Metabolic strategies in freshwater teleost under stress – Consequences of feeding and swimming

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List of Abbreviation

J_{amm}	Ammonia excretion
AQ	Ammonia Quotient
U_{crit}	Critical swimming speed
MS222	Ethyl-3-aminobenzoate methanesulfonic acid
h-PI	Hour-Post Implant
GR1	Glucocorticoid Receptor – 1
GR2	Glucocorticoid Receptor – 2
MR	Mineralocorticoid Receptor
MO_2	Metabolic oxygen consumption
NKCC	$Na^+/K^+/Cl^-$ co-transporter
H^+ ATPase	Proton pump
RIA	Radioimmunoassay
Na^+/K^+ ATPase (NKA)	Sodium pump
SDA	specific dynamic action
T_{amm}	Total ammonia (NH_3/NH_4^+)
J_{urea}	Urea excretion

Contents

Chapter 2

Feeding and swimming modulate iono-and-hormonal regulation differently in goldfish, *Carassius* auratus and common carp, *Cyprinus carpio*

Chapter 3

Cortisol emphasizes the metabolic strategies employed by common carp, *Cyprinus carpio* at different feeding and swimming regimes

Chapter 4

Ion homeostasis in common carp, *Cyprinus carpio*: interactive effects of cortisol, feeding and swimming

Chapter 5

Cortisol signaling induces non-genomic metabolic and ionoregulation responses before genomic changes occur in common carp, *Cyprinus carpio* fed at different rations

Research Summary

As both feeding and swimming are essential for growth and survival in teleosts, any stress associated with these key factors may compromise performance and induce physiological changes to adapt or acclimatize to the new situation. Such acclimatization processes are modulated by endocrine regulation. This thesis "***Metabolic and ionoregulation strategies in freshwater teleosts – consequences of feeding and swimming***" focuses on two main objectives. The first aim was to perform a comparative study between goldfish and common carp on their swimming performance, metabolic strategies, and iono-and-hormonal regulation under different feeding and exercise levels. Fish were grouped into fasting (7 day food deprivation) and feeding (2% body weight (BW)) groups and were conditioned to resting, low aerobic swimming, high aerobic swimming or exhaustive swimming conditions. These findings are described in chapter 1 and 2. A second aim was to unravel the effect of cortisol implantation on these processes in common carp fed at a low and high (0.5% and 3.0% BW) ration. These results are described in chapters 3, 4 and 5.

Chapter 1: Fasting had no significant effect on swimming performance (U_{crit}) of either species. Feeding and swimming profoundly elevated ammonia (J_{amm}) excretion in both species. In goldfish, feeding metabolism was sacrificed to support swimming metabolism with similar oxygen consumption (MO_2) at U_{crit} between fasted and fed fish, whereas in common carp feeding increased MO_2 at U_{crit} to sustain feeding and swimming independently.

Chapter 2: Due to the osmorespiratory compromise, U_{crit} induced increases in gill Na^+/K^+ ATPase (NKA) activity in both species, resulting in stable levels of plasma ions. In contrast to our expectations, this only occurred in fed fish and feeding itself increased NKA activity, especially in carp. We concluded that this was more related to ammonia excretion than ion uptake. Fasting fish were able to maintain ion balance without increasing NKA activity. As expected, this increase in NKA activity coincided with a cortisol elevation in goldfish, but surprisingly no significant change in cortisol levels was seen in carp. This chapter showed that both species, whether fed or fasted, are able to sufficiently adapt their osmorespiratory strategy to minimize ion losses while maintaining gas exchange under exhaustive swimming.

Common carp were able to perform both feeding and swimming metabolism independently while maintaining ion levels relative stable without cortisol increment. With that, common carp was selected to further investigate how carp respond during a cortisol elevation. However, total starvation may not always reflect the reality in nature. Therefore, the feeding rate was set to low (0.5% BW) and high (3.0% BW) and fish were implanted with a physiological cortisol dose. The study focussed on the effect of hypercortisol on metabolic strategies and on the osmorespiratory compromise under these circumstances.

Chapter 3: Swimming, feeding and cortisol all induced aerobic metabolism by increasing oxygen consumption, J_{amm} and urea excretion (J_{urea}) and stimulated protein metabolism as demonstrated by the increased ammonia quotient and endogenous nitrogenous waste level. Hypercortisol stimulated ammonia self-detoxifying mechanisms by enhancing J_{amm} and J_{urea}, especially during exhaustive swimming. At a high swimming level, higher branchial clearance rates in cortisol treated fish succeeded in eliminating the elevation of endogenous ammonia production and resulted in reduced plasma ammonia levels.

Chapter 4: Feeding granted readily available energy for ionoregulation leading to a stable ion balance. Hypercortisol provoked gill NKA and H^+ ATPase activities in high feeding fish, which was most likely associated with ammonia excretion rather than ion uptake per se. Low feeding fish maintained their ion level by upregulating kidney NKA and H^+ ATPase activities to enhance ion reabsorption. Upregulation of gill and kidney NKA and H^+ ATPase activities during hypercortisol confirmed the role of cortisol in ionoregulation in freshwater fish.

With these physiological results in mind, research was continued to further review the effect of hypercortisol on non-genomic and genomic responses in carp. This study was only focused on resting fish fed at different rations.

Chapter 5: Cortisol implants elevated plasma cortisol, glucose and lactate levels. Plasma osmolality and ion levels remained unchanged facilitated by increased gill and kidney ionoregulatory (NKA and H^+ ATPase) activities. As seen in chapter 4, gill ionoregulatory activities were greater in high feeding carp. In kidney, NKA was increased to a comparable level in both feeding groups, whereas H^+ ATPase activity was greater only in low feeding fish. Upregulation of Rhcg-1 enhanced branchial ammonia excretion efficiency. Cortisol induced glucocorticoid (GR1 and GR2) and minerolocorticoid (MR) receptors expression in both kidney and liver. Despite the increasing trends in the gill, GR2 and MR were upregulated in high feeding carp and only GR2 was upregulated in low feeding carp. Cortisol significantly induced non-genomic and metabolic responses to compensate acute stress (12h-PI), followed by genomic responses with upregulation of ionoregulatory and cortisosteroid receptors (24h-PI onward).

Overall, this thesis demonstrated that goldfish and common carp clearly showed different physiological responses, even though both are close family members from the Cyprinidae. With compensation strategies such as (i) aerobic-anaerobic metabolic trade-off to access energy reserves at different degrees; (ii) osmorespiratory compromise likely increasing gill functional surface area to improve gas exchange while reducing gill permeability for ion losses (Na^+ and Cl^-) during swimming; (iii) increased gill ammonia clearance and upregulated gill NKA and H^+ ATPase activities as ammonia self-detoxifying strategy, especially at high dietary protein intake; and (iiii) minimizing gill NKA and H^+ ATP activities while up-regulating kidney NKA and H^+ ATPase activities for ion reabsorption enabling carp to maintain ion levels during food deprivation. In carp, cortisol induced immediate non-genomic actions followed by genomic action to compensate or re-strategize metabolic needs. In fed fish, compensatory responses in ionoregulation were more important in gills, while kidney played a more prominent role in fish on a low feeding ration.

'Feeding is the main key in aquaculture and swimming is a key element to ensure fish welfare. Knowledge about the combination of these two key factors on teleosts allows to improve aquaculture and aids to set a step further towards sustainable aquatic animal welfare in fisheries management and aquaculture'

Introduction

1.1. Overview research justification and aims

Much traditional research focused on the impact of fasting in fish in order to provide a better understanding of the implication of fasting for aquatic animal welfare and good aquaculture practice. Fasting occurs when fish are not ingesting food and rely exclusively on endogenous physiological fuel stores to meet their basal metabolic demands (McCue, 2010). The ecophysiological effects of fasting in fish demonstrates a downregulation of physiological functions, thereby incurring a lower maintenance cost to sustain prioritized metabolic needs (Larsson and Lewander, 1973; Jobling, 1980; Moon and Johnston, 1980; Mehner and Wieser, 1994; Collins and Anderson, 1997; Shimeno et al., 1997; Guderley et al., 2003; Martinez et al., 2003; Fu et al., 2005; McCue, 2010; Zeng, et al., 2012). In fact, the critical impact of feeding on physiological functions has been routinely ignored in other ecophysiological and ecotoxicological studies. In laboratory, fasting is often applied for the sake of experimental convenience (Wood et al., 2005), although the approach is often justified based on the need to normalize the metabolic state and avoid excessive ammonia excretion and faeces under confined experimental setups (Wood, 2001). Under natural scenarios, fish mostly experience temporary feeding limitations rather than total fasting such as during overwintering hibernation, seasonal spawning or pollution events. Temporary feeding limitation refers to a situation where fish forego an opportunity to eat in order to allot their time and energy for other activities such as predator avoidance, feeding competition or hierarchy associate under both free living and captive conditions (McCarthy et al., 1993; Wendelaar-Bonga, 1997; Doucett et al., 1999; Ashley, 2006; McCue, 2010).

In fact, feeding is essential in the daily lives of fish. Most recent studies have found that feeding not only provided essential nutrition for growth and survival but also stimulated better compensation strategies when challenged with a stressor which were absent in fasting fish (Perry et al., 2006; Bucking and Wood, 2008; Pang et al., 2010; Wood et al., 2010; Bucking et al., 2011; Liew et al., 2012, 2013a). In the aquatic environment, successive feeding is determined by swimming performance. Most of the top predators are carnivorous or so called ‘opportunistic feeders’ and consume large meals at irregular intervals followed by gradual ingestion (Wood et al., 2010; Liew et al., 2013a) while maintaining swimming at a lower level. Contrary, their prey species e.g. low class predators, herbivorous or omnivorous fish need to feed frequently and swim continuously to find their food and avoid their predators.

Feeding is followed by an ingestion and digestion process that increases metabolic rate and thus requires extra oxygen expenditure known as ‘specific dynamic action (SDA)’ (Beamish and Trippel, 1990; Brown and Cameron, 1991; Lyndon et al., 1992; Jobling, 1994). When fish are forced to swim after feeding, the processes of digestion and swimming must compete for the oxygen supply (Hicks and Bennett, 2004). In order to achieve both these metabolic demands, the cardiorespiratory system must be designed to accomplish these tasks simultaneously. Alsop and Wood (1997) proposed a hypothesis emphasizing three possible interaction scenarios which may occur between feeding and swimming trade-offs in fish: (1) with sufficient oxygen supply both SDA and swimming metabolisms may proceed simultaneously, thus the SDA induced metabolic increment would be maintained during swimming; (2) a prioritizing preference strategy if swimming is the priority and therefore SDA metabolism is sacrificed or (3) vice versa. This hypothesis recently received extensive

attention to address priority metabolism in teleosts (Altimiras et al., 2008; Dupont-Prinet et al., 2009; Fu et al., 2009; Gingerich et al., 2009; Caruso et al., 2010; Li et al., 2010; Jourdan-Pineau et al., 2010; Marshall, 2010; Pang et al., 2010, 2011; Liew et al., 2012; Zhang et al., 2010, 2012). According to Fu et al. (2009) the effect of feeding on the swimming performance also depends on foraging strategy and meal size, which both have profound effects on metabolic strategies.

Over the past +60 years, swimming performance and physiology have been intensively studied, discovering physiological and biochemical aspects of exhaustive swimming in fish such as time and cost of recovery, behavior, ion and acid-base balance, and metabolic and hormonal responses (Black, 1955; Brett, 1964; Beamish, 1978; Wood and Perry, 1985; Wood, 1991; Moyes and West, 1995; Milligan, 1996; Kolok, 1999; Kieffer, 2000; Nelson et al., 2002; Gilmour et al., 2005; Peake and Farrell, 2006; Farrell, 2007; 2008; McKenzie et al., 2007; Tudorache et al., 2007, 2008, 2009, 2010a; Peake, 2008; Kieffer and Cooke, 2009). Few studies attempted to integrate fitness capacities with ecological relevance in fish (Plaut, 2001; Kieffer, 2010), although recently it has been proven that physiological fitness indicators give relevant ecological information on migration success (Clarck et al, 2011, Miller et al, 2011; Cooke et al, 2012). Critical assessment on the role of cortisol status on the post-swimming recovery dynamic in fish was examined as well. Wood (1991) and Milligan et al. (2000) have examined the interaction between post-aerobic swimming and metabolic recovery status with cortisol in rainbow trout. They found that fish allowed to swim aerobically during recovery from exhaustive swimming had lower cortisol levels and recovered their post-swimming lactate levels about two times faster than fish held in still water. A similar observation was replicated for Pacific salmon (Farrell et al., 2001) and largemouth bass (Suski et al., 2007). Elevation of plasma cortisol levels associated with the exhaustive swimming delays the restoration of metabolite and acid-base status to pre-swimming levels would alter physiological change (Pagontta et al., 1994; Eros ad Milligan, 1996). To our knowledge, only Pang et al. (2011) and Zhang et al. (2012) conducted two studies on cyprinid species. Their research mainly focused on the direct metabolic expenditures during active swimming in response to different temperature and oxygen levels. But there is not much information available on metabolic trade-off, ionoregulation and hormonal regulation under post-feeding circumstances. Thus, there is a need to further investigate and understand post-feeding physiological strategies in cyprinids when swimming.

