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

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The Adrenergic Control of Hepatic Glucose and FFA Metabolism in Rainbow Trout (*Oncorhynchus mykiss*); Increased Sensitivity to Adrenergic Stimulation with Fasting.

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Abstract

The adrenergic control of glucose and FFA release was studied in hepatocytes of Rainbow trout (*Oncorhynchus mykiss*), which were either normally fed or fasted for 3 weeks. Isolated hepatocytes were incubated with adrenaline, noradrenaline or isoproterenol (nonselective β-agonist). To identify the adrenoceptor involved, isoproterenol incubations were combined with atenolol (selective β_1-antagonist) or ICI 118,551 (selective β_2-antagonist). In this study, pharmacological tools were used in fish to both identify a β-adrenoceptor and to quantify the difference in its affinity for adrenaline and noradrenaline. The affinity of the β_2-adrenoceptor, mediating the glucose release, 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). A significant effect of β-adrenoceptor stimulation on FFA release was also found, although only at high concentrations (i.e. 10^{-6} and 10^{-5} M). Again the β_2-adrenoceptor appeared to mediate the stimulation of hepatic FFA release. Upon fasting, both the basal glucose and FFA release were strongly decreased. The ratio between glucose and FFA release decreased from 15.4 to 4.3 upon fasting and at this ratio the energy output for both metabolites became equal. The mobilisation of FFA upon adrenergic stimulation was relatively conserved, namely -35% upon fasting, as opposed to a decrease of 89% in mobilisation of glucose. This indicates that upon fasting FFA gain importance in hepatic metabolism. For the first time, it is demonstrated that the hepatic sensitivity to adrenergic stimulation is enhanced upon fasting.

Introduction

In mammals, the catecholamines, adrenaline and noradrenaline, mediate a strong increase in plasma FFA levels (Saleh *et al*., 1999). For fish however, opposing adrenergic effects have been reported in literature. Adrenaline induced a rise in plasma FFA concentrations in eel (*Anguilla anguilla*; Larsson, 1973), lamprey and scorpion fish (Plisetskaya, 1980), but a decrease in goldfish (*Carassius auratus*; Minick and Chavin, 1973), pike (*Esox lucius;* Ince and Thorpe, 1975) and Rosy barb (*Puntius conchonius*; Khanna and Singh, 1984). Noradrenaline, on the other hand, consistently lowered plasma FFA levels (Farkas, 1967a, b; Ince and Thorpe, 1975; Minick and Chavin, 1973; Van den Thillart *et al*., 2001). These potentially opposing effects were well demonstrated by Van Raaij *et al*. (1995) in cannulated carp (*Cyprinus carpio*), in which plasma FFA levels were stimulated by adrenaline but decreased by noradrenaline.

The physiological relevance of opposing adrenergic effects on FFA metabolism in fish is that different stress conditions can mediate different metabolic effects through a differentiated catecholamine release. During stress situations such as exhaustive swimming, where there is an increased demand for energy metabolites like FFA, the dominating catecholamine in trout (*Oncorhynchus mykiss*) is adrenaline (Butler *et al*., 1986; Tang and Boutilier, 1988). During stress situations such as hypoxia, where plasma FFA levels need to be reduced to protect against lipid poisoning (Van den Thillart *et al*., 2002), noradrenaline is the dominating catecholamine in carp (Van Raaij *et al*., 1996b) and tilapia (Vianen *et al*., 2002) mediating a reduction in plasma FFA levels. In hypoxic trout, adrenaline is generally the dominating catecholamine (Fiévet *et al*., 1990; Reid and Perry, 1994, 2003; Thomas *et al*., 1991), although some studies found predominantly noradrenaline (Montpetit and Perry, 1998; Van Raaij *et al*., 1996b). However, plasma FFA levels (Van Raaij *et al*., 1996a; Vianen, 1999) and/or FFA mobilisation (Haman *et al*., 1997) are reduced in hypoxic trout is, most likely due to an inhibition of lipolysis in adipose tissue (Van den Thillart *et al*., 2002; Vianen *et al*., 2002). Vianen *et al*. (2002) showed that β-adrenergic stimulation in isolated tilapia adipocytes (*Oreochromis mossambicus*) significantly decreased the FFA release. Because both adrenergic effects on FFA metabolism in carp were mediated by β-adrenoceptors, Van den Thillart *et al*. (2001) hypothesised that a stimulatory β_2-adrenoceptor was present on the liver, while an inhibitory β_1-adrenoceptor was present on the adipose tissue. Thus far, only one study demonstrated a catecholamine-mediated increase in hepatic lipolysis in fish, namely in Coho salmon (*Oncorhynchus kisutch*, Sheridan, 1987).

A classic response to catecholamine administration in fishes 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; Reid *et al*., 1992; Wright *et al*., 1989). *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; Fabbri *et al*., 1995a; Moon *et al*., 1993). In trout, only one β-adrenoceptor is present on the liver, identified as a β_2-adrenoceptor (Dugan and Moon, 1998; Fabbri *et al*., 1995a; McKinley and Hazel, 1993; Nickerson *et al*., 2001, 2003; Reid *et al*., 1992).

In mammals, both catecholamines stimulate β_1- as well as β_2-adrenoceptors. There is, however, a clear difference in affinity; adrenaline has a higher affinity for β_2-adrenoceptors while noradrenaline for β_1-adrenoceptors (Milligan *et al*., 1994). To potentiate opposing adrenergic effects on FFA metabolism in fish, there has to be an affinity difference comparable to mammals. Such a difference in affinity has been demonstrated for the β-adrenoceptor on trout erythrocytes (Tetens *et al*., 1988). Based on this affinity difference, this adrenoceptor was typed as of the β_1-subtype (Tetens *et al*., 1988), but based on molecular data, it appeared to belong to the β_3-subtype (Nickerson *et al*., 2003). It is not known whether the same difference in adrenoceptor affinity exists for the β_2-adrenoceptor found on trout hepatocytes.

