters between treatments were analyzed using the t-test (α = 0.05).

RESULTS AND DISCUSSION

Fish Health and Growth

Exposure to fipronil did not appear to influence the health of rainbow trout. There was no mortality, and coloration and behavior of the fipronil exposed fish were consistent with control fish. The growth rate of fipronil exposed fish was 0.015 d^{-1} (Figure 1) and was similar to that of control fish, 0.017 d^{-1}.

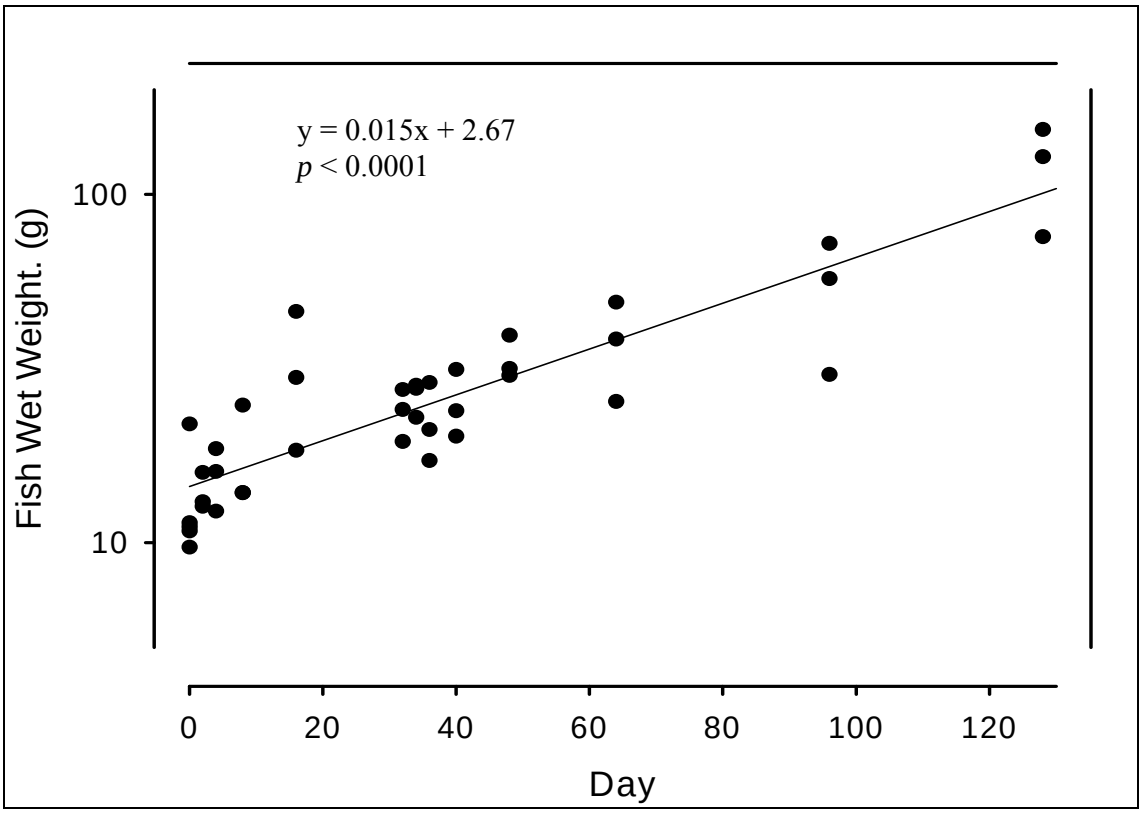


Figure 1. Growth rate (0.015 d^{-1}) of fipronil exposed fish, weight is natural log; $r^2 = 0.78$,