Furthermore, cortisol elevation is known to affect spontaneous locomotion, behavior and feeding performance in teleosts (Gregory and Wood, 1999; Overli et al., 2002). Therefore, it will subsequently alter intermediary energy metabolism (Wendelaar Bonga, 1997; Mommsen et al., 1999; De Boeck et al., 2001) and induce gluconeogenesis and hyperglycemia as glucose serves as readily accessible energy for fish when stressed (Vijayan et al., 1991; 1997). Cortisol also plays a significant role in ionoregulation in freshwater fish by upregulating gill Na^+/K^+ ATPase (NKA) expression and activity (Overli et al., 2002; Zhou et al., 2003; Marshall and Grosell, 2006; McCormick e al., 2008; Babitha and Peter, 2010). Mommsen et al. (1999) reviewed that there are inconsistencies in the literature regarding the action of cortisol during stress. Much of the confusion probably arises owing to differences among species (Vijayan and Moon, 1994), methods employed to raise cortisol levels (Gamperl et al., 1994), sampling procedures (Iwama et al., 1989) or nutritional conditions (Vijayan et al., 1993; Reddy et al., 1995; Barcellos et al., 2010). One of the underlying assumptions in several studies is that elevated plasma cortisol level is deleterious fish health (Barton and Iwama, 1991; Pottinger, 1998; Ruane et al., 2002; Wojtaszek et al., 2002; Stolte et al., 2008; Pankhurst, 2011).

However, most of the research mentioned above were performed on salmonids as a model (Kieffer, 2010), not many of the studies focused on cyprinid fish (Knudsen and Jensen, 1998; Metz et al., 2003; Stolte et al., 2008), although zebrafish are gaining importance as model due to their fully annotated genome. Therefore, comparative research on swimming physiology and biochemistry on cyprinid species is necessary. Additionally, most metabolic rate studies are misleading since they were performed on fasting fish. This is perhaps more a philosophical statement than a rigorous hypothesis, nevertheless, most analyses of oxygen consumption use data for fasted fish rather fed fish for practical reasons. Obviously, this is far away from ecological reality (Wood, 2001). The SDA effect of feeding is well-known in virtually all vertebrates, but it is not widely appreciated that it causes fundamental changes in all kinds of metabolic strategies (Alsop and Wood, 1997). Altogether, it is therefore *unclear in what way cortisol elevation in combination with nutritional status and swimming level affects metabolic strategies and ionoregulation in cyprinid fish. In other words, is cortisol elevation an additional strain on swimming capacity in cyprinids when feeding is limited or in abundance? And does cortisol elevation induce cyprinids to re-direct their metabolic strategy to cope with the demand for extra energy expenditure or are some physiological processes amplified or simply impaired under these circumstances?*

To address these questions, a series of objectives were targeted:

i. Our first aim was to investigate in a comparative study which metabolic pathways were prioritized, which metabolic trade-offs existed, and how iono-and-hormonal regulation in goldfish and common carp were influenced under different feeding and swimming regimes. We hypothesised that fasting fish would suffer more from exhaustive swimming due to the limited dietary energy and ion intake, and since fasted fish cannot compensate ion and energy losses swimming capacity would be impaired. Whereas fed fish could spend greater metabolic expenses to sustain swimming and suffer less ion losses. On the contrary, fed fish could experience endogenous ammonia overload due to the combination of feeding and swimming (*Chapter 1 & 2*).

ii. Our second aim was to evaluate the effect of hypercortisol on metabolic strategies and ionoregulation in common carp subject to different feeding and swimming regimes. We hypothesized that hypercortisol may exaggerate metabolic rate due to stress, thus induce increased MO_2, hyperglycemia and endogenous ammonia production that might impair swimming performance when fish were fed at high ration (*Chapter 3*). Our previous study found that carp were able to sufficiently adapt their osmorespiratory strategy by increasing gas exchange during active swimming while minimizing gill ions losses. The increase of gill NKA activity was related to ammonia excretion rather than ion uptake per se in fed fish. Thereby, we hypothesized that swimming fish would improved gill ammonia excretion and clearance rates during hypercortisol, and thus subsequently would induce gill NKA and H^+ ATPase activities in comparison to low aerobic swimmung or resting fish. As high feeding granted sufficient dietary ion intake to maintain basal ion levels, kidney NKA and H^+ ATPase activities would remain unchanged. On the contrary, low feeding limited dietary ion intake, and therefore hypercotisol would upregulate kidney NKA and H^+ ATPase activities to maintain basal ions level (*Chapter 4*).

iii. Finally, we aim to assess the effects of hypercortisol on non-genomic and genomic responses of common carp fed different feeding regimes. We hypothesized that (i) hypercortisol immediately induces metabolic trade-offs, induces gill NKA and H^+ ATPase activities in the gill to improve ammonia excretion in high feeding fish, and promotes renal ion reabsorption capacity in low feeding fish, and (ii) gill Rhesus glycoproteins expression level would be greater in high feeding fish to facilitate ammonia excretion, therefore plasma ammonia level would be maintained either low or comparable to low feeding fish; and finally (iii) hypercortisol would induce GR1, GR2 and MR expression and the effect would more prominent in liver and kidney of low feeding fish to signalling glycogenolysis or gluconeogenic actions compared to high feeding fish. Immediate response such as metabolic trade-offs were assessed as non-genomic responses to hypercortisol.

With all these knowledge background, the ultimate aim of this research was to investigate the impact of feeding, swimming and cortisol induction on metabolic and ionoregulation strategies of cyprinid fish.

1.2 The cyprinids – goldfish, *Carassius auratus* and common carp, *Cyprinus carpio*

Both goldfish and common carp are ecological and economical important species worldwide. The production of carp has increased markedly in Asia mainly in China and India for local consumption as well as in some Eastern Europe countries (Naylor et al., 2000). Additional to their role in food supply, cyprinid fish such as the goldfish and koi are both highly symbolic in Chinese and Japanese culture. This species had been cultured in some city aqua-gardens not only to attract tourists but also to raise people's awareness to maintain a healthy and clean environment. This spirit had been spread to other Asian countries (e.g. Korea, Malaysia, Singapore and Taiwan). Furthermore, scientifically these cyprinids also provide an excellent research model to understand how they respond to a combination of environmental challenges as they adapt relatively easy to laboratory conditions. Thus, juvenile goldfish and common carp were selected as experimental species in the present study.

1.3 Swimming - The critical swimming speed, U_{crit}

Swimming is a life essential action to ensure optimal survival in aquatic animals and is needed for searching food, meeting mated and avoiding predators. In general, swimming performance is classified into three categories: sustained, prolonged and burst swimming (Beamish, 1978). Sustained swimming speed defines those speeds that can be maintained by a fish for long periods >240 min (Beamish, 1966) or >200 min (Brett, 1967), and that are fuelled aerobically. Prolonged swimming speed is also fuelled aerobically, but is of shorter duration 20 s - 200 min (Beamish, 1978) than sustained, and ends in fatigue of the fish. Burst swimming speed is the highest speed of which fishes are capable, and can be maintained only for short periods <20 s (Beamish, 1978), and is considered to be fuelled anaerobically (Plaut, 2001).

Swimming energetic is determined by the gait transition from steady cruising to burst-and-glide swimming modes (Videler, 1993). The aerobically driven red muscles are used to power cruise swimming, while when switching to burst-and glide swimming the anaerobically white muscles are engaged (Videler, 1993; Tudorache et al., 2010a). The transition swimming mode is typically characterized by the large and discrete increase in upstream motion; increased tail-beat amplitude and increased tail-beat frequency (Tudorache et al., 2007, 2010a). The transition swimming modes are also applied to reveal both ecological and physiological fitness (Peake, 2008, Tudorache et al., 2007, 2010a).

In the laboratory, fish swimming capacity is often determined by measuring critical swimming speed (U_{crit}) which is performed by progressive increments in water velocity in a swimming flume at a constant time interval until exhaustion occurs (Brett, 1964; Tudorache et al., 2007, 2010a). U_{crit} is widely applied to investigate swimming capacity in relation to the impact of nutritional status, environmental stress or disease (Hammer, 1995; Kieffer, 2000; Nelson et al., 2002; Lurman et al., 2007). The U_{crit} test allows to measure maximum O_2 consumption (Gregory and Wood, 1999), aerobic and anaerobic metabolic scopes (Lauff and Wood, 1996, 1997; Reidy et al., 2000; Lurman et al., 2007), metabolic waste excretion capacity (Alsop and Wood, 1997; Kieffer et al., 1998; Liew et al., 2012), effects on ion balance (Wang et al., 1994), endocrine status (Gamperl et al., 1994; Wang et al., 1994; Milligan, 1996) and behaviour (Tudorache et al., 2007, 2008, 2010a, 2010b) as well as to examine the 'osmorespiratory compromise' (Wood and Randall, 1973a; 1973b; Jones and Randall, 1978; Gonzalez and McDonald, 1992, 1994; Postlethwaite and McDonald, 1995; Liew et al., 2013a).

Although, the U_{crit} test has its limitations (Tudorache et al., 2007) in predicting real performance in nature (Nelson et al., 2002), it provides a simple and direct evaluation tool to examine the fitness of fish experiencing environmental challenges (Alsop and Wood, 1997; Gallaugher et al., 2001; Lee et al., 2003; McKenzie et al., 2003; De Boeck et al., 2006; Farrell, 2007, 2008; Tudorache et al., 2010a).

1.4 Feeding

Physiologically, feeding is followed by an increase in metabolic rate known as SDA which represents all the oxygen expenditure for ingestion, digestion, absorption and transformation of food and somatic development (Jobling, 1981; Alsop and Wood, 1997; Secor et al., 2007). SDA is known to be influenced by meal size (Jobling, 1980; Fu et al., 2009), body mass (Hunt von Herbing and White, 2002), temperature (Pang et al., 2010) and dissolved oxygen level (Zhang et al., 2012).

Gastrointestinal macronutrients absorption and the capacity of fish gut to respond to diet composition are constitutive differences among fishes (Clements and Raubenheimer, 2006). Buddington et al. (1987) demonstrated that the ratio of amino acids/glucose uptake decreased in the order carnivores > omnivores > herbivores fed with a similar dietary formula. Fish can absorb a range of carbohydrate monomers (e.g. glucose, fructose, galactose and digestion product of chitin, N-acetyl-glucosamine (Gutozska et al., 2004). The glucose is transported across the basolateral membrane in the gut lumen facilitated by a group of glucose transporters through blood and stored in tissue, especially liver (Soengas and Moon, 1998). The large proportion of protein dietary intake is hydrolyzed by cytoplamic enzymes and absorbed across the apical and basolateral membranes of enterocytes into circulatory system (Sire and Vernier, 1992). However, the absorption capacity varies among species, gut system and time of ingestion (Clements and Raubenheimer, 2006). Overall lipid is categorized according to long, medium or short-chat fatty acids and stored in adipose tissues (Tocher, 2003). The structure and properties of fatty acids chain influence their absorption rate across the intestine, where absorption rate increases with chain-length and degree of saturation (Sigurgisladottir et al., 1992). Portions of these absorbed macronutrients are stored in specific tissue as energy reserves, while others are used for basal metabolism, growth and reproduction. The use of energy reserves is influence by physical activity, environmental challenge as well as species-specific influences (Lauff and Wood, 1996; De Boeck et al., 2001; Liew et al., 2012).

