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The combined effect of hypoxia and nutritional status on metabolic and ionoregulatory responses of common carp (*Cyprinus carpio*)

Sofie Moyson [a,*], Hon Jung Liew [a,b], Marjan Diricx [a], Amit Kumar Sinha [a], Ronny Blust [a], Gudrun De Boeck [a]

[a] Systemic Physiological and Ecotoxicological Research, Department of Biology, University of Antwerp, Groenenborgerlaan 171, BE-2020 Antwerp, Belgium
[b] Institute of Tropical Aquaculture, University Malaysia Terengganu, 21030 Kuala Terengganu, Terengganu, Malaysia

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ABSTRACT

Since hypoxia is a common event in aquatic environments, oxygen has been a major driving force in the evolution of fish. When fish are simultaneously faced with food deprivation in their natural habitat, this poses an even greater challenge to energy and ion homeostasis. In the present study, the combined effects of hypoxia and nutritional status (fed versus fasted) were examined in common carp (*Cyprinus carpio*), a relatively hypoxia tolerant cyprinid. Fish were either fed or fasted and were exposed to hypoxia (1.5–1.8 mg O_2 L^{-1}, 16–19% saturation at 18 °C) at or slightly above their critical oxygen concentration (1.4 mg O_2 L^{-1}, 16.5% saturation at 20 °C) during 1, 3 or 7 days followed by a 7 day recovery period. Fasted fish had lower ventilation frequencies than fed fish but under both feeding regimes, ventilation initially increased during hypoxia. In fed fish, ventilation returned to control levels during hypoxia, while in fasted fish recovery only occurred after reoxygenation. Due to this, *C. carpio* managed, at least in part, to maintain aerobic metabolism during hypoxia: muscle and plasma lactate levels remained relatively stable although they tended to be higher in fed fish (despite its higher ventilation rates). However, during the recovery phase compensatory responses differed greatly between both feeding regimes: plasma lactate in fed fish increased with a simultaneous breakdown of liver glycogen indicating increased energy use, while fasting fish seemed to economize energy and recycle decreasing plasma lactate levels into increasing liver glycogen levels (Cori cycle). Protein was used under both feeding regimes during hypoxia and subsequent recovery: protein levels reduced mainly in liver for fed fish and in muscle for fasting fish. Overall, nutritional status had a greater impact on energy reserves than the lack of oxygen with a lower hepatosomatic index and lower glycogen stores in fasted fish. Fasting fish transiently increased Na^+/K^+-ATPase activity under hypoxia, but in general ionoregulatory balance proved to be only slightly disturbed, showing that sufficient energy was left for ion regulation.

1. Introduction

Dissolved oxygen (DO) is necessary to sustain the life of aquatic animals relying on aerobic respiration. However, anthropogenic activities induce a more frequent appearance of hypoxic and anoxic habitats due to increasing surface temperatures and water column stratification as well as eutrophication, both in fresh water and coastal systems worldwide (Diaz, 2001).

Fish species vary greatly in their ability to tolerate and survive hypoxia (Diaz, 2001; Bickler and Buck, 2007). In general, fish respond to hypoxia through a wide range of physiological, biochemical, molecular and behavioural responses (e.g. Hattink et al., 2005; Lewis et al., 2007; Sloman et al., 2008; Richards, 2011). Fish can increase O_2-uptake by morphological transformation of the gills (Sollid et al., 2003, 2005; Sollid and Nilsson, 2006; Richards, 2011) and by altering respiration patterns (Glass et al., 1990; Soncini and Glass, 2000; Scott et al., 2008). Furthermore, hematocrit and/or hemoglobin binding affinity for oxygen can increase to maintain oxygen uptake and delivery to tissues (Lai et al., 2006; Wells, 2009).

To maintain the cellular energy balance, fish can reduce metabolic demands through a controlled suppression of metabolic rate and/or alter the pathways of metabolic energy production by activation of the anaerobic metabolism (Almeida-Val et al., 2000; Boutilier, 2001; Hochachka and Somero, 2002; Richards, 2009). However, because only glycogen and high-energy phosphates can be used for anaerobic ATP production and their lower ATP yield, these fuels can be quickly exhausted. Since anaerobic metabolism also produces metabolic wastes such as lactate, its accumulation can have harmful effects on tissues (Richards, 2011). In general, an accumulation of lactate and a depletion of glycogen and creatine stocks are observed during anaerobiosis (Van Den Thillart and Van Waarde, 1985; van Ginneken et al., 1998; Richards et al., 2007; Genz et al., 2013). Suppression of metabolic rate is another strategy to reduce cellular energy consumption and is enhanced in hypoxia-tolerant species (e.g. van Waversveld et al., 1989a, 1989b; Johansson et al., 1995; van Ginneken et al., 1998). Reduction of

* Corresponding author. Tel.: +32 32 653482; fax: +32 32 653497.
E-mail address: Sofie.Moyson@uantwerpen.be (S. Moyson).

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metabolic needs is the result of a strong downregulation of protein turnover, Na^+ pumping, gluconeogenesis and urea synthesis (Richards, 2009). However, it is impossible to draw general conclusions about the relation between the capacity to suppress metabolic rate and hypoxia tolerance at present (Richards, 2011).

Since branchial ionoregulation is an energetically expensive process (1–20% of the total ATP demand), it is important to reduce this cost as much as possible (Febry and Lutz, 1987; Buck et al., 1993a, 1993b; Evans and Claiborne, 2009). By reducing their membrane permeability, 'channel arrest' (Hochachka, 1986) enables organisms to maintain membrane potentials and concentration gradients in the ion channels without the use of ATP.

In the absence of food, animals use endogenous energy stores and several behavioural, physiological and structural responses to reduce their metabolic rate. Protein synthesis and the expression of several metabolic genes are reduced (Wang et al., 2006). So, the absence of food may induce similar physiological responses and molecular genetic pathways as oxygen deprivation (Iranon and Miller, 2012).

In addition to branchial uptake routes food can also play an important role as a source of ions, especially when the energy available for branchial uptake processes is limited e.g. under severe hypoxia. In Amazonian Oscars, *Astronatus ocellatus*, fasted fish had higher branchial ion rates under normoxic conditions, which was associated with an increased number and surface area of MRCs. During hypoxic exposure, both fed and fasted fish reduced this branchial ionoregulation, gill MRC density and surface area but fasted fish were able to respond more quickly to lower oxygen levels, and reduced branchial permeability more effectively (De Boeck et al., 2013), indicating another possible interactive effect between hypoxia and food availability. Additionally, critical oxygen tension (Pcrit) was reduced, suggesting a better hypoxia resistance.

Cyprinus carpio is an important ecological and economical fish species worldwide, and can tolerate moderate levels of hypoxia (Lardon et al., 2013). Therefore, this species was chosen to examine their responses on a combination of environmental challenges. Common carp were either fed (2% body weight) or fasted and exposed to normoxic or hypoxic conditions. *C. carpio* already respond to relatively mild levels of hypoxia (50–60% saturation) by hyperventilating (Glass et al., 1990; Soncini and Glass, 2000). An earlier study in our lab using similar sized common carp showed that critical oxygen tension (Pcrit) ranged around 1.4 mg L^{-1} or 16.5% saturation at 20 °C (De Boeck et al., 1995). We aimed at exposing our fish to the same oxygen level since at this oxygen tension fish are clearly affected being no longer able to control oxygen consumption rates. Due to the lag time in our exposure system, measured exposure concentrations remained at or just above this Pcrit (dissolved oxygen levels 1.5–1.8 mg L^{-1}, 16–19% saturation at 18 °C). Responses in ventilation rates, energy metabolism and ionoregulation were examined. It was postulated that fasted fish would be more affected by hypoxia than fed fish, with increased energy store depletion and disturbed ionoregulation.