For many fish species in the temperate zone, fasting is a regular occurring situation, as most species do not or hardly feed during the winter period (Jezierska *et al*., 1982). During these fasting periods in trout, lipids are a major energy source (Pottinger *et al*., 2003). Hepatic lipids are mobilised only in the first stage of fasting while FFA from the mesenteric fat depots are mobilised throughout fasting (Jezierska *et al*., 1982). As glucagon and insulin are important modulators of hepatic metabolism during fasting (Navarro and Gutierrez, 1995), changes in the responsiveness of fish hepatocytes to these hormones during fasting has frequently been studied (Foster and Moon, 1993; Gutierrez and Plisetskaya, 1991; Harmon and Sheridan, 1992a; Klee *et al*., 1990). However, changes in the responsiveness to catecholamines, which are very potent stimulators of hepatic glucose release (Fabbri *et al*., 1998a), have received little attention.

In this study, we tested the hypothesis that adrenergic stimulation of isolated trout hepatocytes leads to an increase in FFA release; the well-known increase in glucose release serves as a reference. To enable opposing *in vivo* effects of adrenaline and noradrenaline, a clear difference in adrenoceptor affinity has to be present. Therefore, hepatocytes were incubated with 3 different catecholamines (adrenaline, noradrenaline and isoproterenol) and the β-adrenoceptor

mediating these effects was identified by combined incubations with the nonselective β-agonist, isoproterenol, and selective antagonists, atenolol (β_1-antagonist), or ICI 118,551 (β_2-antagonist). The same experiments were conducted with hepatocytes from trout fasted for 3 weeks to evaluate if the adrenergic control of hepatic metabolism was altered upon fasting.

Materials and methods

Experimental animals

Rainbow trout (*Oncorhynchus mykiss*) of 151.7 ± 2.0 gr were obtained from a commercial trout farm (Hol Viskwekerij, Vaassen, The Netherlands). The fish were kept in a well-aerated recirculation system in 400-l tanks at a water temperature of 17.0ºC. The fish were fed from 11 a.m. to 13 p.m. at a feeding rate of 1.5% BW/day. After 20 days of acclimatisation to these conditions, the group was split randomly into two groups. Hepatocyte isolations were started in the first group of 16 fish, which were normally fed. The other group of 13 fish was fasted for 3 weeks. Hepatocyte isolations were performed 2 days before and 3 days after the end of this 3-week fasting period. All experiments were approved by the board on Experimentation on Animals of the Leiden University.

Isolation and incubation procedure

Hepatocytes were isolated according to the procedure described by Moon *et al*. (1985). In short, the procedure was as follows. A modified Hanks' medium was used which was pregassed with 5% CO_2 in O_2, the pH was adjusted to 7.6 at 20ºC and contained in mM: 136.9 NaCl, 5.4 KCl, 0.80 $MgSO_4$, 0.33 Na_2HPO_4, 0.44 KH_2PO_4, 5.0 Hepes, 5.0 Hepes.Na, and 5.0 $NaHCO_3$. This medium was used to prepare all other solutions as listed in Table 1.

For each experiment, one or two fish were stunned by a blow to the head and killed by cutting the spinal cord. The vena hepatica was cannulated and the liver was rinsed by perfusion with rinsing solution for approximately 5 min. Subsequently, the liver was perfused for 10-15 min with the collagenase solution to enzymatically digest the collagen bonds between the hepatocytes. When the liver showed signs of degradation, the perfusion was stopped. The liver was manually chopped to pieces and filtered with rinsing solution through a 250-µm mesh followed by a 72-µm mesh. Next, the cells were washed and centrifuged at 50-60 g in

Table 1. Solutions which were used in hepatocyte isolation of trout and were based on a modified Hanks' medium.

Solution as named in text	Additions to the modified Hanks' medium
Rinsing solution	+ 1.0 mM EGTA
Collagenase solution	+ 95 IU/ml collagenase type IV
4% BSA solution	+ 4% BSA (w/v), + 1.5 mM $CaCl_2$
Incubation medium	+ 2% BSA (w/v), + 1.5 mM $CaCl_2$, + 5.5 mM Mannitol, + 0.05 mg/ml $Na_2S_2O_5$ as antioxidant

subsequently, rinsing solution, rinsing solution mixed 1:1 with 4% BSA solution, 4% BSA solution alone, and 4% BSA solution mixed 1:1 with incubation medium. After this last step, the cells were resuspended in incubation medium and given a 60-min metabolic pause. After this pause, the supernatant was removed and the cells were resuspended in incubation medium. Right away, portions of on average $1.02 \pm 0.17 \cdot 10^6$ cells were incubated for 6 h with or without agonist/antagonists in 1 ml incubation medium. All incubations were done in triplicates in a waterbath of 17.0ºC gassed with 5% CO_2 in O_2, under continuous shaking at 60 rpm. Samples of the medium for FFA and glucose measurements were put immediately in a –80ºC freezer and measured within 1 day.

To establish the basal FFA and glucose release by the hepatocytes, the incubation medium was sampled after 0, 1, 2, 4 and 6 h. Different pharmacons were added using incubation medium without BSA as a vehicle. To evaluate the effect of the different pharmacons the medium was sampled at the end of the 6-h incubation period. As agonists, adrenaline, noradrenaline and isoproterenol (nonselective β-agonist) were added in concentrations ranging from 10^{-10} to 10^{-5} M. As antagonist the selective β_1-antagonist, atenolol, and the selective β_2-antagonist, ICI 118,551, were used at 10^{-6} M in combination with isoproterenol. In a combined incubation, the antagonist was added 15 min prior to the agonist.

