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

Heeswijk, J.C.F. van

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Diel Fluctuations in Blood Metabolites in Cannulated African Catfish (*Clarias gariepinus*, Burchell 1822).

J.C.F. van Heeswijk, G.J. Vianen, and G.E.E.J.M. van den Thillart.

Department of Integrative Zoology, Institute of Biology Leiden, Leiden University, PO Box 9516, 2300 RA, Leiden, the Netherlands.

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Abstract

African catfish were cannulated in the dorsal aorta to study diurnal changes in blood metabolites. Cannulation of the branchial artery was tested but proved to be less successful. A clear diurnal fluctuation in the two major blood metabolites, free fatty acids (FFA) and glucose, was observed. As compared to the initial value at 8.30 in the morning, the plasma FFA levels dropped around 50% within 2 h, after which the FFA concentration stayed relatively constant. 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. The most astonishing finding of our study was the almost complete absence of glucose in the plasma of African catfish (0.05 ± 0.03 mM), a never reported phenomenon for any fish species. This study demonstrates the relatively low level of control of plasma glucose levels as compared to plasma FFA levels in African catfish.

Introduction

Classic blood sampling in fish involves catching and handling, thereby leading to strongly elevated catecholamine levels and thus changes in a variety of metabolic parameters (Mazeaud and Mazeaud, 1981). These stress effects are reduced when using cannulation techniques as cannulated fish have lower catecholamine levels than classically sampled fish (Woodward, 1982). Additionally, cannulation offers the opportunity for multiple sampling within a single individual, thereby reducing the required number of fish and the statistical effect of variation between individuals. Cannulation has thus become a routine technique in the study of fish metabolism (see review by Iwama and Ishimatsu, 1994). The most commonly cannulated vessel is the dorsal aorta as it is a large vessel with a known location and with easy access through the mouth. The disadvantage of dorsal aorta cannulation is that it is a blind cannulation as opposed to cannulation of a branchial artery, which can be visually located when the gill cover is lifted.

Clarias species are interesting for comparative physiology, as they are known air-breathing fishes and can thus provide us with more information on the physiological impact of this mode of breathing. However, no cannulation procedure for this species has been described in literature. Based on the description of cannulation of the non-related but anatomical comparable channel catfish (*Ictalurus punctatus*; Kitzman *et al*., 1988) and on the well-described anatomy of the blood circulation of *Clarias* species by Olson *et al.* (1995), commonly used cannulation techniques appear to be feasible for use in this species.

Diurnal rhythms in blood metabolites are common in vertebrates. As such rhythms include major changes in metabolic parameters, they are essential for evaluation of metabolic experiments, but are often not taken into account (Laidley and Leatherland, 1988). In most fish species, a diurnal fluctuation in plasma glucose is found (see review by Boujard and Leatherland, 1992a). Only a few studies report on the presence of diurnal fluctuations in free fatty acids (FFA) concentration in fish, mainly salmonids (Leatherland *et al*., 1974; Leatherland and Nuti, 1982; Boujard and Leatherland, 1992b; Boujard *et al*., 1993). In Seabass (*Dicentrarchus labrax*), a diurnal fluctuation in glycerol levels was found but its presence depended heavily on the type of diet and the moment of feeding (Carillo *et al*., 1982; Perez *et al*., 1988). In goldfish (*Carassius auratus*), a diurnal fluctuation in FFA was absent (Minick and Chavin, 1972).

African catfish were either cannulated in the branchial artery or in the dorsal aorta. Both techniques were evaluated on changes in blood parameters and on the number of patent cannulae. To obtain solid data from African catfish, diurnal fluctuations in blood parameters with special emphasis on glucose and FFA, were determined from catfish fitted with indwelling cannula.

