Response of blood parameters of the Antarctic fish *Notothenia coriiceps* (Richardson, 1844) to warming and hypoxia

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Introduction

There are at least 274 species of fish that are regarded as "endogenous Antarctic"; 95 of these are notothenioids, which constitute a major proportion of Southern Ocean fish biomass (Eastman 1993). All extant notothenioids are closely related and believed to have evolved from a benthic ancestor trapped beneath the ice cover over times of intense glaciation (Eastman 1993). Isolated for more than 25 million years as the Southern Ocean progressively cooled, Antarctic fish have evolved into a highly cold-adapted phenotype (Peck et al. 2005) including antifreeze proteins (Chen et al. 1997) and plasma osmolality, twice as high as that of temperate marine fish (Gonzalez-Cabrera et al. 1995). Moreover, these fish have apparently lost the inducible heat shock response (Hofmann et al. 2000).

Constant low temperatures and high oxygen concentration in Antarctic waters, have, moreover, led to special adaptations of the haematological characteristics of Antarctic fish, such as reduced haematocrits, low haemoglobin concentrations, and haemoglobin multiplicity with low haemoglobin oxygen affinity (Feller & Gerday 1997, Cocca et al. 1997). The decrease in haematocrit counteracts high blood viscosity at low temperature and, thereby, reduces cardiac work load. The notothenioid heart generates a large stroke volume, but only at low pressures and at very low vascular resistance (Davison et al. 1997).

An increase in the concentration of circulating catecholamines in order to maintain cardiovascular and respiratory function, and thus, adequate levels of oxygen in the blood, is the prime stress response of fishes. Interestingly, Antarctic teleosts lack this important stress response (Reid et al. 1998). Only under extreme heat stress on exposure to 10°C for 10 min, catecholamines release to the blood occurred in two notothenioid species, the pelagic *Pagothenia borchgrevincki* and the benthic *Trematomus bernacchii* (Forster et al. 1998). Also, handling stress did elevate heart rate and ventral aortic pressure in *T. bernacchii* (Davison et al. 1997). In *P. borchgrevincki* an extreme increase to over 110% in haematocrit was observed after exercise, which could be mainly attributed to the release of erythrocytes from the spleen (Franklin et al. 1993). Together, these observations suggest that Antarctic fish display the capacity to adapt their oxygen transport system to varying demands. Although environmental hypoxia is rarely occurring in pelagic Antarctic regions, Antarctic fish may suffer from functional oxygen limitation during exhaustive exercise and have obviously conserved a basic response to hypoxia.

To test this hypothesis, we studied the response of several blood parameters to different time periods of experimental warming to 5°C and to hypoxia (20% air saturation, representing critical PO_2 conditions in the Antarctic fish *Notothenia coriiceps*). Below this PO_2 the fish oxygen uptake decreases, and presumably anaerobic energy conservation starts and involves lactate production.

Material & Methods

Animal collection and maintenance

Notothenia coriiceps were fished with baited traps in 15 m water depth in Potter Cove, King George Island, Antarctic Peninsula (62° 14`S; 58° 40`W) in the Antarctic campaign November 2005 to March 2006. The animals were transported to the station and kept in an aquarium system with natural seawater, constant aeration and a natural day-light cycle at 0.7°C. Fishes were fed pieces of frozen fish for the first time five days after capture and afterwards every ten days. They were maintained at least for seven days (ten days in case of determination of critical PO_2) in the aquaria, before experimentation started. Mean fish length was 31.5 ± 3.0cm at a mean weight of 427 ± 129g.

Warm acclimation (5°C)

For the warm acclimation animals were directly transferred (two at a time) to a separated aquarium with natural seawater, thermostated to 5°C, and incubated for 12, 24, 48, 96 (= 4 days), 168 (= 7 days), 336 (= 14 days) hours. The water was kept fully aerated. For sampling, the fish were narcotized by a blow on the head, weighed and killed by cutting through the spine. Blood was immediately taken from the *vena cava* with heparinised syringes and stored on ice. Subsequently, various tissues were sampled removed and stored at -80°C. Sex and length of each fish were determined after sampling. Haematocrit values and the concentration of monomeric haemoglobin were measured directly after finalizing the sampling. Blood lactate content was measured in frozen (-80°C) and re-thawed blood samples.