Chemicals and analysis

Bovine Serum Albumin fraction V (BSA), collagenase type IV, isoproterenol-hydrochloride, noradrenaline-bitartrate, adrenaline-bitartrate and ICI 118,551-hydrochloride were obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands). Atenolol-hydrochloride was a kind gift from AstraZeneca (Zoetermeer, The Netherlands). All other chemicals were of analytical grade.

The viability of the cells was assessed by trypan blue exclusion. The FFA concentration in

the incubation medium was measured using a commercial kit according to the NEFA-C method from WAKO chemicals (Instruchemie, Delfzijl, the Netherlands) using palmitic acid as an internal standard. The glucose concentration was measured using an enzymatic testkit (Instruchemie, Delfzijl, the Netherlands) using an internal standard.

Data analysis and statistics

The data are presented as mean ± SEM of 9 experiments for the fed group and 6-7 experiments for the fasted group. Using Sigmaplot 5.00, a regression analysis on the mean release over time was performed according to the equation: $Y= a\cdot(1-e^{-b\cdot X})$.

All data on the incubation with hormones were normalised to the basal release over 6 h. The effect of the hormones was evaluated as the relative effect over the basal release. On the mean of these data, a sigmoïdal regression was performed; based on the derived equations pD_2-values were calculated as indicators of the apparent affinity of the adrenoceptors. The pD_2-values are defined as the negative logarithm of the ED_{50}; the ED_{50} is the concentration at which 50% of the maximal increase is reached. The pD_2-values were calculated as this parameter is commonly used in pharmacological studies and, as opposed to ED_{50}-values, a higher pD_2-value implies a higher apparent affinity. As pD_2-values could only be calculated on the mean values, no statistical analysis could be performed.

Statistical differences ($p<0.05$) in general characteristics between both groups were tested with a student's t-test. Differences within a group between treatments and the basal release, and differences between treatments without and with antagonists were tested with a repeated measures Anova on ranks according to Dunnett's method. Differences within a group between the 3 different agonists were tested with a repeated measures Anova on ranks according to Student-Newman-Keuls method. Differences between groups in isoproterenol effect were tested with a Mann-Whitney Rank sum test. Statistical analysis was performed with the program Sigmastat 2.0.

Results

General data

The body weight of fasted fish was significantly lower than that of fed fish (Table 2). The

Table 2. General data of trout hepatocytes isolated from either fed fish (*n*=16) or fish fasted for 3 weeks (*n*=13).

	Fed fish	Fasted fish	P (t-test)
Body weight [gr]	173.4 ± 7.9	144.2 ± 6.5	0.008
Liver weight [gr]	2.81 ± 0.22	1.20 ± 0.09	<0.001
HSI [%] [1)]	1.60 ± 0.09	0.83 ± 0.05	<0.001
Viability [%] [2)]	87.3 ± 2.1	71.7 ± 4.1	0.006

1: Hepatosomatic index as percentage of the body weight.
2: *n*=9 for fed fish and *n*=7 for fasted fish.

liver weight had significantly decreased but to a larger extent than the body weight as the relative liver weight of fasted fish (HSI) was significantly different from that of fed fish. The viability of hepatocytes from fasted fish was significantly lower than that of hepatocytes from fed fish.

Basal glucose and FFA release

Both the glucose and FFA concentration in the medium increased over time in both feeding states (Fig. 1). The basal release over 6 h for trout hepatocytes from fed fish was 590.2 ± 147.5 nmol glucose and 42.0 ± 9.6 nmol FFA/10^6 cells. For trout hepatocytes from fasted fish, the basal release rate over 6 h was 39.7 ± 16.3 nmol glucose and 9.4 ± 1.3 nmol FFA/10^6 cells.

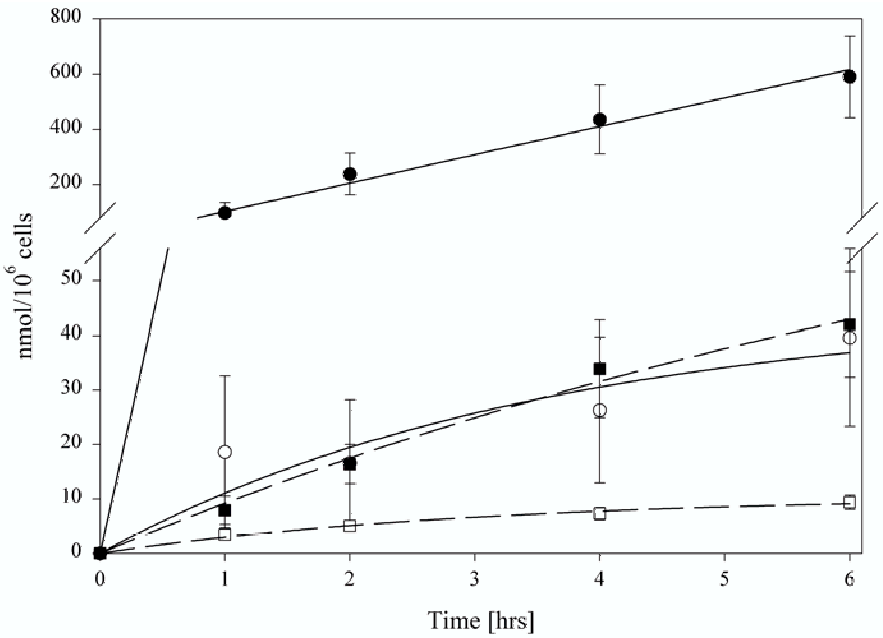

Fig. 1. Glucose (○) and FFA (□) release over time per million hepatocytes of trout, which were either fed (*n*=9; black) or fasted for 3 weeks (*n*=7; white). Exponential regressions were based on the formula: $Y= a\cdot(1-e^{-b\cdot X})$; R^2 for the glucose release by hepatocytes of fed and fasted fish was 0.990 and 0.892, respectively, and for the FFA release 0.993 and 0.991.

