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PII: S0044-8486(17)31455-2
DOI: doi: 10.1016/j.aquaculture.2017.09.008
Reference: AQUA 632810

To appear in: *aquaculture*

Received date: 21 July 2017
Revised date: 4 September 2017
Accepted date: 6 September 2017

Please cite this article as: Jyotsna Shrivastava, Amit Kumar Sinha, Sarah Cannaerts, Ronny Blust, Gudrun De Boeck , Temporal assessment of metabolic rate, ammonia dynamics and ion-status in common carp during fasting: A promising approach for optimizing fasting episode prior to fish transportation, *aquaculture* (2017), doi: 10.1016/j.aquaculture.2017.09.008

Temporal assessment of metabolic rate, ammonia dynamics and ion-status in common carp during fasting: a promising approach for optimizing fasting episode prior to fish transportation

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Abstract

In aquaculture practices, fish are routinely fasted prior to live transport to reduce metabolic rate and excretion. Prolonged periods of fasting often diminish the performance of transported fish and limit the ability to recover in their new environment. Therefore, the main objective of this study was to assess the ideal fasting period prior to the transport. For this purpose, common carp (*Cyprinus carpio*) were progressively fasted for 2, 4, 6, 8, 10, 12 and 14 days. Temporal effects of fasting on oxygen consumption rate (MO_2), ammonia excretion rate (J_{amm}), plasma ammonia (T_{amm}), plasma ions (Na^+, Cl^- and K^+), branchial Na^+/K^+-ATPase (NKA) and H^+-ATPase activity, as well as branchial mRNA expression of NKA, H^+-ATPase, Na^+/H^+ exchanger (NHE-3) and Rhesus (Rh) glycoproteins were assessed. Results show that MO_2 and J_{amm} were considerably depressed from 4-6 days of fasting onwards. Resting plasma T_{amm} were lowered during the first days of fasting preceding the reduced excretion rates, but returned to control levels at day 6 when excretion rates declined. In fasted fish, the basal expression levels of Rhcg-a and Rhcg-b were maintained up to 6 and 8 days respectively, thereafter down-regulation occurred. Plasma [Na^+] and [Cl^-] were temporarily reduced during 4-8 days of fasting followed by a recovery to control levels, while an augmented [K^+] was evident from 6 days onward. The transcript level of NHE-3 was raised in 12–14 day fasted fish which, along with up-regulation in Na^+/K^+-ATPase activity and mRNA expression, most likely facilitated the recovery of [Na^+] to control levels. Gill H^+-ATPase activities and mRNA levels were not affected by feed deprivation. First signs of depletion of liver energy stores, especially glycogen mobilization, were apparent from day 8 onwards. Overall, these data suggest that the desired reduction of metabolic rate (MO_2 and J_{amm}) was obvious from day 6 of fasting onwards, while at this time negative effects were not yet present: fish were still able to regulate ammonia and ion transport efficiently, and energy stores were not yet compromised.

Keywords: Fish transport, ammonia excretion, gene expression, Rhesus (Rh) glycoproteins, energy budget.

1. Introduction

The ability to transport live fish to the market to be sold as fresh product, to re-stock in aquaculture facilities as well as in the wild, or to deliver to processing plants for slaughter, is a challenge for aquaculture producers and marketers. Most of the labor and production costs are invested at the fish harvest phase, and any fish loss during transport due to death, injury or stress severely affects the profit margin. Aerial or ground hauling of live fish in a limited volume of water (typically in closed transport systems) results in rapid water quality deterioration throughout the transport period. The significant degradation of water quality and the onset of stress during transport can result in mortality. The major water quality issues experienced by fish during transport are- depletion of dissolved oxygen, accumulation of carbon dioxide owing to respiration, pH drop due to the carbon dioxide build-up, rise in ammonia levels, mucus accumulation and in case of recent feeding, faeces and regurgitated food in the transport medium. Reducing the water/fish volume ratio further aggravates many of these water quality problems (Harmon, 2009; Paterson et al., 2003). Among different water variables affecting the fish survival and fitness during transport, the increment in levels of ammonia resulting from excretion of nitrogenous excretory wastes from fish is one of the major concerns besides oxygen depletion. Elevated levels of ammonia are toxic for fish and can result in mass mortality. Ammonia accumulates easily in transport medium because it is the principal waste product of fish metabolism, primarily excreted through the gills by diffusion. Many studies suggested that accumulation of ammonia can be minimized by fasting the fish prior to the shipment (Harmon, 2009; Nemoto, 1957; Phillips and Brockway, 1954). It was documented for salmonids that a fasting period of 48 h–72 h prior to the transport reduces the ammonia accumulation in the holding tank (Wedemeyer, 1996). Likewise, trout fasted for 63 h produced half as much ammonia as fed fish (Phillips and Brockway, 1954). Recently, we also reported that fasting goldfish (*Carassius auratus*) and common carp (*Cyprinus carpio*) for 7 and 10 days respectively, significantly reduced the ammonia excretion rate relative to the corresponding fed fish (Diricx et al., 2013; Liew et al., 2012;

Sinha et al., 2012). As such, the practice to fast the fish prior to transport lowers their overall metabolism rate and thus reduces oxygen consumption and waste production. However, it must be noted that long-term fasting prior to transport can be stressful, lead to weight loss and compromise the ability of fish to perform essential life functions (Barton et al., 1980; Harmon, 2009; Schreck et al., 1989; Sprecker and Schreck, 1980). For example fasting common carp for longer periods reduces their ability to cope with increased levels of environmental ammonia and reduces energy stores such as protein and glycogen (Diricx et al., 2013; Liew et al., 2013). Therefore, it is important that ideal fasting period would be determined when fish metabolisms become low enough to keep oxygen consumption as well as ammonia production minimal, while still being able to regulate ammonia-transport so that it enables them to cope with ammonia stress in aquaculture facilities.

A series of cellular biochemical pathways produce ammonia in fish. Amino acid catabolism is one of the major pathways for ammonia synthesis that fish typically eliminate across their gills. Typically for fasting fish, muscle proteins act as a source of amino acids, which are catabolized for the production of ATP with ammonia as by-product (Houlihan et al., 1995). The current proposed model for ammonia excretion in gills of freshwater fish, the 'Na^+/NH_4^+ exchange complex or metabolon' involves several membrane transporters (H^+-ATPase, Na^+/H^+ exchanger, Na^+ channel) and Rhesus (Rh) glycoproteins working together in facilitating both ammonia excretion and Na^+ uptake (Wright and Wood, 2009). The key involvement of Rh glycoproteins in diffusive NH_3 efflux has been documented in fish (Nakada et al., 2007; Nawata et al., 2007). These appear to function as ammonia channels, binding NH_4^+ but facilitating the diffusion of NH_3 (Nawata et al., 2010). Apical Rhesus glycoprotein (Rhcg) transports NH_3 down the diffusion gradient. The Rhcg-driven NH_3 efflux is coupled to the efflux of H^+ through an apical H^+-ATPase and a Na^+/H^+ exchanger (NHE-2). In essence, these protons rapidly ionize NH_3 into NH_4^+, helping to create a positive gradient from blood to water (Evans et al., 2005; Wright and Wood, 2009). Augmentation in mRNA expression of Rhcg2, NHE-2, and H^+-ATPase were observed during feeding in juvenile trout (Zimmer et al.,

2010), suggesting that these transporters involved in ammonia handling are manipulated by nutritional status. It is therefore reasonable to speculate that keeping the fish fasted for an extended period might significantly reduce their ability to regulate ammonia handling and make the fish vulnerable to new environments.

In addition, ammonia excretion pathways in fish gills are closely linked with ion-regulation as a number of transporters/exchanger and coordinated mechanisms assisting ammonia handling are also involved in ion transport (Evans et al., 2005; Wilkie, 1997, 2002). The Na^+/K^+-ATPase (NKA) present in the basolateral membrane of branchial cells creates a chemical gradient for Na^+ contributing to the driving force to take up Na^+ from the water in exchange for ammonia *via* the apical Na^+/H^+ (or Na^+/NH_4^+) exchanger (Avella and Bornancin, 1989; Patrick and Wood, 1999; Randall and Tsui, 2002; Wilkie, 1997), and together with the apical H^+-ATPase an electro-chemical gradient for Na^+ uptake *via* the apical epithelial Na-channel (ENaC) (Lin and Randall, 1991; Perry, 1997). Therefore, besides playing a major role in the transport of Na^+ across the gill membranes, NKA and H^+-ATPase also appear to be involved in the ammonia excretion mechanism. It is documented for a number of fish species that feed deprivation can result in energy shortage, thereby reduces the capacity of fish for regulating ion homeostasis (Bucking and Wood, 2006a,b; Gaylord et al., 2001; Peterson and Small, 2004; Small, 2005; Small and Peterson, 2005; Small et al., 2002). Consequently, it is very likely that inefficient regulation of ammonia handling and ion-regulation following a chronic fasting episode might decline the overall fitness of fish.

In this study, we hypothesize that an ideal window for fasting might exist, where metabolism is reduced to a maximum extent without compromising energy stores, ammonia handling and ion-regulation. Therefore, with the overall aim to determine the most appropriate fasting period for fish transport, we assessed the dynamics of ammonia regulatory pathways, aerobic metabolism as well as ion homeostasis in the course of a series of fasting episodes in juveniles of the common carp. This is an important freshwater culture species and is widely distributed throughout the world. It is therefore

of great commercial and ecological importance.

To achieve our goals we measured (i) oxygen consumption rates and ammonia quotient as prime markers of aerobic metabolism; (ii) ammonia metabolism and excretion dynamics by quantifying ammonia excretion rate, its accumulation in plasma, and expression pattern of ammonia and related ion transporters (Rh isoforms, Na^+/H^+ exchanger, H^+-ATPase); (iii) ion status by determining ion (Na^+, K^+, Cl^-) levels in plasma and branchial NKA activity; and (iv) glycogen, lipid and protein levels in hepatic tissue as indicators of available energy stores.

2. Materials and methods

2.1. Experimental set-up

Common carp juveniles were obtained from the fish hatchery at the Wageningen University, The Netherlands. Fish were kept at the University of Antwerp in aquaria (1000 L) for at least a month before the experiment started. Thereafter, a total of 120 carp were distributed into six 200 L tanks (BW= 21-23g, N= 20 per tank). Each of these tanks was equipped with a recirculating water supply. Room temperature was set at 17°C and photoperiod was 12 h light and 12 h dark. Water quality was ensured through an additional bio-filter containing wadding, activated charcoal and lava stones. Water parameters were: pH 7.6 ± 0.2, dissolved oxygen 6.9-7.4 mg/L, ammonia 0.006-0.009 mM, nitrite 0.0015- 0.0021 mM, nitrate 0.015-0.042 mM, Ca^{2+} 0.8–1.0 mM, Mg^{2+} 0.19-0.21 mM, Na^+ 1.2-1.4 mM, K^+ 0.09-0.10 mM, Cl^- 0.9-1.2 mM, titratable alkalinity 1.6-1.8 mM and hardness 225 mg $CaCO_3$/L. Fish were acclimated to the above mentioned constant temperature and photoperiod for 2 weeks prior to the experiment and were fed with commercial pellets (‘Hikari Staple’, Kyorin Food Ind. Ltd., Japan, 0.65 % NaCl) at a rate of 2% of their wet body weight/day.