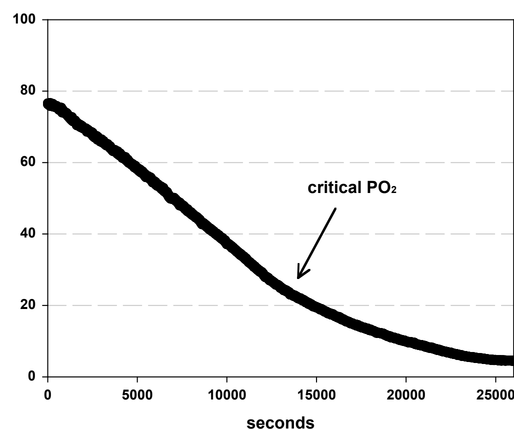

Figure 1
depicts a typical respiration measure (air saturation *versus* time) and demonstrates that at 20% air saturation *N. coriiceps* cannot longer keep constant the oxygen consumption with decreasing oxygen content, i.e. the critical PO_2.

Determination of critical PO_2

Prior to experimentation, the fishes were kept without food for ten days in order to eliminate impact of specific dynamic action (increase of respiration rate due to food digestion) on respiration rates. The critical PO_2 was determined in a pre-experiment, in which fishes were transferred to a respiration chamber in which the air saturation was recorded using oxygen microoptodes connected to a Microx TX 2-array (PreSens GmbH). The respiration chambers were Perspex cylinders of different volumes (9 to 10l), adjustable to the size of the animals. Water temperature was kept at 0.7°C. Microoptodes were calibrated to 100% and 0% air saturation at that

temperature. Fishes were allowed to accommodate to the chambers over night in the open system at 100% air saturation. For the actual measurement of the respiration rate of the fish, the system was closed and the decrease air saturation, respectively PO_2 in the chamber recorded. Fig. 1 depicts a typical respiration curve with a break at 20% air saturation, the PO_2 where *N. coriiceps* cannot longer keep its respiration rate constant.

Hypoxia exposure (20% air saturation)
For the hypoxia exposure, fish were transferred to a separate incubation basin with natural seawater, thermostatted to 0.8°C (= control temperature) and adjusted to 20% air saturation, corresponding to the critical PO_2. Air saturation was maintained constant by bubbling a gas mixture from a gas mixing pump (Wösthoff), into the aquarium. The fishes were incubated (three at a time) for 12, 24 or 48 hours before sampling was carried out as described above. Longer hypoxia incubations could not be carried out due to the limited amount of gas bottles that could be shipped to Antarctica.

Determination of blood parameters
Haematocrit values were determined in capillaries using a haematocrit centrifuge. The monomeric haemoglobin concentration was determined spectro-photometrically using the absorbances at 540 and 570 nm according to Antonini & Brunori (1972). Blood lactate levels were determined using an Accutrend® Lactate test (Roche Diagnostics, Mannheim, Germany) following the instruction manual.

Statistics
Differences between experimental groups were analysed by ANOVA and Bonferroni/Dunn PostHoc test, using StatView 5.0 with a p-level of 5%.

Results

No differences were found between male or female individuals, with respect to neither length nor weight or the hepatosomatic index so that data of both sexes were pooled. Further, neither body mass indices nor condition factors varied between different captures throughout the campaign, or with the time of maintenance in the aquaria.