Furthermore, feeding causes dramatic changes in osmo-and-ionoregulatory responsesand acid-base balance via the gastrointestinal system (Bucking and Wood, 2006a 2007, 2008; Barcellos et al., 2010). It is clearly demonstrated that feeding is beneficial to achieve osmotic balance with net water loss in freshwater fish (Bucking and Wood, 2006b) and it stimulates gastric acid (hydrochloride acid, HCl) secretion causing a substantial post-prandial alkaline tide (Wood et al., 2005). Nevertheless, different species exhibit distinct strategies to cope with different feeding regimes including varying use of proteins, carbohydrates and lipids from different body compartments (Bandeen and Leatherland, 1997; Schjolden et al., 2005), and differences in endocrinology status where some species showed no effect of starvation on cortisol levels (Sumpter et al., 1991; Vijayan et al., 1993; Holloway et al., 1994; Reddy et al., 1995; Jørgensen et al., 1999), while other studies report decreased cortisol levels in fasted fish (Farbridge and Leatherland, 1992; Small, 2005) and/or increased cortisol levels in either fasted or fed fish (Blom et al., 2000; Kelley et al., 2001; Pottinger et al., 2003; Peterson and Small, 2004). Additionally, feeding induces positive effects on nitrogenous waste excretion by the induction of 'Rhesus glycoprotein' as self-detoxification strategy from endogenous ammonia elevation (Nawata et al., 2007; Zimmer et al., 2010; Liew et al., 2013b).

1.5 Osmo-and-ionoregulation

Freshwater fish live in a hypotonic environment. By diffusion and osmosis, freshwater fish inevitably lose ions to the surrounding water and gain water from their environment across their permeable surfaces. The fish gill and kidney all play an important role in acid-base balance and osmo-and-ionoregulatory strategies in order to compensate ion losses to the dilute environment. Freshwater fish need to extract ions actively from water through the gill or from dietary intake via the gastrointestinal tract and control losses by active ion reuptake in the kidney by the functioning of numerous ion-translocating proteins. Water balance is achieved by the production and excretion of large volumes of dilute urine.

1.5.1 The gill

The fish gill is the multifunctional organ responsible for gas exchange and ion uptake (osmo-and-ionoregulation). It is a complex interface organ designed to separate the external and internal fluid by form a large surface area that is highly permeable (Perry et al., 2003). Fish gills contain numerous numbers of mitochondrion-rich cells in the epithelium which are the main location for ion transporting Na^+/K^+ ATPase (NKA) (Hwang and Lee, 2007) and a site of movement of salt and water (Evans et al., 2005). The NKA is a membrane bound enzyme that transport two K^+ into and three Na^+ out of animal cells by using energy from hydrolysis of one molecule ATP (Lingwood et al., 2006).

During active swimming, fish increase O_2 consumption which may lead to proportionately elevated diffusive ion losses as both are dependent upon gill functional surface area (Randall et al., 1972). However, a large highly permeable gill membrane is required for efficient gas transfer and small impermeable epithelium is needed to minimize diffusive ion losses (Randall et al., 1972; Gonzalez and McDonald, 1992). This results in a phenomenon called the 'osmorespiratory compromise' by which any increase in gill functional surface area to promote oxygen uptake ($\dot{M}O_2$) would accelerate Na^+ efflux (J^{Na}_{out}), while reductions of gill functional surface area to lower ion losses would reduce the ability of the gill to take up oxygen (Gonzalez and McDonald, 1992).

Besides for gas exchange and ion uptake, fish gills also play a role in nitrogenous waste excretion. Unlike other vertebrates, teleost produce large amounts of ammonia with approximately one molecule ammonia for every five molecules of O_2 consumed as consequence of protein breakdown/catabolism from either dietary intake or active swimming and they excrete it solely through the gills. Therefore, teleost not only need to deal with the osmorespiratory compromise but they also need to enhance ammonia excretion (J_{amm}) that is facilitated by Na^+/NH_4^+ exchanger and the $Na^+/NH_4^+/2Cl^-$ co-transporter both driven by the Na^+ gradient created by NKA as well as by Rhesus glycoprotein (Nawata et al., 2007). Na^+ uptake across the apical membrane occurs via channels energetically coupled to a vacuolar H^+ ATPase. The active extrusion of H^+ across the apical membrane serves to create a favourable electrochemical gradient that allows the inward diffusion of Na^+ through selective Na^+ channels (Perry et al., 2003). The mechanism involved had been reported as an electrogenic H^+ translocation ATPase coupled with Na^+ conductive channel that transport Na^+ and H^+ in opposite direction (Lin and Randall, 1991). Provide a negative potential in apical membrane by uptake Na^+ through Na^+ channel and actively release H^+ (Lin and Randall, 1993). The performance of H^+ ATPase is also influenced by environmental stress (Lin and Randall, 1993) such as hypercapnia

(Goss et al., 1992). Other than that, H^+ ATPase plays an important role in freshwater fish for ammonia excretion (Zare and Greenaway, 1998; Alam and Frankel, 2006).

Interestingly, Perry et al. (2006) demonstrated that high internal salt loading by feeding is able to induce various elements of the seawater gill phenotype in freshwater fish such as the cystic fibrosis transmembrane conductance regulator (CFTR), the $Na^+/K^+/2Cl^-$ co-transporter (NKCC1) and NKA, which are essential for ionic regulation in seawater, and the appearance of chloride cell-accessory cell complexes, which are normally restricted to fish inhabiting seawater. They enhance Na^+ excretion in freshwater fish due to high dietary NaCl intake. This NKA activity is influenced by nutritional status (Polakof et al., 2006) and endocrine control such as cortisol (Laiz-Carrion et al., 2003), prolactin (Kelly et al., 1999; Mancera et al., 2002), growth hormone (Sakamoto and McCormick, 2006), and insulin-like growth factor-I (Seidelin and Madsen, 1999).

1.5.2 The kidney

Kidney of freshwater fish acts as a final ion absorber prior releasing hypotonic urine to environment. The kidney consists of distal renal tubules connecting the proximal segments to the collecting duct via collecting tubules (Hickman and Trump, 1969). Unlike marine fish that need to prevent water loss and excrete ions, freshwater fish have essentially the opposite osmoregulatory strategy, and need to conserve and gain essential ions such as Na^+ and Cl^- while preventing excessive water loading across permeable body surfaces due to osmosis induction (Cutler et al., 2009). To maintain homeostasis, freshwater fish excrete large volumes of dilute urine to about 3 - 4 ml/kg/h which is 10 times largest than marine fish with only about 0.3 ml/kg/h (Marshall and Grosell, 2006). In the kidney, most of the filtered and secreted NaCl is reabsorbed in conjunction with solutes in the late proximal tubule, distal tubule or urinary bladder. The NaCl reabsorption in the distal tubule of freshwater fish is thought to occur via NKCC co-transporter located in the apical membrane and NKA in the basolateral membrane of tubule cells (Dantzler, 2003). The apical NKCC allows for cellular Cl^- accumulation above the thermodynamic equilibrium and Cl^- transport across the basolateral membrane via K^+/Cl^- co-transporter or Cl^- channels and K^+ is recycled across the apical membrane via apical K^+ channels. Additionally, Na^+ is absorbed via Na^+/H^+ exchanger across the apical membrane (Dantzler et al., 2003; Marshall and Grosell, 2006) and H^+ ATPase creates positive potential gradient also contribute to Na^+ reabsorption (Perry et al., 2000).

1.6 Endocrinology - cortisol

The magnitude of behavioral and physiological responses to stress not only varies among species, but also differs among strains and individuals. Parameters such as growth, reproduction, osmoregulation, metabolic homeostasis, and mobilization of energy stores are under endocrine control (Pickering, 1993; Schjolden et al., 2005). The increase in plasma cortisol levels is a stress indicator in fish (Czesny et al., 2003) and leads to increased levels of plasma glucose and plasma free fatty acids (Casillas and Smith, 1977; Mazeaud et al., 1977), which supply energy for increased metabolic demands during stress (Sheridan and Mommsen, 1991).

Cortisol is characterized as a multifunctional hormone involved in metabolic regulation (Barcello et al., 2010). The cortisol or corticosteroid is synthesized in the interrenal tissue of the head kidney in teleosts (Bury and Sturm, 2007) and it ultimately exerts its function by the transcription factors known as glucocorticoid receptors (GRs) and mineralocorticoid receptor (MR) that signal to

specific targets (Alsop and Vijayan, 2008; Stolte et al., 2008). In fish, glucocorticosteroids play a key regulatory role in stress responses, growth, metabolism, reproduction, immunity, development, behaviour and responses of the cardiovascular system (Wenderlaar Bonga, 1997; Mommsen et al. 1999; Charmandari et al., 2005; Bury and Sturm, 2007). Mineralocorticoid is involved in the regulation of water and mineral balance at a systemic and cellular level for restoration of homeostasis (Bern and Madsen, 1992; Gilmour, 2005; Bury and Sturm, 2007) and is usually considered as seawater-adapting hormone by increasing chloride cell proliferation and stimulating gill NKA expression and activity for osmoregulatory processes (Madsen, 1990; McCormick, 1995). McCormick (2001) suggested the action of cortisol was in cooperation with prolactin to increase ion uptake in hypo-osmotic environments. Seidelin and Madsen (1997) suggested that interaction of cortisol and prolactin on salt secretory capacities may occur in non-branchial tissues such as the gastrointestinal tract and prolactin could reverse the ability to increase hypo-osmoregulatory effects induced by cortisol, but it did not affect the capacity of cortisol to increase gill NKA activity. On the other hand, cortisol has been found to rapidly decrease the release of prolactin from the tilapia pituitary (Borski et al., 1991). Prolactin and cortisol act synergistically in order to promote transepithelial resistance and potential as has been demonstrated by using *in vitro* gill cell preparation, with a positive interaction for ionic balance in freshwater fish (Parwez and Goswami, 1985; Eckert et al., 2001; Zhou et al., 2003).

In conclusion, the control of the osmoregulatory system of teleost involves several hypophysial hormones such as cortisol, which are crucial for osmo-and-ionoregulation (McCormick, 1995, 2001; Sakamoto and McCormick, 2006). It is a well-established fact that prolactin has an important role in the freshwater acclimation teleost. In recent years, evidence also suggests the role for cortisol in ion uptake in low-salinity water-adapted fish or freshwater. This new evidence suggest the role of 'dual-osmoregulatory' action with the classic role of stimulation of ion secretion in hyperosmotic media in cooperation with growth and insulin-like growth factor-I. While cooperate with prolactin, cortisol act to induce increase ion uptake in hypo-osmotic environments.

Chapter 1

Fasting goldfish, *Carassius auratus* and common carp *Cyprinus carpio* use different metabolic strategies when swimming

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Abstract

Fish need to balance their energy use between digestion and other activities, and different metabolic compromises can be pursued. We examined the effects of fasting (7 days) on metabolic strategies in goldfish and common carp at different swimming levels. Fasting had no significant effect on swimming performance (U_{crit}) of either species. Feeding and swimming profoundly elevated total ammonia (T_{amm}) excretion in both species. In fed goldfish, this resulted in increased ammonia quotients (AQ), and additionally plasma and tissue ammonia levels increased with swimming reflecting the importance of protein contribution for aerobic metabolism. In carp, AQ did not change since oxygen consumption (MO_2) and T_{amm} excretion followed the same trend. Plasma ammonia did not increase with swimming suggesting a balance between production and excretion rate except for fasted carp at U_{crit}. While both species relied on anaerobic metabolism during exhaustive swimming, carp also showed increased lactate levels during low aerobic swimming. Fasting almost completely depleted glycogen stores in carp, but not in goldfish. Both species used liver protein for basal metabolism during fasting and muscle lipid during swimming. In goldfish, feeding metabolism was sacrificed to support swimming metabolism with similar MO_2 and U_{crit} between fasted and fed fish, whereas in common carp feeding increased MO_2 at U_{crit} to sustain feeding and swimming independently.