After being acclimatized at the mentioned conditions for 2 weeks, feeding was withheld in four of the six tanks while feeding (2% body weight/day) was continued in the remaining two tanks.

Feeding was adjusted based on the weight and the number of fish remaining in the tank after each sampling period. Fasted fish groups were kept unfed for 2, 4, 6, 8, 10, 12 and 14 days. Control groups (feeding) were set up in parallel to the first (2 days) and the last sampling period (14 days) and were sampled in an identical way as for the fasted groups. To avoid the ammonia build-up in the tanks, recirculation through bio-filter containing wadding, activated charcoal and lava stones was assured, and additionally 40–60% of the water was renewed every 2 days.

All animal experiments were approved by the local ethics committee (University of Antwerp, Permit Number: 2011-05), and conducted according to the guidelines of the Federation of European Laboratory Animal Science Associations.

2.2. Determination of oxygen consumption and ammonia excretion rates

For each time point, 8 fish (4 from each tank) from control or fasted groups were netted and transferred individually into respective respirometers. The fish were placed in Blazka-style swimming respirometers (≈3.9 L) 12 h prior to sampling (the evening before measurement) to minimize handling stress. Water composition and temperature in the recirculation system containing the respirometers was identical to the water composition of the tanks. Water speed was set at 5 cm/s which allowed the fish to orient themselves against the water current and swim gently overnight.

At the start of the oxygen measurements, initial water samples were collected from the eight respirometers containing one fish each. Water circulation through the respirometers was cut off, air bubbles were carefully removed through the outlets without stressing the fish, the oxygen electrodes (WTW, Weilheim, Germany) were inserted and the system was made airtight so that water leakage from the respirometers was negligible. Final water samples were collected to measure ammonia excretion after an interval of 3 h, during which oxygen concentration (mg/L) at the above mentioned swimming speed was continuously recorded. Following 3 h end point, the oxygen concentrations ranged between 3.1 to 2.6 mg/L.

Oxygen consumption rate MO_2 (µmol/g/h) was calculated according to Ultsch et al. (1980) method as

$MO_2 = (\Delta O_2 i - O_2 f) \times V \times 1000 \times (1/O_{2MW}) \times (1/BW) \times T$

where O_2i is first oxygen concentration (mg/L) and O_2f is second oxygen concentration (mg/L); V is total water volume in respirometer (3.9 L); O_{2MW} is molecular weight of oxygen (32); BW is body weight (g) and T is time (h).

Water total ammonia was determined colorimetrically by using the salicylate–hypochlorite method (Verdouw et al., 1978). Ammonia excretion rates J_{amm} (µmol/kg/h) were calculated as:

$J_{amm} = ([Amm]_i - [Amm]_f) \text{ X } V/(t \text{ X } M)$

where $[Amm]_i$ and $[Amm]_f$ are the initial and final concentrations of ammonia in the water (in µmoles/L) obtained from comparison to a standard curve. V indicates volume (L), t time (h), and M mass (kg).

2.3. Sampling procedure

For biochemical and molecular analysis, fish were sampled from the respirometers after measurement of O_2 consumption. Following the last water sampling, the animals were terminally anaesthetized with a lethal dose of neutralized (with 2 parts NaOH) MS-222 (ethyl 3- aminobenzoate methane-sulphonic acid, 1 g/L, Acros Organics, Geel, Belgium), blotted dry and weighed. Subsequently, a blood sample was collected from the caudal blood vessel using a heparinized syringe. Blood was immediately centrifuged (for 1 min at 16,000 rpm at 4°C), and aliquots of plasma were frozen in liquid nitrogen and stored at −80°C for later analysis. Fish were dissected on ice, gills and liver tissue were removed, frozen in liquid nitrogen, and stored at −80°C for further biochemical and enzymatic assays. In addition, one portion of gills was added to five volumes of RNA*later* (Qiagen, Hilden, Germany) and stored at 4°C for later molecular analysis.

2.4. Analytical techniques

Ammonia levels in plasma were determined using an enzymatic kit (R-Biopharm AG, Darmstadt, Germany). Plasma [Na^+], [Cl^-] and [K^+] were analysed using an AVL 9180 Electrolyte Analyzer (AVL, Roche Diagnostics, Belgium). Samples of liver were analyzed for protein content by Bradford's method (Bradford, 1976), glycogen content by using the anthrone reagent (Roe and Dailey, 1966) and lipid content was measured following Bligh and Dyer (1959).

NKA activity and H^+-ATPase activity were measured in crude gill homogenates using previously published methods (McCormick, 1993; Lin and Randall, 1993), as modified by Nawata et al. (Nawata et al., 2007).

Total RNA was isolated from gill samples using Trizol (Invitrogen, Merelbeke, Belgium) according to the manufacturer's instructions. The extracted RNA samples were DNase treated to avoid genomic DNA contamination. The quantity of the RNA was evaluated by using Nano-Drop spectrophotometry (NanoDrop Technologies, Wilmington, DE, USA). The purity was checked by measuring the OD260/OD280 absorption ratio (>1.95).

2.5. Real-time PCR

For quantitative real-time PCR (qPCR), a starting amount of 1µg RNA was transcribed into first-strand cDNA using the Revert Aid H minus First strand cDNA synthesis kit (Fermentas, Cambridge, UK). mRNA expression of NKA (α-subunit), H^+-ATPase (vacuolar, B-subunit), Rhcg-a, Rhcg-b, Rhbg and NHE-3 in the gills was quantified by qPCR using the specific primers listed in Table 1. The primer sequences were adopted from Sinha et al. (2013), with the exception of NHE-3 (Bradshaw et al., 2012). qPCR analyses were performed on an Mx3000P QPCR System (Agilent Technologies, Belgium). Reactions containing 5µL of 5×diluted cDNA, 10pmol each of forward and reverse primers, 0.3µL ROX dye (1:500 dilution) and 10µL Brilliant III Ultra-Fast SYBR Green qPCR Master Mix (Agilent) were performed in a four-step experimental run protocol: a denaturation

program (3 min at 95°C); an amplification and quantification program repeated 40 times (15 s at 95°C, 20 s at 60°C); a melting curve program (60–95°C with a heating rate of 0.10°C/s and a continuous fluorescence measurement) and finally a cooling step. Melt curve analyses of the target genes and reference genes resulted in single products with specific melting temperatures. In addition, 'no-template' controls (i.e. with water sample) for each set of genes were also run to ensure no contamination of reagents, no primer–dimer formation, etc.

Comparison of several reference genes (β-actin, glyceraldehyde-3-phosphate dehydrogenase and elongation factor-1α) favoured β-actin as the most stable gene across the samples (20 random samples were tested) and was used as endogenous standard to calculate relative mRNA expression by the standard curve method. Standard curves for each set of primers were generated by serial dilution of a random mixture of control cDNA samples.

2.6. Statistical analysis

All data have been presented as mean values ± standard error (S.E.). The normality of the data was checked using the Shapiro-Wilk test. For comparisons between different experimental groups a one-way analysis of variance (ANOVA) was performed followed by the least significant difference (LSD) test. Student's two-tailed t-test was used for single comparisons. The data were analyzed by Statistical Package for the Social Sciences (SPSS) version 20.0. A probability level of 0.05 was used for rejection of the null hypothesis. Principal component analysis (PCA) was performed by using OriginLab 9 software (OriginLab, Northampton, MA, U.S.A.). All measured parameters were subjected to PCA to investigate the overall effect fasting on the physiological and gene expression profile. The standardized scores of the first two components which explained the highest variation were used to make biplots.

No significant differences were found between any of the control values at 2 days and 14 days. Therefore, pooled controls for each experimental group are shown for clarity of the figures.

3. Results

3.1. In vivo parameters

In comparison to the control (fed fish) value of 8.33 μmol/g/h, a significant reduction in MO_2 was noted after 4 days of fasting and MO_2 remained lower up to 14 days, except for a non-significant decline at day 6 (Fig. 1). Among all the fasting episodes, the lowest value was recorded after 2 weeks where MO_2 dropped by 34% ($P < 0.001$) compared to the control value. Ammonia excretion rate (J_{amm}) declined following fasting from day 6 onwards and remained lower until the end of the experiment (14 days) (Fig. 2). The reduction was largest at 14 days of fasting, with a decline of J_{amm} from a control value of −1070 μmol /kg/h to −211 μmol /kg/h ($P < 0.001$). The ammonia quotient (AQ) represents the mole to mole ratio of ammonia excretion to oxygen consumption. Due to the relatively larger reductions in J_{amm} compared to MO_2, values of AQ reduced considerably in 12 and 14 day fasted fish by 59% ($P < 0.01$) and 70% ($P < 0.001$) respectively, clearly indicating the effect of feed deprivation (Fig. 3). Moreover, AQ can also be used to estimate the relative contribution of protein mobilization for the oxidative metabolism (relative to other fuels) at various fasting time points.

3.2. Metabolites and ions

Plasma total ammonia (T_{amm}) was significantly reduced during the first days of fasting; levels in fasted fish at 2 and 4 days were 58% ($P < 0.01$) and 62% ($P < 0.01$) lower relative to the fed fish (Fig 4). Thereafter, levels returned to the control levels. A significant decline in plasma [Na^+] compared to the control was noted for 6 and 8 day fasted fish. Thereafter [Na^+] restored to the control level (Table 2). Similarly, the effect of fasting was also observed for [Cl^-] in plasma; a reduction of 13% ($P < 0.001$) and 15% ($P < 0.05$) was seen respectively for 4 and 6 day fasted fish in comparison to the control (Table 2). Conversely, [K^+] augmented when fish were subjected to

feed deprivation (Table 2). An increment was recorded following 6 to 12 days of fasting ($P < 0.05$), and returned to the control value at the last fasting period.

Not surprisingly, a distinct effect of fasting was observed on glycogen content in liver tissue with a progressive reduction ($P < 0.05$) with 27-30% from 8 days onwards (Fig. 5A). Protein content in liver tissue also reduced significantly in fasted fish. Following 12–14 days of feed restriction, protein content reduced by 28% and 35% ($P < 0.01$ %) respectively (Fig. 5B). A drop in lipid level was observed from 10 days onwards (Fig. 5C). Lipid content in fasted fish at 10, 12 and 14 days were 21% ($P < 0.05$), 30% ($P < 0.01$) and 28% ($P < 0.01$) lower relative to the control.

3.3. Activity and gene expression of transporters

No prominent effect of fasting was observed for branchial NKA activity. A steady increase led to a significant increment at 12 days ($P < 0.05$) which then recovered to control levels (Fig. 6). The increase in NKA activity was preceded by an activation of the transcript level; a significant 2- fold increment in NKA mRNA expression was seen at 10 days of fasting (Fig. 7). In the present study, the branchial H^+-ATPase activity remained unchanged throughout the fasting periods. Similar to the activity response, the expression level of H^+-ATPase remained unchanged in all fasting periods (results not shown). NHE-3 expression in gill tissue displayed an up-regulation in response to the feed deprivation from 12 days onwards (Fig. 8). The up-regulation after 12 and 14 days was 2.22 fold ($P < 0.001$) and 2.07 fold ($P < 0.01$), respectively.