Bioaccumulation parameters

Figure 2 shows the uptake and elimination of fipronil over time. After two days, fipronil was detected in fish exposed to the spiked food and accumulated rapidly during the uptake phase at concentrations ranging from 1.6 to 3.7 µg/g lipid in carcass tissue, reaching steady state starting on day eight. Fipronil was rapidly eliminated from fish and was not detected after day 34 of depuration. The calculated depuration half-life of fipronil in fish was 0.58 d indicating the rapid clearance of this compound. The low equilibrium BMF of 0.05 indicated that fipronil will not biomagnify in the food chain at higher trophic levels, due again to its rapid clearance. There are no similar studies with which to compare our bioaccumulation values for fipronil in fish; however, invertebrates either fed or exposed in aqueous solution to fipronil indicated a high accumulation potential (Chaton *et al*. 2002, Chaton *et al*. 2001). Similar studies investigating the toxicokinetics of fipronil at lower trophic levels

would determine whether this compound is transferable to higher trophic species such as rainbow trout. The dietary exposure regimen used in this study involved a high exposure concentration to rainbow trout with no observable effects, yet concentrations obtained in fish carcass tissue reached a level 100 fold above the LC50 of 246 µg/L determined for this species (US EPA 1996). This resistance in toxicity may be explained by fipronil not efficiently reaching the target site (i.e., GABA receptor) through dietary exposure because of sequestration in fat reserves, where fipronil is likely to accumulate. This sequestration combined with a high-rate of detoxification by metabolism for rainbow trout may lead to a tolerance for elevated fipronil concentrations. It should be noted that fipronil was not detected in control fish carcass throughout the study, as a result of not being detected in control food.

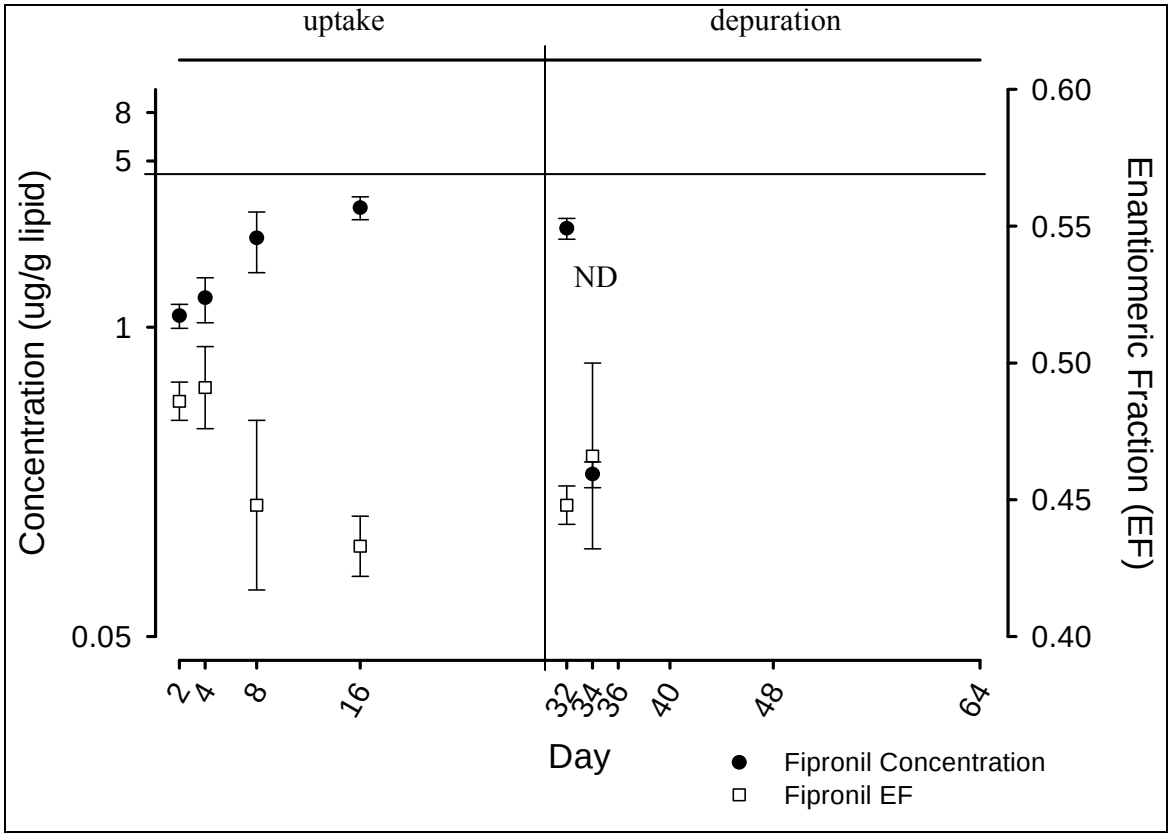


Figure 2. Dietary accumulation and depuration curve for fipronil in rainbow trout. Fipronil was not detected (ND) after day 36 with a depuration half-life of 0.58 d. Each circle represents the mean concentration ± SE of three fish. Each square represents the mean EF ± SE of three fish.