Although release rates of both metabolites were significantly lowered upon fasting, the decrease in glucose release was both in absolute and relative numbers significantly larger than the decrease in FFA release. Thus, the ratio between the glucose and FFA release significantly changed from 15.1 ± 4.0 for fed fish to 4.3 ± 1.8 for fasted fish.

Adrenergic effect on glucose release

In fed fish, all three catecholamines had a significant dose-response effect on the hepatic glucose release (Fig. 2). The maximal adrenergic effect on glucose release by hepatocytes from fed trout was an increase of 104.4 ± 5.9%. The dose-response curves of adrenaline and isoproterenol were rather close to each other as follows from pD_2-values of 8.3 and 7.9, respectively. At 3 concentrations (10^{-8}, 10^{-7} and 10^{-6} M), the curve of noradrenaline lay below the curves of

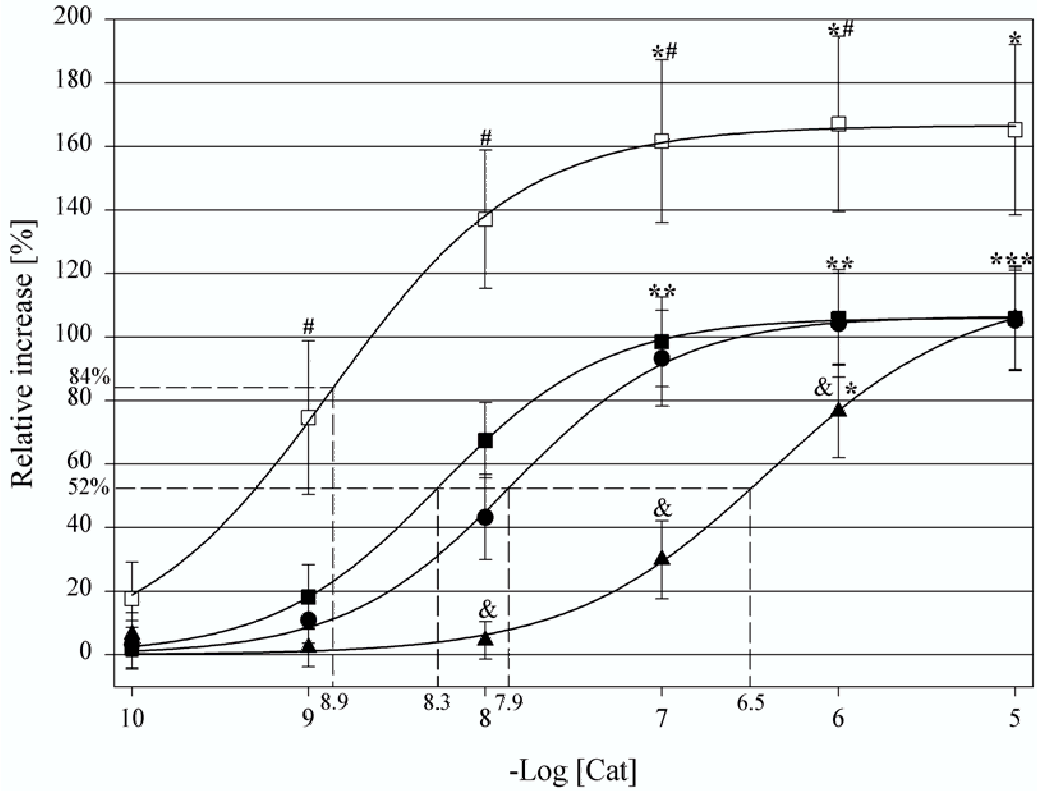

Fig. 2. Relative effect of adrenergic stimulation on the glucose release by trout hepatocytes (normalised to the basal release over 6 h). Hepatocytes were isolated from fed fish (*n*=9; black symbols; basal release of 590.2 ± 147.5 nmol/10^6 cells/6 h) or from fish fasted for 3 weeks (*n*=6, white symbols; basal release of 39.7 ± 16.3 nmol/10^6 cells/6 h). Cells were incubated with adrenaline (●), noradrenaline (▲) or isoproterenol (nonselective β-agonist, ■,□). Dotted lines indicate pD_2-values. *: $p<0.05$ vs. basal release; &: $p<0.05$ noradrenaline vs. isoproterenol and vs. adrenaline; #: $p<0.05$ isoproterenol effect in fed vs. fasted fish.

adrenaline and isoproterenol. Hence, the dose-response curve of noradrenaline showed a right shift of almost 2 decades (pD_2-value of 6.5).

When fish were fasted, the absolute increase in hepatic glucose release mediated by isoproterenol was much smaller than in fed fish. However, the relative increase upon β-adrenoceptor stimulation was significantly larger at the whole concentration range (10^{-9} to 10^{-6} M); the maximal adrenergic effect on glucose release by hepatocytes from fasted trout was 167.1 ± 27.6% at 10^{-6} M. The sensitivity to adrenergic stimulation was also higher in hepatocytes from fasted fish as indicated by an increase of the pD_2-value for isoproterenol by half a unit from 8.3 to 8.9.

Blockage of the β_1-adrenoceptor in hepatocytes of fed fish (Fig. 3A) had no significant effect on the dose-response curve of isoproterenol. The pD_2-value for the β_1-blockage curve was 8.3, which is identical to isoproterenol. Blockage of the β_2-adrenoceptor resulted in a significantly lowered glucose release at isoproterenol concentrations of 10^{-9} to 10^{-7} M. The dose-response curve had clearly shifted to the right as indicated by a lower pD_2-value of 7.4.