Subjecting the fish to feed deprivation incited a downregulation in branchial Rhcg-a expression level (Fig. 9A). The effect was detected from 8 days onwards ($P < 0.05$) and continued until the end. Rhcg-b expression level was significantly reduced from 10 days onwards (Fig. 9B). A 4.18-fold ($P < 0.001$), 1.72- fold ($P < 0.05$) and 2.19- fold ($P < 0.01$) reduction was noted for fasted fish at 10, 12 and 14 days respectively. Unlike Rhcg isoforms, expression levels of Rhbg did not change considerably across the fasting periods (Fig. 9C).

3.4. Principal component analysis (PCA)

The PCA biplot depicts a clear separation of experimental groups, mainly along the first two components (PC1 and PC 2), together explaining 76% of data variability (Fig. 10). These two components showed a clear separation indicating differential responses among various fasting groups. The prevailing PC1 component (54% of the data variance) clustered MO_2, J_{amm}, Rhcg-a and Rhcg-b gene expression and energy store (protein, lipid and glycogen) with experimental fish groups that were fasted for 2, 4, 6 days as well as control fish. PC2 (22% of the data variance) clustered 12-14 days fasted fish with T_{amm}, $[Na^+]$, $[Cl^-]$ and NHE-3 expression on the one hand, and $[K^+]$, NKA activity and NKA expression with 8-10 days fasted fish at the other hand.

4. Discussion

In this study we have focused on finding the ideal pre-transport fasting period by evaluating temporal ammonia dynamics and associated metabolic trade-off. Oxygen consumption is widely used as a primary indicator of metabolic activity in fish. In the present study, a significant reduction of MO_2 from day 4 onwards indicates a depression in metabolic rate with up to 34% under feed deprivation. Likewise, feed deprivation in juvenile rainbow trout (*Oncorhynchus mykiss*) and largemouth bass (*Micropterus salmoides*) were documented to have respectively 30% and 41% lower MO_2 relative to the conspecifics fed with a maintenance ration (Alsop and Wood, 1997; Gingerich et al., 2010). These difference between fed fish and fasted fish might relate to the specific dynamic action (SDA), a characteristic increase in MO_2 following feeding (Randall and Tsui, 2002; Wicks and Randall, 2002). Our MO_2 measurements were probably too far apart to asses any peak SDA, but a decrease in metabolic activity at day 4 matches with the observation that common carp digest slowly and often still have food in the intestine 24- 48 hours after a meal. However, fasted

juvenile rainbow trout have demonstrated a lack of metabolic declines wherein resting oxygen was not affected after periods of 5 days starvation (Scarabello et al., 1991). Inconsistencies in the response of metabolic rates to fasting events across studies may therefore be explained by species-specific differences, culture conditions, water characteristics (e.g. temperature), food quality, animal density and/or differences in experimental protocol.

During transport, ammonia builds up in the transport water, mainly due to digestion and protein metabolism of the fish. The production of ammonia by fish can be decreased by shipping fish only after food has been withheld long enough to empty the stomach and intestine. Similar to our previous experiment with goldfish (Sinha et al., 2012), ammonia excretion rate was lower in fasted fish from day 6 onwards and the decrease continued until the end of the experiment, which would lead to a lower ammonia accumulation in the water. The reduction almost paralleled the drop in MO_2 in fasted fish. Fish have been shown to reduce activities of aerobic enzymes (Sullivan and Somero, 1983; Yang and Somero, 1993) and ventilation rates following periods of fasting (Millidine et al., 2008) which could explain both a reduction in MO_2 and a consequent decline in the ammonia production and thus excretion rate. Decreased ammonia excretion could not only originate from reduced protein metabolism, but could also be caused by an inhibition of excretion. However, the responses of J_{amm} in fasted fish were preceded by reductions in plasma T_{amm} indicating reduced catabolism of protein. Only after the consequent reduction in J_{amm} in 6-14 days fasted fish, a restoration of plasma T_{amm} to the basal level was observed. The reduced dependence on protein catabolism was also confirmed looking at the ammonia quotient. In fish, maximal AQ for 100% aerobic protein degradation is 0.33 (Kutty, 1978) and this value becomes lower when other substances are used. We found that the AQ was between 0.08 and 0.13 in most experimental groups indicating that protein was not the main energy source that was mobilized. In starved fish AQ remained quite stable up to 10 days, since the reduction in ammonia excretion concurred proportionate with the decline in MO_2 within this time frame. This is confirmed by the relatively

stable liver protein stores, which only started to deplete after glycogen and lipid stores were significantly affected. At day 12 and 14, AQ reduced even further to 0.03. This suggests that under the experimental conditions, these fish do not primarily rely on protein catabolism for the total energy production, but rely on carbohydrate and lipid metabolism. In general, during starvation fish utilize the energy reserves stored in hepatic glycogen first with the lipid reserves following next and finally proteins are mobilized (Alliot et al., 1984; Echevarría et al., 1997; Pastoureaud, 1991), except perhaps in anoxia resistant species such as crucian carp and goldfish which preserve liver glycogen stores for possible anoxic insults (Nilsson, 1990). A previous study using a 7 day fasting period also showed that common carp primarily rely on liver and muscle glycogen, in contrast to goldfish that mainly used protein (Liew et al., 2012). In that study, protein stores were also reduced in common carp, although to a lesser extent, and lipid stores remained stable. Liver is the primary site for the glycogen mobilisation, and glycogen metabolites typically glucose serves as the major metabolic fuel for the cellular homeostasis and ion-regulatory processes (Tseng and Hwang, 2008). In the present study, fasting was seen to deplete glycogen stores, possibly to meet the energy demand of a suite of physio-biochemical process. Energy reserve in hepatic tissue revealed differential patterns of mobilization; glycogen depletion occurred fairly earlier (from day 8) compared to lipid (day 10) and protein (day 12). This confirms that glycogen was the main energy source for basal metabolism in all fasted common carp. This observation is consistent with the results of Liew et al. (2012), in which 7 days fasted common carp prioritize glycogen mobilization over lipid or protein for energy source. Likewise, European sea bass (*Dicentrarchus labrax*; Gutierrez et al., 1991), Amazonian oscars (*Astronotus ocellatus*; De Boeck et al., 2013), brown trout (*Salmo trutta fario*; Navarro et al., 1992), white sturgeon (*Acipenser transmontanus*; Hung et al., 1997) and juadia (*Rhamdia quelen*; Barcellos et al., 2010) were also reported to rely on glycogen as preferred energy source when confronted with prolonged starvation. In freshwater fish, the maintenance of ionic balance is an energetically expensive biochemical process due to the concentration gradient that exists between plasma and

fresh water (Gingerich et al., 2010; Gonzalez and McDonald, 1992; Randall et al., 1972). Therefore, to retain the ions-homeostasis, liver metabolism needs to be enhanced to meet the fuel demand of energy dependent ion-transporters such as NKA and H^{+}-ATPase. A reduction in glycogen content coincided with the numerical increment of NKA which facilitated Na^{+} homeostasis in plasma, suggesting prioritization towards ion balance at the cost of glycogen reserve. Similar consequences were also documented for starved largemouth bass wherein a reduction in energy stores was associated with the maintenance of Na^{+} concentration in plasma (Gingerich et al., 2010).

In teleosts, the majority of ammonia transport is thought to be through NH_3 diffusion at the gills, either directly or facilitated by Rh glycoproteins (Nakada et al., 2007; Nawata et al., 2007). The mechanistic ammonia excretion model in freshwater teleost gills suggests that Rhcg present on the apical membrane of the gill epithelium facilitates ammonia efflux out of the gills and appears to be coupled to H^{+} excretion (through H^{+}-ATPase and/or NHE) and Na^{+} uptake (Wright and Wood, 2009). In the present study, the expression profiles of two Rhcg homologs (Rhcg-a and Rhcg-b) were quantified. The expression kinetics showed a significant down-regulation for Rhcg-a and Rhcg-b in response to fasting during 8–14 days and 10–14 days respectively. PC analysis confirmed that these two Rh homologs clustered with the control group with 2, 4 and 6 day fasted fish where expression rates still remained high. Interestingly, the time frame for the down-regulation of these Rh proteins does not completely correspond with the depression of J_{amm}. Downregulation occurred after excretion rates started to reduce. This indicates that initially, the reduced excretion rate was caused by the reduced plasma ammonia levels. The lesser need for ammonia transport led to the downregulation of the Rh glycoprotein and a restoration of plasma ammonia levels. This data set suggests that prolonged fasting can reduce the functional role of Rhcg isoforms. It was also reported that nutritional status can modulate the expression profile of branchial Rhcg2 in rainbow trout; Rhcg2 was up-regulated during feeding in juvenile trout (Zimmer et al., 2010).

In branchial epithelium, Rhbg is localized on the basolateral membranes. In zebrafish larvae, Rhbg was reported to be highly expressed and plays an important role in ammonia excretion (Braun et al., 2009). Likewise, an elevated Rhbg transcript level was also documented in goldfish when exposed to elevated environmental ammonia (Sinha et al., 2013), underlining its role in the regulation of ammonia transportation in gills of these freshwater teleost. However, in our present study on common carp, no change in Rhbg expression level in response to feed deprivation was found. In the former studies also no change in branchial Rhbg expression level in response to environmental stressors (e.g. high ammonia, copper toxicity) was found for common carp (Sinha et al., 2013, 2016) or rainbow trout (Nawata et al., 2007; Sinha et al., 2013) in contrast to the changes seen in goldfish (Sinha et al., 2013). This connotes that at least in common carp Rhbg is not primarily involved in ammonia transport and that functional significance of Rhbg might be highly species specific. Overall, it was apparent from the present study that in course of fasting episodes the fish were able to depress ammonia excretion metabolism from day 6 onwards, whilst maintaining basal transcript levels of the ammonia transporter Rhcg-a and Rhcg-b at least up to this time point.

It is well understood that in freshwater teleosts, the uptake of ions from the water is mainly attributed to mitochondria rich cells (MRC) which also contain a number of transporters and co-ordinated mechanisms that are involved in maintenance of ion-regulation. In the extensive tubular membrane system of MRCs, NKA are present which are believed to provide the major source of energy driving Na^+ influx from the external water into the blood. In response to 6-8 days fasting, $[Na^+]$ in plasma declined considerably which appears to be compensated afterwards by a momentary increase in NKA activity. Moreover, relative to the fed fish, the fasted fish displayed higher resting NKA activity for most of the time points. The increase in NKA activity among fasted fish was accompanied by a parallel activation of the transcript level of NKA. An activated NKA gene expression and activity successive to the reduction in Na^+ pool in the plasma might help the fish to drive Na^+ uptake from the surrounding water, evident by the restoration of Na^+ at the control level

following 10 -14 days of feed deprivation. Similar with our results, a study on goldfish showed that Na^+ level in plasma were lower in the 7 day fasted group (compared to the fed fish), which coincided with a relative higher resting NKA activity in fasted fish (Liew et al., 2013).