Materials and Methods

Experimental animals

African catfish (*Clarias gariepinus*, Burchell 1822) were purchased from a commercial catfish farm (Fleuren, Someren, The Netherlands). The fish were kept in groups in a well-aerated recirculation system at 25 ± 0.5ºC with the following characteristics: total water volume of 4 m^3, O_2-content of >80% air-saturation, NH_3 <0.25 mg/l, NO_2 <0.3 mg/l and NO_3 <100 mg/l. The fish were fed once a day between 10 and 11 a.m. at maintenance level (~7 gr/kg BW) with a commercial catfish diet (Biomeerval, Trouvit, Putten, The Netherlands). The fish were kept at a light-dark cycle of 12:12 h with lights on at 8 a.m. The fish were acclimatised to these conditions for at least 2 weeks.

Experiment 1: Cannulation techniques

The experiments were conducted in flow chambers supplied with well-aerated water of 25ºC from a recirculation system of 2.2 m^3 with a water quality as stated in the previous section. The flow rate through the flow chambers was ~1 l/min. The flow chambers (lxbxh: 55x10x18 cm) were closed with a darkened lid to prevent startling of the fish by outside movements. The flow chambers contained ~2 cm of air to allow air-breathing. The fish were 40 to 45 cm long and could thus move back and forth freely. To prevent damaging of the cannula, they could not turn around.

A 5-day protocol as described by Van Raaij *et al*. (1996a) was applied as this protocol minimised effects of handling, anaesthesia and surgery. In short, three days before cannulation the fish were placed individually in a flow chamber for acclimatisation and were deprived of food since then. After cannulation, there was a 2-day period for recovery from the operation.

One group of fish was cannulated in a branchial artery (n= 28; mean weight of 1146 ± 60 gr) according to the method described by Vianen *et al*. (2002). Preferentially, the fourth afferent gill artery was cannulated as this is the largest vessel in diameter in *Clarias batrachus* (Olson *et al*., 1995). A second group was cannulated in the dorsal aorta (n=22; mean weight of 1116 ± 49 gr) according to the method described by Soivio *et al*. (1975). Because dissection and anatomical studies (Nawar, 1955; Olson *et al*., 1995) showed that the dorsal aorta could only be punctured far back in the mouth, the cannulation needle was inserted at an approximately 20º angel directed caudally. Both cannulation procedures were normally completed within 20 min. To prevent blood

clogging, the cannula (PE50, Rubber, Hilversum, The Netherlands) was at all times filled with a Ringer saline solution (Wolf, 1963) with 1% (w/v) of the anticoagulant sodium citrate. During the recovery period, the cannula was flushed with this anticoagulant solution 3 times a day.

After 1 and 2 days post-cannulation, the number of fish with running cannula was recorded and four blood parameters were measured as described by Vianen *et al*. (2002), namely hematocrit, hemoglobin, MCHC (Mean Cellular Hemoglobin Content) and plasma lactate concentration. Blood samples of 270 µl were taken with gastight microliter syringes containing 30 µl of a 4% (w/v) sodium citrate-saline solution as anticoagulant. Thereafter, the volume of extracted blood was replaced with Ringer saline. Finally, the cannula was refilled with a 1% sodium citrate-saline solution. In total, 540 µl of whole blood was sampled, about 1% of the total blood volume.

Experiment 2: Diurnal fluctuations

In a separate experiment, 8 fish (mean weight of 1432 ± 27 gr) were placed individually in 100-l aquaria, which were supplied with well-aerated water of 25ºC from a recirculation system of 2.2 m^3 with a water quality as stated in the previous section. The fish were not fed in these aquaria. After a 3-day acclimatisation period, the fish were cannulated in the dorsal aorta as described above, and allowed a 2-day recovery period. During this period, the tip of the cannula was filled with a PVP (Poly-Vinyl-Pyrrolidon, Merck, Amsterdam, The Netherlands) solution with 2% sodium citrate-saline.

After the 2-day recovery period, an initial blood sample was taken at 8.30, 30-min after onset of light. Repetitive blood samples were taken at 1-h intervals until 19.30, 30 min before dark. 24 Hours after the initial sample, a blood sample was taken to evaluate fluctuations between both days. Blood sampling was performed as described in the previous section, albeit that now 360 µl of whole blood was mixed with 40 µl of 4% sodium citrate-saline solution. Blood samples were analysed for hematocrit, hemoglobin, MCHC, plasma lactate, plasma glucose and plasma FFA concentration as described by Vianen *et al*. (2002). In total, 4.68 ml of whole blood was sampled per fish. Based on a total blood volume of 47 ml/kg in *Clarias batrachus* (Pandey *et al*., 1978), the total blood volume of the catfish was at least 61 ml (based on the smallest fish of 1311 gr); hence, the total sample volume stayed well below 10% of the total blood volume.