Warm acclimation of Notothenia coriiceps
Upon acclimation of *N. coriiceps* to 5°C, blood lactate content increased significantly during the first 48 hours and returned to control level during the following 5 days. (fig. 2; p-values: 0.0269 / 12 h, 0.0011 / 24 h, 0.0004 / 48 h, 0.0028 / 4 days, 0.2517 / 7 days). After 14 days of warm acclimation blood lactate had increased again ($p = 0.0002$). By contrast, the haematocrit remained unchanged within the first 4 days of warm acclimation (p-values ≥ 0.2444) and was elevated after 7 days ($p < 0.0001$), when blood lactate was already back to control level (fig. 3). After 14 days, also the haematocrit was back to control level ($p = 0.5045$). Thus, blood lactate and haematocrit demonstrated an alternating response to warm acclimation. However, plotting lactate content against the haematocrit in different fish revealed no significant correlation between both parameters (StatView 5.0, R_ = 0.034, $p = 0.3176$). The concentration of monomeric haemoglobin showed the same pattern as the haematocrit values, however, with higher variation between individuals in several

experimental groups (fig. 4). There was no significant change of the ratio of haemoglobin / haematocrit (p-values ≥ 0.4306) indicating that the amount of haemoglobin per blood cell remained constant within two weeks of warm acclimation (fig. 5).

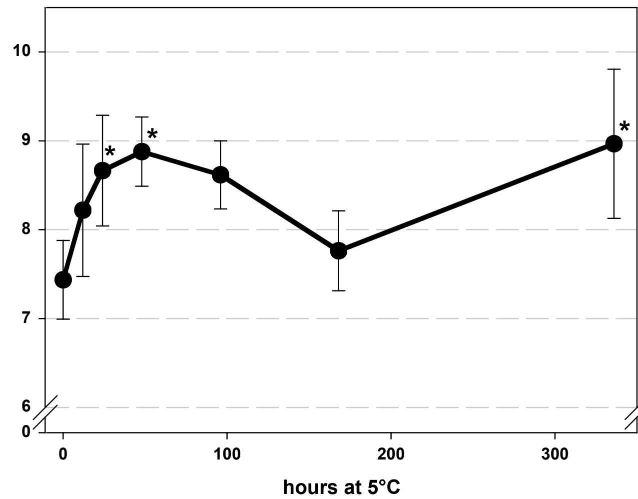

Figure 2
Depicts the blood lactate content of *N. coriiceps* upon two weeks of warm acclimation to 5°C. n = 4-8, p-level 5%, * indicates significant difference to unstressed controls (0 hours).

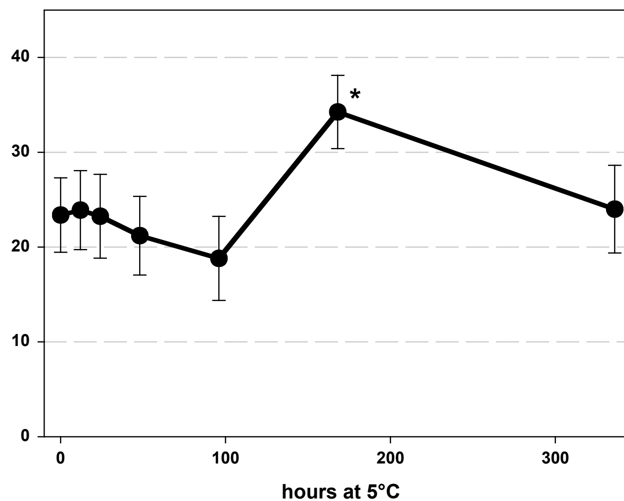

Figure 3
Depicts the haematocrit value of *N. coriiceps* upon two weeks of warm acclimation to 5°C. n = 4-8, p-level 5%, * indicates significant difference to unstressed controls (0 hours).