2. Materials and methods

2.1. Fish maintenance

Common carp (*C. carpio*) juveniles were obtained from the fish hatchery at the University of Wageningen, The Netherlands. Fish were raised to 16–21 g in softened tap water (pH 6.5–8.5, Ca^{2+}: 1.52 mM, Mg^{2+}: 0.29 mM, Na^+: 1.40 mM, K^+: 0.12 mM, 23–25 °C) in the aquaria facilities of the laboratory of 'Systemic Physiological and Ecotoxicological Research' at the University of Antwerp. Water quality was checked every day using Standard Tetra Test Kits (Visocolor, Macherey-Nagel, Germany) and values remained <30 μmol L^{-1} NH_3/NH_4^+; <45 μmol L^{-1} NO_2^- and <800 μmol L^{-1} NO_3^-. Water was filtered through biological filters containing wadding, activated charcoal and lava stones. Fish were fed ad libitum with commercial minipellets ('Hikari Staple', Kyorin Food Ind. Ltd., Himeji, Japan) once a day.

Two weeks prior to the start of the experiment, 128 fish with a body mass of 18.6 ± 0.2 g (mean ± S.D.) were transferred from their maintenance tanks into a climate chamber set at 18 °C with a photoperiod of 15 L:9 D. Fish were randomly distributed into 50–60 L glass aquaria, filled with 40 L well oxygenated water, with a density of 8 fish per aquarium. A filter, filled with lava stones and wadding, ensured the oxygen supply. Black plastic shielding minimized visual disturbance. Water quality was checked as above and 80% of the water was replaced every two days.

2.2. Experimental design

The experimental setup consisted of 8 hypoxic experimental groups (DO: 1.5–1.8 mg L^{-1}) and 4 normoxic control groups (DO: 7–8 mg L^{-1}), divided over two feeding regimes (fed at 2% BW or fasted). For every control group we had 2 tanks with 8 fish each. The feeding regime of 2% BW was determined by a pre-experiment which indicated that this amount of food was close to the maximum consumption for the hypoxic fish. The food was divided over 2 equal portions a day at 9 h30 and 15 h30. No feeding occurred on the day of sampling. After 2 weeks of acclimatization, fasted fish were not fed anymore. Control groups of both feeding regimes were sampled after 3 or 5 weeks of acclimation to normoxia (control 3 and control 5). After 3 weeks of acclimatization, experimental groups of both feeding regimes were exposed to hypoxia during 1 day, 3 days or 7 days. To examine the effect of 7 days of normoxic recovery, fish of both feeding regimes were also exposed to hypoxia for 7 days followed by a normoxic recovery period (Fig. 1).

In the hypoxic aquaria levels of dissolved oxygen were monitored and controlled continuously by the R362 Controller system for pH/mV/conductivity/oxygen (Consort, Turnhout, Belgium). The electrode of the controller was placed in the middle of the aquarium, obliquely along with the flow in order to avoid air bubbles or dirt remaining on the electrode. The R362 system controls the predetermined oxygen level by opening or closing two valves connected to a nitrogen or air supply. At least twice a day the oxygen level, measured by the R362 oxygen electrodes, was manually checked by an additional calibrated oxygen electrode (WTW oxi 3310, Weilheim, Germany). The oxygen level in the experimental aquaria was gradually lowered from the normoxic level (7–8 mg O_2 L^{-1}, 75–85% saturation) to the hypoxic oxygen concentration range of 1.5–1.8 mg O_2 L^{-1} (16–19% saturation). The hypoxic experiment started when the determined hypoxic oxygen levels were reached. Each tank was covered by a plastic lid, to avoid possible diffusion of oxygen from the air.

2.3. Ventilation frequency

Ventilation frequency was determined by visually counting the number of opercular movements during 1 min. This was carried out twice a day (after 5, 23, 29, 47, 53, 71 … h) for all 8 fishes in the aquarium. A waiting period of at least 5–10 min was applied to allow fish to get used to the presence of the observer. For experimental groups, the first counting took place after 5 h, when hypoxic oxygen levels were reached. Ventilation frequencies were determined using the 7 days hypoxia/7 days normoxic recovery and the second series of control groups.

2.4. Sampling procedure

After exposure to normoxia, hypoxia or normoxic recovery, carp were netted and quickly anesthetized with 1.1 g L^{-1} ethyl-3-aminobenzoate methanesulfonic acid (MS-222, Acros Chemicals, Geel, Belgium) neutralized with KOH (Merck Eurolab nv/sa, Leuven, Belgium). Fish were blotted dry and weighted. Before decapitation, a

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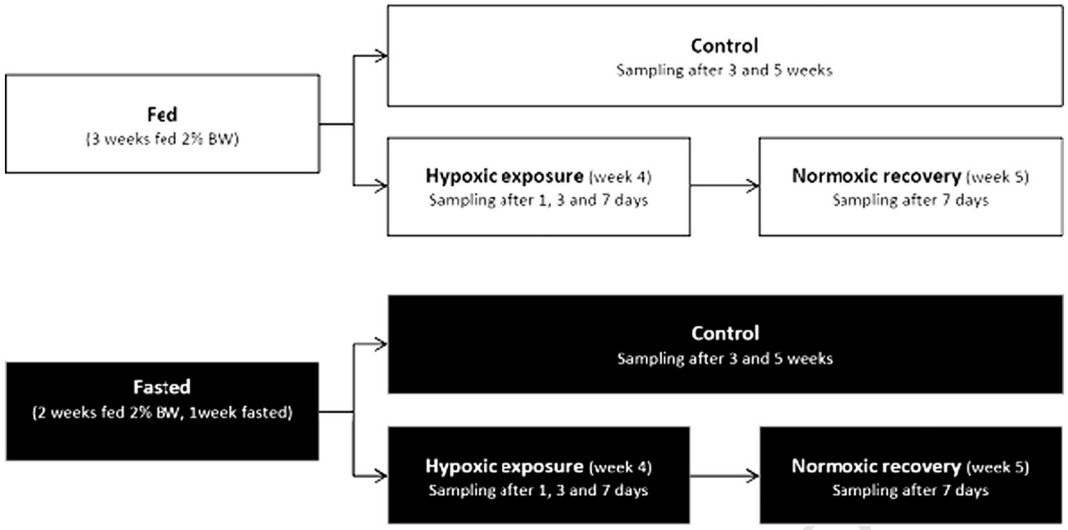

Fig. 1. Schematic diagram of study groups. Sample size of each group was 8 fish.

blood sample was taken from the caudal blood vessel using a heparinised syringe (heparin from Sigma-Aldrich, co, St.Louis, USA). Blood was immediately centrifuged for 2 min at 13,200 rpm at 4 °C. Plasma was carefully pipetted into cryogenic vials and frozen in liquid N_2. Subsequently, fish were killed by severing their spinal cord prior to organ sampling. Gills, liver and white muscle tissues were excised on ice, frozen in liquid nitrogen and stored at −80 °C until further analysis. The whole liver mass was recorded and the hepatosomatic index was calculated as HSI = (LM/BW) × 100, where LM is referred as liver mass.

2.5. Biochemical analysis

Plasma ammonia, plasma lactate and muscle lactate were determined using commercial Enzymatic Kits (R-Biopharm AG, Darmstadt, Germany). Plasma ion concentrations of Na^+, K^+ and Cl^- were analyzed using an Electrolyte Analyzer 9180 (AVL Scientific corporation, GA, USA). Na^+/K^+-ATPase activity in gill samples was measured using the method of McCormick (1993). Samples of liver and muscle were analyzed for glycogen concentration using Anthron reagent (Roe and Dailey, 1966), for total lipid content (Bligh and Dyer, 1959) and for protein concentration using Bradford reagent (Bradford, 1976).

