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

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Summary

Both in mammals and fish, stress, and thus also hypoxia, evokes a so-called classical stress response. The function of this physiological response is to prepare the animal to cope with the stressor. A part of this response is a strong surge of the catecholamines adrenaline and/or noradrenaline. These hormones mediate an optimisation of cardiovascular and respiratory functions, and a mobilisation of energy reserves. Both in mammals and in fish, this means that the heart and ventilation rate go up, and that plasma glucose levels rise to meet an increased energy demand. There is, however, a fundamental difference between mammals and fish in how plasma free fatty acid (FFA) levels are affected by hypoxic stress. The enhanced catecholamine levels strongly stimulate lipolysis in mammals but, due to the oxygen shortage, the β-oxidation of fatty acids is impaired. These processes enhance each other and thus hypoxia results in elevated plasma FFA levels in mammals. High levels of FFA, however, can cause disruption of cell membranes resulting in cell leakage and tissue damage. In mammals, environmental hypoxia does not normally occur. Some fish species, on the other hand, can frequently encounter environmental hypoxia, because water is a relatively poor source of oxygen and, by nature, often has strongly fluctuating oxygen levels. Although catecholamine levels in fish are elevated during hypoxia, FFA levels fall rapidly in many fish species under hypoxia. This suppression of plasma FFA levels is mediated specifically by noradrenaline, and is believed to be a protective mechanism against fatty acid poisoning in fish under hypoxia.

As the effect of noradrenaline on lipid metabolism is different between mammals and fish, it implies that during evolution, the role of noradrenaline has changed. When considering hypoxic stress, a crucial difference between mammals and fish is the mode of breathing, air- and water-breathing, respectively. Therefore, the central hypothesis of this thesis is that the difference in the role of noradrenaline between fish and mammals is connected to the transition from water to air-breathing. There are, however, many fish species that have developed air-breathing. Like mammals, air-breathing fish have access to air, which contains a constant, high percentage of oxygen. Therefore, our working hypothesis is that the suppressive effect of noradrenaline, protecting the fish against fatty acid poisoning, is redundant in air-breathing fish.

Our model species is African catfish (*Clarias gariepinus*), one of the best known air-breathing fish species. To study the role of noradrenaline in this species, there were two requirements, which are described in Chapter 2. First, a suitable cannulation technique had to be selected for use in African catfish. Cannulation is a sampling technique, where the plasma

catecholamine concentration remains at a basal level, and is thus preferred over classical blood sampling techniques, which are known to trigger endogenous catecholamine release. For African catfish, we found that cannulation of the dorsal aorta was more successful than cannulation in a branchial artery in terms of lower blood loss and higher numbers of running cannulae.

A second requirement was to establish a possible diurnal fluctuation in metabolic parameters in African catfish. Diurnal rhythms in blood metabolites are common in vertebrates and such rhythms include major changes in metabolic parameters. Therefore, they are essential for evaluation of metabolic experiments, but are often not taken into account. In African catfish, cannulated in the dorsal aorta, a clear diurnal fluctuation in the two major blood metabolites, FFA and glucose, was observed. Compared to the initial value at 8.30 in the morning, plasma FFA levels dropped around 50% within 2 h. Minimum values of 0.26 ± 0.04 mM were reached at 12.30. The FFA concentration recovered to the initial value within the following 3 h. The fluctuation in plasma glucose levels showed a comparable course, but there was a phase-shift by 2 h. However, the most astonishing finding was the almost complete absence of glucose in the plasma of African catfish at 14.30 in the afternoon (0.05 ± 0.03 mM), a phenomenon never reported before for any fish species. This was the first study using cannulated fish for studying diurnal variations in plasma metabolites. Cannulation appears to be very suitable for this kind of study as it offers frequent sampling within an individual. This in turn allows detection of rapid changes in blood composition.

Although many fish species have developed air-breathing, hypoxic energy metabolism has never been properly studied in air-breathing fish. Generally, air-breathing fish react to low aquatic oxygen tensions by increasing the amount of oxygen extracted from the air. As plasma lactate levels remain low, 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. There is disagreement in the literature about whether African catfish is a facultative or obligate air-breather. However, in our view, aerial oxygen most likely represents an important source of oxygen for this species. Thus, we hypothesised that without access to aerial oxygen, i.e. asphyxia, African catfish becomes hypoxic.

