The Role of Noradrenaline on the Lipid Metabolism of Water- and Air-Breathing Fish Species.

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Free Fatty Acid Metabolism in the Air-breathing African Catfish (*Clarias gariepinus*) during Asphyxia.

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Accepted for publication by *Comparative Biochemistry Physiology 141A: 15-21 (2005).*

Abstract

In several water-breathing fish species, hypoxia induces a decrease in plasma free fatty acid (FFA) levels in contrast to the increase induced by hypoxia in air-breathing mammals. We hypothesised that this change is coupled to the mode of breathing. Therefore, we followed the metabolic response of cannulated air-breathing African catfish to an 8-h asphyxia period. The hematocrit and hemoglobin increased significantly upon asphyxia. No change, however, was observed in the mean cellular hemoglobin concentration, thus indicating that more erythrocytes were brought into circulation. A continuous increase in plasma lactate concentration during asphyxia showed permanent activation of anaerobic glycolysis, pointing to a persistent oxygen shortage. Plasma glucose levels did not change, but FFA levels decreased significantly upon asphyxia with a concomitant increase in plasma noradrenaline levels. Thus, these results suggest that, in the air-breathing African catfish, noradrenaline has a suppressive effect on plasma FFA levels similar to that in water-breathing fish species.

Introduction

In mammals, the hypoxia-induced release of catecholamines strongly stimulates lipolysis (Fain and Garcia-Sainz, 1983), while the oxidative breakdown of free fatty acids (FFA) is impaired during hypoxia (Moore, 1985). Both processes enhance each other, resulting in elevated plasma FFA levels (Roberts *et al*., 1996). Too high FFA levels can cause disruption of cell membranes resulting in cell leakage and tissue damage, as seen in the ischemic heart (Katz and Messineo, 1981). In mammals, however, hypoxia normally does not occur at the organismal level, as opposed to many fish species for which hypoxia is a natural occurring phenomenon (Van den Thillart, 1982). Plasma FFA levels are not elevated but rather depressed in hypoxic carp (*Cyprinus carpio*, Van Raaij *et al*., 1996a) and tilapia (*Oreochromis mossambicus*, Vianen *et al*., 2002). Vianen *et al*. (2002) clearly showed that a surge in plasma noradrenaline mediated a strong reduction in adipose lipolysis and hence a depression of plasma FFA levels. This suppression of plasma FFA levels by noradrenaline is believed to be a protective mechanism against fatty acid poisoning in fish under hypoxia (Van den Thillart *et al*., 2002).

Under hypoxia anaerobic pathways are activated (Van den Thillart and Van Waarde, 1985); while glucose can be broken down anaerobically to lactate, the breakdown of fatty acids via the β-oxidation is impaired when there is no oxygen. Therefore, in hypoxic conditions there is generally a strong increase in plasma lactate levels. Hypoxia implies a low oxygen tension, but the effect of hypoxia on metabolism, however, depends on the PO_2 as well as on the condition of the animal (Van den Thillart and Van Waarde, 1985). A more useful criterion is the critical oxygen level at which the lactate response shows a switch from a transient to permanent accumulation (Vianen *et al*., 2001). At that oxygen level, the animal will not be able to recover from the hypoxic challenge.

Many fish species have developed air-breathing, originally thought of as a way to counteract the negative effects of low aquatic oxygen tensions (Graham, 1997). Remarkably however, hypoxic energy metabolism has never been studied with air-breathing fish species. Several studies showed that in air-breathing fishes low aquatic oxygen tensions induce an increase in air-breathing (e.g. Babiker, 1979; Johnston *et al*., 1983; Kind *et al*., 2002). When exposed to hypoxic water, the plasma lactate levels of the Australian lungfish (*Neoceratodus forsteri*) remained low (<1 mM; Kind *et al*., 2002). Hence, it can be assumed that the oxygen uptake from the air by these fishes is sufficient to sustain complete aerobic metabolism even at low aquatic oxygen tensions. This implies that these fishes were metabolically not in a hypoxic condition. Recently,