2.6. Statistical analysis

All data are presented as mean values ± standard error (SEM). Data were analyzed with the statistical program 'R', version 2.13.1, with a 5% level of significance. Normality was checked by the Shapiro–Wilk test. The Bartlett test was used to verify the homogeneity of variances. If the requirements for ANOVA were not fulfilled, a log-transformation of data was applied. The main effects of hypoxia and feeding and their interaction were analyzed by a two-way ANOVA (Table 1). Comparisons within treatment were carried out by a one-way ANOVA. Subsequently, a Tukey HSD test was used to determine the differences between groups. Data were compared to the closest control: 1 and 3 day hypoxic exposure was compared with control 3; data of 7 day hypoxia were compared with controls 3 and 5, while results of 7 day hypoxic exposure followed by normoxic recovery were compared with control 5 only. Repeated measurements ANOVA was used for analyzing significant effects on ventilation frequency, which was also followed by a Tukey HSD test to determine significant differences between groups.

3. Results

3.1. Ventilation frequency

3.1.1. Effect of feeding

Feeding regime had a significant effect on ventilation frequency of both normoxic and hypoxic groups ($P < 0.001$). At the start of the observation period, no difference in ventilation frequency was observed between fed carp and fish that had been fasted for a week at that time. However, for fasted normoxic groups, a significant reduction in ventilation frequency started to emerge from 101 h, or approximately 11 days of fasting, onwards ($P < 0.05$), with an exception at 191 h (Fig. 2). At 101 h, fasted normoxic fish had a 17% lower ventilation frequency than fed normoxic carp. Hereafter, the difference between both normoxic groups continued to increase and after 335 h, fasted fish had a 79% lower frequency than fed fish. In contrast to the decline in ventilation frequency in fasted fish, ventilation frequency remained stable over the entire experimental period in fed normoxic fish. Also during hypoxia, the ventilation frequency of fed fish was significantly higher than that of fasted fish ($P < 0.01$). This difference faded by the end of the hypoxic exposure (after 71, 119, 125, 143, and 149 h of exposure) but resurfaced during the normoxic recovery period (Fig. 2). After 5 h

Table 1
Effect of treatment and feeding pattern and their interaction on physiological parameters of common carp.

		Treatment		Feeding pattern		Treatment × feeding pattern	
	N	*F*-value	*P*-value	*F*-value	*P*-value	*F*-value	*P*-value
HSI	127	5.254	0.000	203.909	0.000	5.119	0.000
Muscle lactate	125	3.841	0.003	43.099	0.000	9.683	0.000
Plasma lactate	126	2.675	0.025	152.160	0.000	21.764	0.000
Liver glycogen	124	3.785	0.003	193.108	0.000	16.290	0.000
Muscle glycogen	117	4.276	0.001	64.422	0.000	3.199	0.010
Liver lipid	127	4.474	0.001	1.509	0.222	3.803	0.003
Muscle lipid	125	4.716	0.001	11.351	0.001	0.639	0.670
Liver protein	126	32.998	0.000	0.472	0.493	4.086	0.002
Muscle protein	123	5.497	0.000	0.422	0.517	1.548	0.181
Plasma sodium	120	3.550	0.005	15.251	0.000	6.460	0.000
Plasma potassium	108	2.166	0.064	2.495	0.118	5.225	0.000
Plasma chloride	119	4.240	0.001	5.247	0.024	2.583	0.030
Plasma ammonia	116	0.833	0.529	6.118	0.015	2.391	0.043
Na^+/K^+-ATPase	122	33.628	0.000	18.376	0.000	13.371	0.000

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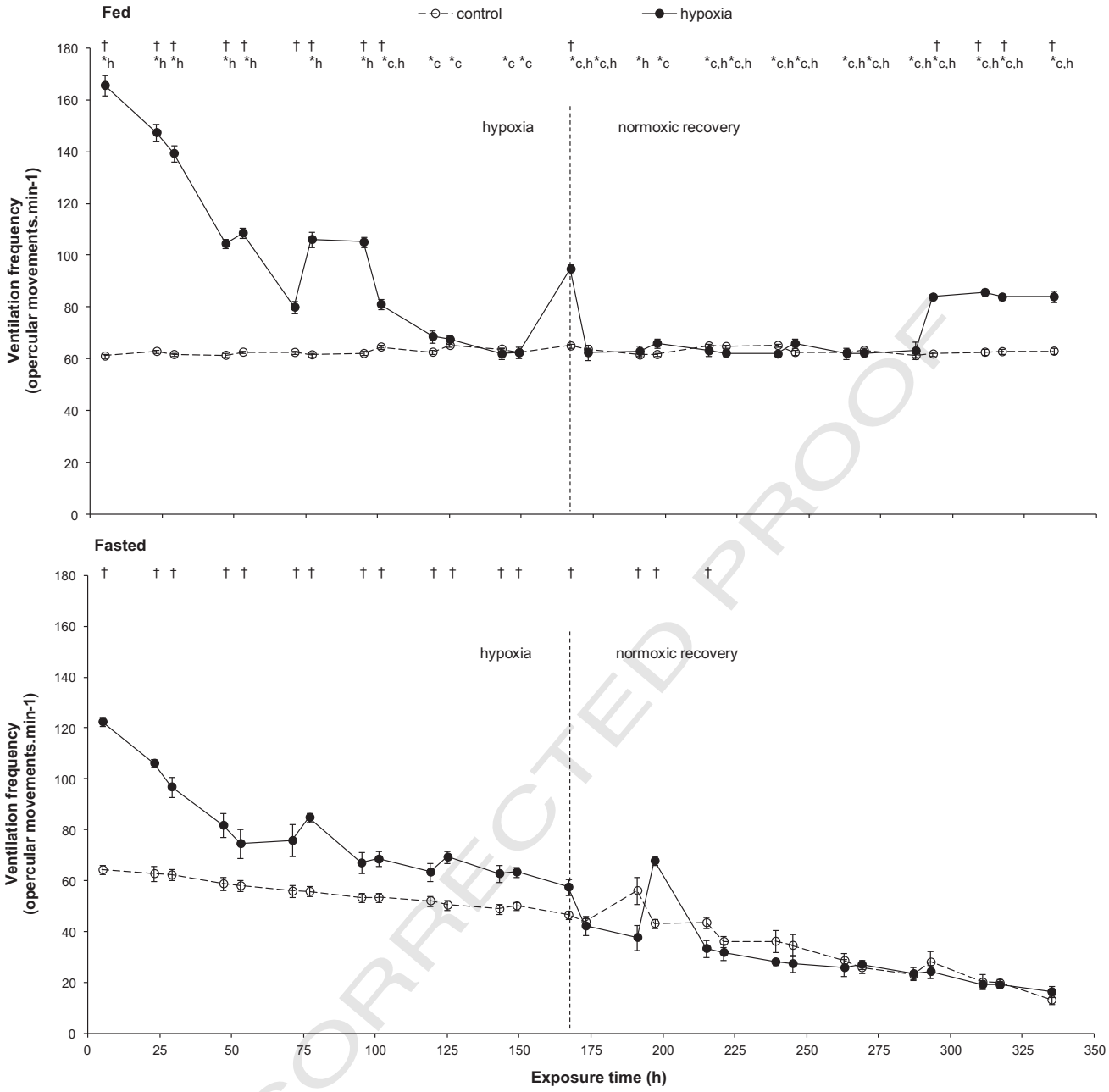

Fig. 2. Ventilation frequency of fed and fasted common carp during a weeklong hypoxic exposure, followed by 7 days of normoxic recovery (• hypoxia) and their respective controls (o control) that remained normoxic (mean ± SEM). Sample size of each group was 10, except for the fed hypoxic group with 8 ventilation measurements. Different symbols denote significant differences ($P < 0.05$) between feeding patterns (*) and treatments (†). Letters c and h denote significant differences between feeding patterns in control groups and between feeding patterns in exposed groups respectively.

of hypoxia fasted fish had a 26% lower ventilation frequency and at the end of the normoxic recovery period this was increased to 81%.