In fasted fish (Fig. 3B) blockage of the β_1-adrenoceptor had no effect on the dose-response curve, while blockage of the β_2-adrenoceptor resulted again in a significantly lower glucose release (isoproterenol: 10^{-9} - 10^{-7} M). The pD_2-value of the β_2-blockage curve was 7.7 as opposed to 8.9 for the isoproterenol curve.

Adrenergic effect on FFA release

All 3 catecholamines had equal effects on the FFA release in fed fish (Table 3): at 10^{-5} M, the stimulatory effect on the FFA release was significant as compared to the basal FFA release. In starved fish, however, the effect of β-adrenoceptor stimulation was significant at 10^{-6} and 10^{-5} M isoproterenol. The maximal increase in FFA release by hepatocytes from fed trout was 35.1 ± 4.7%, while the maximal increase by hepatocytes from starved fish was 100.7 ± 21.9%. As no pharmacological dose-response curves were found, no pD_2-values were calculated.

Blockage of either of the two β-adrenoceptors in hepatocytes from fed fish had no significant effects on the isoproterenol effect; again, FFA release was significantly stimulated at 10^{-5} M. In hepatocytes from fasted fish, blockage of the β_1-adrenoceptor had no effect. Blockage of the β_2-adrenoceptor, on the other hand, showed a significant lower FFA release at 10^{-5} M isoproterenol.

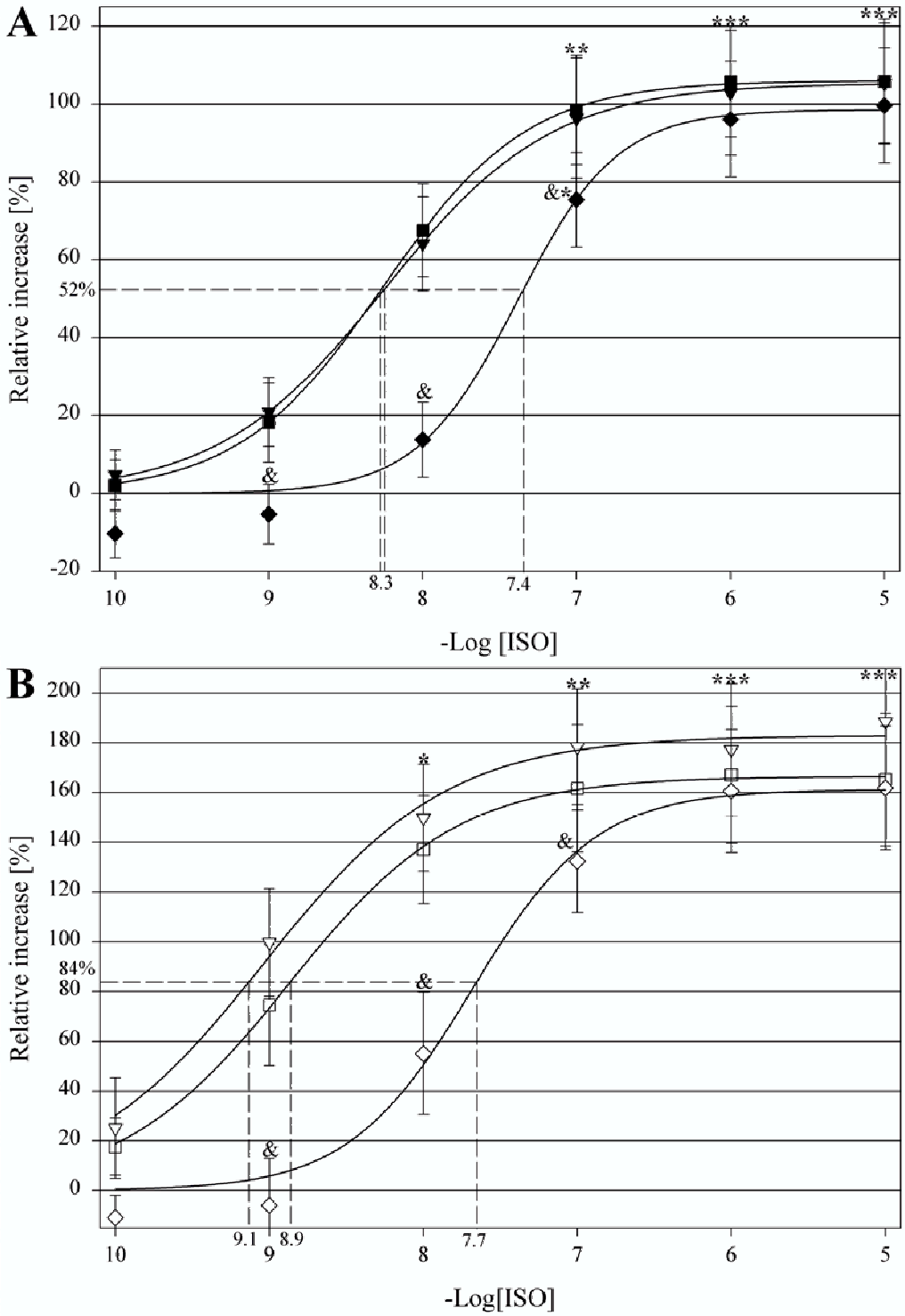

Fig. 3. Relative effect of β-adrenoceptor stimulation on the glucose release by trout hepatocytes (normalised to the basal release over 6 h). Hepatocytes were isolated from fed fish (A; *n*=9; basal release of 590.2 ± 147.5 nmol/10^6 cells/6 h) or from fish fasted for 3 weeks (B; *n*=6; basal release of 39.7 ± 16.3 nmol/10^6 cells/6 h). Cells were incubated with isoproterenol (nonselective β-agonist, □,■), isoproterenol with atenolol (1 µM, selective β_1-antagonist, ▽,▼), and isoproterenol with ICI 118,551 (1 µM, selective β_2-antagonist, ◇,◆). Dotted lines indicate pD_2-values. *: $p<0.05$ vs. basal release, &: $p<0.05$ atenolol + ISO or ICI 118,551 + ISO vs. isoproterenol alone.