Keywords: *Carassius auratus*, *Cyprinus carpio*, energy budgets, feeding, metabolism, plasma metabolites, fasting, U_{crit}

1. Introduction

Physiological processes in teleosts respond to environmental factors such as water currents, food availability, temperature, dissolved oxygen and its combinations (Kieffer et al., 1998). Under all these circumstances, feeding is important to supply energy and maintain routine metabolism for survival, growth and reproduction. Swimming occurs simultaneously not only for migration, but also to ensure sufficient feeding and leads to increased oxygen uptake and energetic costs. Additionally, feeding itself increases oxygen consumption (MO_2), known as the specific dynamic action (SDA), a phenomenon associated with digestion. Recent studies have shown that feeding can impair swimming capacity due to the limitation of oxygen delivery to the tissues. Hence, it affects the aerobic locomotion performance (Li et al., 2010; Pang et al., 2010; Zhang et al., 2010). Alsop and Wood (1997) proposed three possible physiological compromises that can occur in the competition between SDA and swimming for oxygen needs. The first metabolic mode is that both SDA and swimming proceed independently. This would then inevitably lead to increased MO_2 and is only possible if there is no diffusion or circulatory constraints. The other two possibilities are that either digestion or swimming is sacrificed to sustain the prioritised metabolism. Alsop and Wood (1997) showed that in rainbow trout feeding caused the sacrifice of swimming due to the irreducible SDA. However, a recent study showed that southern catfish shifted from prioritising SDA under moderate hypoxia to swimming metabolism under more severe hypoxia (Zhang et al., 2010). Therefore, the aim of present study was to investigate which metabolic mode would be prioritised in goldfish and common carp under different feeding and swimming regimes by respirometric approach. As both goldfish and common carp are ecological and economical important species worldwide, these species also provide an excellent research model to understand how they respond to a combination

of environmental challenges. Thus, juvenile goldfish and common carp were selected as experimental species in the present study. This approach has been widely applied to investigate aerobic metabolism in aquatic animals swum up to critical swimming speed (U_{crit}) (Lauff and Wood, 1996; Milligan et al., 2000; Gallaugher et al., 2001; De Boeck et al., 2006; Pang et al., 2010). Besides swimming performance (U_{crit}), MO_2 and total ammonia excretion (T_{amm}), we also analysed the use of metabolic energy stores in our study. Additionally, we looked at accumulation of lactate and ammonia in plasma and muscle of goldfish and common carp under the imposed conditions.

2. Materials and methods

2.1 Fish management

The goldfish, *Carassius auratus* were purchased from a local fish supplier (Aqua Hobby, Heist op den Berg, Belgium). Common carp, *Cyprinus carpio* were obtained from University of Wageningen, The Netherlands. Both species were kept in the aquaria facilities of the Systemic Physiological and Ecotoxicological Research at the University of Antwerp, Belgium with Antwerp City water. Fish were fed at 2% body weight (BW) with commercial pellets ('Hikari Staple', Kyorin Food Ind. Ltd., Japan) twice a day. The feed used in the experiment contained of ≥35% protein, ≥3.0% lipid, 5.0% fibre, 13.0% ash, 1.0% phosphorus and 10% mixture of vitamins and minerals. After 2 months pre-acclimatisation, 80 fish from each species were transferred to a climate room (goldfish 16.4±1.6 g; common carp 20.0±0.3 g (Mean±SEM)). Fish were distributed equally into eight 150 L rectangular fibreglass tanks and allowed to acclimatise to the experimental conditions for one month prior to the experiment. The room temperature was set to 17^{o} C maintaining the water temperature at 16.7±0.3^{o} C. A constant photoperiod regime was set at 14L:10D. Water was filtered through biological filters containing wadding, lava stones (0.8-16.0 mm) and activated carbon (charcoal). About 80% of the water was replaced twice a week. Water quality was monitored regularly by using the Standard Tetra Test Kits (Germany) and values remained <0.1 mg/L of NH_3/NH_4^+; <0.03 mg/L of NO_2^- and <25 mg/L of NO_3^-.

2.2 Experimental design

After one month acclimation, fish were divided in two feeding regime, feeding groups that remained at 2% BW were divided over 2 equal portions a day at 08:30h and 18:30h and fasted groups that were subjected to short-term fasting stress (7 days). Subsequently, these fish were divided into resting, low aerobic swimming and exhaustive swimming groups. Resting fish were sampled directly from the holding tank. For low aerobic swimming, fish were swum at ¼ U_{crit} for 3 h in respirometers ($MO_{2\text{-low aerobic}}$). The exhaustive swimming test were performed in two sets of fish: a swimming performance test in which fish were swum up to U_{crit} and subsequently sampled, and another separate set of fish for respirometry measurements ($MO_{2\text{-high aerobic}}$) in fish swum at ¾ U_{crit} for 1 h. Each sampling group consisted of eight fish.

2.3 Swimming performance, U_{crit}

Eight Blazka-style swimming respirometers with a volume of 3.9 L (outer tunnel (length × diameter) 50 × 11 cm and inner tunnel 35 × 6 cm) were placed into 4 analogous flow through experimental tanks, each consisting of one 110 L reservoir tank, one 80 L tank holding 2 respirometers and one 60 L head tank with the total volume of 250 L. Water was pumped directly from the reservoir to the head tank after which it was flowing back by gravity through the respirometers and into the reservoir. The 8 fish were randomly placed into the respirometers 14 h prior to the experiment to reduce the handling stress. During this acclimation period, respirometers were provided with air-saturated water from the overflow head tank at 4 L/min to ensure water quality and oxygen saturation. Fish were swimming at a low velocity of 10 cm/s during acclimation. For U_{crit} measurement, water velocity was increased with 5 cm/s every 20 min interval until fatigue. The point of fatigue was determined as the moment when the fish was incapable to swim against the water current, and was swept downstream against the mesh screen at the end of tunnel (De Boeck et al., 2006). At this point water velocity was reduced in order to allow the fish to recommence swimming. Fish were considered totally fatigued when they were swept downstream for the second time within the same 20 min interval. The performance test was then terminated. U_{crit} was calculated as $U_{crit} = U_i + [U_{ii}(T_i/T_{ii})]$, where U_i is referred to highest velocity sustained for the whole interval, U_{ii} is the velocity increment (5 cm/s), T_i is the time elapsed at fatigue velocity and T_{ii} is the interval time (20 min) (Brett, 1964; Tudorache et al., 2007). The absolute values (cm/s) were converted to relative swimming speeds in body lengths per second (BL/s) by factoring the absolute values with their body length. Physical parameters were measured individually from all fish, blood and tissue samples were dissected. The condition factor was calculated as $K = (BW/BL^3) \times 100$.

2.4 Oxygen consumption, MO_2

Fish were allowed to acclimatise to the respirometers overnight at a speed of 10 cm/s as described above. Respiration measurements were conducted on low aerobic swimming fish ($MO_{2\text{-low aerobic}}$) swum at ¼ U_{crit} for 3 h. To ensure that all fish would be able to complete their respirometry at a speed close to U_{crit}, a separate set of fish were swum for 1h at ¾ U_{crit} and were called high aerobic swimming fish ($MO_{2\text{-high aerobic}}$). Calibrated oxygen electrodes (Oximeter Oxy-340, Wissenschaftlich-Technische Werkstatten & Co KG, Weilheim, Germany) were inserted and all outlets were sealed with rubber bungs to prevent water and gas exchange. Oxygen consumption rate (MO_2) was recorded by using Windmill Logger (Windmill Software Limited, Manchester, UK) with a reading frequency of 26 readings per minute. MO_2 was calculated according to Ultsch et al. (1980) method as $MO_2 = (\Delta O_{2i} - O_{2f}) \times V \times 1000 \times (1/O_{2MW}) \times (1/BW) \times T$, 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); and expressed as µmol/g/h (De Boeck et al., 2006). A one ml water sample was sampled from each of the respirometers prior and post MO_2 determination to measure the ammonia excretion (T_{amm}). T_{amm} was determined by using the phenol-nitroprusside method (Verdouw et al., 1978). The NH_4^+ excretion rate was calculated as T_{amm} excretion = $(\Delta NH_4^+{}_f - NH_4^+{}_i) \times V \ (1/NH_4^+{}_{MW}) \times (1/BW) \times (1/T)$, where $NH_4^+{}_i$ is referred as initial ammonia concentration (µg/L); $NH_4^+{}_f$ is the final ammonia concentration (µg/L); V as the total water volume in respirometer (3.9 L); $NH_4^+{}_{MW}$ as the molecular weight of ammonia (17); BW as body weight (g); T is time (h) and expressed as µmol/g/h. Ammonia

quotient (AQ) of each fish was determined as AQ = T_{amm}/MO_2. The theoretical maximum AQ in teleost is 0.33 and this theoretical maximum AQ is known as maximum protein contribution during aerobic metabolism (Kutty, 1978; De Boeck et al., 1995; 2000a; Kunwar et al., 2009). After measurement, fish were removed from respirometers, anaesthetised, blotted dry for measurement of physical parameters. Subsequently, only blood and tissue samples from the low aerobic swimming fish were sampled for further biochemical analysis.

2.5 Sampling procedure

Overall, resting fish from the holding tank, low aerobic swimming fish swum at ¼ U_{crit} from the respirometry experiment and exhaustive swimming fish swum up to U_{crit} from the swimming performance experiment were dissected for further biochemical analysis. Fish were anaesthetized with Ethyl-3-aminobenzoate methanesulfonic acid (MS222) at 0.5 g/L (Acros Organics, Geel, Belgium) neutralized with NaOH. Fishes were blotted dry for biometric measurement and blood was drawn via the caudal vessels using a heparinised needle and a syringe within 1 min. The blood was immediately centrifuged to 13,200 rpm at 4^{o} C for 2 min. Plasma was carefully pipetted into cryogenic vials and flash frozen in liquid N_2. Fishes were killed by severing their spinal cord prior to organ sampling. Whole liver mass was recorded and hepatosomatic index was calculated as HSI = (LM/BW) × 100, where LM is referred as liver mass. Collected liver and muscle samples were immediately flash frozen in liquid N_2 and stored at -80^{o} C for further analysis.

2.6 Biochemical analysis

Muscle ammonia was measured according to Wright et al. (1995). Plasma ammonia and lactate were determined using commercial Enzymatic Kits (R-Biopharm AG, Darmstadt, Germany). Tissue energy such as glycogen, protein and lipid contents were determined in both liver and muscle tissues. Glycogen was measured using Anthron reagent and a glycogen standard curve (Roe and Dailey, 1966). Protein was determined according to Bradford (1976) using a standard curve of bovine serum albumin. Total lipid was extracted by methanol-chloroform and measured with a tripalmitin standard curve (Blingh and Dyer, 1959).

2.7 Statistics analysis

Normality was checked prior to the analysis by Shapiro-Wilk test. Log-transformed data were applied if the requirements for ANOVA were not fulfilled. The differences between fed and fasted fish on U_{crit} were assessed by unpaired two-tail student *t*-test. In addition, when swimming had no significant effect, data were pooled to examine the feeding regime. Effect of feeding and swimming on MO_2, T_{amm} excretion, AQ, plasma metabolites, muscle ammonia, and energy budgets in liver and muscle tissue under resting, routine and exhaustive swimming were assessed by one-way ANOVA. If the ANOVA indicated a significance at $P<0.05$, a Tukey multiple post-hoc test was done. Two-way ANOVA was used to compare overall interactive effects. All data were expressed as mean values with standard error (Mean±SEM; $n = 8$).

3. Results

3.1 The effect of feeding regime on biometrics and U_{crit}

The short swimming regimes (resting, low aerobic swimming and exhaustive swimming) did not affect biometric characteristic, therefore all data in Table 1 were pooled. As expected, a week of fasting did not cause any significant changes in body weight, body length or condition factor (*K*-factor). However, there was an effect on the liver mass leading to a significantly reduced in HSI in fasted fish for both species. Under all circumstances, the HSI of goldfish was two-fold higher than the common carp.

Table 1 Biometric data of goldfish and common carp at different feeding regimes.

	Goldfish		Common carp	
Characteristics	Fasted	Fed	Fasted	Fed
Body weight (g)	17.13±0.02	16.06±0.05	20.22±0.11	20.06±0.56
Body length (cm)	8.09±0.04	8.05±0.06	11.23±0.08	11.22±0.15
K-factor	3.29±0.04	3.13±0.09	1.41±0.04	1.39±0.06
Hepatosomatic index	3.91±0.18	*4.73±0.07	1.24±0.04	*1.98±0.21

Asterisk (*): Significant difference between the fasted and fed group (P<0.05).