3.1.2. Effect of hypoxia

Hypoxia exposure induced an increase in ventilation frequency in fish of both feeding regimes ($P < 0.001$). Fasted hypoxic fish had a 90% higher ventilation frequency after 5 h of hypoxia exposure than the normoxic group ($P < 0.001$). The ventilation frequency remained elevated compared to fasted normoxic fish over the entire hypoxic exposure period ($P < 0.01$) (Fig. 2). During the normoxic recovery period, ventilation rates of previously exposed and normoxic fasted fish were very similar and either one or the other was slightly increased or decreased compared to the other (Fig. 2). Compared to fed control, fed hypoxic fish had a 171% higher ventilation frequency after 5 h of exposure ($P < 0.001$) and ventilation remained elevated up to 101 h of hypoxia after which it recovered to control levels, with an exception at 167 h of hypoxia and at the end of the normoxic recovery period ($P < 0.001$) (Fig. 2). After 7 days of hypoxia exposure followed by 7 days of reoxygenation, fed hypoxic fish had a 34% higher ventilation frequency compared to fed normoxic fish ($P < 0.001$).

3.2. Energy metabolism

3.2.1. Lactate in muscle and plasma

Compared to fed fish, fasted fish had 56%, 52% and 32% lower muscle lactate concentration after 1 day ($P < 0.001$), 3 days ($P < 0.001$) and

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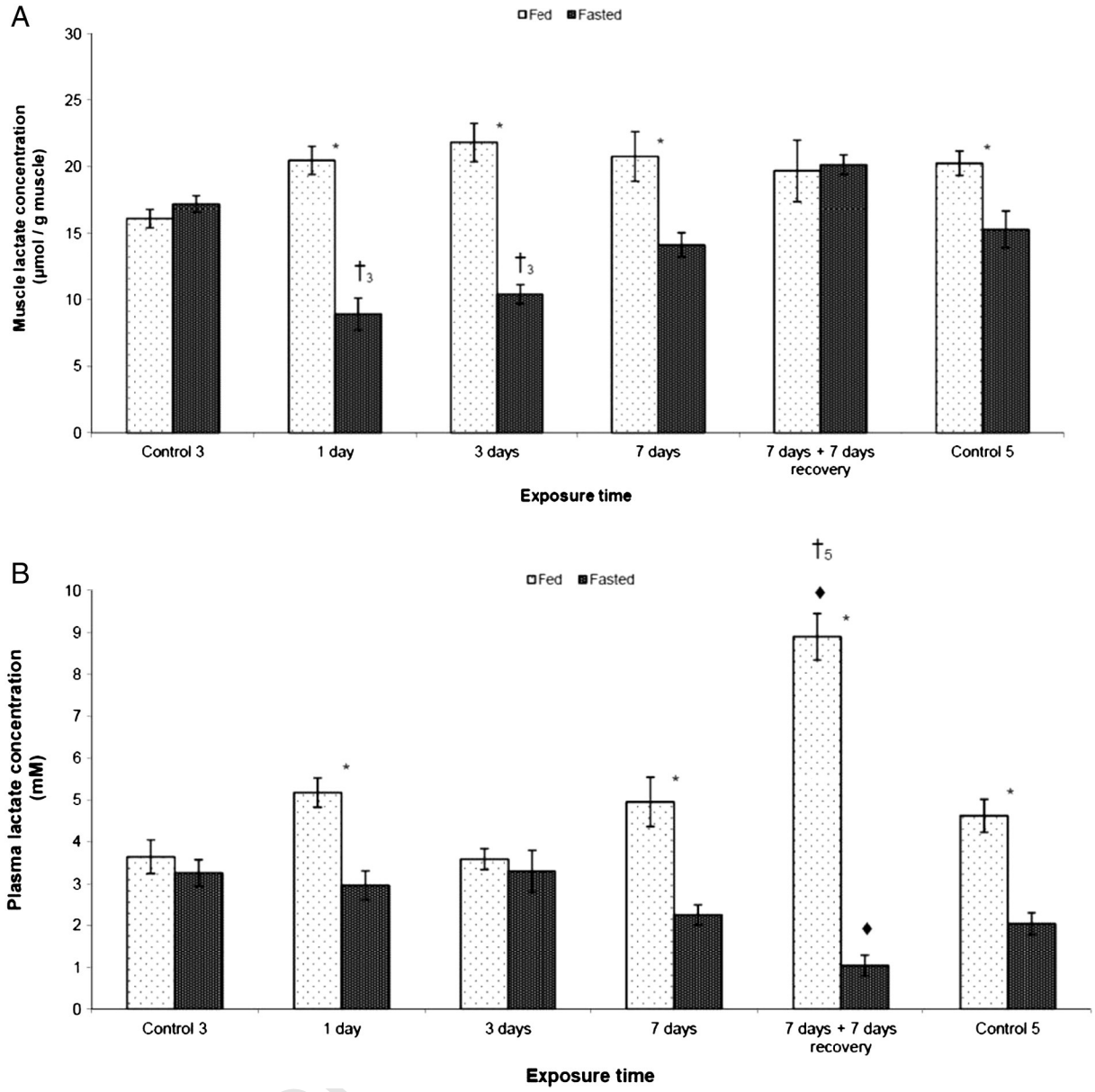

Fig. 3. Lactate accumulation in fed and fasted common carp during hypoxic exposure in (A) muscle and (B) plasma. Values are mean ± SEM. Different symbols denote significant differences ($P < 0.05$) between feeding patterns (*), treatments (†) and between 7 days of hypoxic exposure followed by 7 days of reoxygenation and without normoxic recovery (♦). The numbers (3 and/or 5) refer to significant differences with the corresponding control group (control 3/control 5).

7 days ($P < 0.05$) of hypoxia respectively (Fig. 3A). After 5 weeks of normoxic control conditions muscle lactate of fasted fish was also 25% lower than of fed fish ($P < 0.05$). Hypoxia induced a significant decrease in muscle lactate of fasted fish after 1 day ($P < 0.001$) and 3 days ($P < 0.01$). The concentration of muscle lactate in fed fish remained surprisingly stable during the exposure period but levels tended to be higher than in controls. No significant difference was observed from normoxic recovery. However, a significant effect of treatment, feeding and interaction between hypoxic treatment and feeding regime was observed (Table 1).

Besides a significant interaction between treatment and feeding regime, feeding and hypoxia also had a significant effect on plasma lactate. Plasma lactate concentrations of fed fish were significantly increased compared to fasted carp after 1 day of hypoxia (75%, $P < 0.05$), 7 days of hypoxia (120%, $P < 0.001$), 7 days of hypoxia with 7 days of normoxic recovery (756%, $P < 0.001$) and 5 weeks of normoxia (127%, $P < 0.001$) (Fig. 3B). Only the fed group exposed to 7 days of hypoxia and 7 days of normoxia differed significantly from control 5. Normoxic reoxygenation played a significant role in both fed and fasted carp ($P < 0.05$). Plasma lactate of the fasted group decreased after 7 days of normoxic recovery ($P < 0.05$) in contrast with fed fish where it increased with 80% ($P < 0.05$).