Enantiomer Fractions

Calculated enantiomeric fractions (EFs) showed that relative abundance of fipronil enantiomers changed quickly over time in this study (Figure 2). After two days, the (-) enantiomer is more prominent indicating a greater enantioselective biotransformation rate of the (+) enantiomer. This EF trend continued throughout the uptake phase of the experiment before a possible slight increase in EF during the depuration phase. The non-racemic residues found here are a strong indication of metabolism over time and can serve as a tracer of exposure time. For example, if native fish were collected and their EFs were determined, this value could give an indication of when initial exposure to fipronil had occurred and likewise how long before complete elimination of this compound was likely.

The changes in EFs are most likely due to biotransformation by the rainbow trout. Enantioselective uptake of fipronil is unlikely because the transfer from GI tract into the body through mixed micelle vesicles is a passive transport process for hydrophobic compounds and is not considered to be enantioselective (Drouillard and Norstrom 2000, Landoni *et al*. 1997). Likewise, elimination of fipronil, such as excretion through feces, is considered a passive process and not expected to be stereospecific (Thomann 1989). Similarly, elimination through the gills is not enantioselective, involving passive partitioning between the gill surface and water (Thomann 1989). The observed EFs suggest that enantioselective metabolism started early during the uptake phase of the experiment and proceeded in such a way that residues were non-racemic until complete biotransformation of fipronil.

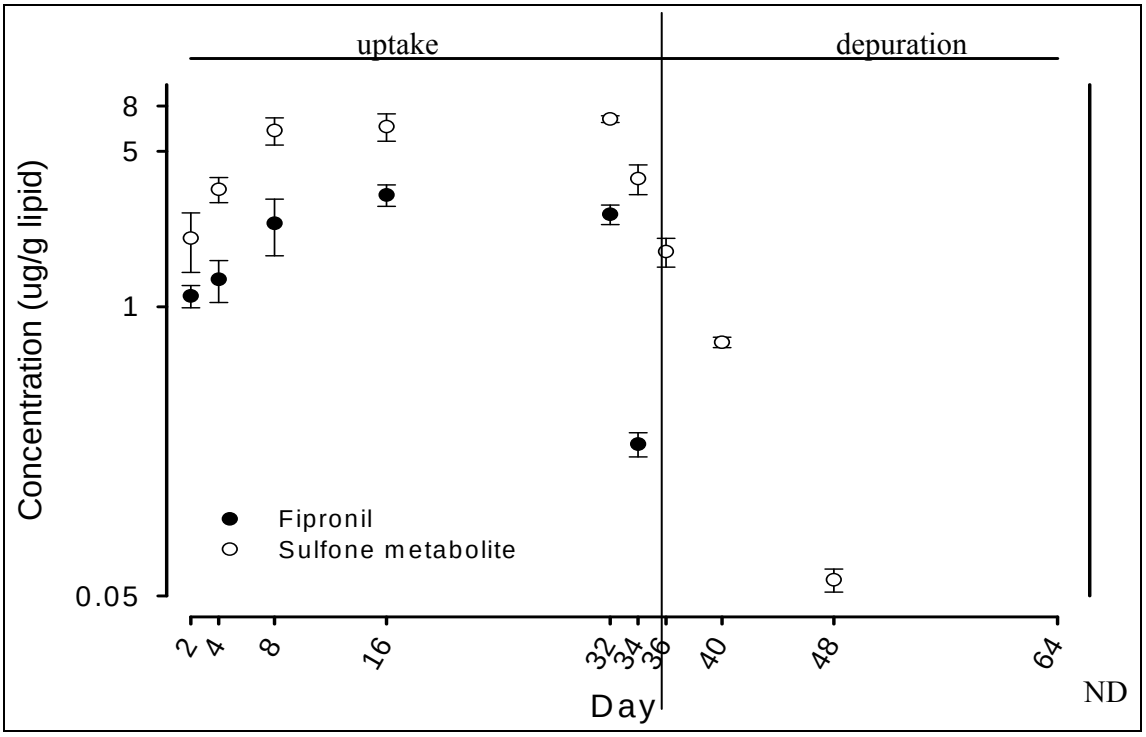


Figure 3. Concentrations of fipronil and fipronil sulfone, a metabolite of fipronil, in rainbow trout. Fipronil sulfone was not detected (ND) after day 64 with a depuration half-life of 2.36 d. Each data point represents the mean concentration ± SE of three fish.

