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

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GENERAL INTRODUCTION

1. Hypoxic energy metabolism.

Both mammals and fish show a so-called classic stress response to various stressors including hypoxia (Wendelaar Bonga, 1997; Fabbri *et al*., 1998a). The function of this physiological response is to prepare the animal to cope with the stressor (Wendelaar Bonga, 1997). A part of this response is a strong surge of catecholamines, adrenaline and/or noradrenaline.

The elevated catecholamine levels mediate an optimisation of cardiovascular and respiratory functions, and a mobilisation of energy reserves (Randall and Perry, 1992; Fabbri *et al*., 1998a). Both in mammals and in fish, this results in an increased heart and ventilation rate, leading to a higher and faster oxygen and metabolite delivery to the tissues. To meet an increased energy demand, glycogen stores are mobilised leading to elevated plasma glucose levels. How plasma free fatty acids (FFA) are affected by hypoxic stress is, however, fundamentally different between mammals and fish. The enhanced catecholamine levels strongly stimulate lipid mobilisation in mammals (Fain and Garcia-Sainz, 1983; Smith, 1983). Fatty acids can only be broken down via the β-oxidation as opposed to glucose, which can be broken down anaerobically to lactate. Therefore, in case of oxygen shortage the consumption of FFA is impaired (Moore, 1985; Van der Vusse *et al*., 1992), which enhances the increase in plasma FFA levels due to the increased FFA mobilisation. Hypoxia thus results in strongly elevated plasma FFA levels in mammals (Vogel and Hannon, 1966; Stock *et al*., 1978; Meerson *et al*., 1994; Roberts *et al*., 1996). High levels of FFA, however, can cause disruption of cell membranes resulting in cell leakage and tissue damage, as seen in the ischemic heart (Katz and Messineo, 1981; Hagve *et al*., 1990; Hütter and Soboll, 1992). Besides such pathological situations, hypoxia normally does not occur in mammals at the organismal level. Some fish species on the other hand can frequently encounter environmental hypoxia, as water is a relatively poor source of oxygen and, by nature, often has strongly fluctuating oxygen levels (Van den Thillart, 1982). Although catecholamine levels in fish are elevated during hypoxia, FFA levels fall rapidly in fish (Van Raaij *et al*., 1996a; Van Ginneken *et al*., 1998; Vianen *et al*., 2002). This suppression of plasma FFA levels is mediated specifically by noradrenaline (Van den Thillart *et al*., 2001; Vianen *et al*., 2002) and is believed to be a protective mechanism against fatty acid poisoning in fish under hypoxia (Van den Thillart *et al*., 2002).

As the effect of noradrenaline on lipid metabolism is different between fish and mammals, 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. There are, however, many fish species that have developed a form of air-breathing, originally thought of as a way to counteract the negative effects of low aquatic oxygen tensions (Graham, 1997). Like mammals, air-breathing fish in their natural surroundings always have access to air, which contains a constant, high percentage of oxygen of around 21%. Therefore, it is highly likely that environmental hypoxia is not a threat to air-breathing fish species. It is, however, unknown what the role of noradrenaline is in the stress metabolism of air-breathing fishes.

2. Adrenergic control of lipid metabolism.

Lipid metabolism and its hormonal regulation in fish have received relative little attention (Van den Thillart *et al*., 2002). Liver, adipose tissue and muscle are quantitatively the major sites involved in lipid metabolism in fish. While the liver functions mainly as a processing site in receiving dietary lipids and excreting lipoproteins, and storage of lipids in the muscles serves to meet local demands (Sheridan, 1994), the adipose tissue is the dominant and most dynamic site for storage and mobilisation of FFA (Van den Thillart *et al*., 2002).

2.1. Adrenergic effects *in vivo*.

As mentioned above, plasma FFA levels in hypoxic fish such as the hypoxia-tolerant carp (*Cyprinus carpio*; Van Raaij *et al*., 1996a; Van Ginneken *et al*., 1998; Vianen, 1999) and tilapia (*Oreochromis mossambicus*; Vianen *et al*., 2002) are decreased. However, also in the hypoxia-intolerant trout (*Oncorhynchus mykiss*; Van Raaij *et al*., 1996a; Vianen, 1999), there is a significant decrease in plasma FFA levels upon hypoxia. In tilapia, noradrenaline levels increased during progressive hypoxia while adrenaline levels remained low (Vianen *et al*., 2002). They concluded therefore, that noradrenaline mediated the decrease in plasma FFA levels.

Of course besides the catecholamines, many other neuro-endocrine factors are involved in adaptation to hypoxia (Wendelaar Bonga, 1997). In hypoxic carp for instance, there are significant changes in glucagon, insulin and thyroid hormone levels (Vianen, 1999). Therefore, to identify if the observed changes in hypoxic fish are a direct effect of the surge in

catecholamines or that this surge in catecholamines is an indirect secondary effect, valuable information may be derived from direct administration of catecholamines.