3.2.2. Hepatosomatic index and glycogen, protein and lipid content in liver and muscle

Fasting led to a significant reduction in HSI (Table 1) in both control groups ($P < 0.001$) and in the groups exposed to hypoxia for 1 day ($P < 0.001$), 7 days ($P < 0.001$) and 7 days with re-oxygenation ($P < 0.001$) (Fig. 4). After 7 days of hypoxic exposure, HSI of the fed group was significantly increased ($P < 0.001$), compared to control 5. Recovery did not have a significant effect.

Due to fasting glycogen content in liver was reduced in normoxic (control 3: $P < 0.001$; control 5 $P < 0.05$) and hypoxic groups (1 day, 3 days: $P < 0.001$) (Fig. 5A). Compared to fed fish, liver glycogen content

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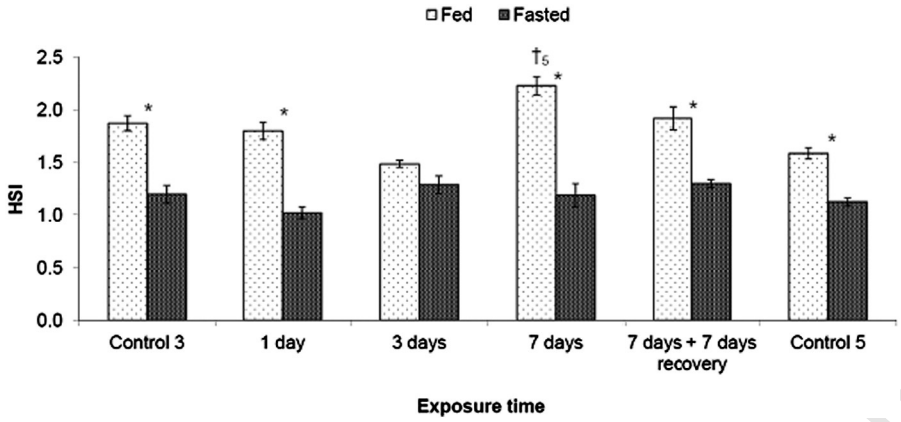

Fig. 4. Hepatosomatic index of fed and fasted common carp during hypoxic exposure. Values are mean ± SEM. Different symbols denote significant differences ($P < 0.05$) between feeding patterns (*), treatments (†) and between 7 days of hypoxic exposure followed by 7 days of reoxygenation and without normoxic recovery (♦). The numbers (3 and/or 5) refer to significant differences with the corresponding control group (control 3/control 5).

of fasted fish was 84%, 75%, 69% and 43% lower respectively. After 1 day of hypoxic exposure glycogen content of the fasted fish slowly started to increase: after 7 days of hypoxia glycogen was 189% higher ($P < 0.001$). Furthermore, glycogen level of control 5 was 125% higher compared to control 3 ($P < 0.001$). In fed fish, glycogen concentration in liver remained almost constant during the exposure period. However, liver glycogen concentration of control 5 was significantly lower compared to control 3 ($P < 0.01$). Overall, significant effects of feeding regime, treatment and of interaction between treatment and feeding regime were observed (Table 1).

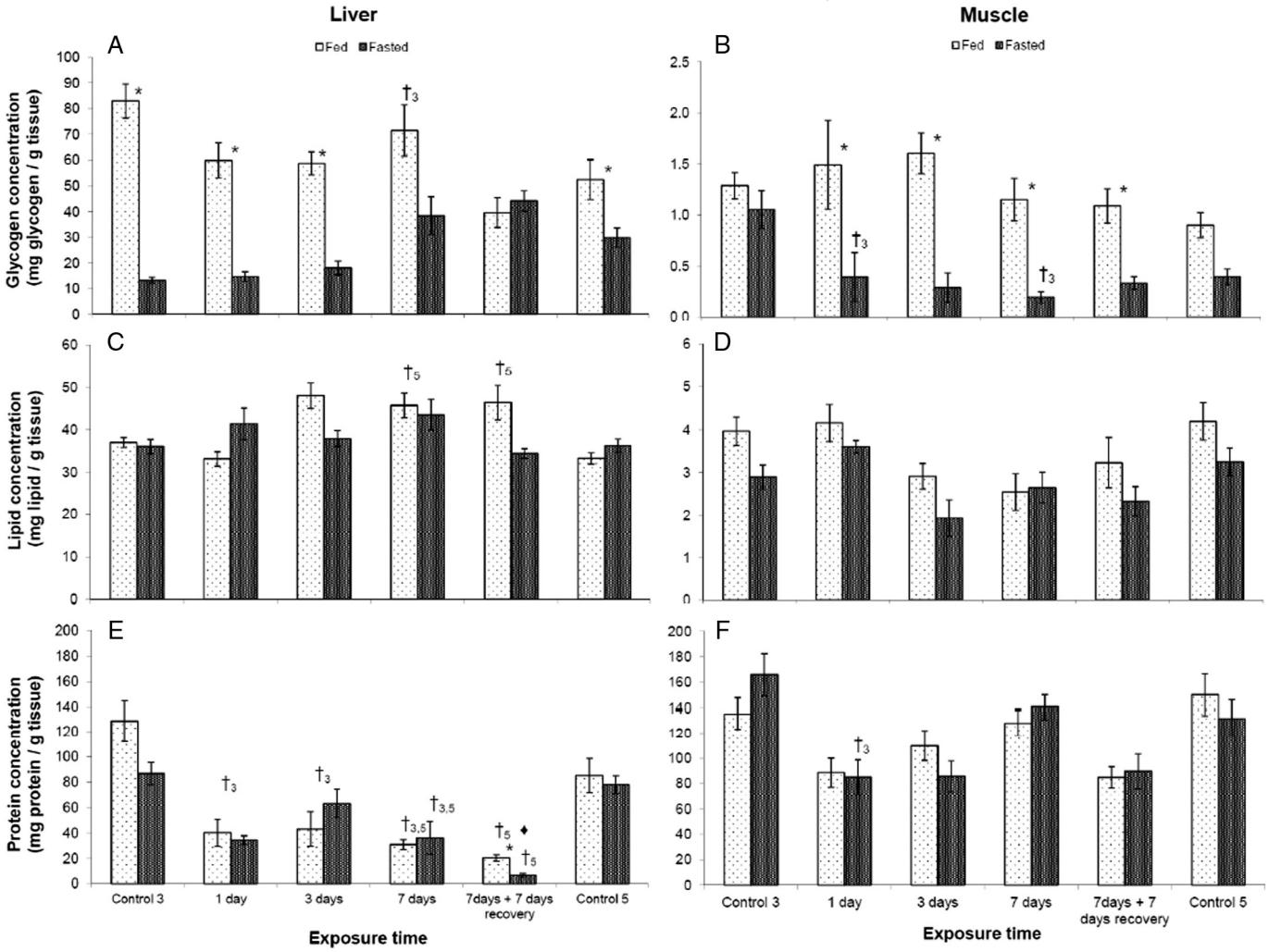

Fig. 5. Liver and muscle glycogen (A, B), lipid (C, D) and protein (E, F) content in fed and fasted common carp during hypoxic exposure. Values are mean ± SEM. Different symbols denote significant differences ($P < 0.05$) between feeding patterns (*), treatments (†) and between 7 days of hypoxic exposure followed by 7 days of reoxygenation and without normoxic recovery (♦). The numbers (3 and/or 5) refer to significant differences with the corresponding control group (control 3/control 5).