Furthermore, as per the proposed "Na^+/NH_4^+ exchange complex" scheme (see introduction) the deprotonation of NH_4^+ at the intracellular binding site of the Rhcg channel releases H^+ to the external water by either or both H^+-ATPase and the Na^+/H^+ exchangers (NHE). Both these mechanisms would provide a coupling to Na^+ uptake by exchange of Na^+ versus H^+ (via NHE) and by providing the necessary electromotive force (via H^+-ATPase) to power the uptake of Na^+ from the water through a Na^+– selective channel. Therefore, in freshwater fish besides the potential involvement of NKA, the gradient needed for sodium uptake is also believed to linked with Na^+/H^+ exchangers (NHE) and the apical H^+-ATPase (Weihrauch et al., 2009; Wright and Wood, 2009). Along with pronounced NKA activity (and expression), an elevated NHE-3 transcript level in fasted fish could explain in part the upsurge and restoration of $[Na^+]$ in plasma of 10- 14 days feed deprived fish to the basal level. These scenarios also predict that the concomitant augmentation in NKA and NHE-3 level during fasting episodes would be a compensatory response of fish to maintain intracellular Na^+ level.

H^+ efflux (via either H^+-ATPase and/or the NHE) would acidify the gill boundary layer, thereby providing the 'diffusion-trapping' of NH_3 as NH_4^+. However, in the present study the expression level as well as the activity of H^+-ATPase in all fish groups subjected to feed deprivation remained unchanged. This corroborates with our earlier results in common carp wherein neither the activity nor the expression level of H^+-ATPase was affected under environmental stresses (Shrivastava et al., 2016; Sinha et al., 2013, 2016). Therefore, it is reasonable to acknowledge that common carp do not rely on H^+-ATPase for boundary layer acidification and ammonia trapping.

In addition to Na^+ imbalance, fasting episodes also altered circulating Cl^- in plasma, marked by a decline relative to the control. This might relate to the decreased CO_2 production rates as a

consequence of the depressed metabolic rate in fasted fish from day 4 onwards. CO_2 serves as a substrate for the carbonic anhydrase (CA), which catalyses the hydration of CO_2 into H^+ and HCO_3^- (Perry et al., 2003; Vitale et al., 1999). This HCO_3^- is exchanged for Cl^- via Cl^-/HCO_3^- exchanger at the apical side of the branchial epithelium. It is tempting to speculate that an anticipated lower production of CO_2 in unfed fish would reduce the CA activity, which in turn would limit the amount of HCO_3^- available for exchange with Cl^-, thereby lowering Cl^- uptake.

Furthermore, augmented NKA activity in fasted fish group would facilitate the movement of K^+ from plasma to the intracellular compartment. However, we found an increased level of K^+ in the plasma under fasting conditions, and consequently K^+ clustered in a different co-ordinate of PCA than Na^+ and Cl^-. The increment in $[K^+]$ observed for fasted fish corroborates with earlier findings in goldfish that showed significantly higher K^+ level in plasma following 7 days of fasting (Liew et al., 2013). The probable reason for such an observation could be attributed to transport of K^+ from other tissues (typically RBC) to plasma due to electrolyte imbalances. Nevertheless, it is also probable that RBC become more fragile (and ruptures) following prolonged fasting. In addition, the ability of fasted fish to elevate plasma K^+ level could also be facilitated by the active K^+ uptake via K^+ channels in gills and/or reabsorption in kidney. Eddy (1985) also found that fasting rainbow trout were able to regulate K^+ independently from food (under low salinity or in freshwater), mainly through the gill.

Overall, this temporal analysis of ion homeostasis and the associated ions status in plasma suggest that fish are able to sustain ion-homeostasis approximately up to 4 days of feed withdrawal; a more prolonged fasting period prior to fish transport can result in transient hyponatremic, hypochloremic and hyperkalemic plasma.

5. Conclusion

To find an ideal fasting period for fish prior to the shipment, we have determined a suit of physiological, biochemical, ammonia metabolic and ion-regulatory status in common carp when subjected to a series of fasting episode (2-14 days). We observed many, but not all, responses started to amend by 6 days of feed deprivation. Firstly, at this point the metabolic rate oxygen (MO_2) and ammonia excretion rate (J_{amm}) were depressed, two factors that are extremely important to sustain a good water quality when transporting fish. These temporal responses were also preceded by lower ammonia accumulation in plasma. Secondly, Rh glycoprotein which are implicated as a putative mechanism of ammonia transport, maintained normal expression levels (typically Rhcg-a) until 6 days of fasting. This means that common carp fasted for a maximum of 6 days are still capable of efficient ammonia handling in case environmental ammonia would increase due to deteriorating water quality. Lastly, the glycogen store in the liver could efficiently fuel aerobic tissues up to 6 days of feed deprivation, as no significant declines were seen in resting liver glycogen store up to 8 days of fasting. Together our results suggest that an ideal fasting period exists around 6 days. However, it should be noted that factors affecting metabolism, such as water temperature and fish size are also important variables to consider in future studies.

Acknowledgments

The technical assistance of Karin Van den Bergh, Steven Joosen, Eline Verrostte, Surjya Datta and Nemo Maes is gratefully acknowledged. Jyotsna Shrivastava is a research fellow supported by a PhD Grant [BOF-DOCPRO-31444] from the University of Antwerp Research Council. Amit Kumar Sinha worked as a research fellow supported by the Research Fund - Flanders [FWO Grant12A8814N].

References

Alliot, E., Djabali, M., Pastoureaud, A., Thebault, H., 1984. Changes in the biochemical composition of tissues in juvenile sea bass during forced starvation. Biochem. Syst. Ecol. 12, 200–213.

Alsop, D., Wood, C.M., 1997. The interactive effects of feeding and exercise on oxygen consumption, swimming performance and protein usage in juvenile rainbow trout (*Oncorhynchus mykiss*). J. Exp. Biol. 200, 2337–2346.

Avella, M., Bornancin, M., 1989. A new analysis of ammonia and sodium transport through the gills of the freshwater rainbow trout (*Salmo gairdneri*). J. Exp. Biol. 142, 155–175.

Barcellos, L.J.G., Marqueze, A., Trapp, M., Quevedo, R.M., Ferreira, D., 2010. The effects of fasting on cortisol, blood glucose and liver and muscle glycogen in adult jundia *Rhamdia quelen*. Aquaculture 300, 231–236.

Barton, B.A., Peter, R.E., Paulencu, C.R., 1980. Plasma cortisol levels of fingerling rainbow trout (*Salmo gairdneri*) at rest, and subjected to handling, confinement, transport, and stocking. Can. J. Fish. Aquat. Sci. 37, 805-811.

Bligh, E.G., Dyer, W.J., 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. 37, 911–917.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of micro-gram quantities of protein utilizing the principle of protein–dye binding. Anal. Biochem. 72, 249–254.

Bradshaw, J.C., Kumai, Y., Perry, S.F., 2012. The effects of gill remodeling on transepithelial sodium fluxes and the distribution of presumptive sodium-transporting ionocytes in goldfish (*Carassius auratus*). J. Comp. Physiol. B 182, 351–366.

Braun, M.H., Steele, S.L., Ekker, M., Perry, S.F., 2009. Nitrogen excretion in developing zebrafish (*Danio rerio*): a role for Rh proteins and urea transporters. Am. J. Physiol. 296, F994-F1005.

Bucking, C., Wood, C.M., 2006a. Water dynamics in the digestive tract of the fresh-water rainbow trout during the processing of a single meal. J. Exp. Biol. 209, 1883–1893.

Bucking, C., Wood, C.M., 2006b. Gastrointestinal processing of Na^+, Cl^-, and K^+ during digestion: implications for homeostatic balance in freshwater rainbow trout. Am. J. Physiol. Regul. Integr. Comp. Physiol. 29, 1764–1772.

De Boeck, G., Wood, C.M., Iftikar, F.I., Matey, V., Scott, G.R., Sloman, K.A., da Silva, M.D.N.P., Almeida-Val, V.M., Val, A.L., 2013. Interactions between hypoxia tolerance and food deprivation in Amazonian oscars, *Astronotus ocellatus*. J. Exp. Biol. 216, 4590– 4600.

Diricx, M., Sinha, A.K., Liew, H.J., Mauro, N., Blust, R., De Boeck, G., 2013. Compensatory responses in common carp (*Cyprinus carpio*) under ammonia exposure: additional effects of feeding and exercise. Aquat. Toxicol. 142–143,123–137.

Echevarría, G., Martínez-Bebiá, M., Zamora, S., 1997. Evolution of biometric indices and plasma metabolites during prolonged starvation in European sea bass (*Dicentrarchus labrax*, L.). Comp. Biochem. Physiol., Part A: Mol. Integr. 118,111–123.

Eddy, F.B., 1985. Uptake and loss of potassium by rainbow trout (*Salmo gairdneri*) in fresh water and dilute sea water. J. Exp. Biol. 118, 277–286.

Evans, D.H., Piermarini, P.M., Choe, K.P., 2005. The multifunctional fish gill:dominant site of gas exchange, osmoregulation, acid-base regulation, and excretion of nitrogenous waste. Physiol. Rev. 85, 97–177.

Gaylord, T.G., MacKenzie, D.S., Gatlin III, D.M., 2001. Growth performance, body composition and plasma thyroid hormone status of channel catfish (*Ictalurus punctatus*) in response to short-term feed deprivation and refeeding. Fish Physiol. Biochem. 24, 73–79.

Gingerich, A.J., Philipp, D.P. and Suski, C.D., 2010. Effects of nutritional status on metabolic rate, exercise and recovery in a freshwater fish. J. Comp. Physiol. B 180, 371-384.

Gonzalez, R.J., McDonald, D.G., 1992. The relationship between oxygen consumption and ion loss in a freshwater fish. J. Exp. Biol. 163, 317–332.

Gutierrez, J., Perez, J., Navarro, I., Zanuy, S., Carrillo, M., 1991. Changes in plasma glucagon and

insulin associated with fasting in sea bass (*Dicentrarchus labrax*). Fish Physiol. Biochem. 9, 107–112.

Harmon, T.S., 2009. Methods for reducing stressors and maintaining water quality associated with live fish transport in tanks: a review of the basics. Reviews in Aquaculture 1, 58-66.

Houlihan, D.F., Carter, C.G., McCarthy, I.D., 1995. "Protein turnover in animals," in Nitrogen Metabolism and Excretion, eds P. J. Walsh and P. Wright (Boca Raton, FL: CRC Press), 307–395.

Hung, S.S.C., Lui,W., Li, H., Storebakken, T., Cui, Y., 1997. Effect of starvation on some morphological and biochemical parameters in white sturgeon, *Acipenser transmontanus*. Aquaculture 151, 357–363.

Kutty, M.N., 1978. Ammonia quotient in sockeye salmon (*Oncorhynchus nerka*). J. Fish. Res. Board Can. 35, 1003–1005.

Liew, H.J., Sinha, A.K., Mauro, N., Diricx, M., Blust, R., De Boeck, G., 2012. Fasting goldfish, *Carassius auratus*, and common carp, *Cyprinus carpio*, use different metabolic strategies when swimming. Comp. Biochem. Physiol., Part A: Mol. Integr. 163, 327-335.