Injection of noradrenaline induced a decrease in plasma FFA levels in every fish species tested so far, all being freshwater species: carp (Van Raaij *et al*., 1995; Van den Thillart *et al*., 2001), bream (*Abramis brama*; Farkas, 1967a,b), goldfish (*Carassius auratus*; Minick and Chavin, 1973) and pike (*Esox lucius*; Ince and Thorpe, 1975). Injection of adrenaline on the other hand gave many conflicting results. In carp (Van Raaij *et al*., 1995), eel (*Anguilla anguilla*, Larsson, 1973), lamprey (*Lampetra* sp.) and scorpion fish (*Scorpionides* sp., Plisetskaya, 1980) a clear rise in plasma FFA levels was observed. In trout however, adrenaline injection appeared to have no effect at all (Perrier *et al*., 1972). In goldfish (Minick and Chavin, 1973), pike (Ince and Thorpe, 1975) and Rosy barb (*Puntius conchonius*; Khanna and Singh, 1984), adrenaline mediated a decrease in plasma FFA levels.

One problem in interpreting experiments, in which stress hormones are administered, is the endogenous release of catecholamines in reaction to the experimental protocol. Most of these protocols include catching and anaesthesia prior to injection of the hormone, followed by recovery and again catching and injection for blood sampling. Even when fish are only briefly handled, catecholamine levels can rise up to 10^{-7} M (Mazeaud and Mazeaud, 1981; McDonald and Milligan, 1992; Gerwick *et al*., 1999). Such an endogenous release of catecholamines, which will also occur in a control treatment, can obscure and mask the effect of exogenous catecholamines. Resting catecholamine levels in cannulated fish are significantly lower than in such ”classically” sampled fish (Woodward, 1982). Therefore, the use of cannulated fish is preferable in physiological experiments. Van Raaij *et al*. (1995) administered adrenaline as well as noradrenaline via a cannula to carp, thereby excluding any significant endogenous catecholamine release. While noradrenaline mediated a reduction in plasma FFA levels, adrenaline strongly stimulated FFA levels. Van den Thillart *et al*. (2001) applied the same technique for identification of the receptors mediating these apparently conflicting responses. In mammals, α_2-adrenoceptors are known to mediate an inhibition of lipolysis (Fain and Garcia-Sainz, 1983; Lafontan *et al*., 1992), and were thus likely to mediate the reduction of plasma FFA levels in fish. However, Van den Thillart *et al*. (2001) demonstrated that an α_2-effect was most likely indirect, e.g. by a reduced blood flow through the adipose tissue via vasoconstriction. Surprisingly, β-adrenoceptor stimulation, known to enhance lipolysis in mammals, completely mimicked the effect of noradrenaline. Van den Thillart *et al*. (2001) therefore, concluded that β-adrenoceptor stimulation was mainly responsible for the decrease in plasma FFA levels.

However, when following identification of the subtypes of the β-adrenoceptor, β_2-adrenoceptors proved to be stimulatory and β_1-adrenoceptors were inhibitory. Van den Thillart *et al*. (2001) hypothesised that lipolysis-stimulating β-adrenoceptors are present on the liver, while lipolysis-inhibiting β-adrenoceptors are present on the adipose tissue. The actual physiological effect of a certain catecholamine concentration is determined by the affinity of the receptors involved. In mammals, adrenaline has a higher affinity for the β_2-adrenoceptor and noradrenaline for the β_1-adrenoceptor. This is in corroboration with the observations of Van Raaij *et al*. (1995) and Van den Thillart *et al*. (2001). It should be noted, however, that for fish such a difference in affinity has not been demonstrated yet, although many studies assumed it to be comparable to mammals.

2.2. Adrenergic effects *in vitro*: adipose tissue.

As stated earlier, two tissues are involved in mediating plasma FFA levels: the liver as a central processing organ and the adipose tissue as the most dynamic and quantitatively most important storage site. Isolation of mammalian adipocytes has become a classically used technique for studying adipose tissue lipolysis, since Rodbell (1964) published a suitable isolation procedure. Therefore, there is a vast amount of information on how the adrenergic control on lipolysis in mammalian adipocyte is organised; a schematic diagram of the signal transduction pathway in adipocytes is given in fig.1. For more detailed information on the signal transduction pathway, we refer to several reviews on this subject (Fain and Garcia-Sainz, 1983; Smith, 1983; Lafontan and Berlan, 1993; Holm, 2003).