In Chapter 3, we followed the metabolic response of dorsal aorta cannulated African catfish to an 8-h asphyxia period. There was a clear and non-transient surge in plasma lactate in African catfish under asphyxia as observed in many fish species under hypoxia. This clearly indicates that African catfish can not extract sufficient oxygen from the water. Hence, normal air-saturated water induced functional hypoxia for the air-breathing African catfish, when denied access to

aerial oxygen. Plasma glucose levels did not change during hypoxia, but FFA levels decreased significantly upon asphyxia with a concomitant increase in plasma noradrenaline levels. Plasma adrenaline, however, remained at basal levels. Thus, these results suggest that, in the air-breathing African catfish, noradrenaline had a suppressive effect on plasma FFA levels similar to the effect in water-breathing fish species.

In Chapter 4, an alternative approach was applied to elucidate the role of noradrenaline in African catfish. Noradrenaline was infused via a cannula, which was also used for blood sampling. As previous studies have shown that the effect of noradrenaline is mediated by stimulation of β-adrenoceptors, we additionally infused the nonselective β-agonist isoproterenol. Furthermore, the subtype of the adrenoceptor mediating the response was identified by combining the isoproterenol infusion with injection of selective antagonists for β_1- and β_2-adrenoceptors, namely atenolol and ICI 118,551. Both noradrenaline and isoproterenol led to an expected rise in glucose concentration. Isoproterenol combined with either a β_1- or β_2-antagonist led to higher glucose concentrations than isoproterenol alone. At first sight, this seems paradoxical. Possible explanations could be that there are stimulatory β-adrenoceptors on the liver, that differ from the β_1- and β_2-subtype. It also implies that the β_1- and β_2-adrenoceptors mediated a reduction in plasma glucose. There are two possible pathways. First, inhibitory β_1- and β_2-adrenoceptors could be present on the liver of African catfish. However, to the best of our knowledge, such inhibitory β-receptors have never been described in the literature. A second possibility is supported by the literature, namely a $\beta_{1,2}$-adrenoceptor mediated stimulation of the insulin release by the pancreas. Insulin is a known hypoglycemic hormone in fish. The observed plasma glucose concentration after β-adrenergic stimulation is then a result of an increase due to direct stimulation of hepatic glucose release and a decrease mediated by insulin, released by the pancreas after stimulation of $\beta_{1,2}$-adrenoceptors. In case of infusion of only isoproterenol, there is an increase in hepatic glucose release, but the effect on the plasma levels is suppressed by the simultaneous increase in plasma insulin levels. When β-blockers were injected, the hepatic glucose release would be equal, but the suppression by insulin would have been less. As a result, glucose levels increase more when $\beta_{1,2}$-adrenoceptors were blocked than when only isoproterenol was infused.

Both noradrenaline and isoproterenol had comparable suppressive effects on plasma FFA levels in African catfish, suggesting that in this species, as well as in others, it is mainly a β-adrenoceptor mediated process. When the isoproterenol infusion was preceded by a β_1-antagonist, the decrease in plasma FFA levels was identical to that seen when only isoproterenol

was infused, indicating that β_1-adrenoceptors had no major role. Injection of a β_2-antagonist, on the other hand, delayed the decrease in plasma FFA levels significantly. This rightward shift in the time-response curve indicates that the decrease in plasma FFA levels in African catfish was mediated by β_2-adrenoceptors. The results presented in Chapter 4 clearly demonstrate that, as in water-breathing fishes, noradrenaline suppresses plasma FFA levels in African catfish by stimulation of β-adrenoceptors.