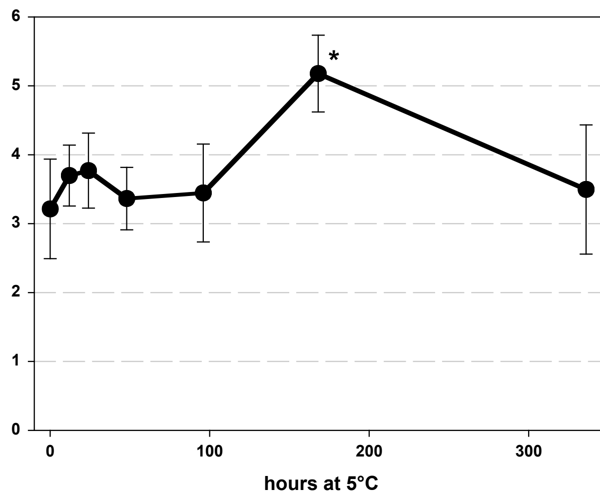

Figure 4
Depicts the content of monomeric haemoglobin of *N. coriiceps* upon two weeks of warm acclimation to 5°C. n = 4-8, p-level 5%, * indicates significant difference to unstressed controls (0 hours).

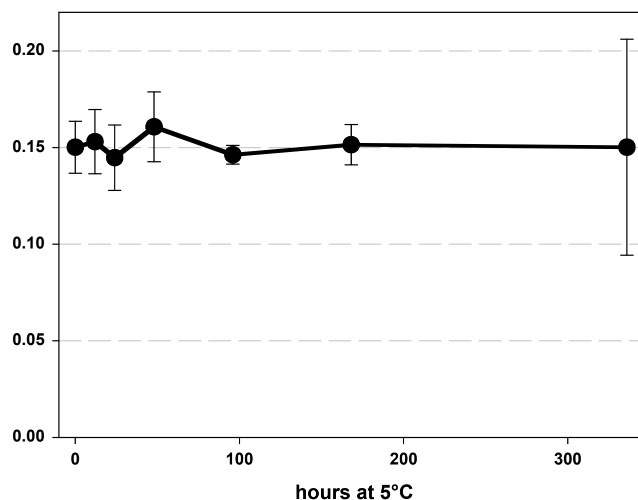

Figure 5
Depicts the ratio of haemoglobin / haematocrit, indicating the amount of respiratory pigment per red blood cell of *N. coriiceps* upon two weeks of warm acclimation to 5°C. n = 4-7, p-level 5%, * indicates significant difference to unstressed controls (0 hours).

Hypoxia exposure

Hypoxia exposure to critical PO_2 conditions (20 % air saturation) led to a significant increase in blood lactate within the first 12 hours. Subsequently, the blood lactate content started to decrease steadily, without reaching control level again within 48 hours (fig. 6; $p < 0.0001$ / 12 h, < 0.0001 / 24 h, 0.0059 / 48 h). The haematocrit increased significantly during the first 12 h of hypoxic exposure and remained elevated until the end of the experiment after 48 h hypoxia (fig. 7; $p \leq 0.0002$). The haemoglobin content followed the same pattern as the haematocrit, however the changes did not reach significance ($p \geq 0.0194$). The ratio of haemoglobin / haematocrit was lower in all hypoxia exposed groups compared to control fish (fig. 9; $p \leq 0.0034$) indicating lower amount of haemoglobin per red blood cell in hypoxia exposed than in unstressed fish.

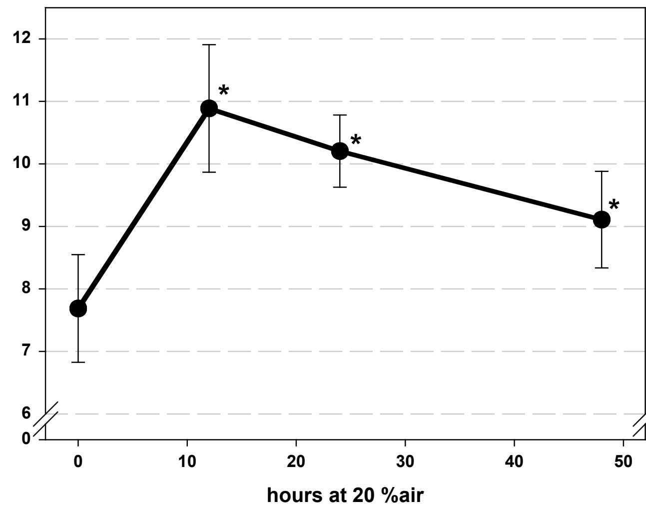

Figure 6
Depicts the blood lactate content of *N. coriiceps* upon 48 hours of hypoxia exposure to 20% air saturation (= critical PO_2 conditions). n = 3-8, p-level 5%, * indicates significant difference to unstressed controls (0 hours).