Catecholamines mediate their effect by coupling to membrane bound receptors, called adrenoceptors. These receptors are divided into three classes: α_1-, α_2- and β-adrenoceptors. The α_1-adrenoceptors appear to have no direct effect on adipose lipolysis; rather they act indirectly by stimulating glycogen phosphorylase (Fain and Garcia-Sainz, 1983; Lafontan and Berlan, 1993). The α_2- and β-adrenoceptors, on the other hand, control the lipolytic rate via activation of membrane bound G-proteins (guanine-nucleotide binding protein). The α_2-adrenoceptors couple to inhibitory G-proteins, while β-adrenoceptors couple to stimulatory G-proteins. Subsequently, these G-proteins regulate the activity of the enzyme adenylcyclase, which converts ATP into cAMP. The concentration of cAMP regulates the activity of protein kinase A and subsequently phosphorylation of hormone-sensitive lipase (HSL), which is the rate-limiting step in triglyceride degradation (Coppack *et al*., 1994). Although it is generally accepted that the phosphorylation

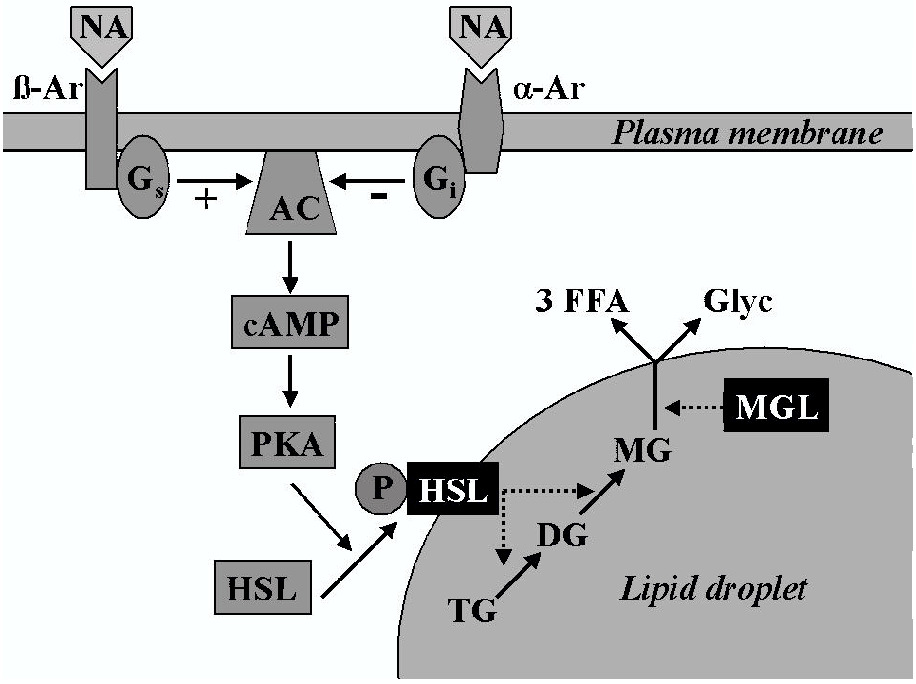

Fig.1. Schematic overview of the adrenergic signal transduction pathway in an mammalian adipocyte (After Holm, 2003). See text for details. Abbr.: NA: noradrenaline; β-Ar : β-adrenoceptor; α-Ar : α-adrenoceptor; $G_{s/i}$: stimulatory or inhibitory G-protein; AC: adenylcyclase; cAMP: cyclic AMP; PKA: protein kinase A; HSL: hormone-sensitive lipase; P: phosphorylated; TG: triglycerides; DG: diglycerides; MG: monoglycerides; MGL: monoglyceride lipase; FFA: free fatty acids; Glyc: glycerol.

status of HSL plays a major role in controlling the lipolytic rate, the maximal activity of HSL is insufficient to account for the maximal lipolytic rate in adipose tissue (Clifford *et al*., 2000). This suggests that other processes are involved in regulating lipolysis, such as translocation of HSL to the lipid droplet (Egan *et al*., 1992), access to the lipid droplet regulated by the protein perilipin (Østerlund, 2001) and binding to a protein called adipocyte lipid-binding protein (ALBP; Shen *et al*., 1999).

HSL hydrolyses triglycerides to di- and subsequently monoglycerides, while monoglyceride lipase (MGL) hydrolyses the thus formed monoglycerides to glycerol (Vaughan *et al*., 1964). Complete hydrolysis of triglycerides results in a FFA:glycerol ratio of 3:1. The hydrolysed FFA and glycerol may diffuse out of the adipocyte into the circulation. Adipocytes essentially lack the enzyme glycerokinase (Margolis and Vaughan, 1962) and thus glycerol can not be recycled in adipocytes. FFA can be used for reesterification as long as there is glycerol-3-phosphate, for instance formed out of glucose via glycolysis. In adipose tissue, both lipolysis and esterification occur simultaneously resulting in triglycerides-FFA cycling (Edens *et al*., 1990). Hence, the FFA:glycerol ratio is generally well below 3 (Vaughan and Steinberg, 1963) as the large majority

of released FFA are reesterified (Wolfe *et al*., 1990). Also in trout *in vivo*, the rate of appearance of glycerol was higher than of FFA, 8.1 and 4.9 μmol/kg/min, respectively. As 8.1 μmol of glycerol is equivalent to 24.3 μmol of FFA, a large majority of around 80% of the hydrolysed FFA was recycled in trout (Bernard *et al*., 1999).