Liew, H.J., Sinha, A.K., Mauro, N., Diricx, M., Darras, V.M., Blust, R., De Boeck, G., 2013. Feeding and swimming modulate iono-and-hormonal regulation differently in goldfish, *Carassius auratus* and common carp, *Cyprinus carpio*. Comp. Biochem. Physiol., Part A: Mol. Integr. 165, 13-21.

Lin, H., Randall, D.J., 1991. Evidence for the presence of an electrogenic protonpump on the trout gill epithelium. J. Exp. Biol. 161, 119–134.

Lin, H., Randall, D.J., 1993. H^+-ATPase in crude homogenates of fish gill tissue: inhibitor sensitivity and environmental and hormonal regulation. J. Exp. Biol. 180, 163–174.

McCormick, S.D., 1993. Methods for the nonlethal gill biopsy and measurements of Na^+:K^+-ATPase activity. Can. J. Fish. Aquat. Sci. 50, 656–658.

Millidine, K.J., Metcalfe, N.B., Armstrong, J.D., 2008. The use of ventilation frequency as an accurate indicator of metabolic rate in juvenile Atlantic salmon (*Salmo salar*). Can. J. Fish. Aquat. Sci. 65, 2081-2087.

Nakada, T., Westhoff, C.M., Kato, A., Hirose, S., 2007. Ammonia secretion from fish gill depends on a set of Rh glycoproteins. FASEB J. 21, 1067–1074.

Navarro, I., Gutierrez, J., Planas, J., 1992. Changes in plasma glucagon, insulin and tissue metabolites associated with prolonged fasting in brown trout (*Salmo trutta fario*) during two different seasons of the year. Comp. Biochem. Physiol. 102, 401–407.

Nawata, C.M., Hung, C.C.Y., Tsui, T.K.N., Wilson, J.M., Wright, P.A., 2007. Ammonia excretion in rainbow trout (*Oncorhynchus mykiss*): evidence for Rh glycoprotein and H^+-ATPase involvement. Physiol. Genom. 31, 463–474.

Nawata, C.M., Wood, C.M., O'Donnell, M.J., 2010. Functional characterization of Rhesus glycoproteins from an ammoniotelic teleost, the rainbow trout, using oocyte expression and SIET analysis. J. Exp. Biol. 213, 1049–1059.

Nemoto, C.M., 1957. Experiments with methods for air transport of live fish. Prog. Fish Cult. 19, 147-157.

Nilsson, G.E., 1990. Long-term anoxia in crucian carp: changes in the levels of amino acid and monoamine neurotransmitters in the brain, catecholamines in chromaffin tissue, and liver glycogen. J. Exp. Biol. 150, 295-320.

Pastoureaud, A., 1991. Influence of starvation at low temperatures on utilization of energy reserves, appetite recovery and growth character in sea bass, *Dicentrarchus labrax*. Aquaculture 99, 167–178.

Paterson, B.D., Rimmer, M.A., Meikle, G.M., Semmens, G.L., 2003. Physiological responses of the Asian sea bass, *Lates calcarifer* to water quality deterioration during simulated live transport:

acidosis, red-cell swelling, and levels of ions and ammonia in the plasma. Aquaculture 218, 717-728.

Patrick, M.L., Wood, C.M., 1999. Ion and acid-base regulation in the freshwater mummichog (*Fundulus heteroclitus*): a departure from the standard model for freshwater teleosts. Comp. Biochem. Physiol., Part A: Mol. Integr. 122, 445–456.

Perry, S.F., 1997. The chloride cell: structure and function in the gills of freshwater fishes. Annu. Rev. Physiol. 59, 325–347.

Perry, S.F., Shahsavarani, A., Georgalis, T., Bayaa, M., Furimsky, M., Thomas, S.L., 2003. Channels, pumps, and exchangers in the gill and kidney of freshwater fishes: their role in ionic and acid-base regulation. J. Exp. Zool. A 300, 53–62.

Peterson, B.C., Small, B.C., 2004. Effects of fasting on circulating IGF binding proteins, glucose, and cortisol in channel catfish (*Ictalurus punctatus*). Domest. Anim. Endocrinol. 26, 231–240.

Phillips, A.M., Brockway, D.R., 1954. Effect of starvation, water temperature, and sodium amytal on the metabolic rate of brook trout. Prog. Fish Cult. 65–68.

Randall, D.J., Baumgarten, D., Malyusz, M., 1972. The relationship between gas and ion transfer across the gills of fishes. Comp. Biochem. Physiol., Part A: Mol. Integr. 41, 629–637.

Randall, D.J., Tsui, T.K., 2002. Ammonia toxicity in fish. Mar. Pollut. Bull. 45, 17–23.

Roe, J.H., Dailey, R.E., 1966. Determination of glycogen with the anthrone reagent. Anal. Biochem. 15, 245–250.

Schreck, C.B., Solazzi, M.F., Johnson, S.L., Nickelson, T.E., 1989. Transportation stress affects performance of coho salmon *Oncorhynchus kisutch*. Aquaculture 82, 15-20.

Scarabello, M., Wood, C.M., Heigenhauser, G.J.F., 1991. Glycogen depletion in juvenile rainbow trout as an experimental test of the oxygen debt hypothesis. Can. J. Zool. 69, 2562–2568.

Shrivastava, J., Sinha, A.K., Datta, S.N., Blust, R., De Boeck, G., 2016. Pre-acclimation to low

ammonia improves ammonia handling in common carp (*Cyprinus carpio*) when exposed subsequently to high environmental ammonia. Aquat. Toxicol. 180, 334-344.

Sinha, A.K., Liew, H.J., Diricx, M., Blust, R., De Boeck, G., 2012. The interactive effects of ammonia exposure: nutritional status and exercise on metabolic and physiological responses in goldfish (*Carassius auratus* L.). Aquat. Toxicol. 109, 33–46.

Sinha, A.K., Liew, H.J., Nawata, C.M., Blust, R., Wood, C.M., De Boeck, G., 2013. Modulation of Rh glycoproteins, ammonia excretion and Na^+ fluxes in three freshwater teleosts when exposed chronically to high environmental ammonia. J. Exp. Biol. 216, 2917–2930.

Sinha, A.K., Kapotwe, M., Dabi, S.B., da Silva Montes, C., Shrivastava, J., Blust, R., De Boeck, G., 2016. Differential modulation of ammonia excretion, Rhesus glycoproteins and ion-regulation in common carp (*Cyprinus carpio*) following individual and combined exposure to waterborne copper and ammonia. Aquat. Toxicol. 170, 129-141.

Small, B.C., Soares Jr., J.H., Woods, L.C., Dahl, G.E., 2002. Effect of fasting on pituitary growth hormone expression and circulating growth hormone levels in striped bass. N. Am. J. Aquac. 64, 278–283.

Small, B.C., 2005. Effect of fasting on nychthemeral concentrations of plasma growth hormone (GH), insulin-like growth factor I (IGF-I), and cortisol in channel catfish (*Ictalurus punctatus*). Comp. Biochem. Physiol., B: Biochem. Mol. Biol. 142, 217–223.

Small, B.C., Peterson, B.C., 2005. Establishment of a time-resolved fluoroimmunoassay for measuring plasma insulin-like growth factor I (IGF-I) in fish: effect of fasting on plasma concentrations and tissue mRNA expression of IGF-I and growth hormone (GH) in channel catfish (*Ictalurus punctatus*). Domest. Anim. Endocrinol. 28, 202–215.

Sprecker, J.L., Schreck, C.B., 1980. Stress responses to transportation and fitness for marine survival in coho salmon (*Oncorhynchus kisutch*) smolts. Can. J. Fish. Aquat. Sci. 37, 765-769.

Sullivan, K.M., Somero, G.N., 1983. Size and diet related variations in enzymatic activity and tissue composition in the sablefish, *Anoplopoma Fimbria*. Biol. Bull. 164, 315–326.

Tseng, Y.C., Hwang, P.P., 2008. Some insights into energy metabolism for osmoregulation in fish. Comp. Biochem. Physiol., Part C: Toxicol. Pharmacol. 148, 419–429.

Ultsch, G.R., Ott, M.E., Heisler, N., 1980. Standard metabolic rate, critical oxgyen tension, and aerobic scope for spontaneous activity of trout (*Salmo gairdneri*) and carp (*Cyprinus carpio*) in acidified water. Comp. Biochem. Physiol., Part A: Mol. Integr. 67, 329–335.

Verdouw, H., Van Echteld, C.J.A., Dekkers, E.M.J., 1978. Ammonia determination based on indophenol formation with sodium salicylate. Water Res. 12, 399–402.

Vitale, A.M., Monserrat, J.M., Castilho, P., Rodriguez, E.M., 1999. Inhibitory effects of cadmium on carbonic anhydrase activity and ionic regulation of the estuarine crab *Chasmagnathus granulata* (Decapoda *Grapsidae*). Comp. Biochem. Physiol., Part C: Toxicol. Pharmacol. 122, 121–129.

Wedemeyer, G.A., 1996. Physiology of Intensive Culture Systems. Chapman and Hall, New York.

Weihrauch, D., Wilkie, M.P., Walsh, P.J., 2009. Ammonia and urea transporters in gills of fish and aquatic crustaceans. J. Exp. Biol. 212, 1716–1730.

Wicks, B.J., Randall, D.J., 2002. The effect of feeding and fasting on ammonia toxicity in juvenile rainbow trout, *Oncorhynchus mykiss*. Aquat. Toxicol. 59, 71–82.

Wilkie, M.P., 1997. Mechanisms of ammonia excretion across fish gills. Comp. Biochem. Physiol., Part A: Mol. Integr. 118, 39–50.

Wilkie, M.P., 2002. Ammonia excretion and urea handling by fish gills: present understanding and future research challenges. J. Exp. Zool. 293, 284–301.

Wright, P.A., Wood, C.M., 2009. A new paradigm for ammonia excretion in aquatic animals: role of Rhesus (Rh) glycoproteins. J. Exp. Biol. 212, 2303–2312.

Yang, T.H., Somero, G.N., 1993. Effects of feeding and food deprivation on oxygen consumption,

muscle protein concentration. J. Exp. Biol. 181, 213–232.

Zimmer, A.M., Nawata, C.M., Wood, C.M., 2010. Physiological and molecular analysis of the interactive effects of feeding and high environmental ammonia on branchial ammonia excretion and Na^+ uptake in freshwater rainbow trout. J. Comp. Physiol. B 180, 1191–1204.

Figure Captions

Figure 1. Oxygen consumption rates (MO_2) in control (fed) and fasted fish. Values are mean ± S.E. The asterisk (*) denotes the significant differences between pooled control (*N=16*) and fasted groups (*N=8*) ($^{*}P < 0.05$; $^{**}P < 0.01$;$^{***}P < 0.001$).

Figure 2. Ammonia excretion rates (J_{amm}) in control (fed) and fasted fish. Values are mean ± S.E. The asterisk (*) denotes the significant differences between pooled control (*N=16*) and fasted groups (*N=8*) ($^{*}P < 0.05$; $^{**}P < 0.01$;$^{***}P < 0.001$).