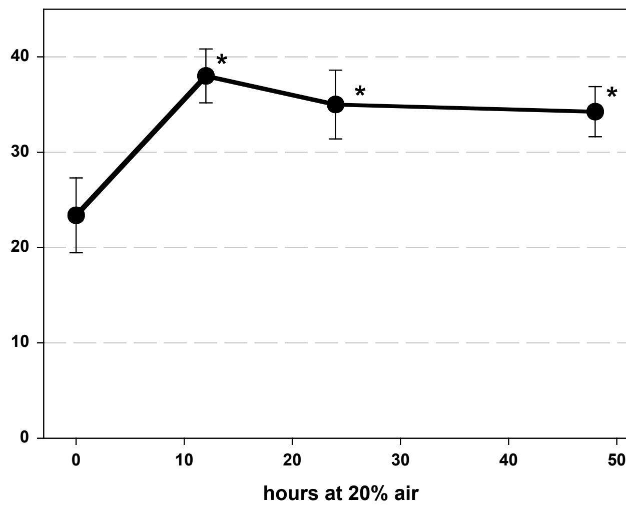

Figure 7
Depicts the haematocrit value of *N. coriiceps* upon 48 hours of hypoxia exposure to 20% air saturation (= critical PO_2 conditions). n = 3-8, p-level 5%, * indicates significant difference to unstressed controls (0 hours).

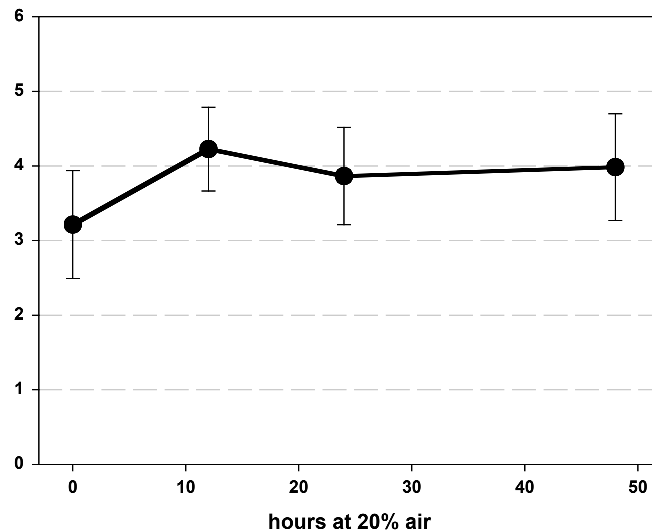

Figure 8
Depicts the content of monomeric haemoglobin of *N. coriiceps* upon 48 hours of hypoxia exposure to 20% air saturation (= critical PO_2 conditions). n = 3-8, p-level 5%, * indicates significant difference to unstressed controls (0 hours).