Perry and co-workers (2004) published a study on the adrenergic response to environmental hypoxia of an air-breathing fish species, the jeju (*Hoplerythrinus unitaeniatus*). When the jeju was allowed air-breathing, lowering the aquatic oxygen content did not mediate an increase in plasma catecholamines. However, when the fish could not breathe air, there was a very marked increase in plasma catecholamines from around 3.3 nM at normoxia to around 60 nM when the aquatic oxygen tension was 10-20 mm Hg. The dominant catecholamine in hypoxic jeju was adrenaline, although the specific levels of both catecholamines were not specified. From these data, they concluded that the key variable for catecholamine secretion is the blood oxygen content. Hence in air-breathing fish, the denial of access to air induces an adrenergic stress response comparable to water-breathing fish species. Unfortunately, this study did not include any measurement of plasma metabolites.

Species from the family of Clariidae are known for their air-breathing capabilities (Graham, 1997). African catfish (*Clarias gariepinus*) are often classified as facultative air-breathers, meaning that they can live indefinitely without aerial oxygen (Magid, 1971; Babiker, 1979). They are, however, also described as obligate air-breathers, implying vital dependence on aerial oxygen (Moussa, 1957; Johnston *et al*., 1983). In the latter two studies, the fish survived asphyxia, depending on the size of the fish, for 11 to over 24 h. Despite these opposing statements in the literature, aerial oxygen without a doubt represents an important source of oxygen for African catfish. Thus, we hypothesised that without access to aerial oxygen African catfish becomes hypoxic.

Our primary objective was to study the hypoxic metabolism of the air-breathing African catfish. Van den Thillart *et al*. (2002) hypothesised that in air-breathing fishes the suppressive role of noradrenaline is redundant because hypoxia is no longer a realistic threat. Hence, we assumed that a noradrenaline-mediated decrease in plasma FFA levels is absent in hypoxic African catfish.

Materials and Methods

Experimental animals

African catfish (*Clarias gariepinus*, Burchell 1822) of an average weight of 1.14 ± 0.10 kg were obtained from a commercial catfish farm (Fleuren, Someren, The Netherlands). The fish were kept in groups in a well-aerated recirculation system (25ºC) with a light-dark cycle of

12L:12D. They were fed once a day with Trouvit Biomeerval (Trouvit, Putten, The Netherlands) at maintenance level (7 gr/kg BW). The fish were acclimatised to these conditions for at least 2 weeks. The experiments were approved by the board on Experimentation on Animals of the Leiden University.

Pre-experimental protocol

The experiments were conducted in flow chambers supplied with well-aerated water of 25ºC (see Vianen *et al*., 2001). The flow rate through the flow chambers was approximately 1 l/min. The fish could move back and forth freely without the possibility to turn around. The flow chambers were closed with a darkened lid to prevent startling of the fish by outside movements. The flow chambers contained approximately 2 cm of air above the water to allow air-breathing.

Before the start of an experiment, the fish were placed individually in the flow chambers and were deprived of food. After 3 days of acclimatisation, the fish were anaesthetised in a MS222 solution (300 mg/ml, tricaine methanesulphonate, Argent Chem. Lab., Redmond, U.S.A.) and cannulated in the dorsal aorta according to Soivio *et al*. (1975). To prevent blood clogging, the cannulae were filled at all times with a Ringer saline solution (Wolf, 1963) containing 1% (w/v) of the anticoagulant sodium citrate. After cannulation, the fish were placed back into the flow chambers and allowed to recover for two days during which the cannula was flushed frequently. This 5 day pre-experimental protocol has been shown to minimise effects of handling, anaesthesia and surgery (Van Raaij *et al*., 1996a).

Experimental protocol

The experiment started by taking 2 initial blood samples at t= -½ and 0 h. After the second sample, one group of fish, referred to as the asphyxia group (*n*= 7), was denied access to air. This was accomplished by increasing the water level within a few seconds, which drove the air out of the flow chamber. The control group, referred to as the normoxia group (*n*= 5), had access to air during the whole experiment. After t= 0 h, blood samples were taken at t=1, 2, 3, 4, 6 and 8 h.

Blood samples of 450 µl were drawn with gastight microliter syringes containing 50 µl of a 4% (w/v) sodium citrate-saline solution as anticoagulant. Thereafter, the volume of extracted blood was replaced with Ringer saline. Finally, the cannula was refilled with a 1% sodium citrate-saline solution.