There is very little information about the adrenergic control in fish adipocytes compared to mammalian adipocytes. Only a few studies have been performed with isolated fish adipocytes, most likely because these cells are very fragile and thus hard to keep viable. Slices of adipose tissue have been used more frequently. The release of lipolytic metabolites by slices is, however, an underestimation of the true lipolytic rate as diffusion is impaired due to interstitial barriers (Angel *et al*., 1971).

Probably the first report on the adrenergic control in fish adipose tissue was by Farkas (1967a,b, 1969a), who studied the effect of catecholamines on the FFA release by slices of adipose tissue. He reported that in four freshwater fish species (carp, bream, pikeperch, *Lucioperca lucioperca,* and shad, *Pelecus cultratus*) catecholamines reduced the FFA production of the adipose tissue. Almost two decennia later, Murat *et al*. (1985) reported that both catecholamines, adrenaline and noradrenaline, had no effect on the release of FFA or glycerol by isolated adipocytes of several fish species (carp, trout, goldfish and snakehead, *Channa maculata*). There are several reasons to doubt the physiological relevance of the results of Murat and co-workers. First, for carp it has been shown *in vitro* that adrenergic stimulation reduces the FFA release by slices of the adipose tissue (Farkas, 1967a). Additionally, catecholamine administration to carp *in vivo* decreased plasma FFA levels (Farkas, 1967b; Van Raaij *et al*., 1995; Van den Thillart *et al*., 2001), suggesting a reduced FFA mobilisation from the adipose tissue. Secondly, Murat and co-workers measured glycerol release using normal spectrophotometry based on NAD-reduction; this is a method, which has a relatively low sensitivity (Hellmer *et al*., 1989). Thirdly, they exposed the adipocytes to both low (4ºC) and high (25ºC) temperatures, which most likely damaged the fragile adipocytes. Also in slices of adipose tissue of tigerfish (*Hoplias malabaricus*), adrenaline had no effect on the FFA release (Migliorini *et al*., 1992). In 2002, Vianen and co-workers published a detailed study, in which the mammalian adipocyte isolation was adapted for use in fish, namely tilapia. This study was also the first pharmacological work on the adrenergic control of lipolysis in fish adipocytes. While the in vivo experiments by Van den Thillart *et al*. (2001) could not eliminate a potential α_2-adrenoceptor mediated inhibition of plasma FFA levels, Vianen *et al*. (2002) clearly showed that the FFA release by tilapia adipocytes was not inhibited by α-adrenoceptor stimulation. A $\beta_{1,2}$-

antagonist (timolol) on the other hand blocked the inhibition of the FFA release by noradrenaline. Conversely, the FFA release could also be inhibited by stimulation with a β-agonist (isoproterenol). This strongly suggests that $\beta_{1,2}$-adrenoceptors mediated this inhibition. Additional experiments indicated that most likely also β_3-adrenoceptors became stimulated at high concentrations ($\geq 10^{-6}$ M). All together, this was a surprising finding as β-adrenoceptor stimulation in mammals is known to strongly stimulate lipolysis (Fain and Garcia-Sainz, 1983).

The signal transduction pathway in fish adipose tissue is largely unknown. No G-proteins have been identified on fish adipocytes. In slices of tigerfish adipose tissue, elevation of the intracellular cAMP levels, either directly or indirectly by theophylline or IBMX (both phosphodiesterase inhibitors), resulted in an increase in FFA release. This suggests that stimulatory G-proteins were present and that increased cAMP levels activated HSL (Migliorini *et al*., 1992). Apparently, the intracellular messenger system was present in the adipocytes of this species and identical to mammals. However, it should be noted that the FFA release in their experiments could not be significantly increased by adrenaline, even at pharmacological doses of 10^{-4} M. They concluded therefore, that either the binding to the adrenoceptors was defective or that there were no or not enough functional receptors to mediate an effect. In adipose tissue of pikeperch, increased cAMP levels by incubation with theophylline, however, reduced the FFA production, like catecholamine incubations did (Farkas, 1969b). This suggests that increased cAMP levels reduced lipolysis and that G_s-proteins are likely to be involved. Farkas (1969a) stated however that no HSL was present in fish adipose tissue as adrenergic stimulation reduced the FFA release, but not the glycerol release. As the addition of glucose to the medium also reduced the FFA release, he concluded that the reduced FFA release mediated by catecholamines was due to an increased reesterification rate. Farkas (1969b) concluded therefore, that cAMP plays no role in mediating lipolysis in fish. He gave no alternative possibility, which fitted the reduced FFA release mediated by increased cAMP levels. Besides a potential inhibitory effect of cAMP on HSL activity, of which Farkas thought that it was unlikely, increased cAMP levels can inhibit glycogen synthase activity in mammalian adipocytes (Fain and Garcia-Sainz, 1983). In this way, cAMP increases the availability of glucose, which is needed to enable an increased reesterification rate mediated by catecholamines as suggested by Farkas.