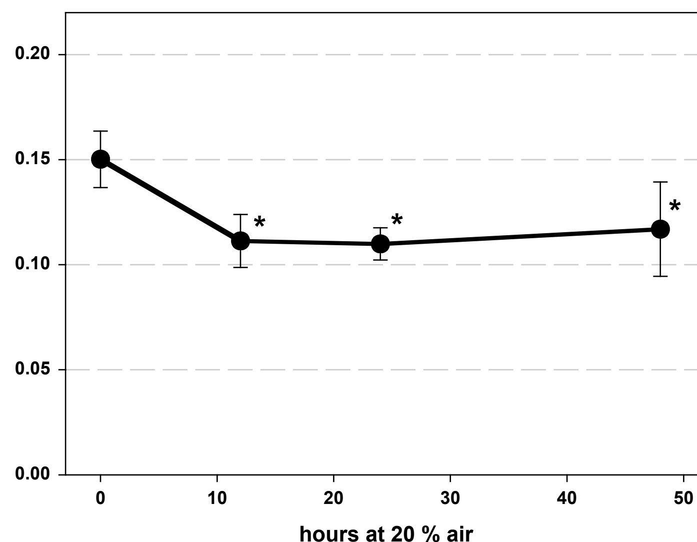

Figure 9
Depicts the ratio of haemoglobin / haematocrit, indicating the amount of respiratory pigment per red blood cell of *N. coriiceps* upon 48 hours of hypoxia exposure to 20% air saturation (= critical PO_2 conditions). n = 3-8, p-level 5%, * indicates significant difference to unstressed controls (0 hours).

Discussion

Not only hypoxia but also the initial phase of warm acclimation to temperatures outside the optimum temperature range is considered to cause oxygen shortage in central organs when the temperature-dependent increase in metabolic rate exceeds oxygen transport capacity (for review see Pörtner 2002). One of the characteristic responses of fishes to oxygen limitation is an increase in haematocrit value (Jensen et al. 1993), which can result from erythrocyte swelling, release of stored red cells, changes in plasma volume, plasma skimming, and new red cell formation

(Gallaugher & Farrell 1998, Nikinmaa 1990, Nikinmaa & Tervonen 2004). Additionally, anaerobic metabolism is induced by an upregulation of lactate dehydrogenase isoform expression (Gracey et al. 2001).
We were interested to see whether in spite of their special haematological adaptations and the absence of environmental hypoxia in their natural habitat, Antarctic red-blooded nototheniids have conserved the basic responses to oxygen limitation known of fish from lower latitudes. Whereas for many Antarctic fish species low haematocrit values between 10 and 18% have been reported (Mark et al. 2002), we found haematocrits of 23±4% in control fish, which is well within the range reported from non-polar fishes (18-38%, Petri et al. 2006, Ribeiro et al. 2005, Jain & Farrell 2003, Moraes et al. 2002, Madison & Wang 2006) and aligns with the comparatively high swimming activity of the investigated species. Wells et al. (1980) have also found higher haematocrit values and haemoglobin concentrations in more actively swimming Antarctic fish compared to slow moving species.
At first sight, 5°C appears as a quite high temperature for an Antarctic fish, especially when taking into account the reports by Weinstein & Somero (1998) that benthic notothenioids survive only few weeks at 4°C. However, more recently, Lowe et al. (2005) have shown an unexpected capacity to warm acclimate in the more active pelagic *Pagothenia borchgrevincki* and Lannig et al. (2005) maintained Antarctic eelpout *Pachycara brachycephalum* at 5°C for as long as nine months without apparent loss of condition factors. We were therefore not too surprised to see that a coastal Antarctic fish like *N. coriiceps* acclimates at 5°C. Especially, as in the Antarctic summer season 2005/2006 water temperatures as high as 2.5°C were reached in 10 m depth in Potter Cove (see also Schloss et al this volume).
The up-regulation of anaerobic lactate formation in the initial 48 hrs of experimental warming can be viewed as a first aid response to bridge energetic deficits (see Hochachka & Lutz 2001), until, following 7 days of warm acclimation, the haematocrit and also the haemoglobin content were elevated, such that higher oxygen transport capacity was reached in the blood. It remains to be shown whether this is due to release of pre-formed blood cells from the spleen or to new synthesis of red blood cells. In contrast to experimental warm acclimation, under hypoxia the blood lactate content increased on much shorter time scale. This indicates that oxygen shortage was more dramatic when exposing the fish to 20% air saturation than to acute warming stress. Also, the decline in blood lactate content after the initial increase during hypoxia occurred much faster than during warm acclimation, which can be explained by the simultaneous rapid increase of the haematocrit within the first 12 hours of hypoxia. Taken that the response in haematocrit was delayed by 7 days during acute warming stress compared to the fast response under hypoxia, the release of pre-formed blood cells or erythrocyte swelling appear more likely than erythrocyte new synthesis. This shows that exposure to 20% hypoxia represents a much more severe stress for these fish than acclimation to 5°C.