Data analysis and statistics

Data are presented as means ± SEM. The MCHC was calculated as hemoglobin concentration divided by the hematocrit. Statistical differences ($p<0.05$) were tested using Sigmastat 2.03. In experiment 1, differences between day 1 and day 2 post-cannulation were tested with a Wilcoxon Signed Rank test. Differences between both cannulation techniques were tested with a Rank Sum test. In experiment 2, differences from initial values were tested with a One-way Anova for Repeated Measures according to Dunnett's method.

Results

Experiment 1: Cannulation techniques

Between both groups, there were no significant differences on day 1 post-cannulation (Table 1). On day 2 post-cannulation, hematocrit, hemoglobin content and MCHC in the dorsal aorta cannulated group were significantly higher than in the branchial artery cannulated group. Between both days, only the hemoglobin content had dropped significantly in both groups. The mean lactate concentrations were in both groups on both days below 1 mM, and did not change significantly. Hence, no indications of anaerobic metabolism were found.

Table 1. Blood values (± SEM) for African catfish cannulated in the dorsal aorta (*n*= 13) or in the branchial artery (*n*= 5) 1 and 2 days post-cannulation (p.c.).

	Cannulation in the dorsal aorta				
	1 Day p.c.		2 Days p.c.		
Hct [%]	25.0 ± 1.0		23.9 ± 0.9	†	
Hb [mM]	4.59 ± 0.21	‡	4.11 ± 0.14	†	
MCHC [mM]	18.55 ± 0.91		17.15 ± 0.39	†	
Lactate [mM]	0.81 ± 0.08		0.62 ± 0.06		
	Cannulation in the branchial artery				
	1 Day p.c.		2 Days p.c.		
Hct [%]	21.4 ± 2.1		18.8 ± 1.6	†	
Hb [mM]	3.98 ± 0.41	‡	2.90 ± 0.22	†	
MCHC [mM]	20.03 ± 2.94		15.50 ± 0.41	†	
Lactate [mM]	0.95 ± 0.17		0.65 ± 0.17		

†: Significant difference ($p<0.05$) between cannulation techniques, ‡: significant difference ($p<0.05$) between days p.c.

Of the 28 fish cannulated in the branchial artery, 29% had patent cannulae after 1 day and 18% after 2 days. Because the fish often moved fiercely during recovery, cannulae were frequently torn out of the gills. Of the 22 fish cannulated in the dorsal aorta, 91% had patent cannulae after 1 day and 64% after 2 days. In almost all cases when cannulae were not open, one solid blood clot around the first 2-3 cm of the cannula blocked the cannula.

Experiment 2: Diurnal fluctuations

The initial hematocrit, hemoglobin content, MCHC and plasma lactate concentration are given in Table 2. These four parameters did not change significantly over the day. After 24 h the hemoglobin concentration had significantly decreased.

The initial plasma FFA concentration at 8.30 was 0.61 ± 0.14 mM and showed a fast decline of over 50% within 2 h (Fig. 1). Afterwards, the FFA concentration decreased slightly to significantly lower values at 11.30 and 12.30 compared to the initial value (0.27 ± 0.08 and 0.26 ± 0.04 mM, respectively). Within the next 3 h, there was a strong recovery to initial values. After 24 h, the FFA level of 0.48 ± 0.11 mM was not significantly different from the initial value.

The initial glucose concentration was 2.65 ± 0.59 mM. The diurnal fluctuation of the plasma glucose concentration showed a comparable trend as the FFA concentration, but there were two major differences. First, there was a phase-shift of around 2 h, as significantly lower values were reached at 13.30 and 14.30. Second, the plasma glucose levels dropped almost 100%, meaning an almost complete absence of glucose in the blood; the plasma glucose at 14.30 was only 0.05 ± 0.03 mM. Afterwards, the plasma glucose levels recovered from zero to close to initial levels within 2 h. After 24 h, the plasma glucose level of 2.61 ± 0.76 mM was not significantly different from the initial value.