Table 3. Relative effect of adrenergic stimulation on the FFA release (normalised to the basal release over 6 h). Trout hepatocytes were isolated from fed fish (n=9; basal release of 42.0 ± 9.6 nmol FFA/10^6 cells/6 h) or from fish fasted for 3 weeks (n=6; basal release of 9.4 ± 1.3 nmol FFA/10^6 cells/6 h). Cells were incubated with adrenaline (ADR), noradrenaline (NA) or isoproterenol (ISO, nonselective β-agonist); isoproterenol incubations were also combined with atenolol (At, 1 μM, selective β_1-antagonist), or ICI 118,551 (ICI, 1 μM, selective β_2-antagonist).

	Fed fish				
[Cat]	ISO	ISO+At	ISO+ICI	ADR	NA
10^{-10} M	0.4 ± 4.2%	2.6 ± 6.2%	5.7 ± 4.3%	11.9 ± 8.7%&	8.9 ± 6.4%&
10^{-9} M	4.9 ± 4.1%	2.2 ± 5.2%	5.1 ± 5.3%	9.9 ± 7.7%	7.9 ± 4.6%
10^{-8} M	4.1 ± 5.5%#	3.1 ± 7.4%	3.3 ± 5.8%	1.7 ± 5.8%	6.0 ± 5.0%
10^{-7} M	10.6 ± 10.7%#	1.5 ± 8.6%	3.9 ± 6.0%	5.3 ± 5.2%	4.7 ± 6.1%
10^{-6} M	13.0 ± 9.1%#	12.9 ± 9.3%	16.7 ± 9.5%	16.3 ± 5.4%&	17.7 ± 7.2%&
10^{-5} M	34.9 ± 14.5%*#	25.4 ± 9.3%*	23.6 ± 7.9%*	38.9 ± 12.0%*	50.6 ± 13.2%*
	Fasted fish				
[Cat]	ISO	ISO+At	ISO+ICI		
10^{-10} M	14.5 ± 8.2%	23.0 ± 9.9%	0.9 ± 5.7%		
10^{-9} M	13.8 ± 8.8%	15.8 ± 7.6%	6.2 ± 6.6%		
10^{-8} M	29.0 ± 11.2%	23.3 ± 9.6%	18.0 ± 8.0%		
10^{-7} M	26.1 ± 6.6%	31.8 ± 9.2%*	15.8 ± 8.4%		
10^{-6} M	56.1 ± 12.0%*	60.0 ± 14.0%*	46.5 ± 15.6%*		
10^{-5} M	100.7 ± 21.9%*	102.6 ± 17.1%*	72.1 ± 20.8%*$		

*: $p<0.05$ vs. basal release; &: $p<0.05$ ADR or NA vs. ISO; #: $p<0.05$ isoproterenol effect in fed vs. fasted fish; $: $p<0.05$ ICI + ISO vs. ISO alone.

Discussion

General data

A reduced liver weight in fasted fish is commonly observed and mainly caused by glycogen and protein breakdown (Blasco *et al*., 1992; Foster and Moon, 1991; Moon *et al*., 1989). From a functional point of view, it is surprising that less viable hepatocytes could be isolated from fasted fish suggesting that these liver cells were more fragile. This corroborates with earlier observed liver cell damage upon fasting (Black and Love, 1986; Blasco *et al*., 1992).

Adrenergic effect on FFA release

The 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 (Table 2). Such high plasma catecholamine concentrations are rarely reached *in vivo* (Randall and Perry, 1992), raising doubt on the physiological relevance of an adrenergic stimulation of hepatic lipolysis in trout. However, noradrenaline concentrations can locally reach the micromolar range due to overflow

from adrenergic nerve terminals (Schömig and Richardt, 1990). In mammalian liver, direct innervation can stimulate hepatic glucose release (Shimazu, 1996); it is, however, unknown if the liver of fish is also directly innervated.

In this study, an adrenergic effect on hepatic lipolysis in trout was only found at pharmacological concentrations. Likewise in hepatocytes of copper rockfish (*Sebastes caurinus*), neither adrenaline nor noradrenaline had any significant effect on hepatic lipolysis (Danulat and Mommsen, 1990). These same authors stated, as unpublished results, that also in Coho salmon there is no adrenergic effect on hepatic lipolysis. These results are in contrast with those of Sheridan (1987), also on Coho salmon liver. This latter study is the only report of increased hepatic lipolysis upon adrenergic stimulation over a physiological relevant concentration range; hepatic lipolysis in Coho salmon was stimulated by noradrenaline at a minimum effective dose of 10^{-9} M and a pD_2-value of around 6.7 (calculated as negative log from an ED_{50} of $2 \cdot 10^{-7}$ M). Based on the stimulatory effect of isoproterenol, FFA mobilisation in this species was 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, differential effects of adrenaline and noradrenaline can be expected as is demonstrated for the hepatic glucose release in trout at a catecholamine concentration of 10^{-8} M (see Fig. 2). Scott-Thomas *et al*. (1992) demonstrated differential effects of adrenaline and noradrenaline at $2 \cdot 10^{-7}$ M on FFA release by hepatocytes of brook charr (*Salvelinus fontinalis*), in which FFA release was decreased (!) by noradrenaline and not by adrenaline.