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Muscle glycogen levels were very low and in fasted fish close to the detection limit. In normoxic fish, both fed and fasted fish showed similar muscle glycogen levels. During hypoxic exposure, fasted fish had lower muscle glycogen than fed fish on days 1 ($P < 0.01$), 3 ($P < 0.001$), 7 ($P < 0.01$) and 7 days with 7 days of normoxic recovery ($P < 0.05$). This was mainly caused by a drop in glycogen in fasted fish; glycogen concentration decreased over time with a reduction of 63% after the first hypoxic day and a drop of 81% after 7 day hypoxia exposure compared to control 3. After 7 days of reoxygenation, the content slightly increased again. In fed groups, levels remained relatively stable. Effects of treatment and feeding were significant in muscle glycogen content, both individually and by interaction (Table 1).

Almost no significant differences were found in liver lipid levels (Fig. 5C). Only the fed groups of 7 day hypoxia and 7 day hypoxia wit normoxic recovery differed significantly from control 5 ($P < 0.05$). Lipid content in muscle showed no significant differences (Fig. 5D).

Overall, different trends were observed in liver and muscle protein. An effect of feeding on liver protein content was only seen after 7 days of normoxic recovery ($P < 0.05$) (Fig. 5E). Hypoxia on the other hand influenced liver protein of fed carp, with significantly lower protein levels compared to the control after 1 day ($P < 0.001$), 3 days ($P < 0.001$) and 7 days (control 3 and 5: $P < 0.001$) and a further reduction after 7 days recovery ($P < 0.001$). For the fasted fish the reduction was only significant in the 7 day hypoxic group (control 3: $P < 0.01$; control 5: $P < 0.05$) and after normoxic recovery ($P < 0.001$). Normoxic recovery induced a significant reduction in protein level of fasted fish ($P < 0.001$).

No significant differences in muscle protein content were observed between the two feeding regimes in any of the sampling periods (Fig. 5F). The only influence of hypoxic exposure occurred in muscle protein of fasted fish where a significant reduction was observed after 1 day ($P < 0.01$). A (non-significant) but very similar trend could be observed in fed fish. Overall, hypoxic exposure had a significant effect on protein content, both in liver and in muscle. The interaction between treatment and feeding regime only also had a significant effect on liver protein content (Table 1).

3.3. Ionoregulation

3.3.1. Plasma ions and ammonia

Under normoxic conditions and after 7 days of hypoxic exposure, fasted fish had a lower Na concentration than fed fish (control 5: $P < 0.001$; 7 days: $P < 0.01$) (Table 2). No effect of hypoxia was seen in fasted fish. However, during the first few days of hypoxic treatment, Na levels in the fed group dropped, which led to significantly lower Na compared to the normoxic group after 3 days ($P < 0.05$). Normoxic recovery induced a 17% increase in Na concentration in fasted fish ($P < 0.01$), but did not have a significant effect in the fed group. In general, marked effects of nutrient status and its interaction with hypoxic treatment were observed (Table 1).

Overall, fasted fish had lower plasma K than fed fish ($P > 0.05$) (Table 2). However, this trend was reversed after seven days of hypoxia, when potassium concentration in fasted fish was higher than in fed fish (60%, $P < 0.01$) or control 3 ($P < 0.05$). In both feeding regimes, normoxic recovery did not affect K concentration. Neither feeding regime, nor hypoxic exposure or normoxic recovery had a significant effect on plasma chloride concentration (Tables 1 and 2), although plasma Cl showed a similar trend as plasma Na. No significant difference was seen in plasma ammonia (Tables 1 and 2).

3.3.2. Na^+/K^+-ATPase activity

Normoxic group 3 showed a striking effect of feeding on Na^+/K^+-ATPase activity: enzymatic activity in fasted carp was 68% lower than in fed normoxic control 3 ($P < 0.001$) (Fig. 6). Hypoxia elevated Na^+/K^+-ATPase activity in fasted fish to the same level as in fed fish, ($P < 0.001$) at days 1, 3 and 7. The enzyme activity of the hypoxic fasted group was also significantly higher then control 5 ($P < 0.001$) after 7 days of exposure. During normoxic recovery after a hypoxic exposure of 7 days Na^+/K^+-ATPase activity decreased, which for fed fish led to a significant difference compared to normoxic fish ($P < 0.05$) and 7 day hypoxic fish ($P < 0.001$) (Fig. 6). A significant combined and separate effect of treatment and feeding regime on enzymatic activity was observed (Table 1).

4. Discussion

4.1. Ventilation frequency

In the present study, hyperventilation was clearly observed during the first days of hypoxic exposure, both in fed and fasted carp (Fig. 2). Hyperventilation is arguably the most important physiological response of fish exposed to low oxygen levels in order to minimize the reduction in arterial oxygen pressure, which is an inevitable result of lower oxygen pressure in the surrounding water (e.g. Wood and Johansen, 1973; Maxime et al., 2000; Soncini and Glass, 2000; Perry et al., 2009). Common carp have been seen to increase ventilation frequency without any modification of ventilation amplitude, even at lower levels of hypoxia (50–60% sat., Glass et al., 1990; Soncini and Glass, 2000) well above the Pcrit of these fish (16.5% sat. at 20 °C, De Boeck et al., 1995). Hereby, the transition from aerobic to anaerobic metabolism can be delayed (Perry et al., 2009). Respiratory responses are initiated rapidly after hypoxic water contacts the gills, (Kinkead et al., 1991; Scott et al., 2008; Perry et al., 2009). After the initial peak in hyperventilation, ventilation frequency of hypoxic groups decreased with time and ventilation frequencies of hypoxic and normoxic groups converged quickly. For fasted fish exposed to hypoxia, respiration frequency approached

Table 2

Sodium, potassium, chloride and ammonia concentration in plasma of common carp under different treatments. Values are mean ± SEM. Different symbols denote significant differences ($P < 0.05$) between feeding patterns (*), hypoxic treatments (†) and between 7 days of hypoxic exposure followed by 7 days of reoxygenation and without normoxic recovery (♦). The numbers (3 and/or 5) refer to significant differences with the corresponding control group (control 3/control 5).

Treatments		Na^+ (mM)		K^+ (mM)		Cl^- (mM)	Total ammonia (mM)
Control 3	Fed	120.60 ± 2.88		3.48 ± 0.19		106.93 ± 1.95	0.84 ± 0.15
	Fasted	115.47 ± 2.50		2.79 ± 0.19		106.13 ± 2.17	0.44 ± 0.11
1 day	Fed	112.42 ± 2.78		3.97 ± 0.40		104.50 ± 2.15	0.90 ± 0.20
	Fasted	110.58 ± 3.29		3.61 ± 0.40		102.71 ± 2.16	0.78 ± 0.15
3 days	Fed	107.21 ± 3.01	†3	3.56 ± 0.53		98.58 ± 1.91	0.94 ± 0.17
	Fasted	115.00 ± 1.90		2.82 ± 0.16		103.33 ± 1.40	0.33 ± 0.08
7 days	Fed	121.83 ± 2.84	*	2.52 ± 0.10	*	107.83 ± 2.49	0.50 ± 0.03
	Fasted	102.67 ± 4.37		4.02 ± 0.33	†3	98.06 ± 3.31	0.61 ± 0.14
7 days + 7 days recovery	Fed	118.17 ± 1.73		3.23 ± 0.06		107.33 ± 1.01	0.38 ± 0.10
	Fasted	120.38 ± 2.65	♦	2.82 ± 0.05		106.10 ± 2.66	0.35 ± 0.06
Control 5	Fed	124.98 ± 1.43	*	3.07 ± 0.17		112.71 ± 1.13	0.70 ± 0.11
	Fasted	110.76 ± 1.71		3.03 ± 0.19		105.81 ± 2.11	0.72 ± 0.18