The detection of fipronil metabolites in exposed fish would confirm that biotransformation was occurring. Analysis for its known metabolite from rat studies, the oxidation product fipronil sulfone, indicated the presence of this compound within fish carcass tissue (Figure 3). It was detected concurrently with the parent compound starting on day two, and at higher concentrations than fipronil throughout the study. Because both compounds were detected in fish throughout the study, it is important to note that any effects, most likely at the neuro-cellular level, may be enhanced by both compounds having the same mode of action. From this data, we were able to establish a depuration half-life of 2.36 days for fipronil sulfone. To our knowledge, no further biotransformation pathways have been identified for fipronil sulfone; apparently this compound was excreted by the fish as is. It should be noted that very low concentrations of fipronil sulfone were detected in the food exposed to

rainbow trout, a result of its presence in the standard used to spike the food. However, concentrations of fipronil sulfone detected in food were approximately 40 times lower than fipronil. The BMF of 4.8 for fipronil sulfone calculated from our fish food and fish tissue concentrations is unlikely for this low log K_{ow} compound (3.68) (Walse *et al*. 2004) that is similar in persistence to fipronil, which has a calculated BMF of 0.05. A BMF of this magnitude means fipronil sulfone would biomagnify to the extent of some of the higher chlorinated PCBs, which is clearly not the case due to its rapid elimination. Therefore, the high concentrations of fipronil sulfone found in our exposed fish are the direct result of biotransformation of fipronil and do not come from the spiked food. This study is the first to our knowledge to show the formation of fipronil sulfone by fish with direct implications for using this compound as a tracer of exposure to fipronil.

CONCLUSIONS

This study shows that the use of chiral compounds can provide insight into biotransformation processes. Through measurement of EFs, we were able to demonstrate the biological metabolism of fipronil by fish, which could not have been observed with traditional achiral analysis. Formation and detection of fipronil sulfone, a metabolite of fipronil, in the fish was further proof of metabolic transformation. Measurements of the EF of chiral compounds may indicate time of initial exposure, an important component in risk assessment of these chemicals.

DISCLAIMER

This paper has been reviewed in accordance with the U.S. Environmental Protection Agency's peer and administrative review policies and approved for presentation and publication. Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

REFERENCES

Borga, K., A.T. Fisk, P.F. Hoekstra, and D.C.G. Muir. 2004. Biological and chemical factors of importance in the bioaccumulation and trophic transfer of persistent organochlorine contaminants in arctic marine food webs. Environmental Toxicology and Chemistry 23: 2367-2385.

Chaton, P.F., P. Ravanel, M. Tissut, and J.C. Meyran. 2002. Toxicity and bioaccumulation of fipronil in the nontarget arthropodan fauna associated with subalpine mosquito breeding sites. Ecotoxicology and Environmental Safety 52: 8-12.

Chaton, P.F., P. Ravanel, J.C. Meyran, and M. Tissut. 2001. The toxicological effects and bioaccumulation of fipronil in larvae of the mosquito *Aedes aegypti* in aqueous medium. Pesticide Biochemistry and Physiology 69: 183-188.

Drouillard, K.G., and R.J. Norstrom. 2000. Dietary absorption efficiencies and toxicokinetics of polychlorinated biphenyls in ring doves following exposure to arochlor® mixtures. Environmental Toxicology and Chemistry 19: 2707–2714.

Fisk, A.T., J. Moisey, K.A. Hobson, N.J. Karnovsky, and R.J. Norstrom. 2001. Chlordane components and metabolites in seven species of Arctic seabirds from the northwater polynya: relationships with stable isotopes of nitrogen and enantiomeric fractions of chiral components. Environmental Pollution 113: 225-238.

Fisk, A.T., R.J. Norstrom, C.D. Cymbalisty, and D.C.G. Muir. 1998. Dietary accumulation and depuration of hydrophobic organochlorines: bioaccumulation parameters and their relationship with the octanol/water partition coefficient. Environmental Toxicology and Chemistry 17: 951-961.