Taken together, the different time scale of change upon hypoxia exposure and warm acclimation in blood parameters may relate to different defence mechanisms: A fast one to ensure survival during critical oxygen shortage in the environmental, but also on exhaustive exercise, and a slower response possibly requiring gene expression and new synthesis of red blood cells, occurring upon warm acclimation. However, as the hypoxia experiment lasted only 48 hours (because of limited amounts of gas bottles; see material & methods) it remains unknown whether this treatment would induce gene expression as well upon longer time scales of several days.

Our future goal is to investigate the molecular mechanisms behind the observed differences in the haematological responses to warm acclimation and to hypoxia exposure.

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References

Brunoni M., Antonini E. 1972, Kinetics of the reaction with oxygen of mixtures of oxy- and carbon monoxide haemoglobin. *J. Biol. Chem*. 247, 4305-4308.

Chen L., DeVries A.L., Cheng C.-H.C. 1997. Convergent evolution of antifreeze glycopeptides in Antarctic notothenioid fish and Arctic cod. *Proc. Natl. Acad. Sci. USA* 84, 3817-3822.

Cocca E., Ratnayake-Lecamwasam M., Parker S.K., Camadella L., Ciaramella M., di Prisco G., Detrich H.W. 1997. Do the hemoglobinless icefishes have globin genes? *Comp. Biochem. Physiol*. 188A, 1027-1030.

Davison W., Axelsson M., Nilsson S., Forster M.E. 1997. Cardiovascular control in Antarctic notothenioid fishes. *Comp. Biochem. Physiol*. 118A, 1001-1008.

Eastman J.T. 1993. Antarctic fish biology. Evolution in a unique environment. Academic Press Inc. San Diego, USA.

Feller G. Gerday C. 1997. Adaptations of the hemoglobinless Antarctic icefish (Channichthyidae) to hypoxia tolerance. *Comp. Biochem. Physiol.* 118A, 981-987.

Forster M.E., Davison W., Axelsson M., Sundin L., Franklin C.E., Gieseg S. 1998. Catecholamine release in heat-stressed Antarctic fish causes proton extrusion by the red cells. *J. Comp. Physiol.* 168B, 345-352.

Franklin C.E., Davison W., McKenzie J.C. 1993. The role of the spleen during exercise in the Antarctic teleost, *Pagothenia borchgrevinki*. *J. Exp. Biol*. 164, 381-386.

Gallaugher P., Farell A.P. 1998. Hematocrit and blood oxygen-carrying capacity. In: Fish respinration, edited by Perry SF and Tufts BL. San Diego: Academic Press, 185-227.

Gonzalez-Cabrera P.J., Dowd F., Pedibhotla V.K., Rosario R., Stanley-Samuelson D., Petzel D. 1995. Enhanced hypo-osmoregulation induced by warm-acclimation in Antarctic fish is mediated by increased gill and kidney Na^+/K^+-ATPase activities. *J. Exp. Biol.* 198, 2279-2291.

Gracey A.Y., Troll J.V., Somero G.N. 2001. Hypoxia-induced gene expression profiling in the euryoxic fish *Gillichthys mirabilis*. *Proc. Natl. Acad. Sci. USA* 98, 1993-1998.

Hochachka P.W., Lutz P.L. 2001. Mechanism, origin, and evolution of anoxia tolerance in animals. *Comp. Biochem. Physiol. B, Biochem. Mol. Biol*. 130, 435-459.