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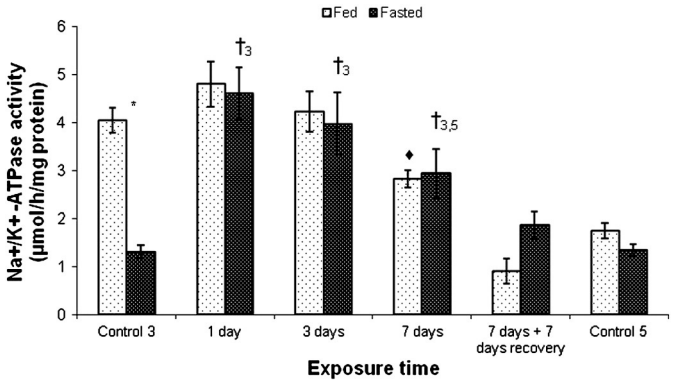

Fig. 6. Gill Na^+/K^+-ATPase activity in fed and fasted common carp during hypoxic exposure (mean ± SEM). Different symbols denote significant differences ($P < 0.05$) between feeding types (*), treatments (†) and between 7 days of hypoxic exposure followed by 7 days of reoxygenation and without normoxic recovery (♦). The numbers (3 and/or 5) refer to significant differences with the corresponding control group (control 3/control 5).

the control value immediately after re-oxygenation, suggesting that they could not fully compensate hypoxic effects before re-oxygenating occurred. However, ventilation frequency of fed hypoxic group already stabilized after about 119 h, meaning that the normoxic value was reached before re-oxygenation started and other compensatory responses must have developed by that time. Hypoxic hyperventilatory response has a high energetic cost (e.g. Steffensen, 1985). As hypoxic exposure is prolonged, several responses are initiated to further enhance O_2 uptake and delivery, such as an increase in Hb-O_2 affinity (Wood and Johansen, 1972; Wood et al., 1975; Rutjes et al., 2007) and a better oxygen transport capacity (Wood and Johansen, 1973; Lai et al., 2006; Rutjes et al., 2007). Gills of carp have a high O_2 extraction capacity and its blood has a very high O_2 affinity (Van Raaij et al., 1996). Acclimation to hypoxic circumstances may affect the gill morphology and the density, size and morphology of gill neuroepithelial cells (the putative O_2 chemo sensors of the fish gill) and change respiratory parameters (Jonz et al., 2004). Although not measured in this study, it is very likely that these effects contributed to the restored ventilation rates. At the end of the recovery period, ventilation rates of the fed fish that had been exposed to hypoxia increased again and stabilized at a new value. This can be attributed to behavioral changes resulting in more aggressive behavior of these fish.

The breathing frequency of the fasted normoxic group gradually decreased over time, while the frequency of the fed normoxic group remained stable (Fig. 2). The ventilation frequencies of fed groups were always higher than those of fasted groups (Fig. 2) due to the postprandial increase of metabolism and the higher activity of these fish. During hypoxic exposure, larger meals require a longer digestion time (Chabot et al., 2001) in order to keep SDA (specific dynamic action) within limits. Jordan and Steffensen (2007) confirmed that the increase of oxygen consumption (MO_2) after a meal was reduced during hypoxia (30% sat., 10 °C), which was accompanied by an increase in the duration of postprandial MO_2. Therefore, transit time of food bolus through the digestive system increases during hypoxic exposure and consequently food intake decreases. In the present study, no reduction in food intake was noticed, but the reduced SDA might have contributed to a reduced energy metabolism and thus the fast decrease in the hyperventilatory response of fed fish.

4.2. Energy metabolism

One would expect lactate accumulation under hypoxia (Richards et al., 2007; Wood et al., 2007) as was seen before in common carp exposed to stepwise decreasing oxygen (van Ginneken et al., 1998). Despite the fact that we were at, or close to, the Pcrit of these fish, there was no significant lactate accumulation in neither fasted nor fed fish. *C. carpio* seems to resist the hypoxic conditions well in the present study, possibly by inducing some metabolic changes. By suppressing ATP consumption/turnover, energy is used more economically, reducing lactate accumulation and hence also metabolic acidosis to a minimum (Hochachka et al., 1996). However, factors determining lactate accumulation might be subtle; exposing common carp to hypoxia (0.9 mg L^{-1}, 1.5% sat. at 5 °C), Lardon et al. found decreases in lactate in all tissues except brain, and only complete anoxia resulted in significant lactate accumulation. It seems that common carp only increase anaerobic metabolism at values well below Pcrit as was also seen in previous studies (Muusze et al., 1998; Lewis et al., 2007; Lardon et al., 2013). Another factor could be that common carp sustain a relatively constant level of anaerobic metabolism that suffices the need for energy supply during hypoxia and thus needs no further increase. Recent studies reported that *C. carpio* maintained high levels of basal plasma [lactate] of approximately 3.4–3.8 µmol/mL in venous blood (Liew et al., 2012; Sinha et al., 2012; Diricx et al., 2013; Genz et al., 2013) which are similar to that measured in the present study (Fig. 3), while arterial blood of *C. carpio* show only 0.8–1.5 µmol/mL lactate in the plasma during normoxia (Vianen et al., 2001; van Ginneken et al., 2004). An explanation might be found in the use of a different working method. In the study of Vianen et al. (2001) a catheter was inserted in the dorsal aorta for collecting arterial blood. In the other studies, however, one collected both arterial and venous blood, which gave rise to higher plasma lactate concentrations. Furthermore, fish of both studies (Vianen et al., 2001; van Ginneken et al., 2004) had a higher biomass and were probably less active since they were placed in small tanks. Interestingly, fed carp showed a higher level of anaerobic metabolism both during hypoxia and normoxia (control 5). This seemed to be independent of the occurrence of hyperventilation. Contrary to what was expected, plasma lactate of fed fish increased during normoxic recovery, while that of fasted fish further decreased. The increase in lactate concentration could be attributed to the higher activity of the fed fish during normoxic recovery, which was clearly noticeable and obviously required more energy. This was also reflected by the simultaneous breakdown of liver glycogen (Fig. 5A), while the decreasing plasma lactate levels matched the increasing liver glycogen levels in fasted fish (Cori cycle).

The effect of feeding regime was most striking in liver of fasted fish: after 1 week of fasting, HSI was reduced with 39% (Fig. 4) and glycogen content was substantially depleted. However, as mentioned above, hypoxia increased glycogen content in fasted fish, but not HSI. The opposite was true in muscle where glycogen stores decreased during hypoxia.

In the current study, lipid concentration in liver (Fig. 5C) was much higher than in muscle (Fig. 5D). As in a study by Wang et al. (2006) there was no increased consumption of hepatic lipids: carp prefer other lipid sources (such as visceral lipid) before using their hepatic reserves. Lipids provide more energy than glucose, but the oxidation of glucose is able to deliver the necessary energy quicker to respond to changing conditions (Dutra et al., 2008). As a result of the hypoxic exposure in the present study, especially liver proteins were used and muscle protein concentration in fasted fish significantly decreased immediately after reducing the oxygen concentration. Food deprivation had no significant effect on lipid concentration in both liver and muscle, although lipid was almost always lower in fasted groups.