Analytical procedures

On whole blood, the hematocrit (2x9 µl) and hemoglobin content (2x10 µl) were determined. The hematocrit was measured by filling heparinized capillaries and centrifugation in a mini-centrifuge (Compur M1100). The hemoglobin concentration was measured using a hemoglobin test kit (Roche, Almere, The Netherlands). Subsequently, the blood was centrifuged for 5 min at 15,000 g and plasma was separated immediately. Aliquots of 50 µl of untreated plasma were stored at –80ºC for FFA measurement. Aliquots of 100 µl of plasma were added to 15 µl of EDTA as anti-oxidant (59 mM) and stored at –80ºC for catecholamine measurement. For glucose and lactate measurements, 100 µl of plasma was added to 400 µl of 6% trichloric acetic acid, mixed and put on ice for at least 20 min to precipitate plasma proteins. After centrifugation at 15,000 g, two aliquots of the supernatant were stored at –20ºC and analysed within a week.

Plasma lactate was measured according to the method of Hohorst (Bergmeyer, 1970) and plasma glucose was measured using an enzymatic test kit (Instruchemie, Delfzijl, The Netherlands). Plasma FFA was measured using an enzymatic test kit from Waco chemicals (Instruchemie, Delfzijl, The Netherlands). The plasma catecholamine concentration was measured according to the HPLC-method of Mitsui *et al*. (1985); the detection limit was 0.1 nmol/l.

Data analyses and statistics

Data are presented as means ± SEM. The mean cellular hemoglobin content (MCHC) was calculated as hemoglobin concentration divided by the hematocrit. Between both groups, there were no significant differences in initial values except in plasma FFA concentration. Therefore, the values of all fish on t= -½ and 0 h were pooled except for plasma FFA values. Statistical differences ($p<0.05$) were tested using Sigmaplot 2.03. Differences with the initial values were tested with a Repeated Measures Anova on Ranks according to Dunnett's method, while differences between the two groups were tested with a Wilcoxon signed rank test.

Results

The initial value for hematocrit was 23.3 ± 0.5 %, for hemoglobin 4.75 ± 0.14 mM and for MCHC 20.65 ± 0.57 mM; all three hematological parameters did not change significantly in the