The triacylglycerol lipase, isolated from adipose tissue of trout, could be activated by a cAMP-mediated phosphorylation (Michelsen *et al*., 1994), and was biochemically comparable to mammalian HSL (Sheridan and Allen, 1984). In adipose tissue of trout, glucagon mediated an increased lipolytic rate (Harmon and Sheridan, 1992c). As the intracellular messenger system of

glucagon is largely identical to the catecholamines (Plisetskaya and Mommsen, 1996; Moon, 1998), these results of Harmon and Sheridan (1992c) with glucagon appear to be conflicting to what is found for the catecholamines (Farkas, 1967a,b, 1969a; Vianen *et al*., 2002). The fact that the stimulatory effect of glucagon and the inhibitory effect of the noradrenaline are both mediated via cAMP, implies that the intracellular pathways of both hormones are spatially separated in fish adipose tissue. Secondly, in mammals phosphorylation of HSL is certainly not the only regulating factor of lipolytic activity and the signal transduction pathway offers more entry points for adrenergic inhibition. So far, it is unknown at which entry points both hormones affect lipolysis and this still remains to be elucidated in fish.

Conclusively, all literature, which demonstrated a significant effect of catecholamines on lipolysis in fish adipose tissue, indicates that adrenergic stimulation reduces the FFA release in fish adipose tissue. There are, however, contradictory results on how this action is mediated intracellularly. The different components of the pathway, as they are known from mammalian adipocytes, appear to be present in fish adipocytes. How these different components are interconnected in fish adipocytes, remains to be determined.

2.3 Adrenergic effects *in vitro*: liver tissue.

There is only one report of an increased hepatic lipolysis upon adrenergic stimulation in fish, published by Sheridan (1987). Hepatic lipolysis in Coho salmon (*Oncorhynchus kisutch*) was stimulated by noradrenaline at a minimum effective dose of 10^{-9} M and ED_{50}-value of around $2 \cdot 10^{-7}$ M. Based on the stimulatory effect of isoproterenol, hepatic FFA mobilisation in this species must be mediated by β-adrenoceptors. Remarkably adrenaline, also a strong β-agonist, had no effect even at a supra-physiological concentration of 10^{-4} M. Only at low catecholamine concentrations can such differential effects of adrenaline and noradrenaline be expected based on receptor affinity, but at high concentrations both catecholamines are expected to have comparable effects. Scott-Thomas *et al*. (1992) demonstrated differential effects of adrenaline and noradrenaline at $2 \cdot 10^{-7}$ M on the FFA release by hepatocytes of brook charr (*Salvelinus fontinalis*); hepatic FFA release decreased (!) following noradrenaline administration while adrenaline had no effect. In hepatocytes of copper rockfish (*Sebastes caurinus*), neither adrenaline nor noradrenaline mediated an effect on hepatic lipolysis (Danulat and Mommsen, 1990). Interestingly, these same authors stated, as unpublished results, that there is also no adrenergic effect on hepatic lipolysis in Coho salmon. These results are in contrast with those of

Sheridan (1987), also on Coho salmon liver. Thus, published studies show sometimes even conflicting results, suggesting a complex mechanism for control of hepatic lipolysis in fish.

In mammals, hepatic lipolysis is not affected by catecholamines as no HSL is present (Debeer *et al*., 1979; Holm *et al*., 1987). Fish on the other hand appear to have a hepatic HSL as trout liver possesses a triacylglycerol lipase with characteristics comparable to HSL from mammalian adipose tissue (Harmon *et al*., 1991). Phosphorylation of this hepatic lipase of trout resulted in a higher catalytic activity, which again suggests HSL-like characteristics (Harmon and Sheridan, 1992c; Harmon *et al*., 1993).

3. Adrenergic control of glucose metabolism

The fish liver plays a key regulatory role in controlling not only plasma FFA levels but also plasma glucose levels. This thesis focuses on the adrenergic control of FFA metabolism, but a brief introduction on the effect of catecholamines on plasma glucose levels and hepatic glucose release is needed. As opposed to the adrenergic control on lipid metabolism, the effect of catecholamines on glucose metabolism is perhaps the best-studied phenomenon in stress metabolism in fish. Therefore, a catecholamine-mediated increase in either plasma glucose levels or hepatic glucose release will frequently be used as a reference reaction in this thesis.