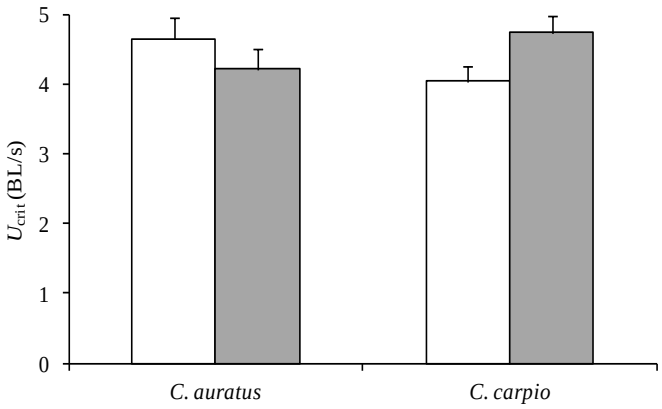

Fig. 1 Critical swimming speed (U_{crit}; body length/s) of fasted and fed *C. auratus* and *C. carpio*. Open bars indicate fasted fish, filled bars indicate fed fish.

Feeding regime had no significant effect on U_{crit} of goldfish and common carp (Fig. 1). U_{crit} of fasted goldfish averaged at 4.66±0.31 BL/s which was not significantly different from fed goldfish 4.23±0.29 BL/s. In common carp, the opposite trend was observed with the U_{crit} of 4.05±0.23 BL/s for fasted fish that lower than the fed fish at 4.75±0.23 BL/s, a result that was close to significance (P=0.051).

3.2 Effects of feeding and swimming on MO_2, T_{amm} excretion and AQ

Feeding effects on $MO_{2\text{-low aerobic}}$ were absent in both species under low aerobic swimming (Fig 2.a and d). When fish were swum close to exhaustion, feeding had no effect on $MO_{2\text{-high aerobic}}$ of goldfish, but it significantly increased $MO_{2\text{-high aerobic}}$ in fed common carp (Fig. 2.d).

At low aerobic swimming, feeding significantly elevated the $T_{amm\text{-low aerobic}}$ excretion in goldfish (Fig. 2.b) but this effect was not significant in common carp (Fig. 2.e). When swum close to exhaustion, fed fish excreted significantly higher $T_{amm\text{-high aerobic}}$ than fasted fish in both species. For goldfish, this resulted in a significant increase of AQ at both swimming speeds, which reflected the increase of protein use for aerobic metabolism (Fig. 2.c). This was not the case in common carp (Fig. 2.f), since the increase in T_{amm} excretion concurred with the increase in MO_2.

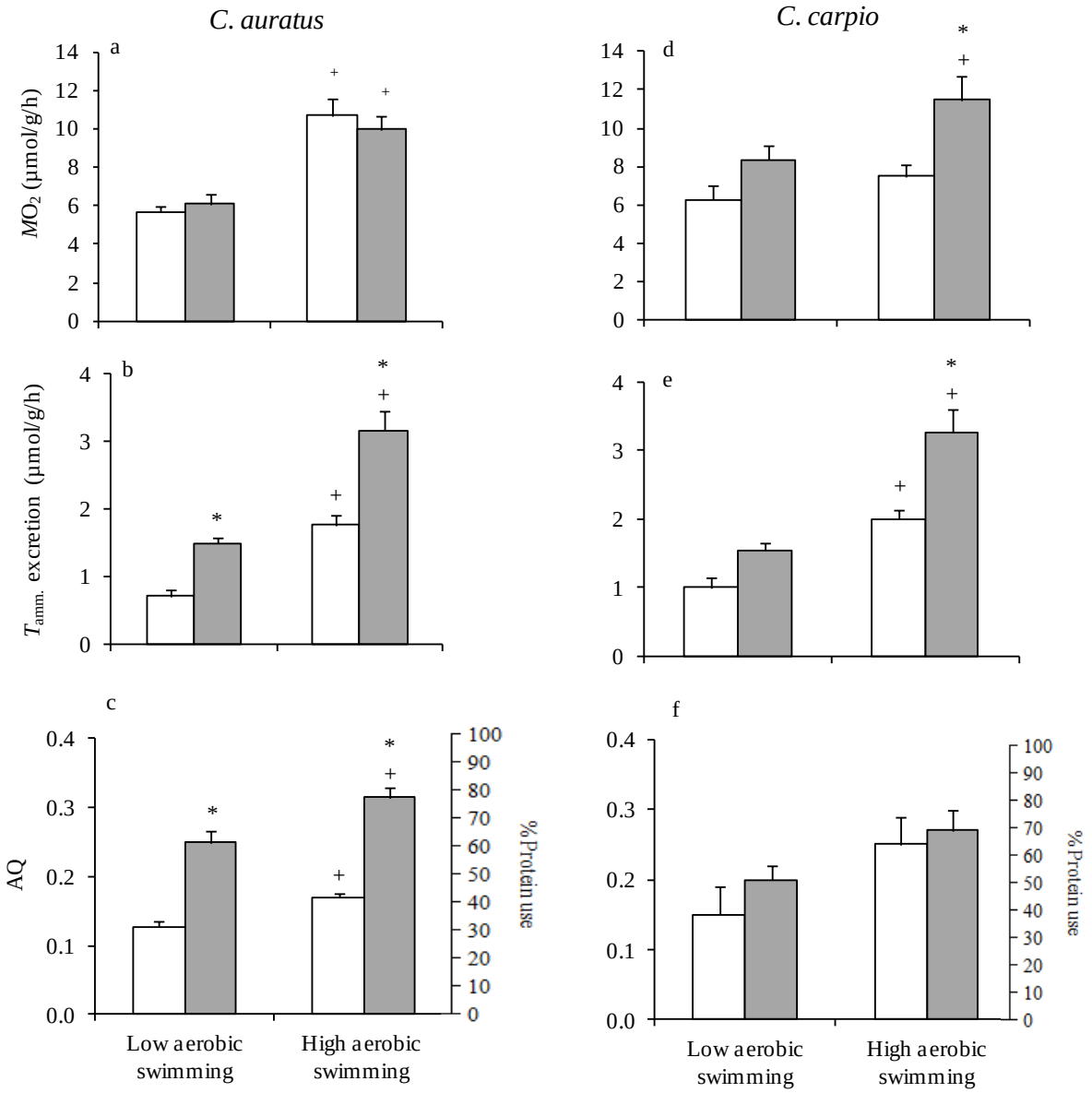

Fig. 2 MO_2, T_{amm} excretion, AQ and % of protein use for aerobic metabolism of fasted and fed *C. auratus* (a-c) and *C. carpio* (d-f) at low aerobic and high aerobic swimming. Open bars indicate fasted fish, filled bars indicate fed fish. An asterisk (*) indicates a significant difference between fasted and fed fish (P<0.05). A plus ($^+$) indicates a significant difference between low aerobic and high aerobic swimming fish (P<0.05).

As expected, high aerobic swimming significantly elevated the MO_2 of goldfish above that of low aerobic swimming fish (Fig. 2.a), but for common carp this was only significant in fed fish (Fig. 2.f). For T_{amm} excretion, increasing swimming levels significantly elevated $T_{amm\text{-}high\ aerobic}$ excretion for both feeding conditions in both species (Fig. 2.b and e). This resulted in a significant effect of both feeding and swimming on AQ of goldfish, either separately or in interaction (Fig. 2.c, Table 2). In common carp, feeding and swimming had no significant effect on AQ (Fig. 2.f), and therefore no interacting effect was detected either (Table 2).

3.3 Effects of feeding and swimming on tissue and plasma metabolites

Overall, different trends were observed in muscle and plasma metabolites of both species (Fig. 3). Effects of feeding were limited to increased plasma ammonia levels in goldfish at all swimming levels (Fig. 3.b), but feeding reduced plasma ammonia level in exhaustively swimming common carp (Fig. 3.e).

Exhaustive swimming significantly increased muscle ammonia accumulation in both fasted and fed goldfish as compared to resting and low aerobic swimming conditions (Fig. 3.a), whereas no effect was found in common carp. Although not significant, overall fasted common carp accumulated numerically higher tissue ammonia than fed fish in all groups (Fig. 3.b). When data of different swimming regimes were pooled, fasted carp accumulated significantly higher muscle ammonia then fed carp. Both feeding and swimming had a significant effect on plasma ammonia in goldfish (Fig. 3.b, Table 2). For common carp, this increase was only limited to fasted fish at exhaustive swimming (Fig. 3.e). A distinct elevation in plasma lactate was recorded in both fasted and fed goldfish swum to exhaustion compared to the other two swimming levels (Fig. 3.c). Plasma lactate increased significantly in both routine and exhaustively swimming common carp compared to resting common carp, and this effect was significantly higher in fed compared to fasted fish at U_{crit} (Fig. 3.f, Table 2).

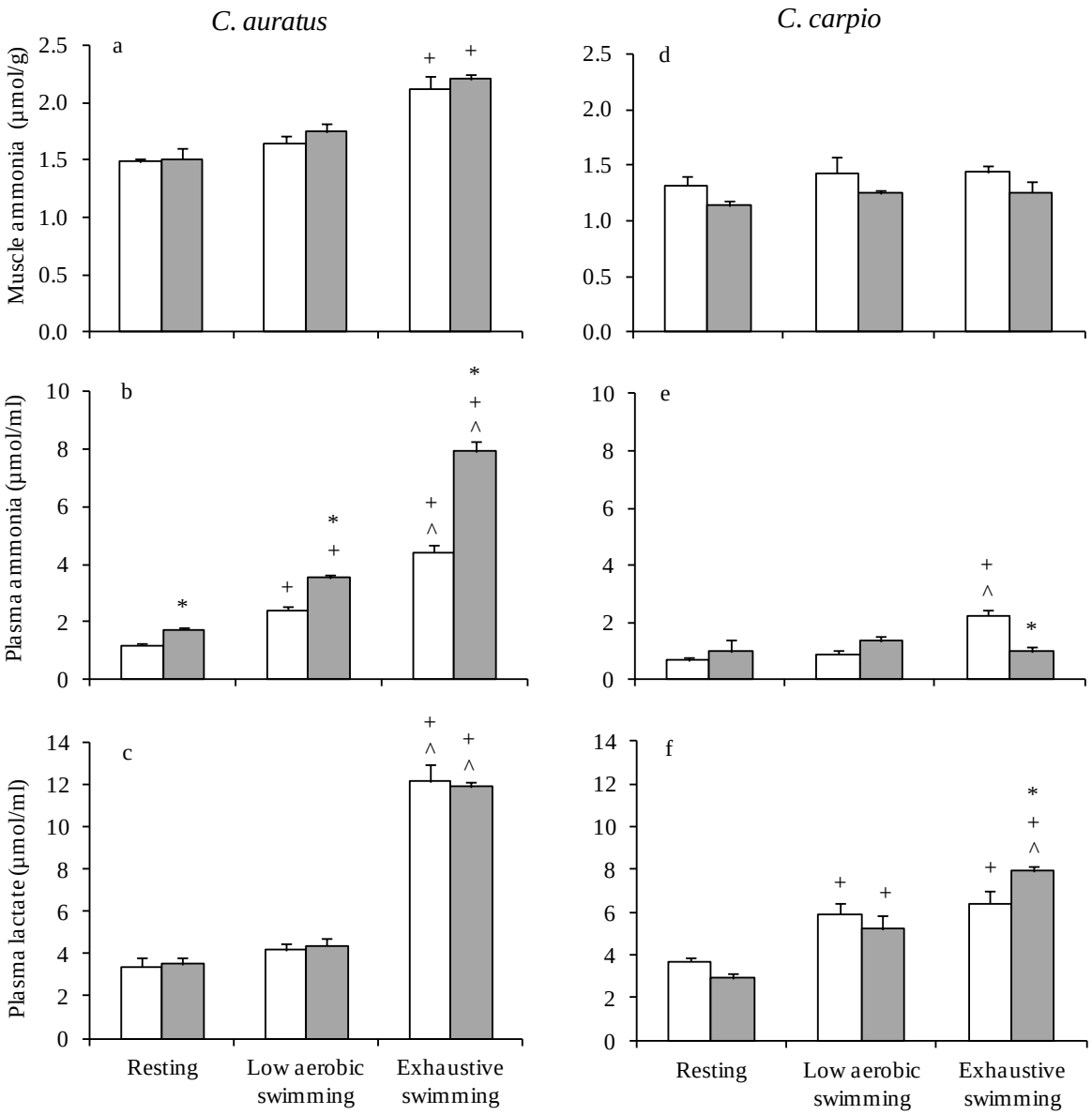

Fig. 3 Muscle ammonia, plasma ammonia and plasma lactate of fasted and fed *C. auratus* (a-c) and *C. carpio* (d-f) at resting, routine and exhaustive swimming. Open bars indicate fasted fish, filled bars indicate fed fish. Significant differences at the $P<0.05$ ($^{*,+,\wedge}$). An asterisk (*) indicates a significant difference between fasted and fed fish ($P<0.05$). A plus ($^{+}$) indicates a significant difference with corresponding resting fish ($P<0.05$). A caret ($^{\wedge}$) indicates a significant difference with corresponding low aerobic swimming fish ($P<0.05$).