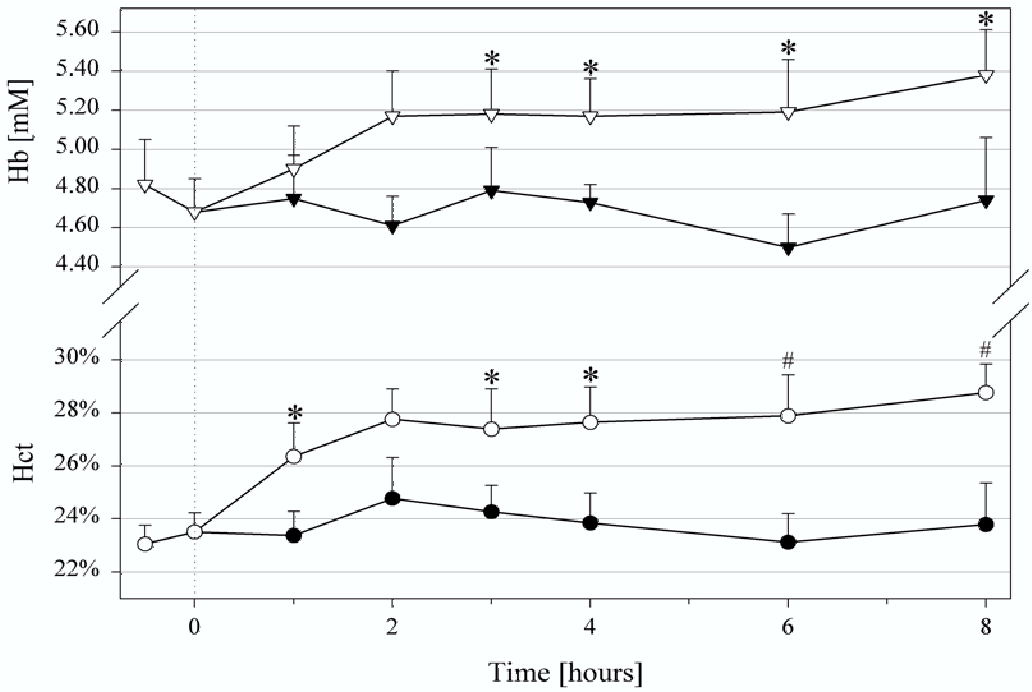

Figure 1. Hematocrit (Hct; circles) and hemoglobin concentration (triangles) in cannulated African catfish during asphyxia (○, ▽; *n*= 8) or normoxia (●, ▼; *n*= 6); the dotted line indicates the start of asphyxia. *: p<0.05 vs. normoxia group; #: p<0.05 vs. normoxia group and vs. initial values.

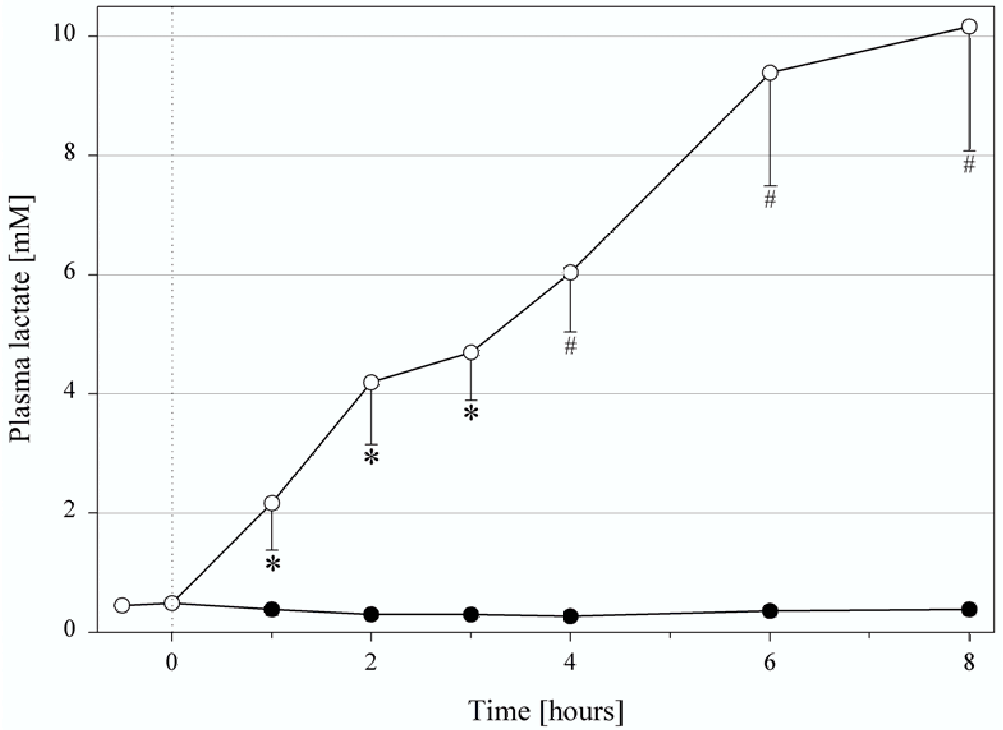

Figure 2. Plasma lactate concentration in cannulated African catfish during asphyxia (○; *n*= 8) or normoxia (●; *n*= 6); the dotted line indicates the start of asphyxia. *: P <0.05 vs. normoxia group; #: p<0.05 vs. normoxia group and vs. initial values.

normoxia group. In the asphyxia group, the hematocrit was significantly higher from the normoxia group on t= 1, 3 and 4 h and also different from the initial value at t=6 and 8 h (Fig. 1). In the asphyxia group, the hemoglobin content was significantly different from the normoxia group from t= 3 to 8 h. In both parameters, a plateau was reached after 2 h. The MCHC levels (not shown) did not differ significantly between both groups on all time points.