A classic response to catecholamine administration is hyperglycaemia, which is observed in many fish species (see review by Fabbri *et al*., 1998a). This is generally accepted to be mainly mediated by β-adrenoceptor stimulated glycogenolysis and β-adrenoceptor inhibited glycolysis in the liver (Birnbaum *et al*., 1976; Janssens and Lowrey, 1987; Mommsen *et al*., 1988; Wright *et al*., 1989; Reid *et al*., 1992). *In vitro*, the presence of stimulatory α_1-adrenoceptors on fish liver has been demonstrated, but their role appears to be minor in comparison to the β-adrenoceptors (Brighenti *et al*., 1987b; Moon *et al*., 1993; Fabbri *et al*., 1995a,b, 1999). The β-adrenoceptors activate the adenyl cyclase/cAMP pathway (Fabbri *et al*., 1992, 1995a,b, 1998b, 2001), which stimulates glycogen phosphorylase and thus the breakdown of glycogen (Brighenti *et al*., 1987a; Ottolenghi *et al*., 1986, 1989; Moon *et al*., 1999). Recently, the presence of stimulatory G-proteins on catfish (*Ictalurus melas*) liver was directly demonstrated (Fabbri *et al*., 2002). Compared to published data on hepatic lipolysis, data on the adrenergic control of hepatic glucose release is consistent and all studies indicate that the intracellular pathway in fish liver is similar to that in mammals.

4. Air-breathing fish

Many fish species have developed air-breathing in addition to the normal gill/water-breathing; this ranges from taking air bubbles into the buccal cavity to the presence of actual lungs (Graham, 1997). Gills however never became redundant in fish, since no fish species has ever lost its gills, even when it is no longer dependent on its gills for its oxygen uptake. The gills appear to be essential for carbon dioxide and ammonia excretion (Janis and Farmer, 1999). Air-breathing in fish can be subdivided into facultative and obligate air-breathing; facultative air-breathers can live on aquatic oxygen indefinitely as opposed to the obligate air-breathers, which drown when prevented to surface for taking in air.

Clariidae (Order of Siluriformes) is one of the best known families of air-breathing fish species (Graham, 1997). All 6 subgenera of the Clariidae possess a suprabranchial respiratory organ (SRO), which originates from the second and fourth gill branch (Teugels, 1982; Olson *et al*., 1995). This organ consists of gill fans, arborescent organs and suprabranchial chambers, all highly vascular (Moussa, 1957). *Clarias gariepinus* (African catfish) starts breathing air at around 2 cm of length (Haylor and Oyegunwa, 1993). This species is most active at night and thus also its air-breathing frequency is higher when dark (Babiker, 1979; Britz and Pienaar, 1992).

The vasculature of the SRO of *Clarias batrachus* (walking catfish) is in parallel with the vascular system of the gill (Olson *et al*., 1995). In contrast to normal water-breathing fish, *C. batrachus* has multiple ventral aortas and an additional vessel connecting the suprabranchial epithelium to the dorsal aorta. There are no other vascular shunts or adaptations to separate the blood flow to the gills and to the SRO. The SRO respiration seems to be additional to the normal branchial respiration and doesn't function as a replacement of the gills (Olson *et al*., 1995). Separation of blood flow to the gills and SRO is likely because otherwise in hypoxic waters the gills would lose oxygen extracted from the air by the SRO. Olson *et al*. (1995) proposed that in *Clarias*, temporal shunting of the blood flow through the gills could be accomplished through direct hypoxic vasoconstriction as is observed in trout and cod. In this way, a greater proportion of the blood is directed to the SRO. Although less detailed information is available for *C. gariepinus* (Nawar, 1955), the organisation of the gill and SRO vasculature appears comparable to *C. batrachus*.

C. batrachus is a facultative air-breather (Jordan, 1976); under normal situations (oxygen saturated water, access to air), 10 to 20% of the oxygen consumption comes from air-breathing.

Singh and Hughes (1971), however, reported that *C. batrachus* is an obligate air-breather with an aerial oxygen consumption of 58%. The same contradiction occurs for *C. gariepinus*: this species is classified as an obligate air-breather (Moussa, 1957; Johnston *et al*., 1983) as well as a facultative air-breather (Magid, 1971; Babiker, 1979). These conflicting statements can be (partially) explained by the fact that multiple factors are involved in the dependence on air-breathing for catfish, i.e. temperature, nutritional state, disturbance, activity level, *et cetera*. Despite these opposing statements in literature, aerial oxygen without a doubt represents an important source of oxygen for *C. gariepinus*.

In the facultative air-breathing *Clarias*, low aquatic oxygen tensions induced an increase in oxygen uptake from the air (Jordan, 1976; Johnston *et al*., 1983). Oxygen uptake from the water was reduced (Johnston *et al*., 1983), although the frequency of gill ventilation increased (Babiker, 1979; Bevan and Kramer, 1987). As a result the total oxygen consumption stayed relatively constant, except at extreme low pO_2-levels (Jordan, 1976; Johnston *et al*., 1983). In an obligate air-breather, such as the African lungfish (*Protopterus aethiopicus*), low aquatic oxygen tensions does not affect the aerial ventilation (Johansen en Lenfant, 1968), while low aerial oxygen tensions induce a strong increase in lung ventilation (Jesse *et al*., 1967; Johansen en Lenfant, 1968).