3.4 Effects of feeding and swimming on energy stores

The glycogen, protein and lipid use from liver of goldfish and common carp is summarized in Fig. 4. Protein was significantly depleted in fasted goldfish in all groups (Fig. 4.b). No significant effect was observed for liver glycogen and lipid stores of goldfish (Fig. 4.a & c) unless data from the different swimming regimes were pooled after which fasted fish exhibited significant depletions of glycogen and protein. A different trend was observed in common carp. Fasting significantly mobilized liver glycogen and protein (Fig. 4.d & e). When results were pooled liver lipid in fasted carp was depleted significantly as well.

Swimming had no effect at all on liver energy stores in goldfish (Fig. 4.a-c), but swimming significantly depleted liver glycogen to a larger extent in common carp (Fig. 4.d). Even though fasted carp already had very low glycogen content, glycogen was still mobilized at U_{crit}. Therefore, interactive effects were only detected in liver glycogen of common carp (Table 2).

Table 2 Significance levels of the impact of feeding, swimming and their interaction in goldfish and common carp.

Goldfish	Feeding		Swimming		Feeding*Swimming	
	F-value	*P-value*	*F-value*	*P-value*	*F-value*	*P-value*
MO_2	0.092	0.759	542.020	0.000	9.096	0.005
T_{amm} excretion	132.948	0.000	210.825	0.000	8.596	0.007
AQ	119.106	0.000	19.431	0.000	0.948	0.338
Muscle ammonia	0.812	0.373	26.123	0.000	0.084	0.919
Plasma ammonia	114.942	0.000	296.970	0.000	31.753	0.000
Plasma lactate	1.100	0.300	233.087	0.000	0.193	0.826
Liver glycogen	15.962	0.000	2.759	0.075	0.312	0.734
Liver protein	24.775	0.000	0.584	0.562	0.101	0.904
Liver lipid	2.859	0.098	0.596	0.556	0.031	0.969
Muscle glycogen	6.422	0.015	18.795	0.000	0.248	0.782
Muscle protein	27.562	0.000	2.037	0.143	0.924	0.405
Muscle lipid	9.783	0.003	9.114	0.001	0.646	0.529
Common carp						
MO_2	1.370	0.253	6.842	0.015	13.525	0.001
T_{amm} excretion	16.991	0.000	37.857	0.000	2.621	0.118
AQ	1.089	0.306	6.422	0.017	0.200	0.658
Muscle ammonia	6.263	0.016	1.199	0.312	0.001	0.999
Plasma ammonia	1.076	0.305	7.141	0.002	10.840	0.000
Plasma lactate	0.011	0.916	60.374	0.000	6.420	0.004
Liver glycogen	1300.513	0.000	54.808	0.000	12.637	0.000
Liver protein	49.737	0.000	2.341	0.109	0.880	0.422
Liver lipid	6.298	0.016	0.494	0.613	0.124	0.884
Muscle glycogen	379.042	0.000	78.307	0.000	46.682	0.000
Muscle protein	1.504	0.227	20.535	0.000	1.001	0.376
Muscle lipid	5.294	0.026	6.106	0.005	1.625	0.209

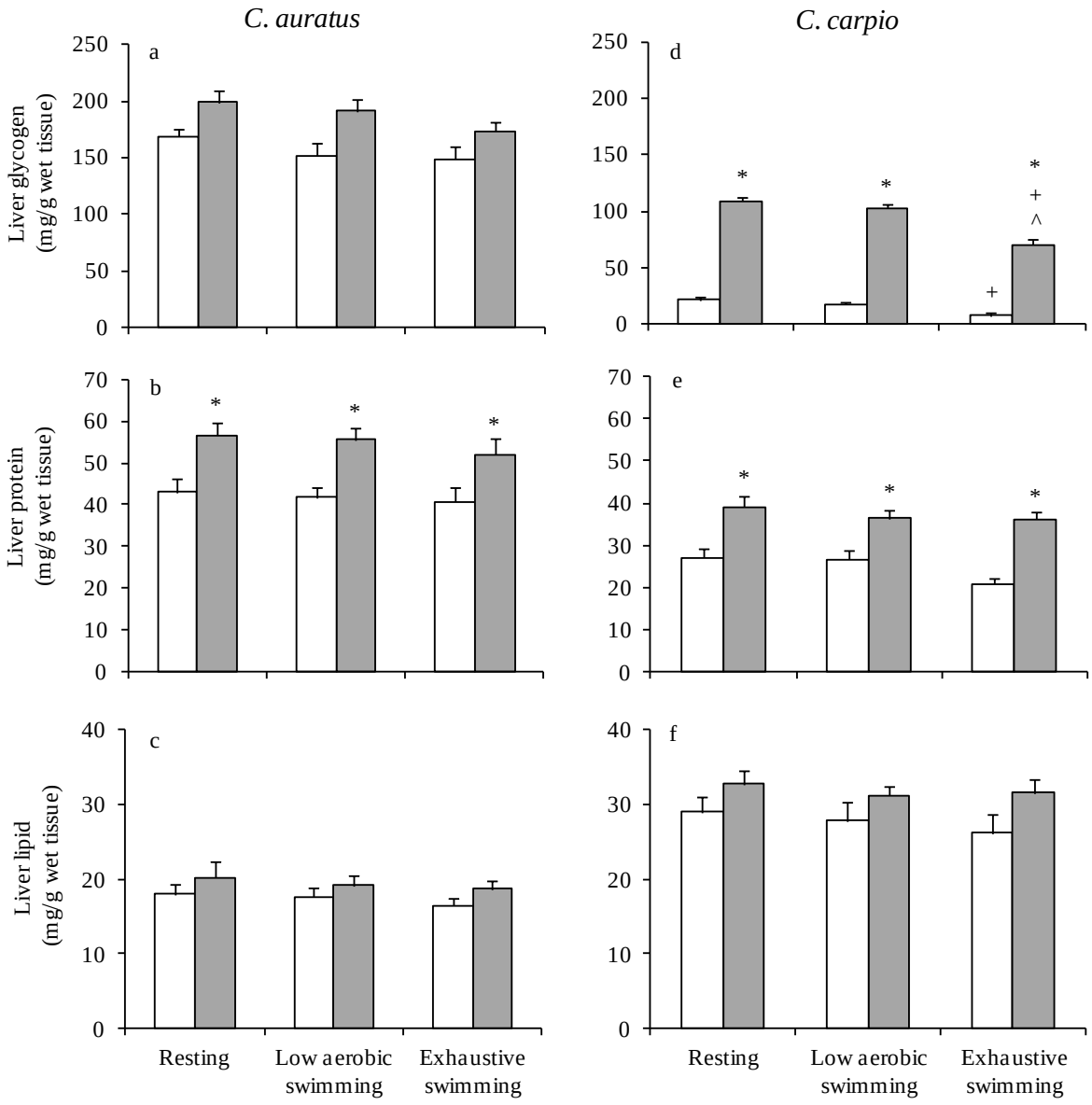

Fig. 4 Liver glycogen, protein and lipid of fasted and fed *C. auratus* (a-c) and *C. carpio* (d-f) at resting, low aerobic swimming and exhaustive swimming. Open bars indicate fasted fish, filled bars indicate fed fish. Significant differences at the $P<0.05$ ($^{*,+,\wedge}$). An asterisk (*) indicates a significant difference between fasted and fed fish ($P<0.05$). A plus ($^{+}$) indicates a significant difference with corresponding resting fish ($P<0.05$). A caret ($^{\wedge}$) indicates a significant difference corresponding with low aerobic swimming fish ($P<0.05$).

The muscle energy content is illustrated in Fig. 5 and interactive effects in Table 2. As in liver, goldfish and common carp exhibited different muscle energy mobilization. Overall fasted fish had lower muscle energy contents then fed fish. Fasting significantly depleted glycogen content in fasted goldfish than fed goldfish when swum to exhaustion (Fig. 5.a). Depletion of muscle protein in fasted fish was significant at routine and exhaustive swimming (Fig. 5.b), while a difference in muscle lipid was only observed at low aerobic swimming (Fig. 5.c). For common carp, fasted fish had spectacularly lower muscle glycogen levels in all swimming regimes (Fig. 5.d) and muscle lipid was lower at U_{crit} (Fig. 5.e). Although no significant effect was found in muscle protein in the different swimming conditions, the effect of fasting was significant when data were pooled (Fig. 5.e).

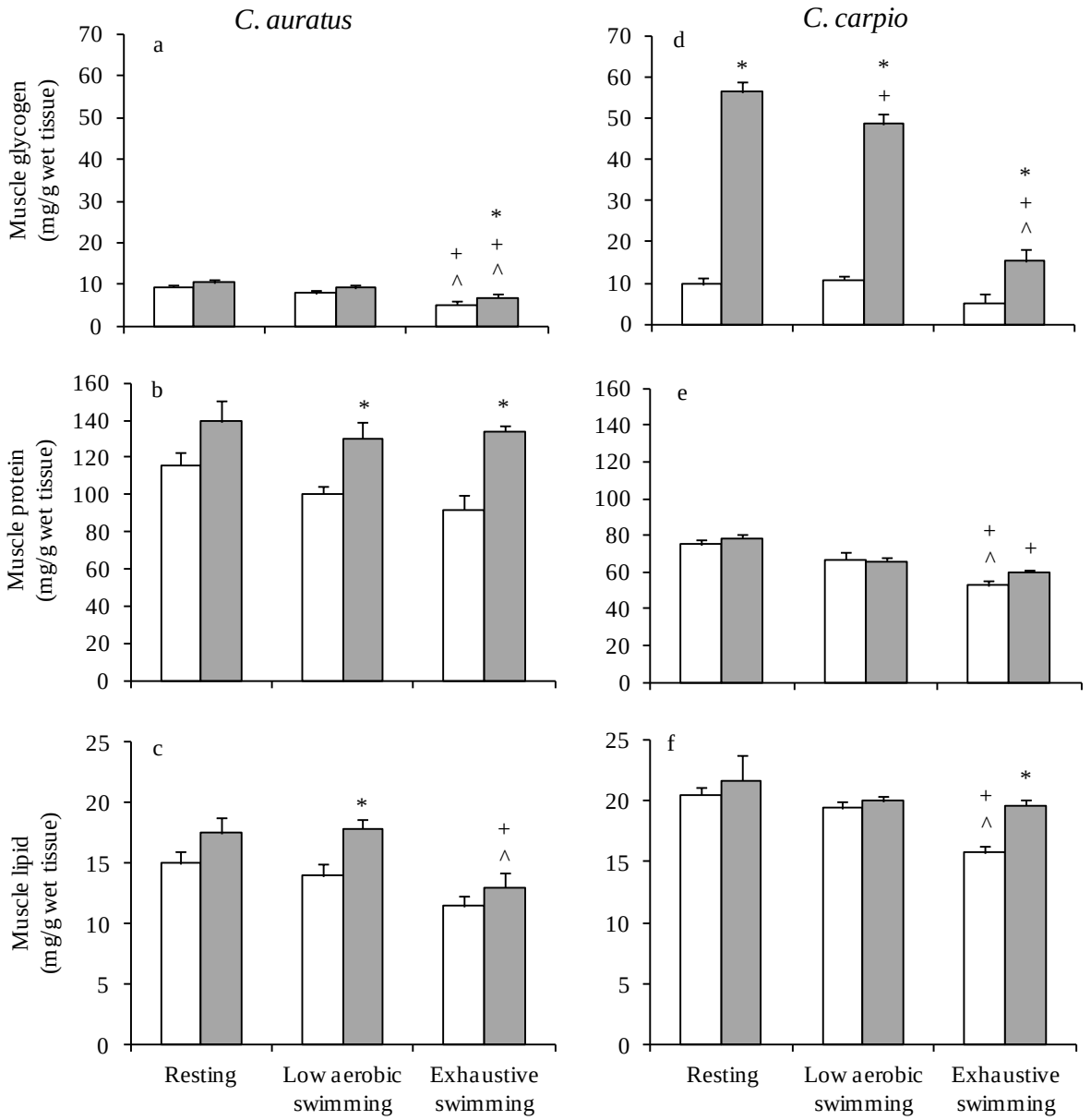

Fig. 5 Muscle glycogen, protein and lipid of fasted and fed *C. auratus* (a-c) and *C. carpio* (d-f) at resting, low aerobic swimming and exhaustive swimming. Open bars indicate fasted fish, filled bars indicate fed fish. Significant differences at the $P<0.05$ ($^{*,+,\wedge}$). An asterisk (*) indicates a significant difference between fasted and fed fish ($P<0.05$). A plus ($^{+}$) indicates a significant difference with corresponding resting fish ($P<0.05$). A caret ($^{\wedge}$) indicates a significant difference with corresponding low aerobic swimming fish ($P<0.05$).