A previous study demonstrated that the noradrenaline-mediated decrease in plasma FFA levels in tilapia could be attributed to a reduced FFA release by the adipose tissue. Because only FFA concentrations were measured, the only conclusion that could be reached was that the FFA release was inhibited by noradrenaline. Unfortunately, it could not be determined if this was due to reduced lipolysis or to increased reesterification. Hydrolysed FFA can be reesterified to triglycerides when glucose is available; glycerol, on the other hand, cannot be recycled due to a lack of glycerokinase in adipocytes, and therefore, the release of glycerol directly reflects lipolysis. In mammalian adipose tissue, both lipolysis and esterification occur simultaneously, resulting in triglyceride/FFA cycling, which leads to ratios between FFA and glycerol release of lower than the theoretical ratio of 3. In Chapter 5, we tested whether β_2-adrenoceptor stimulation reduced lipolysis in adipocytes isolated from the air-breathing African catfish. Adipocytes were isolated from the mesenteric adipose tissue from African catfish and incubated with the nonselective β-agonist, isoproterenol, combined with selective β_1- or β_2-antagonists, atenolol and ICI 118,551. In a basal situation, 33% of the hydrolysed FFA was recycled resulting in a ratio of FFA:glycerol in adipocytes of African catfish of 2.2. To enable reesterification, adipocytes of African catfish most likely used glucose from the medium for formation of glycerol-3-phosphate. Stimulation of β-adrenoceptors mediated an inhibition of both glycerol and FFA release, thereby showing that in fish adipocytes, catecholamines directly reduced lipolysis rather than stimulated reesterification and that thus hormone-sensitive lipase (HSL) is present in fish adipocytes. The FFA cycling rate stayed relatively constant upon β-adrenergic stimulation, indicating that lipolysis and reesterification were equally reduced. Most likely, the reduced reesterification was due to lower intracellular availability of FFA. The pD_2-value for the FFA release, which reflects the affinity of the receptors, was 8.71 in African catfish. This is considerably higher than the affinity in tilapia, (pD_2-value of 7.77), which is of the same level as the highest value reported for mammalian adipocytes. This difference in β-adrenoceptor affinity between mammals and fish illustrates the vital importance of the adrenergic inhibition of lipolysis in fish. The main adrenoceptor mediating the decrease in lipolytic rate in African catfish adipocytes was clearly the

β_2-adrenoceptor as indicated by a clear right shift in the dose-response curve, and by a decrease in the pD_2-value from 8.71 to 7.41. A minor role for the β_1-adrenoceptor can, however, not be excluded as the pD_2-value shifted to 8.16.

As lipolysis in isolated adipocytes of only two tropical fish species (tilapia and African catfish) was studied thus far, the primary objective in Chapter 6 was to obtain comparative data on the lipolytic rate in adipocytes of two other fish species of a different ecological background: the cold freshwater Rainbow trout (*Oncorhynchus mykiss*) and the warm seawater Gilthead sea bream (*Sparus aurata*). To reduce reesterification to a minimum, no glucose was included in the medium. Adipocytes of African catfish were also isolated to allow comparison with previous experiments. The ratio FFA:glycerol release in trout and catfish adipocytes was over 3, 5.0 ± 0.8 and 7.1 ± 3.4, respectively. This means that less glycerol than FFA was released and thus partial glycerides had accumulated. In mammals, two enzymes are involved in triglyceride degradation: HSL hydrolyses triglycerides to di- and subsequently monoglycerides, which are further hydrolysed by monoglyceride lipase to glycerol. If the same dual enzymatic breakdown is present in fish, monoglyceride lipase was rate-limiting in the trout and catfish adipocytes. Upon stimulation by noradrenaline, the ratio FFA:glycerol produced by adipocytes of catfish was significantly reduced from 7.1 to 3.0. In view of the dual enzymatic breakdown, this means that HSL activity was lowered and was equal to or below the monoglyceride lipase activity. In sea bream adipocytes the ratio between FFA and glycerol was around 3, indicating that the lipid degradation was complete. Large differences were found in the basal lipolytic rate between the three species tested in this study. After temperature correction, basal FFA release by catfish adipocytes (~1100 nmol/ml/h) was about 2 times higher than the release by trout adipocytes (~500 nmol/ml/h), but a massive 25 times higher than the release by sea bream adipocytes (~40 nmol/ml/h). Possible explanations for these differences in lipolytic rate can be found in the nutritional state and/or age of the animals. The extremely low lipolytic rate in the sea bream was surprising. In many seawater species, the liver functions as the main storage site of triglycerides, which suggests a dominant role of the liver over the adipose tissue in marine fish species. A second objective in Chapter 6 was to study the effect of the catecholamines (adrenaline, noradrenaline and isoproterenol) on lipolysis of adipocytes of trout, sea bream and African catfish. In adipocytes of trout and catfish, adrenaline as well as noradrenaline reduced lipolysis. Although adrenaline is a β-agonist like noradrenaline, it is surprising that adrenaline was capable of reducing adipose lipolysis. Previous *in vivo* experiments with carp namely showed that only noradrenaline reduced FFA levels but adrenaline, on the contrary, stimulated FFA levels;

probably stimulatory β-adrenoceptors present on fish liver mediated an enhanced hepatic FFA release. As opposed to the reduction in the fresh water trout and catfish, adrenergic stimulation of sea bream adipocytes did not inhibit lipolysis, indicating that no HSL was functionally present. Hence, another triglyceride lipase had to be present in sea bream adipocytes or HSL was in a state in which its activity could not be affected by hormones. Because the lipolytic rate in the adipocytes of this species was, however, extremely low, a reduction of adipose lipolysis had probably no physiological relevant effect on plasma FFA levels. As stated earlier, the liver is a more important site for lipid mobilisation and, thereby, a more likely site for adrenergic action in sea bream.