Table 2. The initial values (± SEM) of different blood parameters of cannulated African catfish (*n*= 5) at 8.30 a.m. and at the same time 1 day later. *: p<0.05 vs. initial value.

	Hct [%]	Hb [mM]	MCHC [mM]	Lactate [mM]	Glucose [mM]	FFA [mM]
8.30	20.7±1.4	4.00±0.32	18.37±0.17	0.62±0.14	2.65±0.59	0.61±0.14
+1 day, 8.30	20.2±2.3	*3.46±0.30	17.31±0.50	0.60±0.30	2.61±0.76	0.48±0.11

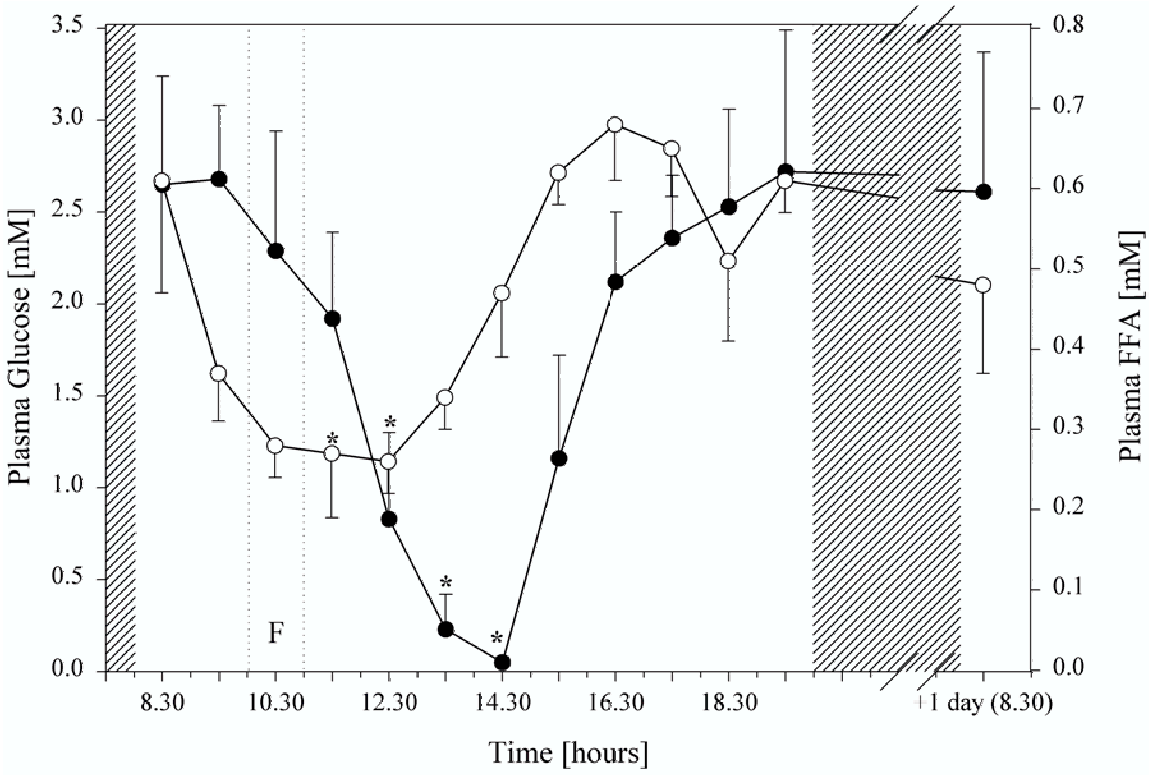

Figure 1. Diurnal fluctuations in plasma glucose (●; left axis) and FFA (○; right axis) concentrations in African catfish (n= 5). The dark period is given as a shaded area; the usual time of feeding (F) is given between dotted lines. *: $p<0.05$ vs. initial value at 8.30.