Hofmann G.E., Buckley B.A., Airaksinen S., Keen, J.E., Somero G.N. 2000. Heat-shock protein expression is absent in the Antarctic fish *Trematomus bernacchii* (family nototheniidae). *J. Exp. Biol*. 203, 2331-2339.

Jain K.E., Farrell A.P. 2003. Influence of seasonal temperature on the repeat swimming performance of rainbow trout Oncorhynchus mykiss. *J. Exp. Biol*. 206, 3569-3579.

Lannig G., Storch D., Pörtner H.-O. 2005. Aerobic mitochondrial capacities in Antarctic and temperate eelpout (Zoarcidae) subjected to warm versus cold acclimation. *Polar Biol*., in press.

Lowe C.J., Seebacher, F., Davison W. 2005. Thermal sensitivity of heart rate and insensitivity of blood pressure in the Antarctic nototheniid fish *Pagothenia borchgrevinki*. *J. Comp. Physiol.* 175B, 97-105.

Madison B.N., Wang Y.S. 2006. Haematological responses of acute nitrite exposure in walleye (*Sander vitreus*). *Aquat. Tox*. 79, 16-23.

Mark F.C., Bock C., Pörtner, H.O. 2002. Oxygen-limited thermal tolerance in Antarctic fish investigated by MRI and ^{31}P-MRS. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 283, R1254-R1262.

Moraes G., Avilez I.M., Altran A.E., Barbosa C.C. 2002. Biochemical and hematological response of the banded knife fish Gymnotus carapo (Linnaeus, 1758) exposed to environmental hypoxia. *Braz. J. Biol*. 62, 633-640.

Nikinmaa M. 1990. Vertebrate red blood cells. Heidelberg: Springer.

Nikinmma M., Tervonen V. 2004. Regulation of blood harmoglobin concentration in hypoxic fish: Proceedings of the 7th International Symposium on Fish Physiology, Toxicology, and Water Quality, edited by Rupp GL and Whte MD, U.S. Environmental Protection Agency, Ecosystems Research Division: Athens, Ga, 243-252.

Peck L.S., Clark M.S., Clarke A., Cockell C.S., Convey P., Detrich H.W., Fraser K.P.P., Johnston I.A., Methe B.A., Murray A.E., Römisch K., Rogers A.D. 2005. Genomics: application to Antarctic ecosystems. *Polar Biol*. 28, 351-365.

Petri D., Glover C.N., Ylving S., Kolas K., Fremmersvik G., Waagbo R., Berntssen M.H.G. 2006. Sensitivity of Atlantic salmon (*Salmo salar*) todietary endosulfan as assessed by haematology, blood biochemistry, and growth parameters. *Aquat. Tox*. 80, 207-216.

Pörtner H.O. 2002. Climate variations and the physiological basis of temperature dependent biogeography: systemic to molecular hierarchy of thermal tolerance in animals. *Comp. Biochem. Physiol.* 132A, 739-761.

Reid S.G., Bernier N.J., Perry S.F. 1998. The adrenergic stress response in fish: control of catecholamine storage and release. *Comp. Biochem. Physiol.* 120C, 1-27.

Ribeiro C.A.O., Neto F.F., Mela M., Silva P.H., Randi M.A.F., Rabitto I.S., Costa J.R.M.A., Pelletier E. 2006. Hematological findings in neotropical fish *Hoplias malabaricus* exposed to subchronic and dietary doses of methylmercury, inorganic lead, and tributyltin chloride. *Environmental Res*. 101, 74-80.

Weinstein, R.B., Somero, G.N. 1998. Effects of temperature on mitochondrial function in the Antarctic fish *Trematomus bernacchii*. *J. Comp. Physiol*. 168B, 190-196.

Wells R.M.G., Ashby M.D., Duncan S.J., Macdonald J.A. 1980. Comparative study of the erythrocytes and haemoglobins in nototheniid fishes from Antarctica. *J. Fish Biol.* 17, 517-527.