Gant, D.B., A.E. Chalmers, M.A. Wolff, H.B. Hoffman, and D.F. Bushey. 1998. Fipronil: action at the GABA receptor. Reviews in Toxicology 2: 147-156.

Garrison, A.W., P. Schmitt, D. Martens, and A. Kettrup. 1996. Enantiomeric selectivity in the environmental degradation of dichlorprop as determined by high-performance capillary electrophoresis. Environmental Science and Technology 30: 2449-2455.

Hainzl, D., L.M. Cole, and J.E. Casida. 1998. Mechanisms for selective toxicity of fipronil insecticide and its sulfone metabolite and desulfinyl photoproduct. Chemical Resources Toxicology 11: 1529-1535.

Hoekstra, P.F., C.S. Wong, T.M. O'Hara, K.R. Solomon, S.A. Mabury, and D.C.G. Muir. 2002. Enantiomer-specific accumulation of PCB atropisomers in the bowhead whale (*Balaena mysticetus*). Environmental Science and Technology 36: 1419-1425.

Hosie, A.M., H.A. Baylis, S.D. Buckingham, and D.B. Sattelle. 1995. Actions of the insecticide fipronil on dieldrin sensitive and resistant GABA receptors of *Drosophila melanogaster*. British Journal of Pharmacology 115: 909-912.

Kaiser, K.L.E. 1974. On the optical activity of polychlorinated biphenyls. Environmental Pollution 7: 93-101.

Kleinow, K.M., M.J. Melancon, and J.J.Lech. 1987. Biotransformation and induction: implications for toxicity, bioaccumulation and monitoring of environmental xenobiotics in fish. Environmental Health Perspectives 71: 105-119.

Landoni, M.F., A.L. Soraci, P. Delatour, and P. Lees. 1997. Enantioselective behaviour of drugs used in domestic animals: a review. Journal of Veterinary Pharmacology and Therapeutics 20: 1-16.

Schlenk, D., D.B. Huggett, J. Allgood, E. Bennett, J. Rimoldi, A.B. Beeler, D. Block, A.W. Holder, R. Hovinga, and P. Bedient. 2001. Toxicity of fipronil and its degradation products to *Procambarus* sp.: Field and laboratory studies. Archives of Environmental Contamination and Toxicology 41: 325-332.

Stegeman, J.J., and P.J. Kloepper-Sams. 1987. Cytochrome P-450 isozymes and monooxygenase activity in aquatic animals. Environmental Health Perspectives 71: 87-95.

Thomann, R.V. 1989. Bioaccumulation model of organic chemical distribution in aquatic food chains. Environmental Science and Technology 23: 699-707.

U. S. Environmental Protection Agency. 2001. Fipronil environmental fate and ecological effects assessment and characterization for a Section 3 for broadcast treatment with granular product to control turf insects and fire ants (addendum). Environmental Fate and Effects Division, Office of Pesticide Programs, Washington, DC, USA.

U. S. Environmental Protection Agency. 1997. Environmental fate and effects Section 3 registration decision for fipronil: Use on rice seed, D235912. Environmental Fate and Effects Division, Office of Pesticide Programs, Washington, DC, USA.

U. S. Environmental Protection Agency. 1996. New pesticide fact sheet. EPA 737-F-96-005. Office of Pesticide Programs, Washington, DC, USA.

Walse, S.S., S.L. Morgan, L. Kong, and J.L. Ferry. 2004. Role of dissolved organic matter, nitrate, and bicarbonate in the photolysis of aqueous fipronil. Environmental Science and Technology 38: 3908-3915.

Walse, S.S., P.L. Pennington, G.I. Scott, and J.L. Ferry. 2004. The fate of fipronil in modular estuarine mesocosms. Journal of Environmental Monitoring 6: 58-64.

Williams, A. 1996. Opportunities for chiral agrochemicals. Pesticide Science 46: 3-9.

Wong, C.S., F. Lau, M. Clark, S.A. Mabury, and D.C.G. Muir. 2002. Rainbow trout (*Oncorhynchus mykiss*) ban eliminate chiral organochlorine compounds enantioselectively. Environmental Science and Technology 36: 1257-1262.