In the armoured catfish (*Glyptoperichtys gibbiceps*), low aquatic oxygen tensions in combination with no air-breathing caused a massive rise in plasma lactate levels from around 1 mM to over 65 mM (MacCormack *et al*., 2003). Although these levels appear unrealistically high, it stresses the importance of access to air in air-breathing fish. When African lungfish were prevented from surfacing, they built up an oxygen debt, which was paid off by a marked hyperventilation when allowed air-breathing again; unfortunately, no plasma lactate levels were measured (McMahon, 1970). In Australian lungfish (*Neoceratodus forsteri*, a facultative air-breather), plasma lactate levels remained at basal levels (<1 mM), when the aquatic oxygen tensions were low (pO_2 of 40 mm Hg) for 2 weeks (Kind *et al*., 2002). From the literature on air-breathing fish, it appears that the oxygen uptake from the air by air-breathing fishes is sufficient to sustain complete aerobic metabolism even at low aquatic oxygen tensions. This implies that at low aquatic oxygen tensions air-breathing fishes are metabolically not in a hypoxic condition.

Recently, Perry and co-workers (2004) published a study on the adrenergic response of an air-breathing fish, the jeju (*Hoplerythrinus unitaeniatus*), to environmental hypoxia. When the jeju was allowed to breathe air, lowering the aquatic oxygen content did not mediate an increase in plasma catecholamines. However, when the fish could not breathe air, there was a marked

increase in plasma catecholamines from around 3.3 nM to around 60 nM when the aquatic oxygen tension was 10-20 mm Hg. From these and other data on water-breathing species they concluded that the key variable for catecholamine secretion is the blood oxygen content without a relation to the mode of breathing. Hence, in this air-breathing fish the denial of access to air induces an adrenergic stress response comparable to water-breathing fish species.

The dominant catecholamine in hypoxic jeju was adrenaline, but no ratio between both catecholamines was provided. Hypoxia was quickly inflicted on the air-breathing jeju, namely by actively removing the oxygen from the water by bubbling nitrogen, and at the same time air-breathing was made impossible. Possibly, the hypoxic challenge was too sudden to induce a gradual adaptation as seen in hypoxic carp (Van Raaij *et al*., 1996a) and tilapia (Vianen *et al*., 2002), where oxygen levels were gradually reduced due to the fishes' own oxygen consumption. The period, in which the pO_2 of the water is reduced, is known to affect the ratio of adrenaline and noradrenaline released into the circulation (Perry *et al*., 1991). Possibly, this resulted in a larger release of adrenaline in comparison to noradrenaline in hypoxic jeju.

In erythrocytes of the air-breathing tarpon (*Megalops cyprinoides*), adrenaline induced an increase in red blood cell volume thereby enhancing the oxygen carrying capacity of the blood during high activity (Wells *et al*., 2003). This effect could be abolished by addition of propanolol, a β-adrenoceptor blocker. This indicates that β-adrenoceptors are involved in erythrocyte swelling in the tarpon as is known from water-breathing fish. Both the study of Perry *et al*. (2004) and Wells *et al*. (2003) indicate that in air-breathing fish the role of catecholamines in optimising the respiratory function is comparable to water-breathing fish. It is, however, still unknown what the adrenergic effects are on the metabolism of air-breathing fishes.

Aims of the investigations

Van den Thillart *et al*. (2002) hypothesised that the suppressive role of noradrenaline on plasma FFA levels was a protective mechanism against fatty acid poisoning under hypoxia. For many fish species, environmental hypoxia is a frequently occurring phenomenon, making such a mechanism vital. Indeed, all studies so far demonstrated a suppressive effect of noradrenaline on plasma FFA levels, while for adrenaline opposing effects are reported. In mammals, on the other hand, noradrenaline and adrenaline both stimulate plasma FFA levels. When considering hypoxic stress, a crucial difference between mammals and fish is the mode of breathing, air- and water-breathing, respectively. The access to aerial oxygen makes environmental hypoxia not a threat to

mammals and apparently the role of noradrenaline on lipid metabolism changed during evolution. The central hypothesis of this thesis is, therefore, that the change in the role of noradrenaline from fish to mammals is connected to the transition from water to air-breathing. There are, however, many fish species that have developed a form of air-breathing, originally thought of as a way to counteract the negative effects of low aquatic oxygen tensions (Graham, 1997). Like mammals, air-breathing fish have access to a high and constant source of oxygen, namely air. It is, therefore, likely that environmental hypoxia does not affect the metabolism of air-breathing fishes, as is observed in water-breathing fishes. Therefore, we hypothesise that the suppressive effect of noradrenaline is redundant in air-breathing fish. The work of this thesis focuses on the role of noradrenaline in the control of FFA metabolism in the air-breathing African catfish (*Clarias gariepinus* Burchell 1822).