McKinley and Hazel (1993) hypothesised that the isolation procedure affected the adrenergic receptors, rendering them incapable of mediating a response. It is unlikely that this is the case in our study in view of the clear pharmacological effect that was found in the hepatic glucose release upon adrenergic stimulation, which is in corroboration with other studies (see review by Fabbri *et al*., 1998a). In mammals, hepatic lipolysis is not affected by catecholamines as no hormone-sensitive lipase (HSL) is present (Debeer *et al*., 1979; Holm *et al*., 1987). Fish, on the other hand, may have a hepatic HSL as trout liver possesses a triacylglycerol lipase with characteristics comparable to HSL from mammalian adipose tissue (Harmon *et al*., 1991). Glucagon stimulated hepatic lipolysis in trout by phosphorylation of triacylglycerol lipase, mediated by an increase in cAMP levels (Harmon and Sheridan, 1992c; Harmon *et al*., 1993). This suggests that the prerequisites for an adrenergic stimulation of hepatic lipolysis in trout over a physiological range of catecholamines concentrations are present: a cAMP-activated lipase, an adrenergic signal transduction system mediating an increase in lipolysis, as demonstrated by this

study, and this adrenergic transduction system can be activated over a physiological concentration range, as demonstrated by this and other studies for glycogenolysis. For unknown reasons, there was a large difference between the adrenergic transduction systems mediating hepatic glycogenolysis and lipolysis. This can only be explained by assuming that the adrenergic signal transduction pathways of both processes are spatially separated.

Affinity of the β_2-adrenoceptor

The β_2-adrenoceptor mediated the hepatic glucose release as indicated by the clear rightward shift in the dose-response curve (Fig.2), which confirms the findings of previous studies (Dugan and Moon, 1998; Fabbri *et al*., 1995a; McKinley and Hazel, 1993; Nickerson *et al*., 2001, 2003; Reid *et al*., 1992). Like hypothesised, the affinity of the hepatic β_2-adrenoceptor for adrenaline was higher than for noradrenaline, which is reflected by a difference in pD_2-values of 1.4 log-units. In Table 4, pD_2-values from other studies are listed, which we calculated from the reported ED_{50}-values. For the β-adrenoceptor present on trout erythrocytes, a comparable but opposite difference in affinity has been demonstrated; the affinity for noradrenaline was higher than for adrenaline, namely pD_2-values of 7.9 and 6.1, respectively. Based on this difference the receptor was typed as a β_1-receptor (Tetens *et al*., 1988). However, on basis of molecular data,

Table 4. Affinity of adrenaline (ADR), noradrenaline (NA) and isoproterenol (ISO) for the β-adrenoceptor mediating the hepatic glucose release in different fish species. Affinity is expressed as a pD_2-value, which is calculated as the negative log of the agonist concentration at 50% response (ED_{50}). For comparison also the affinity for the β-adrenoceptor on trout erythrocytes is given at the bottom of the table.

Fish species	Adrenoceptor	ADR	NA	ISO	Reference
Common carp (*C. carpio*)	β	7.7	5.5 *	8.0	Janssens and Lowrey, 1987
Chinook salmon (*O. tshawytscha*)	β	6.7	6.7 *		Sheridan, 1988
Copper rockfish (*S. caurinus*)	β	6.5	8.2		Danulat and Mommsen, 1990
Rainbow trout (*O. mykiss*)	β_2	8.2 at 5ºC 6.3 at 20ºC			McKinley and Hazel, 1993
Rainbow trout (*O. mykiss*)	β_2	7.9	6.5	8.3	This study
Rainbow trout (*O. mykiss*)	β**	6.1	7.9	8.0	Tetens *et al*., 1988

*: A partial agonist as compared to adrenaline.
**: Originally typed as a β_1-adrenoceptor (Tetens *et al*., 1988), later with molecular tools typed as a β_3-adrenoceptor (Nickerson *et al*., 2003).

this receptor appears to belong to the β_3-subtype (Nickerson *et al*., 2003). Like in our study, the pD_2-value for hepatic glucose release in carp was higher for adrenaline (7.8) than for noradrenaline (5.5; Janssens and Lowrey, 1987) suggesting a β_2-adrenoceptor mediated effect. In copper rockfish, hepatic glucose release is most likely a β_1-adrenoceptor mediated effect as the pD_2-value for noradrenaline was higher than adrenaline, namely pD_2-values of 8.2 and 6.5 (Danulat and Mommsen, 1990). McKinley and Hazel (1993) reported a pD_2-value of 6.3 for adrenaline in trout liver acclimatised at 20ºC which is much lower than the value of 8.3 found at 17ºC in our study. The pD_2-value in the study of McKinley and Hazel increased however to 8.2 when the fish were acclimatised to 5ºC. In Chinook salmon (*Oncorhynchus tshawytscha*), the pD_2-values for adrenaline and noradrenaline were both around 6.7; noradrenaline was, however, only a partial agonist resulting in a lower glucose release than upon stimulation by adrenaline even at a pharmacological concentration of 10^{-3} M (Sheridan, 1988); this implies again a β_2-adrenoceptor mediated effect. A difference in affinity can also be illustrated by differences in minimum effective dose; in brook charr, only adrenaline but not noradrenaline, both at $2 \cdot 10^{-7}$ M, stimulated the hepatic glucose release (Scott-Thomas *et al*., 1992). This matches the dose-response curve as shown in Fig. 2, where noradrenaline had a minimum effective dose of 10^{-6} M.