Discussion

Cannulation techniques

Clearly, the best cannulation procedure for African catfish is cannulation of the dorsal aorta for three reasons. First, a 2-day recovery period, as commonly used for cannulated fish at comparable temperatures (see Van Raaij *et al*., 1996a; Deng *et al*., 2000; Vianen *et al*., 2002), would allow experiments with only 18% of the branchial artery cannulated African catfish as opposed to 64% in dorsal aorta cannulated catfish. The success rate of dorsal aorta cannulated catfish is, however, still low, as in other fish species success rates vary from 80% to 100% (Sohlberg *et al*., 1996; Lo *et al*., 2003). The lower success rate in dorsal aorta cannulated catfish was clearly due to the extremely fast blood clogging. Blood clots were almost always found attached to the tip of the indwelling cannula, when this was blocked.

The second argument for dorsal aorta cannulation in African catfish it that the blood loss was lower than in brachial artery cannulated catfish. There were two moments of significant

blood loss. First, there was blood loss during surgery, which was observed to be higher in branchial artery cannulated catfish, because it was problematic to get the relative flexible cannula into the tough gill tissue. Second, there was blood loss during recovery, which was again higher in the branchial artery cannulated group as indicated by the significantly lower blood values at day 2 as compared to the dorsal aorta cannulated group. To our knowledge, no such comparison between cannulation techniques was reported before in the literature.

The third reason to prefer dorsal aorta cannulation in African catfish is the absence of stressed behaviour as was present in brachial artery cannulated catfish: these fishes frequently tried to remove the cannula by ferociously shaking their head. The higher stress level in brachial artery cannulated catfish matches the significantly lower MCHC on day 2 post-cannulation. This indicates that the erythrocytes were swollen as compared to the erythrocytes in the dorsal aorta cannulated fish. Erythrocyte swelling is a known action of increased plasma catecholamine levels (Soivio and Nikinmaa, 1981).

Diurnal fluctuations

To our knowledge, this is the first time that cannulated fish were used to study diurnal fluctuations in plasma glucose and FFA. Only for establishing diurnal variations in plasma cortisol cannulated fish were used (Bry, 1982). All other studies on diurnal fluctuations used different individuals on different moments thereby requiring large numbers of animals. Working with large, cannulated fish offered us frequent and repetitive sampling with intervals of only 1 h. As can be seen in Fig. 1, this enabled us to detect major changes in blood metabolites, which could have been missed with an interval of 3 to 4 h as is commonly used in studies on circadian rhythms in fish.

The study presented in this paper clearly demonstrates the presence of a diurnal fluctuation in plasma glucose in African catfish, as reported for numerous other fish species (see Boujard and Leatherland, 1992a). The most likely explanation for diurnal glucose fluctuations is the combination of a post-prandial increase due to intestinal uptake and an insulin-mediated decrease. As in mammals, insulin is also in fish a known hypoglycemic hormone (Mommsen and Plisetskaya, 1991) and insulin levels rise just before (Figueiredo-Garutti *et al*., 2002) or after feeding (Sundby *et al*., 1991; Navarro *et al*., 1993). Indeed, most studies on fed fish describe a minimal glucose concentration around the start of feeding followed by an increase after feeding (Laidley and Leatherland, 1988; Boujard and Leatherland, 1992b; Boujard *et al*., 1993; Reddy and Leatherland, 1995). The post-prandial glucose uptake is of course not present in our study as

the catfish were fasted for over 5 days. However, a feeding-entrained insulin release could still have been present as was reported for Seabass after a 7-day fast (Gutierrez *et al*., 1984). It should be noted, however, that not always a causal relation between diurnal insulin and glucose fluctuations was found (Gutierrez *et al*., 1984; Cerda-Reverter *et al*., 1998).