Only at U_{crit}, muscle glycogen was significantly depleted in both fasted and fed goldfish (Fig. 5.a). Swimming had no effect on protein mobilization (Fig. 5.b), while muscle lipid was lower at U_{crit} although the effect was only significant in fed fish (Fig.5.c). For common carp, exhaustive swimming significantly depleted muscle glycogen in fed fish (Fig. 5.d). Since muscle glycogen was already extremely low in fasted fish, the additional reduction was not significant. Furthermore, U_{crit} also led to muscle protein mobilization compared to resting in fed carp and to both resting and low aerobic swimming fish in fasted carp (Fig. 5.e). Similar to muscle protein, muscle lipid was significantly depleted in fasted carp at U_{crit} (Fig. 5.f). Interactive effects were only detected for muscle glycogen of

common carp (Table 2). Since HSI was higher in fed fish, differences in total liver energy content were actually greater than the differences shown on per gram basis.

4. Discussion

4.1 Swimming and respiration of goldfish

Surprisingly, no significant effect of feeding was observed on U_{crit} and MO_2 at both routine and exhaustive swimming fasted and fed goldfish. It seems that the contribution of digestion to the aerobic metabolism is relatively small and that goldfish keep their metabolism at a sufficiently high level to cover the need for both digestion and swimming. This reflected that goldfish have the ability to cover these activities by aerobic metabolism during low levels of activity, which is also shown by the lack of plasma lactate accumulation at low aerobic swimming. Recent studies found that goldfish swimming metabolism is sacrificed to support digestion metabolism at high temperature (Pang et al., 2010) and at higher dissolved oxygen levels (Zhang et al., 2012). Contradictory, the present study found that goldfish sacrificed feeding metabolism to support swimming metabolism. This difference could be due to the different type of food pellet used and temperature throughout the experiment. Previous studies have found that since commercial pellets contain significantly lower moisture content (about 10% as compared to natural prey about 80%) (Kristiansen and Rankin, 2001) this results in the gastrointestinal tract trying to cope with a 'natural degree of the liquefaction' of dry food intake (Buddington et al., 1997; Bucking and Wood, 2006). This appears to delay the whole digestion and ingestion process and resulting in a prolonged SDA process, adding to the slow SDA caused by the low temperature maintained throughout experiment period. At higher swimming speeds, anaerobic metabolism was clearly initiated but again no difference is seen between fed and fasted fish. Energy stores seem large enough to sustain metabolic needs even fish that were fasted for 7 days did not show any dramatic changes in body composition, although fasted fish clearly had a smaller hepatosomatic index, indicating that liver energy stores had been used.

However, this does not mean that there was no difference in metabolic strategy between fed and fasted goldfish. High ammonia production and plasma ammonia accumulation indicated increased protein usage in fed fish with a high AQ as a consequence. Ammonia is the principal end-product of nitrogen metabolism either through breakdown of dietary protein or catabolism from body reserves. Maximum AQ for 100% protein use during aerobic metabolism is 0.33 (Kutty, 1978; De Boeck et al., 1995, 2000a) from protein oxidation via citric acid cycle by deamination and amino acid oxidation (Lyndon et al., 1992). It was suggested previously that protein was the main fuel used by fed fish (Van Der Thillart, 1986; Jobling, 1994). This is supported by our result obtained in fed goldfish, since even at low aerobic swimming, protein played an important role in aerobic metabolism with contributions of >70% at routine and about 90% at U_{crit} (Fig. 2.c). The lower ammonia excretion rate and AQ in the fasted goldfish (Fig. 2) indicated that protein use is higher in fed fish than fasted fish, most likely due to the breakdown of protein from dietary intake. It is clear that exercise induced an increased use of protein in goldfish, possibly in an attempt to conserve glycogen, in contrast to common carp where glycogen seems to be more readily used. Fed goldfish also contained relatively more protein in liver and muscle in all swimming regimes. This is in agreement with Alsop and Wood (1997) who found that rainbow trout fed to satiation exhibited high tissue protein synthesis, as well as channeling of excessive amino acids to be deaminated and oxidized via citric acid cycle to produce energy (Lyndon et al., 1992). Food deprivation resulted in

low levels of protein synthesis in liver and a higher rate of protein degradation (McMillan and Houlihan, 1988). Protein content is mobilized to release free amino acids for both energy and synthesis of new proteins during fasting (Navarro et al., 1997), resulting in weight losses and liver mass depletion in rainbow trout (Martin et al., 2001). This high protein reserves in liver and muscle was paralleled with high glycogen and lipid stores, indicating that fed fish benefited from the dietary protein intake. Protein remained the primary fuel during food deprivation and as a consequence, protein was significantly depleted in liver and muscle of all fasted fish. Also Storer (1967) found that protein was the major fuel to sustain basal metabolism in goldfish during starvation. To a lesser extent, fasted goldfish also mobilized some glycogen and lipid to sustain basal metabolic requirements. Overall, the reduced liver mass in fasted fish reflected the lower energy content.

Even though ammonia excretion was increased, the ammonia levels in plasma still increased in fed goldfish under all swimming regimes. In muscle, ammonia accumulated during exhaustive swimming in both feeding regimes. The substantial increase in plasma ammonia in fed fish reflects insufficient excretion during their high rate of protein use. If feeding up regulated ammonia transporting mechanisms as was seen in trout after feeding (Zimmer et al, 2010), this up regulation was not sufficient to deal with the increased ammonia production rates in goldfish. At U_{crit}, goldfish not only increased $MO_{2\text{-active}}$ for aerobic metabolism but also induced anaerobic metabolism with high plasma lactate accumulation. Plasma lactate accumulation in exhaustive swimming goldfish was approximately 2.5-folds higher than in resting and low aerobic swimming fish. The elevation of plasma lactate reflected that anaerobic metabolism occurred at U_{crit}. The plasma lactate accumulation in the present study seems to be supported by the mobilization of muscle glycogen at high speed (Fig. 3.a). This elevation probably could also be due to the rapid velocity increment in our U_{crit} methodology. As a result, fish might not be in a steady state resulting in high lactate accumulation. This effect has been reported by Hammer (1995) who also observed that swimming speed approaching to U_{crit} by rapid velocity increment induced anaerobic metabolism with the occurrence of metabolic acidosis.

The production of lactate corresponds with the depleted muscle glycogen at U_{crit}. It is striking that liver glycogen was maintained at all swimming conditions, and only slightly affected by fasting. Goldfish, as the closely related crucian carp, are among the few vertebrates that readily survive at anoxia for few hours at room temperature and can produce ethanol instead of lactate when they were exposed to hypoxia or anoxia (Ultsch, 1989, Nilsson and Renshaw, 2004; De Boeck et al., 2006; Sinha et al., 2012). They conserve glycogen by reducing the glycolytic activator fructose 2,6-bisphosphate and increasing the glycolytic inhibitor alanine to inhibit glycolysis pathway (Nilsson, 1990, 1992). Therefore, goldfish, like crucian carp, seem to conserve glycogen at all cost and rely on protein for their metabolism, likely to be prepared for any possible future anoxic event with concomitant anaerobic metabolism. Digestion metabolism was sacrificed to maintain swimming metabolism in goldfish at high swimming levels, whereas at low aerobic swimming goldfish maintained a sufficient metabolic rate to support both digestion and swimming. When food deprived, goldfish tend to mobilize protein for aerobic metabolism and conserve glycogen, possibly anticipating a more severe stress in the future such as hypoxia or prolonged food deprivation.

4.2 Swimming and respiration of common carp

In common carp MO_2 seems to increase with feeding, and overall fed fish had higher MO_2 than fasted fish which became significant at U_{crit}. This strategy illustrates that common carp did not compromise on digestion or swimming. Extra energy was used to provide energy for digestion and swimming metabolism at the same time. The readily available energy from dietary intake, and the swift use of both liver and muscle glycogen in combination with the increased aerobic metabolism, brought the fed carp close to a significantly better swimming performance (P=0.051). Improved swimming performance by feeding was also observed in closely related family member the grass carp; and darkbarbel catfish (Fu et al., 2009) and southern catfish at a temperature of 15 °C (Pang et al., 2010). Furthermore, ammonia accumulation was relatively low, with better excretion rates and considerably lower plasma ammonia in fed fish compared to fasted fish at U_{crit}. High plasma ammonia accumulation leads to decreased U_{crit}, as was observed in common carp when exposed to Cu (De Boeck et al., 2006), and is well-known to reduce swimming capacity in salmonids (Beaumont et al., 1995, 2000a; McKenzie et al., 2003). Therefore, the decrease of U_{crit} in fasted fish might thus not only be caused by the severe depletion of glycogen as a fast energy source for swimming, but also by the high plasma ammonia accumulation indicating insufficient excretion. The decrease in swimming capacity correlates better with plasma than muscle ammonia accumulation (De Boeck et al., 2006) because the distribution of ammonia between intra and extracellular compartment easily leads to muscle depolarization (Beaumont et al., 2000b; McKenzie et al., 2003; De Boeck et al., 2006). Nonetheless, starvation impaired swimming capacity was also reported in other cyprinid species such as danube bleak, european chub and common rudd (Wieser et al., 1992). Besides the elevation in plasma ammonia, fasting diminished spontaneous activity (Jobling, 1994) due to reduction of enzyme activity and concentrations of contractile protein in white muscle that impaired swimming capacity (Houlihan et al., 1988; Lowery and Somero, 1990).

U_{crit} increased cardiac output and ventilation rate to support aerobic metabolism during swimming (Jones and Randall, 1978). The increased of ventilation rate at U_{crit} is likely to enhance MO_2, thereby simultaneously promoting ammonia excretion and to reduce ammonia accumulation in plasma in fed carp. In fact, elevation of endogenous ammonia might stimulate excretory mechanisms such as upregulation of H^+ ATPase activity, Na^+/NH_4^+ exchanger and expression of Rhesus associated glycoprotein (RHAG) in the gills. The Na^+/NH_4^+ exchanger is importance for non-ionic ammonia diffusion to excrete excessive endogenous ammonia across gills (Tsui et al., 2009) and it has been proven that Rhcg 2 was increased in fed compared to fasted rainbow trout when exposed to high environmental ammonia allowing improved ammonia excretion rates (Nawata et al., 2007; Nawata and Wood, 2009; Zimmer et al., 2010).