In both feeding regimes, protein content in muscle (Fig. 5F) was always higher than in liver. Overall, hypoxia induced a reduction of liver and muscle protein content. Since protein synthesis is one of the major energy consuming processes, accounting for 18–26% of the cellular energy costs (Hawkins, 1991), the downregulation of protein turnover is one of the most important factors, contributing to the depression in ATP turnover and metabolic depression at the whole animal level (Hochachka et al., 1996; Guppy and Withers, 1999; Wang et al., 2006; Lewis et al., 2007; Richards, 2011). In our study, protein catabolism exceeded protein synthesis as can be seen from the reduced protein levels (especially in liver), although our results do not allow to discriminate between reduced protein synthesis and/or increased

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catabolism. As was demonstrated by measurement of Na^+/K^+-ATPase activity and Na^+ flux, the reduction in protein turnover in gills was accompanied by a simultaneous decrease in Na^+ pumping and leaking in the gills (Richards et al., 2007; Wood et al., 2007). However, in our study hypoxia induced an initial increase in ion pumping measured as Na^+/K^+-ATPase activity in fasted fish, but as time processed energy was also saved by depression of Na^+/K^+-ATPase.

After normoxic recovery liver protein content of both feeding regimes were significantly lower than the corresponding normoxic groups. As in the case of glycogen and lactate, this indicates that normoxic recovery was still not completed.

Food deprivation seemed to have a larger effect than hypoxia exposure: fasted carp especially depleted their glycogen stores, both in liver and in muscle. Liver proteins, and to a lesser extent muscle proteins, were also used, while lipid content remained relatively constant. Fasted fish also had a lower hepatosomatic index, indicating that liver size was reduced due to use of all energy stores, including lipids. A comparative study of common carp and goldfish also showed that food deprivation almost completely depleted glycogen stores of *C. carpio* but not of *Carassius auratus* (Liew et al., 2012). Both species used liver protein for their basal metabolism during starvation, while carp seemed to maintain muscle protein. This could indicate that common carp is able to synthesize muscle proteins and/or mobilize liver proteins to the muscle (Liew et al., 2012). In general, fasted fish had a lower energy content in muscle than fed fish (Liew et al., 2012), which in the present study was observed in the majority of the groups. The effects of the hypoxic exposure were most striking in liver proteins, as neither of the control groups used this substrate, even after several weeks of fasting.

Despite the small number of long-term studies with hypoxia exposure, it was mentioned several times in literature that the characteristics of anaerobic metabolism tend to disappear when hypoxia is not excessively deep and is prolonged (Wood and Johansen, 1972; Jørgensen and Mustafa, 1980a, 1980b; Smith and Heath, 1980; Johnston and Brenard, 1984; Van den Thillart and Smit, 1984). Hypoxia tolerant organisms activate rescue mechanisms by regulating the expression of several proteins (Hochachka et al., 1996), and common carp probably used other mechanisms to suppress metabolism to a lower level. For a better understanding of the complete process, more parameters should be considered (MO_2, glycogen, lactate, protein, lipid, CrP, glucose, pH, ATP, hematocrit, hemoglobin) and the applied hypoxia should be more severe.

4.3. Ionoregulation

Patterns in sodium and chloride concentration were very similar without any major changes (Table 2). For energetic reasons, enzyme activity was expected to drop during hypoxia exposure, thus reducing the transport of Na^+ ions across the basolateral membrane and resulting in a lower plasma [Na^+]. This, however, did not happen. In the present study, accumulation of plasma ammonia occurred at the beginning of the hypoxic exposure. Increased protein use and the possibility of 'ion channel arrest' offer a possible explanation (Wood et al., 2009) with the inhibition of excretion being greater than the inhibition of ammonia production (Wood et al., 2007). This results in a smaller ammonia efflux across the gills, although the exact mechanism for it remains controversial.

In contrast to the expectations, Na^+/K^+-ATPase activity increased in the present study, while in most studies using acute hypoxia a decrease was observed (Fig. 6). An explanation could be found in the striking similarity between the patterns of the ventilation frequency and the enzyme activity. Hyperventilation indeed increases the risk of fish to lose more ions to their dilute environment (Chippari-Gomes et al., 2005; Scott et al., 2008). As a consequence, fish will have to decide for an osmo-respiratory compromise (Nillson, 2007; Randall et al., 1972). A higher Na^+/K^+-ATPase activity would then contribute to reduce this ion loss and thus to maintain the Na^+ ion homeostasis. In the present study, carp possibly enlarge their gill permeability/area for better gas exchange by changing gill morphology. In goldfish, for example, interlamellar cellular mass (ILCM) disappears (Mitrovic et al., 2009) while in rainbow trout the number and surface of mitochondria rich cells (MRCs) increase (Matey et al., 2011). However, morphological changes were not examined in the current study. The ion loss due to the higher permeability should be compensated by an active ion uptake. The energetic cost of osmotic regulation in freshwater fish was estimated at 2–20% of the overall metabolic rate (Wood et al., 2007). The results of Mitrovic et al. (2009) suggested that the additional metabolic cost of ionoregulation due to an increase in functional lamellar area is not very high.

Although the time effect in the current study was almost never investigated before, the pattern of enzyme activity was very similar to that of goldfish, of which enzymatic activity only fully recovered after two weeks of reoxygenation (Mitrovic et al., 2009). In fasted groups, hypoxia only had a (small) effect on ion concentrations, but enzyme activity significantly increased after hypoxic exposure. In fed groups, there was almost no significant difference in ion concentration or enzyme activity with the normoxic control. This would suggest that hypoxia exposure did not have much effect on ion-exchange and enzymatic activity. Overall, ion concentrations and enzyme activity of fasted groups were lower than of fed fish. Since fasted fish could not obtain ions from their diet, they had to compensate their diffuse ion loss by active ion absorption at the levels of the gills. As for the control groups in a study of Sinha et al. (2012), plasma ammonia concentration was almost always higher in fed fish, since feeding leads to higher endogenous production of ammonia.

In conclusion we can state that, under hypoxia, *C. carpio* only seemed to suffer little of an ionoregulatory imbalance and experienced only a small disturbance in its ammonia regulation.

Food deprivation seemed to have greater impact than hypoxia, but further research is needed to determine whether this will also be the case under more severe hypoxia.

5. Conclusion

The striking differences in hypoxia tolerance of fish and the variety in responses to hypoxia suggest that variation in O_2 levels was an important selection trait. Many fish can tolerate mild hypoxia, but only a few species can survive severe hypoxia or anoxia (Van den Thillart and Van Waarde, 1985). Despite numerous information concerning metabolic and molecular responses to hypoxia of diverse fish species, no uniform concept of important adaptations, underlying hypoxia tolerance, exists. Moreover, for common carp, limited literature is available describing the mechanisms of hypoxia tolerance.

In the present study, food deprivation seemed to have a greater impact than hypoxia, since fasted fish used more substrates and increased enzyme activity more than fed fish. The physiological responses to hypoxia are largely determined by the duration and the severity of the hypoxic exposure. The ventilation frequency of the groups, exposed to hypoxia, recovered to control levels after a few days. It is likely that after these first days carp increased oxygen uptake by increasing the hematocrit value and/or the hemoglobin binding affinity to reduce the energetic cost. Since only a small disturbance occurred in metabolism and ionoregulatory response, the effect of normoxic recovery was difficult to detect. Further research is needed to determine if exposure to more severe hypoxia leads to the same results as in the present study. More parameters have to be examined to get a better view on the physiological mechanisms used by common carp, when encountering hypoxia and/or food deprivation.

6. Uncited references

Q5

Bogdanova et al., 2005

Boutilier and St-Pierre, 2000

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Hochachka and Lutz, 2001
Hylland et al., 1997
Nilsson, 1991
Nilsson, 1992
Piersma and Lindstrom, 1997
Sloman et al., 2006
Watters and Cech, 2003

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