The most astonishing finding of our study is the almost complete absence of glucose in the plasma of African catfish (0.05 ± 0.03 mM) around 14.30, a never reported phenomenon for any fish species. In carp (*Cyprinus carpio*), low glucose levels of around 0.1-0.2 mM were found as part of a diurnal fluctuation (Kühn *et al*., 1986), and in eel (*Anguilla anguilla*), insulin administration reduced glucose levels over 90% to 0.2 mM (Ince and Thorpe, 1974). These fishes were, however, fasted over a prolonged period of time (>1 month) in contrast to the 5-day fast in our experiments and prolonged fasting is known to reduce plasma glucose levels in fish (Navarro and Gutierrez, 1995). Fish are generally considered as glucose-intolerant and carnivorous species display a weaker control of plasma glucose levels than omnivorous species (Moon, 2001). The almost complete lack of glucose in the plasma of African catfish is, however, extreme and demonstrates the low level of control of plasma glucose levels in this carnivorous species. The physiological meaning of the extremely low glucose levels in African catfish is uncertain, but it suggests that glucose is not an important metabolite in this species. In line with this relative unimportance of glucose, the recovery of plasma glucose levels within 2 h (see Fig.1) is fast and abrupt. It suggests that low glucose levels are not the dominant trigger for an increase in glucose release to the blood. Possibly, it is a side-effect of another process, for instance the recovery of plasma FFA levels as FFA concentrations started to increase 2 h earlier but reached initial levels around the same time as the glucose levels, namely around 16.30.

Few studies have reported fluctuations in FFA concentration in fish. In African catfish, the decrease in plasma FFA started before the usual time of feeding (10-11 a.m.) followed by a strong recovery. A similar trend was reported for trout (*Oncorhynchus mykiss*, Leatherland and Nuti, 1982; Boujard *et al*., 1993). Boujard and Leatherland (1992b) stated that plasma FFA levels were minimal during feeding, possibly due to a minimal level of lipolysis. In Seabass, glycerol levels started to decrease before the normal feeding time (Carillo *et al*., 1982). As glycerol truly reflects lipolytic rate, the study of Carillo and co-workers points to a reduced lipolytic rate linked to the moment of feeding. Perez *et al*. (1988) found no significant fluctuation in glycerol levels with the same species; a possible fluctuation could easily have been missed due to the higher temperatures (>22ºC) and sampling at 4-h intervals. Also in Kokanee salmon fed twice a day, a possible rhythmicity in lipolysis could not be detected as both the highest and lowest levels of FFA coincided with feeding (Leatherland *et al*., 1974); double feeding and low temperatures (10-

12ºC) could have hampered quick changes in lipolytic rates. In goldfish at 25ºC and fed 4 times a day, a complete absence of diurnal fluctuations in serum FFA was reported (Minick and Chavin, 1971).

Unfortunately, Boujard and Leatherland (1992b) provided no potential cause for the fluctuation in lipolytic rate, and in our study no hormones were measured as this was outside the scope of the study. A feeding-induced insulin surge could have reduced lipolysis as insulin is a known antilipolytic agent in mammals and probably also in fish (Harmon and Sheridan, 1992c; Harmon *et al*., 1993). However, the decrease in FFA levels started at least 2 h before feeding, making a relation to a feeding-induced insulin surge doubtful and a relation to the onset of light more plausible. In humans, both a nocturnal rise in cortisol (Dinneen *et al*., 1995) and growth hormone (Davidson *et al*., 1988) are linked to an increased lipolytic rate. Also in diurnal fish, cortisol (Kühn *et al*., 1986; Laidley and Leatherland, 1988; Cerda-Reverter *et al*., 1998) and growth hormone (Leatherland *et al*., 1974) appear to peak at the end of the dark phase. For African catfish, such a surge in cortisol and/or growth hormone can be expected to occur late on the day as this species is nocturnal. Hence, lipolysis and FFA levels will be maximal at the end of the day as observed by this study. Evidently, hormone measurements in African catfish will allow better conclusions about the endocrinological basis of the metabolite fluctuations observed in this study.

The presence of a diurnal fluctuation in both plasma glucose and FFA concentrations in African catfish is clearly demonstrated by this study. The dramatic changes in glucose concentrations illustrate the extremely low level of control on plasma glucose concentrations and the higher level of control on plasma FFA concentrations in African catfish.

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