In ***Chapter 1***, a general introduction on the thesis is provided, while chapters 2 to 7 comprise experimental work. Classical sampling techniques are known to induce a release of catecholamines. Therefore, this sampling technique can not be used when studying the role of catecholamines under hypoxia as well as after direct administration. The endogenous release of noradrenaline can be avoided by applying a cannula for blood sampling and infusion of hormones.

In ***Chapter 2***, the selection of a suitable cannulation technique for African catfish is described. Cannulation of the dorsal aorta proved to result in less blood loss than cannulation of a branchial artery. With dorsal aorta cannulated catfish, a clear fluctuation of the blood metabolites, glucose and FFA, was demonstrated, which has to be taken into account in the interpretation of coming metabolic experiments.

Chapter 3 describes the metabolism of African catfish when it is forced to switch from bimodal respiration to only aquatic respiration, i.e. asphyxia. It proved that in asphyxic African catfish there was a permanent accumulation of plasma lactate levels, indicating a switch to an anaerobic metabolism. During asphyxia, there was also a strong decrease in plasma FFA levels with a concomitant rise in plasma noradrenaline levels, while plasma adrenaline levels stayed low. These results suggests that normal water respiration induces a hypoxic metabolism in African catfish when no aerial oxygen is available, and that noradrenaline has a similar suppressive effect on plasma FFA levels, like observed in other, water-breathing fish species.

In ***Chapter 4***, an alternative approach was applied to elucidate the role of noradrenaline in the air-breathing African catfish. Noradrenaline was infused via a cannula fitted in the dorsal aorta, which was also used for blood sampling. As previous studies (Van den Thillart *et al*.,

2001, Vianen *et al*., 2002) have shown that the effect of noradrenaline is mediated by stimulation of β-adrenoceptors, also the nonselective β-agonist isoproterenol was infused. Additionally, the subtype of the adrenoceptor mediating the response was identified by combining the isoproterenol infusion with injection of specific antagonists for the β_1- and β_2-adrenoceptors, namely atenolol and ICI 118,551. The results indicate that noradrenaline as well as isoproterenol significantly reduced the plasma FFA levels. The adrenoceptor mediating this response was identified as the β_2-adrenoceptor. These data indicate that in African catfish noradrenaline reduces plasma FFA levels by stimulation of the β_2-adrenoceptor.

In ***Chapter 5***, adipocytes were isolated from the mesenteric adipose tissue of African catfish because this tissue is the most dynamic storage site of FFA and thus the main organ controlling plasma FFA levels. The same combination of agonist (isoproterenol) and antagonists (atenolol and ICI 118,551) was used as in Chapter 4 and the results confirm that stimulation of the β_2-adrenoceptor on adipose tissue inhibited the FFA release. Previous studies were not able to show whether lipolysis was inhibited or reesterification was stimulated. By measuring both FFA and glycerol release, we concluded that adipose lipolysis was inhibited by stimulation of β-adrenoceptors and that reesterification was concomitantly reduced.

There is very little information on lipolysis and its adrenergic control in fish adipocytes except for tilapia and African catfish (chapter 5). Therefore in ***Chapter 6***, adipocytes were isolated from two fish species from different ecological background: the cold freshwater Rainbow trout (*Oncorhynchus mykiss*) and the warm seawater Gilthead sea bream (*Sparus aurata*). The basal lipolytic rate of trout adipocytes was comparable to African catfish adipocytes, when the nutritional state and age were taken into account. The basal lipolytic rate of sea bream adipocytes was, however, much lower, suggesting a minor role for adipose tissue in this species in supplying FFA as energy metabolite. There was no adrenergic effect on the lipolytic rate in sea bream adipocytes. The lipolytic rate in the trout adipocytes was inhibited by noradrenaline, albeit a to far lower extent than in adipocytes from African catfish. Surprisingly, also adrenaline reduced the lipolytic rate in trout and catfish adipocytes, while on the organismal level adrenaline stimulates plasma FFA levels in carp. This apparent enigma can be explained by hypothesising a clear difference between adrenaline and noradrenaline in receptor affinity and an adrenergic stimulation of hepatic lipolysis.

This hypothesis was tested in ***Chapter 7***. Hepatocytes were isolated from rainbow trout and were incubated with adrenaline, noradrenaline or isoproterenol. To identify the adrenoceptor involved, isoproterenol incubations were combined with atenolol or ICI 118,551. A clear

adrenergic stimulation of hepatic glucose release was demonstrated, which was mediated by the β_2-adrenoceptor. There was a higher receptor affinity for adrenaline than for noradrenaline. Adrenergic stimulation also enhanced hepatic lipolysis albeit only at high, pharmacological concentrations. Hence, opposing effects of adrenaline and noradrenaline on plasma FFA levels can be explained by differences in receptor affinity as we hypothesised.