The plasma lactate concentration in the normoxia group stayed at low levels (maximum of 0.67 mM) throughout the experimental period and did not change significantly. The plasma lactate concentration in the asphyxia group increased dramatically from initial values of 0.47 ± 0.05 mM to 10.2 ± 2.1 mM after 8 h (Fig. 2). Two individuals died before the end of the experimental period, after t= 6 h and after t= 8 h, respectively; the last measured plasma lactate concentrations were 18.7 and 16.4 mM, respectively.

The initial plasma glucose concentration was 3.25 ± 0.17 mM. The plasma glucose levels did not change significantly within and between both groups.

The initial plasma FFA concentration was 0.38 ± 0.03 mM in the normoxia group and 0.34 ± 0.02 mM for the asphyxia group. Immediately after the start of the experimental period, a strong decrease in plasma FFA levels was observed in the normoxia group from 0.45 mM on t= -½ h to 0.27 mM on t= 1 h, followed by a slow recovery (Fig. 3). Also in the asphyxia group, an initial decrease after t= -½ h was observed. This decrease, however, persisted and resulted in an apparent plateau of significantly lower FFA levels of around 0.1 mM from t= 2 h and later.

The initial adrenaline concentration was below the detection limit of 0.1 nM in both groups. In the normoxia group, the adrenaline levels did not rise above the detection limit throughout the experimental period. Also in the asphyxia group, no significant difference in adrenaline concentrations were found, although the adrenaline levels rose above the detection limit from t= 0 h onwards: the highest adrenaline concentration of 0.9 ± 1.0 nM was recorded at t= 2 h.

The initial noradrenaline concentration was 2.4 ± 0.7 nM. In the normoxia group, the noradrenaline concentration did not change significantly throughout the whole experimental period (Fig. 4). In the asphyxia group, the noradrenaline concentration increased directly after start of asphyxia to significantly elevated levels from t= 1 h and later. After 4 h, the noradrenaline concentrations remained at an apparent plateau of around 35 nM; noradrenaline concentrations in this period ranged from 4.1 to 92.5 nM.

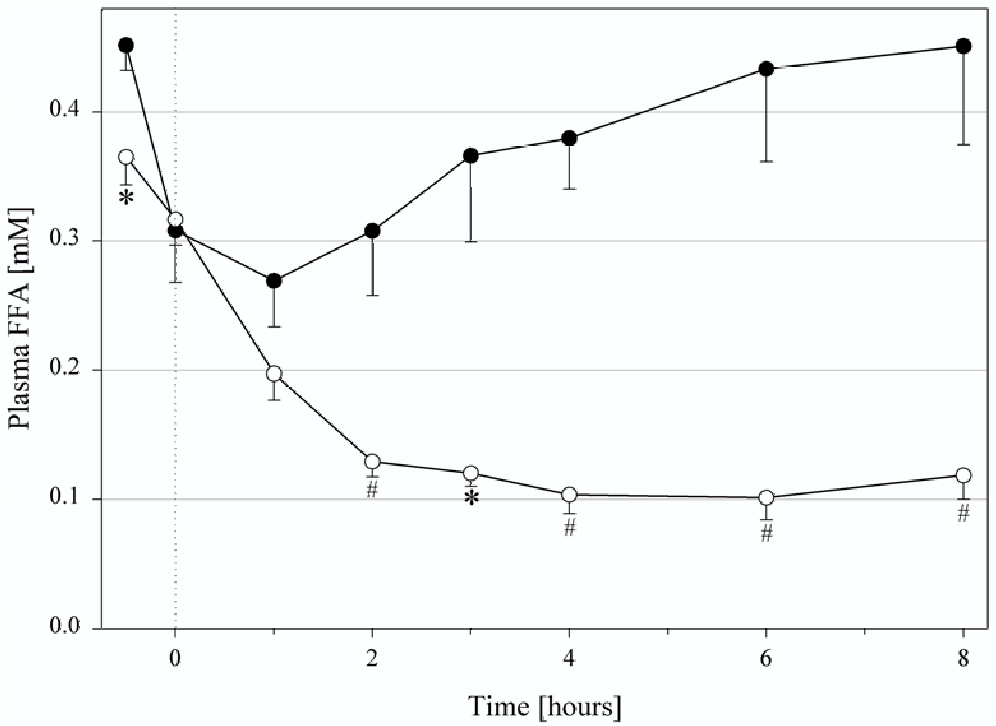

Figure 3. Plasma FFA concentration in cannulated African catfish during asphyxia (○; *n*= 8) or normoxia (●; *n*= 6); the dotted line indicates the start of asphyxia. *: P <0.05 vs. normoxia group; #: p<0.05 vs. normoxia group and vs. initial values.