As no clear pharmacological effect of adrenergic stimulation on FFA release by trout hepatocytes was found, identification of the β-adrenoceptor mediating hepatic lipolysis could not be as solid as the identification of the β-receptor mediating the hepatic glucose release. The blocking effect of ICI 118,551 at 10^{-5} M isoproterenol in hepatocytes of fasted fish (Table 3) suggests that the β_2-adrenoceptor mediates hepatic lipolysis in trout. As stated earlier, the β_2-adrenoceptor was shown to be the only receptor present on trout liver (Dugan and Moon, 1998; Fabbri *et al*., 1995a; McKinley and Hazel, 1993; Nickerson *et al*., 2001, 2003; Reid *et al*., 1992). Also on carp liver, a stimulatory β_2-adrenoceptor was identified using the same pharmacological tools as in this study (Van den Thillart *et al*., 2001). In brook charr (Scott-Thomas *et al*., 1992) and Coho salmon (Sheridan, 1987), hepatic lipolysis was also mediated by β-adrenoceptors, but no specific typing was performed.

The affinity difference for adrenaline versus noradrenaline as established for mammalian β-adrenoceptors (Milligan *et al*., 1994) has frequently been used in fish for β-adrenoceptor typing (e.g. Danulat and Mommsen, 1990; Janssens and Lowrey, 1987; Reid *et al*., 1992; Tetens *et al*., 1988). The use of this typing system in fish was, however, not validated with pharmacological or molecular tools. In this study, pharmacological tools were used in fish to both identify a β-adrenoceptor and to quantify the difference in its affinity for adrenaline and noradrenaline.

Effect of fasting on hepatic metabolism

In our study, the release of glucose and FFA by trout hepatocytes was strongly reduced upon fasting implying a reduced overall energy demand. A reduced energy metabolism appears to be a major fasting strategy in fish (Blasco *et al*., 1992; Foster and Moon, 1991). The large drop in hepatic glucose release as found for fasted trout in this study, has frequently been observed in fasted fish (Foster and Moon, 1991; Pottinger *et al*., 2003; Sheridan and Mommsen, 1991). The rate of glycogenolysis, accounting for the majority of glucose released by hepatocytes (Mommsen *et al*., 1988), is proportional to the amount of glycogen (Foster and Moon, 1991). Hence, the rapid but partial consumption of hepatic glycogen stores in fasted fish (Foster and Moon, 1991; Pottinger *et al*., 2003; Sheridan and Mommsen, 1991) induced a lower glucose release rate, as reported here for fasted trout. The absolute hepatic lipid content in fasting trout drops, but due to a reduction in liver weight, the relative hepatic liver content stays fairly constant (Jezierska *et al*., 1982). Thus, the reduced lipolytic rate in fasted trout is not due to reduced substrate levels, but likely due to a reduced energy metabolism.

After a 3-week fast, the decrease in glucose release was larger than the decrease in hepatic FFA release. This is reflected by a decrease in the ratio between glucose and FFA release from 15.4 to 4.3 upon fasting. Based on an energy content of –2854 KJ/mol for glucose and –9791 KJ/mol for palmitate, this means that the energy output in the form of glucose and FFA is almost equal after a 3-week fast, indicating that lipid metabolism had gained preference in fasted fish. Also in hepatocytes of yellow perch (*Perca flavescens*) after a 7-week fast, FFA had gained preference over glucose as the basal lipolytic rate was constant while the basal glycogenolytic rate was reduced (Foster and Moon, 1991). In Coho salmon (Sheridan and Mommsen, 1991), glycogen reserves were mobilised after 1 week fasting resulting in a transient hyperglycaemia, which had disappeared after 3 weeks fasting; by that time, plasma FFA levels and liver lipase activity had increased.

Remarkably, the mobilisation of FFA upon adrenergic stimulation was relatively conserved after a 3-week fast: in hepatocytes from fasted fish this was 9.5 nmol/10^6 cells/6 h (100.7% of 9.4 nmol/10^6 cells/6 h, Table 3) as opposed to 14.7 nmol/10^6 cells/6 h for hepatocytes from fed fish (34.9% of 42.0 nmol/10^6 cells/6 h), a reduction of 35%. This adrenergic mobilisation capacity for glucose, however, decreased almost 90% from 616.2 to 66.3 nmol/10^6 cells/6 h upon fasting. To our knowledge, our data demonstrate for the first time that upon fasting FFA gain importance in the hepatic metabolism.

The sensitivity of the hepatic signal transduction system in trout was enhanced upon fasting (Fig. 2). This enhanced sensitivity is reflected in an increased pD_2-value from 8.3 to 8.9 after a 3-week fast. The physiological relevance of the enhanced sensitivity of hepatocytes from fasted trout as reported in this study, can be deduced from a study by Vijayan and Moon (1992). These authors showed that fasted trout displayed a stronger and quicker increase in plasma glucose after acute stress, a situation linked to a powerful release of catecholamines. Foster and Moon (1993) showed that sensitivity of sea raven hepatocyte (*Hemitripterus americanus*) to glucagon was increased upon fasting. In catfish (*Ictalurus melas*) after a 1-week fast at 18-24ºC, glucagon had a stronger stimulatory effect on hepatic glucose release as compared to adrenaline at the same concentration (Ottolenghi *et al*., 1994). However, in Chinook salmon hepatocytes at 14ºC, stimulation of the glucose release by glucagon had disappeared after a 3-week fast, while the stimulation by adrenaline was conserved (Klee *et al*., 1990). This suggests that in salmonids catecholamines are more important than glucagon as modulators of hepatic metabolism during prolonged fasting. Our study demonstrates that the hepatic sensitivity to adrenergic stimulation in trout is enhanced upon fasting.

In summary, both hepatic glucose and FFA release in trout appear to be mediated by a β_2-adrenoceptor. The affinity of this β_2-adrenoceptor for adrenaline and isoproterenol was higher than for noradrenaline. After a 3-week fast, the basal glucose and FFA release by trout hepatocytes was strongly reduced. Upon fasting, FFA had gained relative importance in basal hepatic metabolism as well as in stress metabolism. Additionally, the hepatic sensitivity to adrenergic stimulation was enhanced in fasting trout.

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