In Chapter 6, it was demonstrated that adrenaline like noradrenaline reduced lipolysis in adipocytes of two freshwater fish species, namely trout and African catfish. Yet, *in vivo* adrenaline stimulated plasma FFA levels in carp. These apparent contradictory effects of adrenaline may be explained by, first, assuming that adrenaline stimulated lipolysis in the liver. Second, because noradrenaline can stimulate the same receptors like adrenaline, a distinct affinity difference has to exist between β-adrenoceptors in fish, like is known for mammalian adrenoceptors. Due to the difference in receptor affinity, adrenaline and noradrenaline stimulate different β-adrenoceptors at concentration within the physiological range; noradrenaline mainly stimulates β_2-adrenoceptors and adrenaline mainly stimulates β_1-adrenoceptors. Therefore, in Chapter 7, the adrenergic control of FFA release was studied in hepatocytes of Rainbow trout, which were either normally fed or fasted for 3 weeks. Isolated hepatocytes were incubated with adrenaline, noradrenaline or isoproterenol. To identify the adrenoceptor involved, isoproterenol incubations were combined with atenolol or ICI 118,551. As hypothesised, FFA release by trout hepatocytes was stimulated by all three catecholamines at concentrations of 10^{-5} and 10^{-6} M, depending on the feeding status. Such high plasma catecholamine concentrations are, however, rarely reached *in vivo*, raising doubt on the physiological relevance. However, noradrenaline concentrations can locally reach the micromolar range due to overflow from adrenergic nerve terminals. In mammalian liver direct innervation can stimulate hepatic glucose release; it is however unknown if the liver of fish is also directly innervated. For the glucose release, a clear dose-response curve was found for all three catecholamines. The adrenoceptor mediating the glucose release was the β_2-adrenoceptor. The affinity of the β_2-adrenoceptor for isoproterenol and adrenaline (pD_2-values of 8.3 and 7.9) was clearly higher than for noradrenaline (pD_2-value of 6.5). Opposing effects of adrenaline and noradrenaline can thus be explained by differences in receptor affinity as we hypothesised. The release of glucose and FFA by trout hepatocytes was

strongly reduced upon fasting, implying a reduced overall energy demand. The ratio between glucose and FFA release decreased from 15.4 to 4.3 upon fasting. Remarkably, the mobilisation of FFA upon adrenergic stimulation was conserved after a 3-week fast: in hepatocytes from fasted fish, the mobilisation capacity was 3.5 nmol/10^6 cells/h, matching the 3.4 nmol/10^6 cells/h for hepatocytes from fed fish. These data demonstrate for the first time that upon fasting, FFA gain importance in the hepatic metabolism. The sensitivity of the adrenergic signal transduction system in trout hepatocytes was enhanced upon fasting; this enhanced sensitivity is reflected in an increased pD_2-value from 8.3 to 8.9 after a 3-week fast.

The main conclusion from this thesis is that the role of noradrenaline in the air-breathing African catfish is similar to its role in other water-breathing fish species, namely suppression of plasma FFA levels by inhibition of adipose lipolysis. Apparently, the evolution of air-breathing in the catfish lineage was not associated with changes in the adrenergic control of lipid metabolism as we hypothesised. We demonstrated that environmental hypoxia is a potential stress condition for African catfish in its natural surroundings, namely submersion without access to aerial oxygen. Obligate air-breathers, such as adult lungfish (*Protopterus* spp.) on the other hand, are critically dependent on aerial oxygen and can only live when allowed air-breathing. Hence, environmental hypoxia, even when it only means submersion like in African catfish, is per definition not a physiological relevant situation for lungfish, rendering this species as an interesting model fish to test our hypothesis. Air-breathing in fishes has evolved in many fish species on separate occasions. The evolution of air-breathing was originally thought of as a way to survive environmental hypoxia. Some authors have suggested, however, that air-breathing also functions to maintain activity levels when aquatic oxygen levels are low. Therefore, the main driving force for the evolution of air-breathing may not necessarily be survival of hypoxia, but rather coping with hypoxia by sustaining normal activity levels. As the suppressive role of noradrenaline on plasma FFA levels is hypothesised to be a survival mechanism during hypoxia, such an alternative drive for the evolution of air-breathing is in corroboration with our finding that noradrenaline suppresses plasma FFA levels in air-breathing African catfish as in other water-breathing fishes.