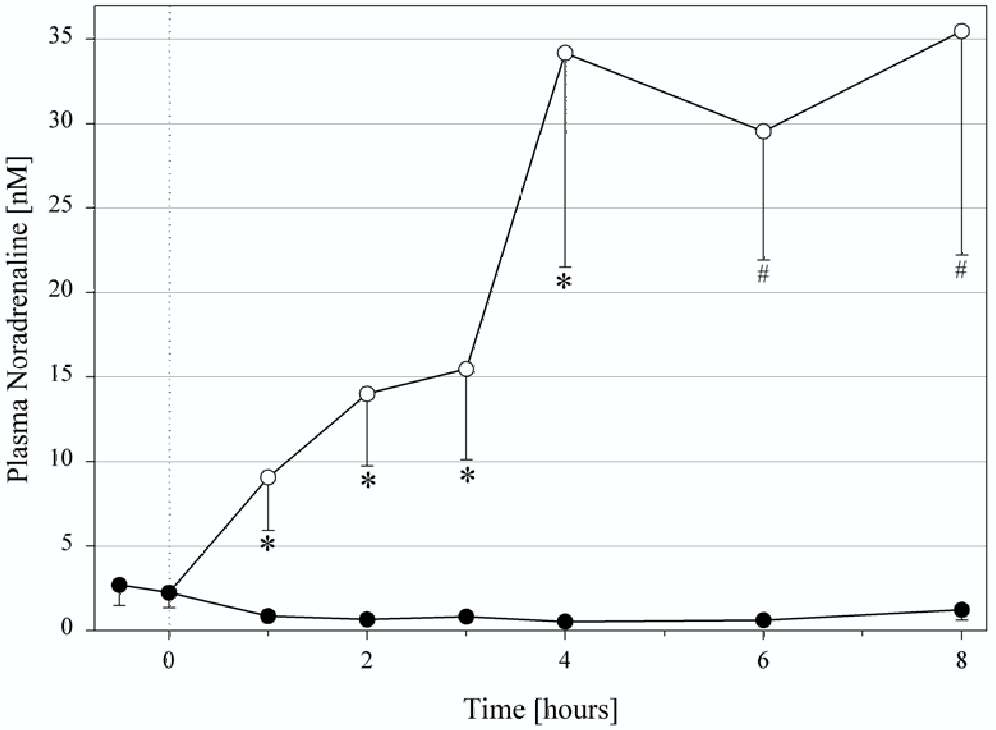

Figure 4. Plasma noradrenaline concentration in cannulated African catfish during asphyxia (○; *n*= 7) or normoxia (●; *n*= 5); the dotted line indicates the start of asphyxia. *: P <0.05 vs. normoxia group; #: p<0.05 vs. normoxia group and vs. initial values.

Discussion

There was a clear and non-transient surge in plasma lactate in African catfish under asphyxia as observed in many fish species under hypoxia (Holeton and Randall, 1967; Jørgensen and Mustafa, 1980; Dunn and Hochachka, 1986, 1987; Van Raaij *et al*., 1996a; Vianen *et al*., 2001, 2002). This clearly indicates the activation of anaerobic glycolysis. The permanent accumulation of lactate shows that African catfish is not able to recover from the exposed challenge, i.e. no aerial oxygen. Hence, normal air-saturated water induces functional hypoxia for the air-breathing African catfish, when denied access to aerial oxygen.