Figure 3. Ammonia quotient in control (fed) and fasted fish. Values are mean ± S.E. The asterisk (*) denotes the significant differences between pooled control (*N=16*) and fasted groups (*N=8*) ($^{**}P < 0.01$;$^{***}P < 0.001$).

Figure 4. Accumulation (mM) of total ammonia in plasma of control (fed) and fasted fish. Values are mean ± S.E. The asterisk (*) denotes the significant differences between pooled control (*N=14*) and fasted groups (*N=7-8*) ($^{**}P < 0.01$).

Figure 5. Energy store of (A) Glycogen (B) Protein and (C) Lipid content in liver tissue of control (fed) and fasted fish. Values are mean ± S.E. The asterisk (*) denotes the significant differences between pooled control (*N=15-16*) and fasted groups (*N=6-8*) ($^{*}P < 0.05$; $^{**}P < 0.01$).

Figure 6. Enzyme activity of Na^+/K^+-ATPase (NKA) in the gills of control (fed) and fasted fish. Values are mean ± S.E. The asterisk (*) denotes the significant differences between pooled control (*N=15*) and fasted groups (*N=7-8*) ($^{*}P < 0.05$).

Figure 7. Expression of Na^+/K^+-ATPase mRNA in the gills of control (fed) and fasted fish. Values are mean ± S.E. The asterisk (*) denotes the significant differences between pooled control (*N=16*) and fasted groups (*N=7-8*) ($^{*}P < 0.05$).

Figure 8. Expression of Na^+/H^+ (NHE-3) exchanger in the gills of control (fed) and fasted fish. Values are mean ± S.E. The asterisk (*) denotes the significant differences between pooled control (*N=16*) and fasted groups (*N=6-8*) ($^{**}P < 0.01$;$^{***}P < 0.001$).

Figure 9. Expression of (A) Rhcg-a (B) Rhcg-b and (C) Rhbg mRNA in the gills of control (fed) and fasted fish. Values are mean ± S.E. The asterisk (*) denotes the significant differences between pooled control (*N=14-16*) and fasted groups (*N=6-8*) ($^{*}P < 0.05$;$^{**}P < 0.01$;$^{***}P < 0.001$).

Figure 10. Principal Component Analysis (PCA) representing the contribution of biochemical parameters. The variable coordination is presented by the complementary cases analysis showing distribution of control (fed) fish and fasted fish (2- 14 days) in the (PC 1 ×PC 2) coordination plane.

Tables

Table 1
qPCR primer list and accession numbers of target and reference genes, and efficiencies of the PCR reactions

Gene	Accession no.	Sequence of Primer (5′→ 3′)	Efficiency (%)
Na^+/K^+-ATPase	JX570881	F: aggtggacaactcctccctg R: atacgacccatgacagtacg	128.0%
H^+-ATPase	JX570880	F: ctatgggggtcaacatggag R: ccaacacgtgcttctcacac	97.5%
Rhbg	JX570877	F: tcccagtttccaggatgttc R: tggaaaaagccctgcataag	114.0%
Rhcg-a	JX570878	F: atcctgaacatcctccatgc R: aacttggccagaacatccac	82.7%
Rhcg-b	JX570879	F: cacaaagccacacacagtcc R: tctttttctcgccgttcttg	109.2%
NHE-3	Bradshaw et al., 2012	F: gtgtcatttggaggctcgtt R: atccatgttggcggtaatgt	89.0%
β-actin	M24113.1	F: cgtgatggactctggtgatg R: tcacggacaatttccctctc	114.4%

The accession number refers to the registered sequence from GenBank. F: forward, R: reverse.

Table 2

Ion concentrations (mmol/L) in plasma of common carp under different treatments.

Treatments	Na^+	Cl^-	K^+
Control	129.6 ± 2.38	113.0 ± 1.65	3.67 ± 0.37
2 days	123.3 ± 4.03	108.6 ± 1.49	3.47 ± 0.36
4 days	120.7 ± 7.55	98.1 ± 2.40***	3.56 ± 0.53
6 days	108.9 ± 5.32**	96.1 ± 5.90 *	4.91 ± 0.31*
8 days	112.4 ± 2.10**	111.7 ± 1.36	4.80 ± 0.35*
10 days	126.8 ± 3.75	112.9 ± 0.55	5.01 ± 0.48*
12 days	128.9 ± 1.50	111.4 ± 0.95	5.11 ± 0.46*
14 days	122.6 ± 4.10	108.6 ± 2.15	4.63 ± 0.47

Figure 1

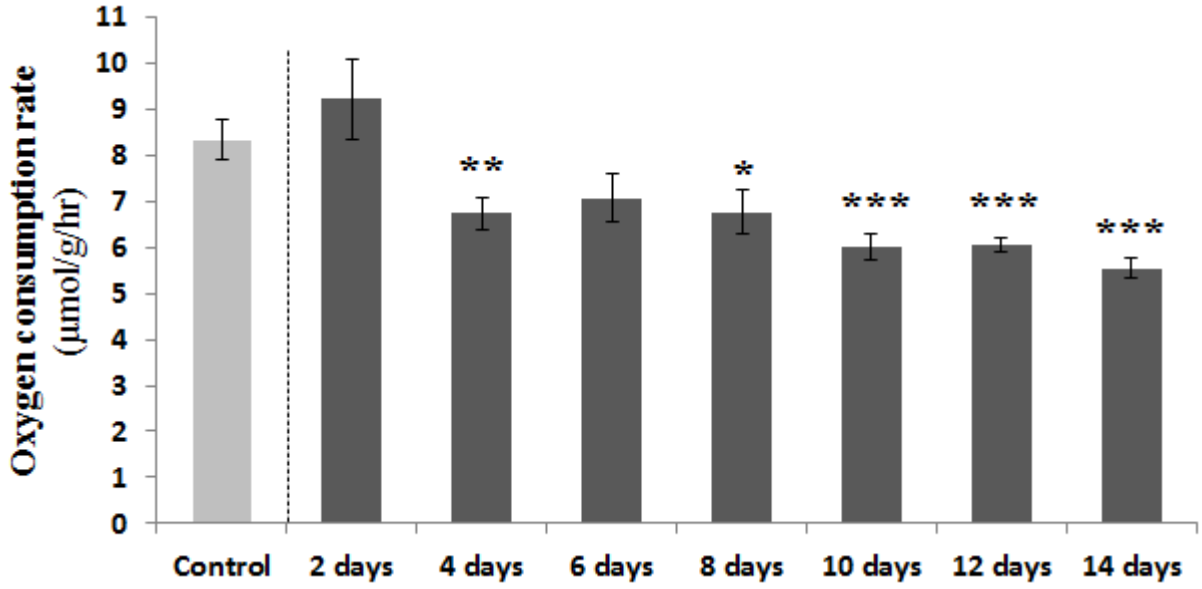


Figure 2

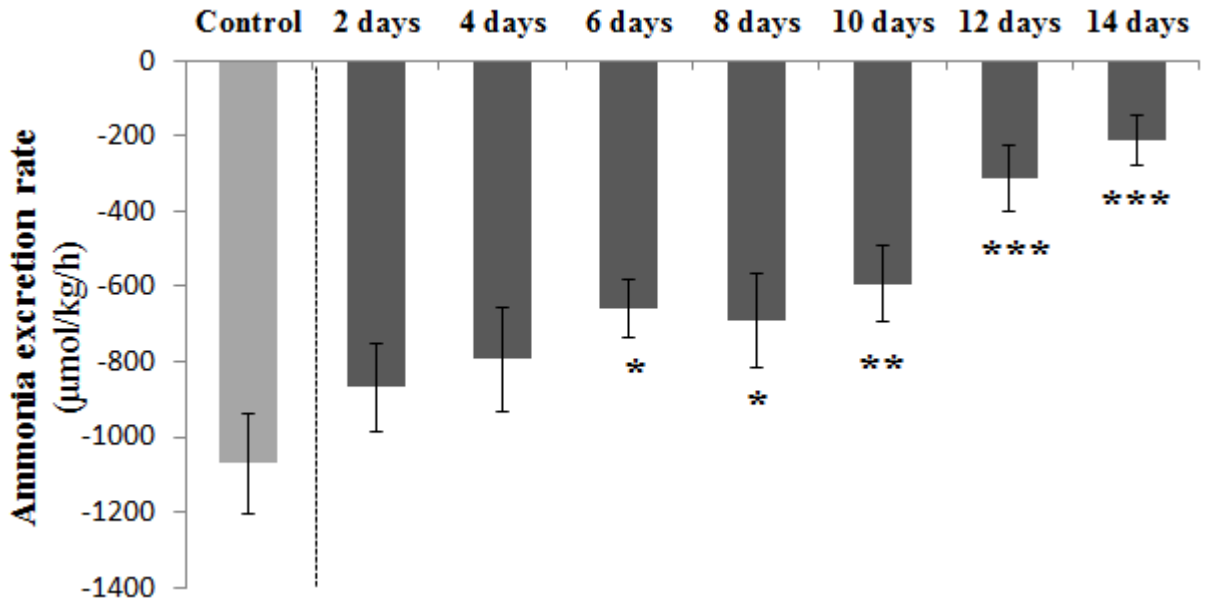


Figure 3

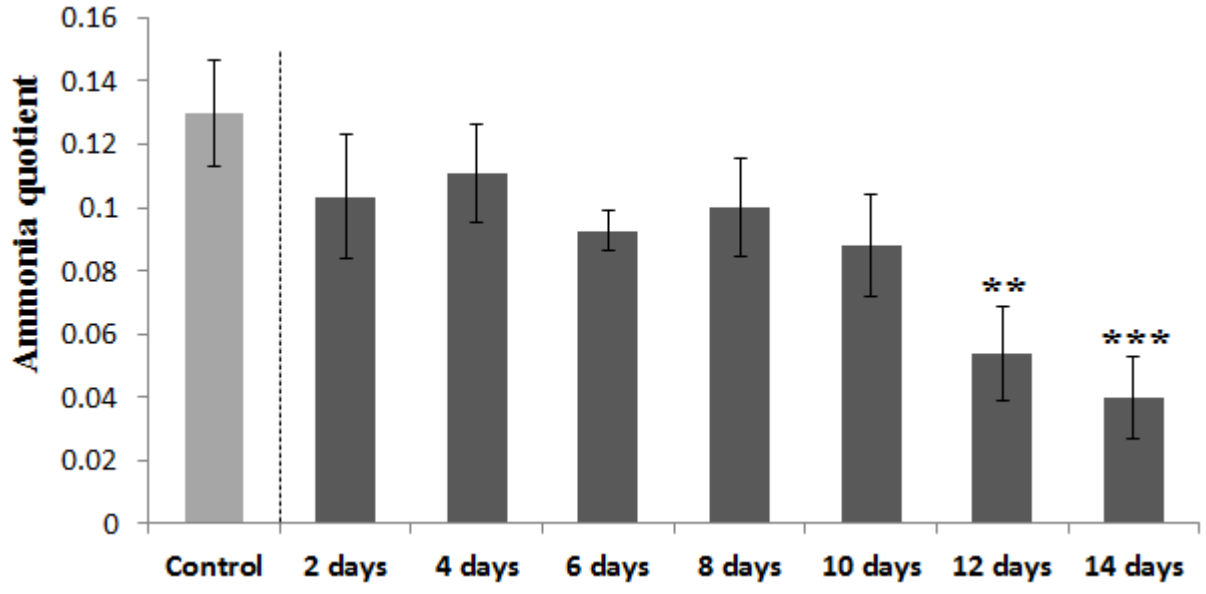


Figure 4

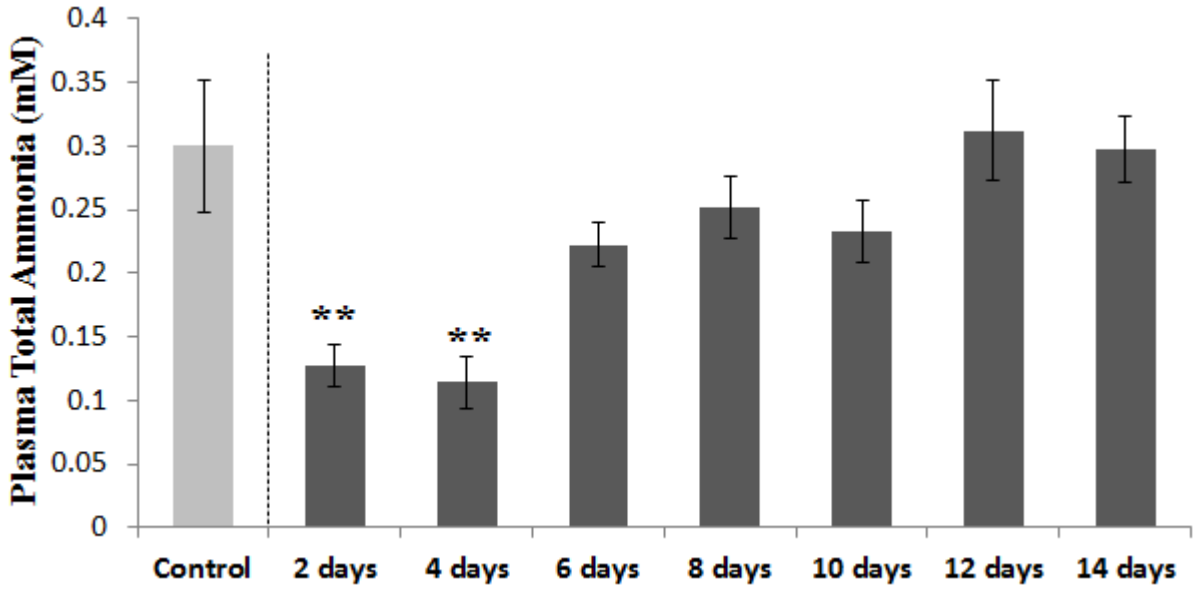


Figure 5

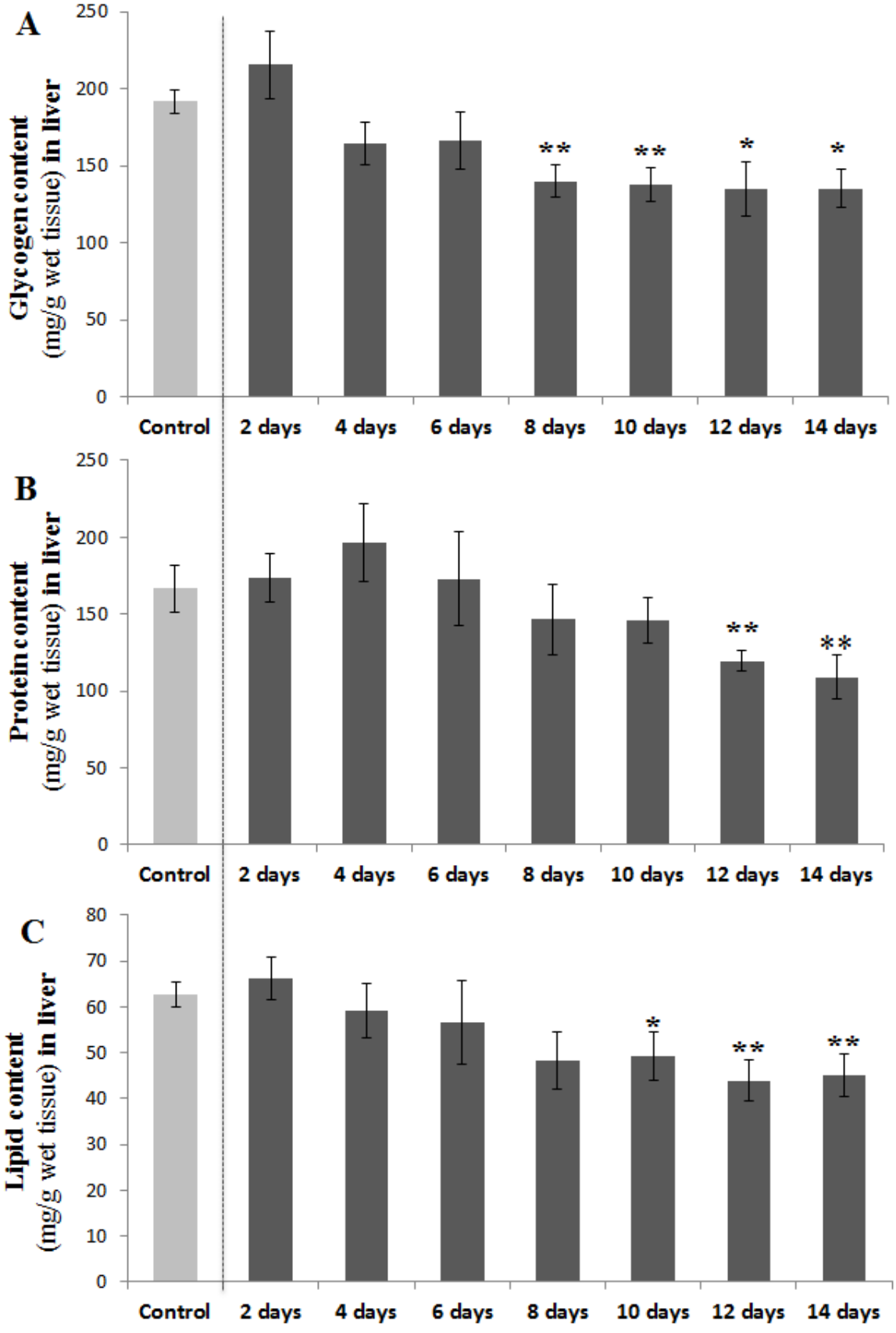


Figure 6

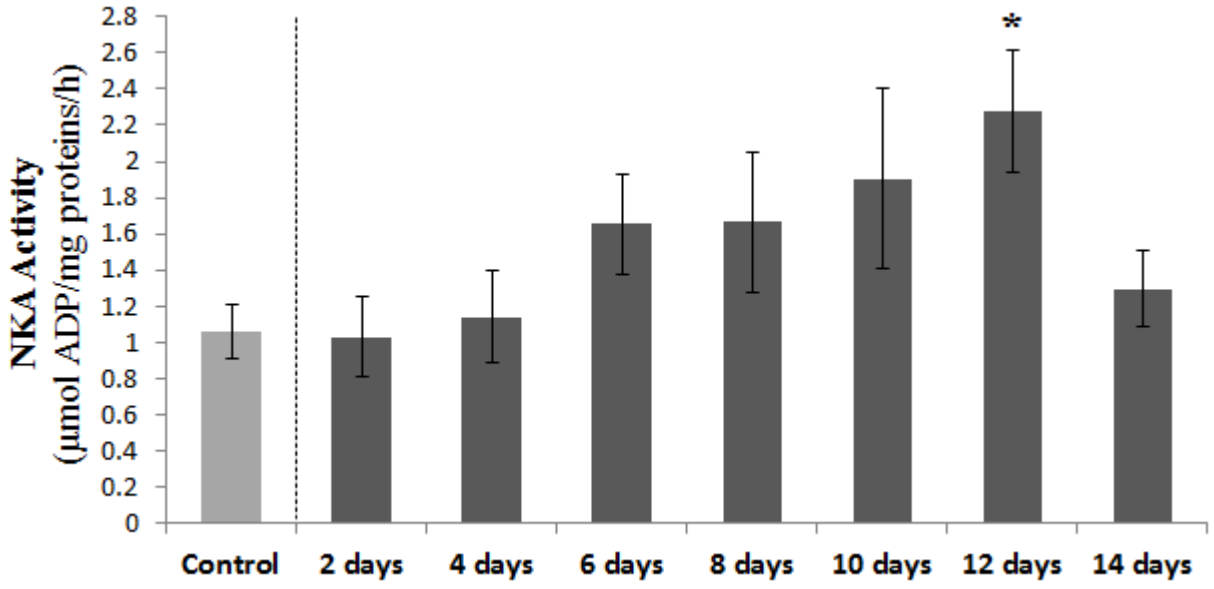


Figure 7

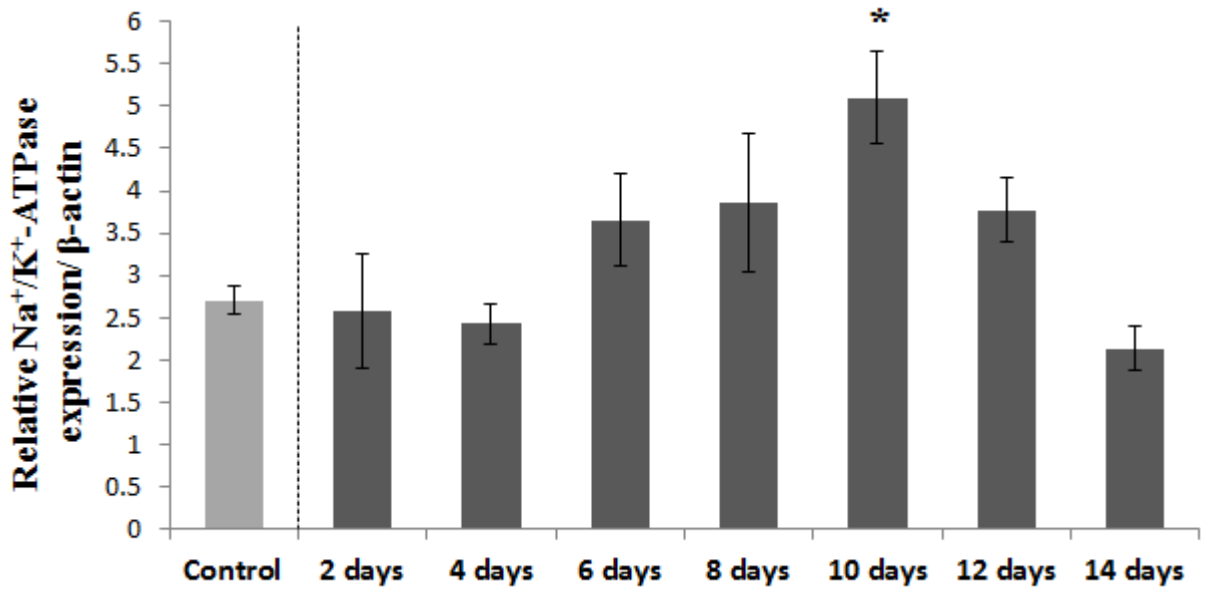


Figure 8

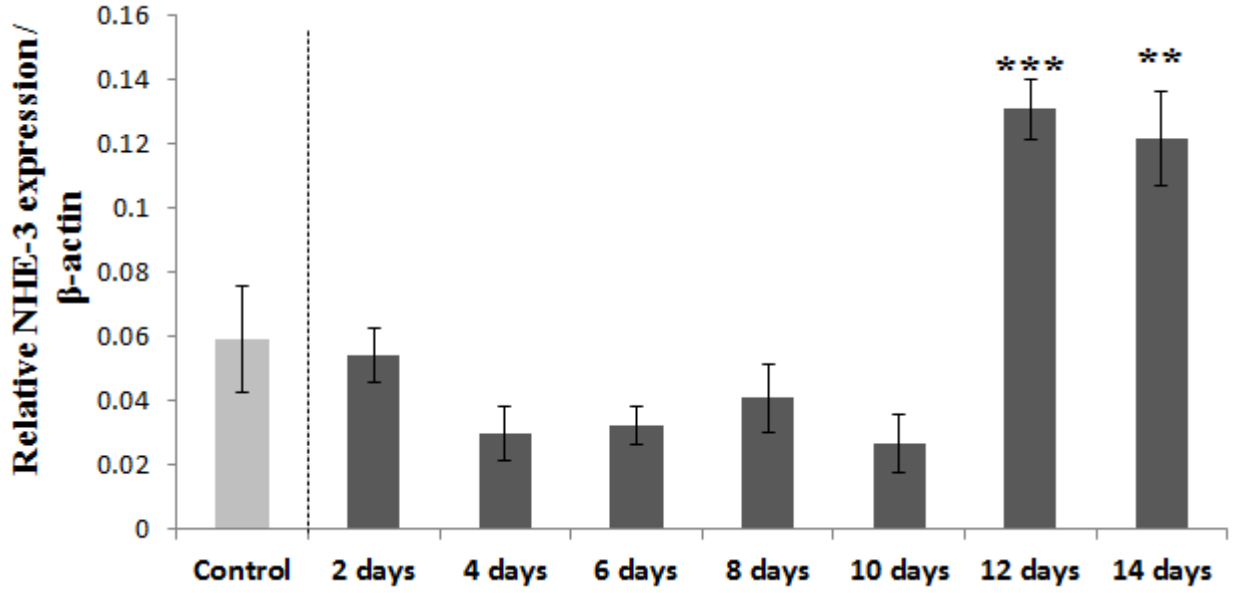


Figure 9

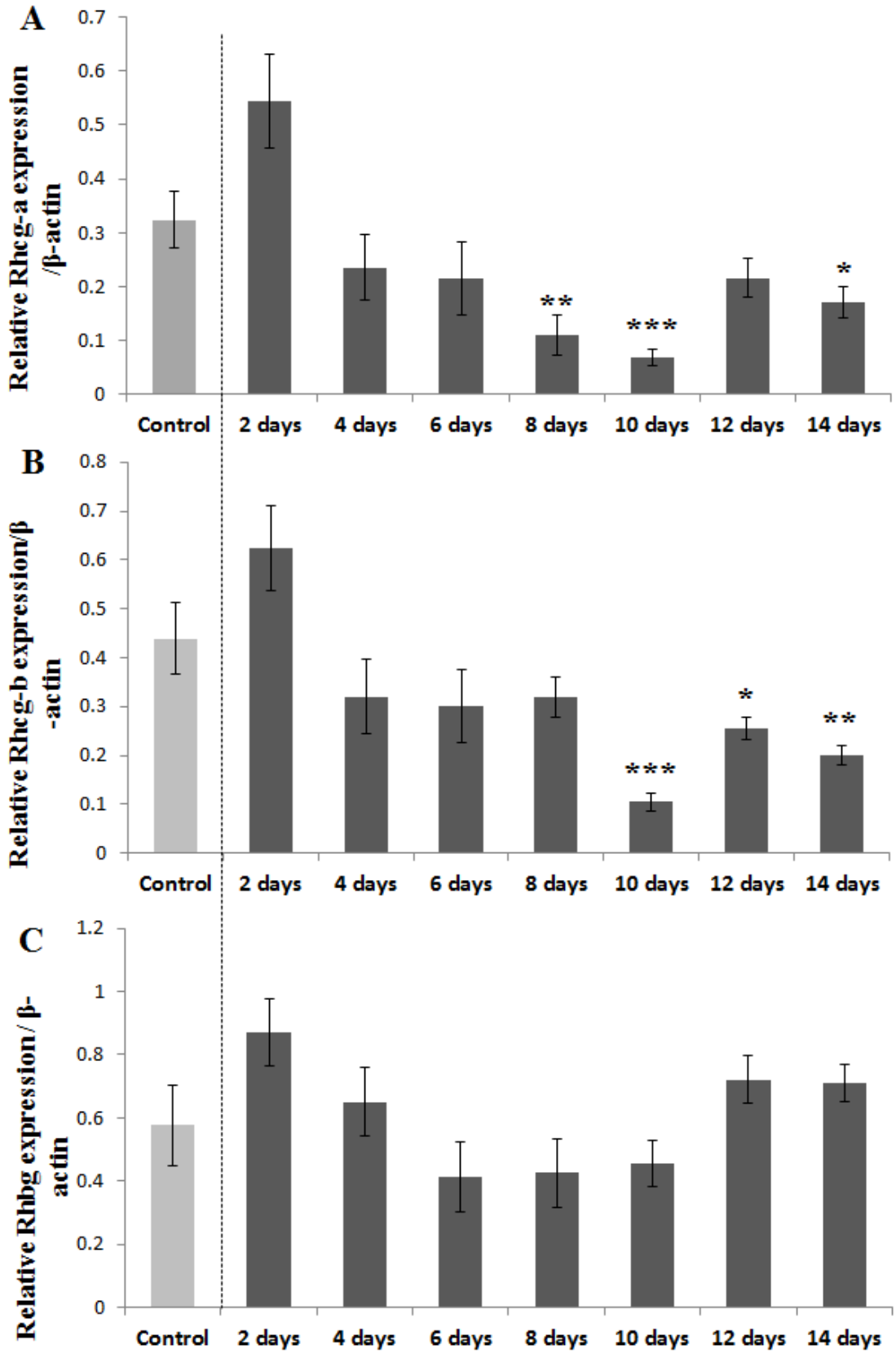
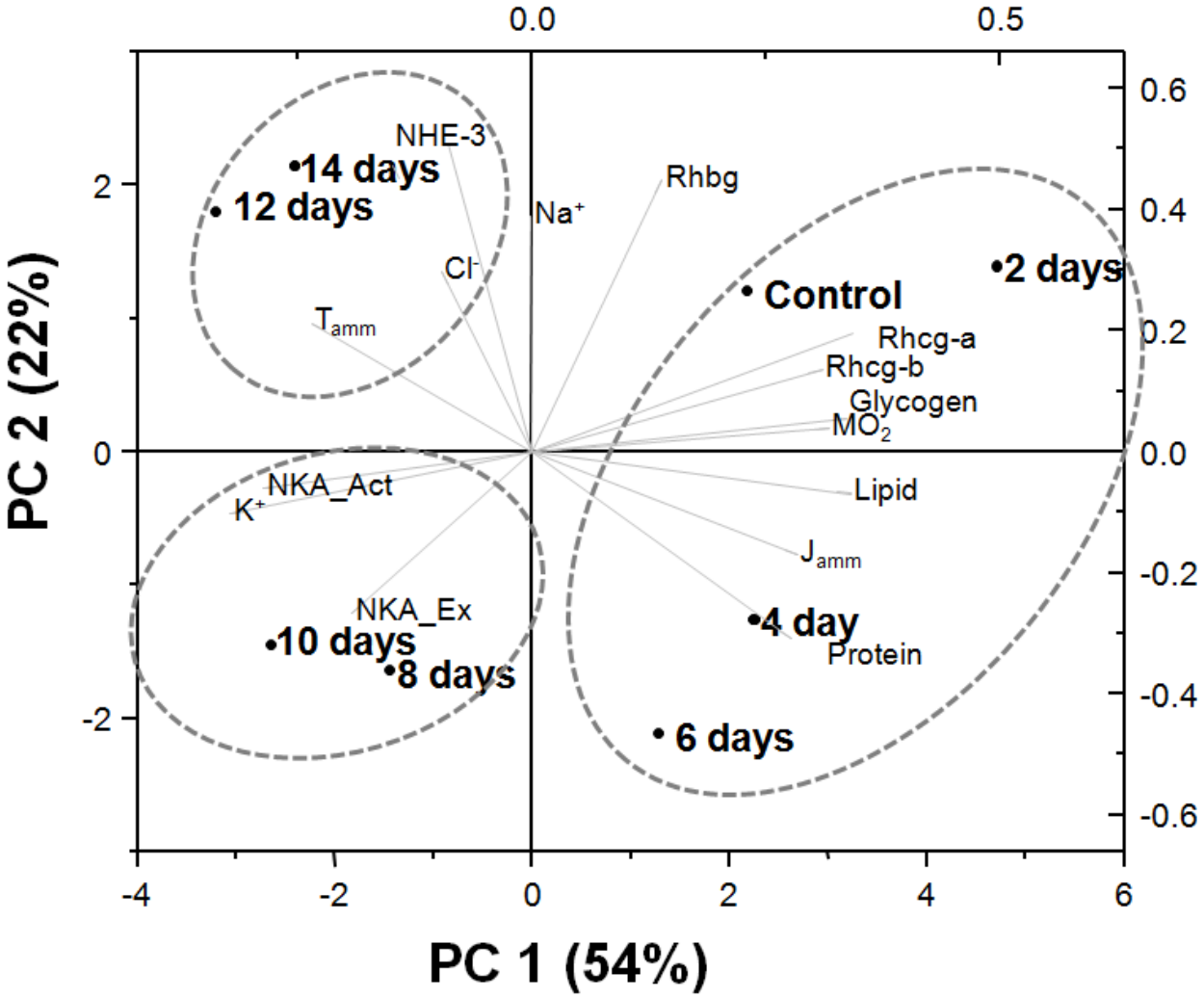


Figure 10



Research highlights

→ An ideal fasting period prior to the fish transport was investigated using common carp as model aquaculture species.

→ We hypothesized, an ideal fasting period exists when metabolisms is minimized but ammonia-transport is still regulated.

→ Aerobic metabolism, ammonia regulatory pathways as well as ion homeostasis in the course of fasting episodes were investigated.

→ Reduction of metabolic rate was evident from day 6 of fasting onwards, while ammonia dynamics, ion status and energy stores were not yet compromised

→ Overall, our results suggest that an ideal fasting period exists around 6 days.