When African catfish shifted from bimodal respiration to only aquatic respiration, hematocrit and hemoglobin increased but the MCHC remained stable. Hence, the oxygen carrying capacity of the blood was enhanced by bringing more red blood cells into circulation. These cells are most likely released from the spleen upon adrenergic and/or cholinergic stimulation (Nilsson and Grove, 1974). As the MCHC did not change in hypoxic African catfish, swelling of red blood cells, a known reaction to hypoxia in salmonids (Soivio and Nikinmaa, 1981), did not occur despite the rising noradrenaline concentrations. Hence, African catfish erythrocytes do not respond to catecholamines by increasing ion influxes. Also in erythrocytes of American eel (*Anguilla rostrata*), catecholamines do not mediate cell swelling, most likely due to a limited number of ion pumps or insensitivity of these pumps to activation by cAMP (Perry and Reid, 1992).

No hyperglycaemia was found in hypoxic African catfish. Also in hypoxic trout (*Oncorhynchus mykiss*), hyperglycaemia was absent while a significant hyperglycaemia was found in hypoxic carp (Van Raaij *et al*., 1996a) and flounder (*Platichthys flesus*; Jørgensen and Mustafa, 1980). The absence of hyperglycaemia under hypoxia is remarkable as catecholamines are known to enhance plasma glucose levels (Fabbri *et al*., 1998a). The presence or absence of an adrenergic effect depends on the dominant catecholamine and on the affinity for the adrenoceptor mediating the response, i.e. hepatic glycogenolysis. The dominant catecholamine in our study was clearly noradrenaline. In carp, the affinity for the hepatic adrenoceptor was much lower for noradrenaline than for adrenaline; noradrenaline was thus far less potent than adrenaline in stimulating hepatic glucose release. The minimum effective dose of noradrenaline was 10^{-7} M in carp (Janssens and Lowrey, 1987). In this study with African catfish, noradrenaline concentrations of on average $0.35 \cdot 10^{-7}$ M were equally ineffective in stimulating glucose release. Additionally, an increased tissue utilisation of glucose under hypoxia was observed in trout

(Dunn and Hochachka, 1987), which could potentially prevent the elevation of plasma levels after adrenergic stimulation of hepatic glycogenolysis. Wright *et al*. (1989) did indeed find depressed plasma glucose levels in hypoxic trout, when the β-adrenoceptors mediating hepatic glucose release were blocked.

This study clearly demonstrates that in hypoxic African catfish plasma FFA levels are decreased like in hypoxic carp (Van Raaij *et al*., 1996a) and tilapia (Vianen *et al*., 2002). Even in the hypoxia-intolerant trout, a significant depression in plasma FFA levels during hypoxia was observed (Van Raaij *et al*., 1996a). In hypoxic tilapia, noradrenaline levels increased during progressive hypoxia while adrenaline levels remained at basal levels (Vianen *et al*., 2002). Also in the air-breathing African catfish, the decrease in plasma FFA levels was accompanied by an increase in noradrenaline concentrations, which remained elevated during the course of hypoxia; plasma adrenaline concentrations, however, remained at low levels. These results strongly suggest that noradrenaline was responsible for mediating a decrease in plasma FFA levels in tilapia and African catfish. Injection of noradrenaline induces, in every fish species tested so far, a decrease in plasma FFA levels (carp, Van Raaij *et al*., 1995; Van den Thillart *et al*., 2001; bream (*Abramis brama*), Farkas, 1967a; goldfish (*Carassius auratus*), Minick and Chavin, 1973; pike (*Esox lucius*), Ince and Thorpe, 1975). Therefore, it appears that the suppressive role of noradrenaline is a general mechanism in fish, as hypothesised by Van den Thillart *et al*. (2002).

The specific role of noradrenaline in the adrenergic response of fishes to hypoxia, is demonstrated by the fact that only noradrenaline but not adrenaline reduced plasma FFA levels in carp (Van Raaij *et al*., 1995). The noradrenaline levels at the moment of a significant decrease in FFA levels were ~3 nM in tilapia (Vianen *et al*., 2002) and ~10 nM in carp (Vianen *et al*., 1995). This matches the noradrenaline level of ~15 nM at t= 2 h with African catfish in this study.

Although African catfish has the ability to breathe air, noradrenaline has in this species a similar suppressive effect on plasma FFA levels as in water-breathing fish species. Apparently, the suppressive role of noradrenaline did not become redundant as we hypothesised.

Acknowledgements

We thank Jan Didden of the Department of Clinical Chemistry (Leiden University Medical Centre) for measuring the catecholamine levels.