In the present study, AQ tended to be higher in fed common carp and at U_{crit} but did not significantly differ. The proteins used seemed to originate from liver protein content only, as these were depleted in all fasted fish. In contrast with goldfish, fasting alone did not lead to a reduction in muscle protein indicating the importance of another energy source. The increased protein usage was mainly mobilized from liver and carp tended to conserve muscle protein, as no remarkable muscle protein mobilization was observed between fasted and fed swum exhaustively (Fig. 5.e). This may reflect that common carp are still capable to synthesis muscle protein and/or mobilize liver protein to muscle and instead mobilize glycogen during fasting to maintain swimming performance. Both digestion and swimming metabolism were performed independently in common carp which is in agreement with recent finding reported by Pang et al. (2011) and Zhang et al. (2012). Impressively,

glycogen depletion occurred in both liver and muscle in fasted carp confirming that glycogen was the main energy source for basal metabolism in all fasted common carp. Glycogen was also used as primary energy source for swimming, as muscle glycogen were being used in fed carp during low aerobic swimming and at U_{crit}; and liver glycogen was mobilized in both fed and fasted carp at U_{crit}. Apparently, common carp easily switch to anaerobic metabolism even at low aerobic swimming, as can be seen from the high plasma lactate compared to resting fish. Significant glycogen depletion was also reported by De Boeck et al. (2000b) in common carp when stressed by salinity challenge leading to food deprivation. In contrast to crucian carp and goldfish, other fish seem to rely on glycogen as energy source more easily when confronted with starvation, as was seen in sea bass (Gutierrez et al., 1991), brown trout (Navarro et al., 1992), white sturgeon (Hung et al., 1997) and juadia (Barcellos et al., 2010). Due to the extreme glycogen depletion, muscle protein and lipid also had to be mobilized at U_{crit} to sustain increasing energy demand for swimming in fasted carp. The use of lipids as a main metabolite during early stages of starvation also was found in gibel carp and it only shifted to protein in later stage of development (Cui and Wang, 2007). In rainbow trout, lipids seem to be the major fuel to sustain aerobic swimming capacity followed by carbohydrates and then protein (Lauff and Wood, 1996). Thus, both feeding and swimming metabolism in common carp proceeded independently by increasing MO_2 to maintain swimming performance. Glycogen was a priority energy source during fasting and swimming. Swimming enhanced ammonia excretion capacity and elevated MO_2 in common carp.

5. Conclusion

We can conclude that feeding and swimming induced different metabolic strategies in goldfish and common carp. It was clear that goldfish highly depended on protein metabolism, and used readily available protein from food to increase the protein contribution during aerobic metabolism with peaking values of AQ and plasma ammonia accumulation at U_{crit}. The conservation of glycogen reserves at all times in goldfish is likely related to their capacity to tolerate anoxia. In common carp, swimming seems to enhance ammonia excretion in fed fish, but not in fasted fish. Carp rely on glycogen as major energy source both under fasting and to sustain swimming. Overall, the insignificant difference in the MO_2 and U_{crit} between fed and fasted goldfish showed that digestion was sacrificed to support swimming while in common carp both digestion and swimming proceeded independently with higher U_{crit} and MO_2 in fed common carp.

Chapter 2

Feeding and swimming modulate iono-and-hormonal regulation differently in goldfish, *Carassius* auratus and common carp, *Cyprinus carpio*

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Abstract

Feeding and swimming can influence ion balance in fish. Therefore we investigated their impact on ionoregulation and its hormonal control in goldfish and common carp. As expected due to the osmorespiratory compromise, exhaustive swimming induced increases in gill Na^+/K^+ ATPase (NKA) activity in both species, resulting in stable levels of plasma ions. In contrast to our expectations, this only occurred in fed fish and feeding itself increased NKA activity, especially in carp. Fasting fish were able to maintain ion balance without increasing NKA activity, we propose that the increase in NKA activity is related to ammonia excretion rather than ion uptake per se. In goldfish, this increase in NKA activity coincided with a cortisol elevation while no significant change was found in carp. In goldfish, high conversion of plasma T_4 to T_3 was found in both fed and fasted fish resulting in low T_4/T_3 ratios, which increased slightly due to exhaustive swimming. In starved carp the conversion seemed much less efficient, and high T_4/T_3 ratios were observed. We propose that thyroid hormone regulation in carp was more related to its role in energy metabolism rather than ionoregulation. The present research showed that both species, whether fed or fasted, are able to sufficiently adapt their osmorepiratory strategy to minimise ions losses while maintaining gas exchange under exhaustive swimming.

Keywords: Aerobic, exercise, cortisol, fasting, gill, osmorespiratory compromise, plasma ion, U_{crit}

1. Introduction

In recent years an increased interest on how feeding impacts other physiological processes in fish has emerged (Fu et al., 2009; Gingerich et al., 2009; Caruso et al., 2010; Marshall, 2010; Pang et al., 2010; Zhang et al., 2012). The hypothesis initiated by Alsop and Wood (1997) on the interaction between feeding and swimming with regards to their competition for oxygen stating that fish either increase their aerobic metabolism to sustain both digestion and swimming or prioritise one over the other if maximum capacity to take up O_2 at the gills and/or to deliver O_2 through the circulatory system is reached, has been tested in recent research, and different species seem to show different strategies (Li et al., 2010; Pang et al., 2010; Zhang et al., 2010; Liew et al., 2012). Beside the fact that digestion competes for priority in aerobic metabolism, recent studies found that feeding offered clear advantages in the ion regulatory homeostatic balance, and ion regulatory mechanisms that were absent in fasting fish were stimulated in fed fish (Bucking et al., 2010; Wood and Bucking, 2011).

Swimming is an important life-time activity to ensure optimum growth and survival for all aquatic animals. In the laboratory, fish swimming capacity is often determined by measuring critical swimming speed (U_{crit}) which is done by progressively increasing water velocity in a flume (Brett, 1964; Tudorache et al., 2007). U_{crit} is widely applied to assess the impact of nutritional status, environmental factors or disease (Hammer, 1995; Kieffer, 2000). It was also used to test physical fitness (Nelson et al., 2002) including maximum O_2 consumption (Keen and Farrell, 1994; Gregory and Wood, 1999), aerobic and anaerobic metabolic scopes (Lurman et al., 2007); effects on ion balance (Wang et al., 1994), endocrine status (Gamperl et al., 1994; Milligan, 1996) and behaviour (Tudorache et al., 2007; 2008; 2010a; 2010b). Although the U_{crit} test has it's limitations (Tudorache et al., 2007) in predicting real performance in nature (Nelson et al., 2002), it provides a simple tool to compare the swimming performance of groups of fish experiencing environmental challenges

(Alsop and Wood, 1997; Gallaugher et al., 2001; Lee et al., 2003; McKenzie et al., 2003; De Boeck et al., 2006; Farrell, 2007; 2008; Tudorache et al., 2010a).

Swimming induces increased ventilation rates, branchial blood perfusion and lamellar recruitment (Jone and Randall, 1978). Consequently, the enhanced functional surface area of gills promotes permeability for gas and ion exchange (Gallaugher et al., 2001) but also results in increased ionic losses compared to resting freshwater fish (Van Dijk et al., 1993). Therefore, freshwater teleosts are required to compensate for the trade-off between increased gill functional respiratory surface area, obtained by changing the water flow over the gills or blood flow inside the gills for gas exchange, and the diffusive ion losses and water gain (Nilsson, 2007; Wood et al., 2009). In general, swimming freshwater fish experience increased water gain and Na^+ losses during the early stages of swimming followed by partial or complete recovery by activation of compensatory mechanisms (Gonzalez and McDonald, 1992; Gonzalez and McDonald, 1994; Postlethwaite and McDonald, 1995). Effects of swimming on ionoregulation have also been discovered in diadromous species and during acclimatisation from freshwater to seawater environment in euryhaline species (Laiz-Carrion et al., 2005; Yang et al., 2009; Vargas-Chacoff et al., 2010). However, underlying mechanisms of osmoregulatory trade-offs still remain to be discovered (Wood et al., 2009). Problems of ion losses in freshwater fish may be extreme during swimming in fasting fish, since they cannot absorb ions from food. Conversely, feeding fish could compensate for ion losses by enhancing ion uptake from dietary intake. Therefore, our interest was to investigate the combined effect of fasting/feeding and swimming on ionoregulation in two cyprinid fish species that show different metabolic strategies under these circumstances (Liew et al., 2012). Whereas goldfish seem to sacrifice digestion to sustain swimming with similar oxygen consumption rates in starved and fed fish, common carp increased their aerobic metabolism to sustain feeding and swimming independently (Liew et al., 2012). To address this question, fish were conditioned to different feeding regimes (fasting for a week and fed at 2% body weight) and exercised at different levels (rest, low aerobic swimming and exhaustive swimming) and plasma ions as well as gill Na^+/K^+ ATPase (NKA) activity were measured. Gill NKA activity and ion uptake are not only affected by nutritional status (Polakof et al., 2006), but also modulated by hormonal balance (Dange, 1986; Madsen, 1990; Shrimpton and McCormick, 1999; Laiz-Carrion et al., 2003; Babitha and Peter, 2010). Therefore, plasma cortisol and thyroid hormones were also assessed.

Cortisol plays an important role in intermediary metabolisms (Mommsen et al., 1999; De Boeck et al., 2001) and glucocorticoid and mineralcorticoid actions also regulate iono-homeostasis (Wendelaar-Bonga, 1997; Colombe et al., 2000). Cortisol is known to play a role in seawater acclimatisation for Na^+ secretion (McCormick, 1995), but the role of cortisol is not just limited to seawater fish and is also important in freshwater fish. In the latter, cortisol appeared to modulate ion homeostasis with other hormones such as prolactin in hypophysectomised freshwater catfish (Parwez and Goswami, 1985) and other freshwater fish (Abo Hegab and Hanke, 1984; Dang et al., 2000; Evens et al., 2005; Babitha and Peter, 2010; Chasiotis and Kelly, 2012). Other hormones such as the thyroid hormone T_3 are known to be crucial for ion homeostasis but are also important in energy metabolism and have been reported to decrease significantly during fasting (Coimbra and Reis-Henriques, 2007). The second goal of our study was to investigate the overall interactive effects of feeding and swimming on plasma cortisol and thyroid hormone status and assess their role in ionoregulation in both goldfish and common carp.

The aim of our research was to assess the role of feeding and swimming on the iono-and-hormonal regulation in goldfish and common carp. With that we hypothesised that ion homeostasis

in fasting fish would suffer more from exhaustive swimming due to the osmorespiratory compromise, since these fish cannot compensate ion losses via ion uptake from the food. Additionally, we hypothesised that fasting goldfish would be challenged more severely than fasting common carp when swimming, since fasting goldfish increased oxygen consumption rates to the same extend as fed goldfish under exhaustive swimming and thus would be more prone to ion loss.

2. Materials and methods

2.1 Fish maintenance

The goldfish, *Carassius auratus* were purchased from Aqua Hobby, Heist op den Berg, Belgium and common carp, *Cyprinus carpio* from Wageningen University, The Netherlands and acclimated to the aquaria facilities of the Systemic Physiological and Ecotoxicological Research, University of Antwerp, Belgium in Antwerp city tap water. Fish were fed at 4% of their body weight (BW) twice per day (2% at 08:30h and 2% at 18:30h) to ensure all fish were fed with 'Hikari Staple' (Kyorin Food Ind. Ltd., Japan with nutrition values of ≥35% protein, ≥3% lipid, 5% fibre, 13% ash, 1% phosphorus and 10% mixture of vitamins and minerals). After two months of pre-acclimatisation, 60 fish from each species (goldfish 16.4±1.6 g BW and 12.2±0.6 cm body length (BL); common carp 20.0±0.3 g BW and 14.4±0.9 cm BL) were transferred to a climate room set to 17.0±0.1 °C and a constant photoperiod at 14L:10D. Fish were distributed equally into six 150 L (50×30×50 inch) rectangular fibreglass tanks and acclimatised to experimental conditions for one month. During this one month feeding rate was reduced to 2% of BW per day divided between 2 equal portions per day fed at 08:30h (1% BW) and 18:30h (1% BW). Water qualities were monitored regularly using the Standard Tetra Test Kits (Germany) to maintain the NH_3/NH_4^+ at <0.1 mg/L; NO_2^- <0.03 mg/L, NO_3^- <25 mg/L, pH 8.3±0.2 (7.8-8.5), aeration was provided and 80% water was renewed twice on a weekly basis.

2.2 Experimental design

After one month of acclimatisation, fish were divided in a feeding and a fasting regime. For feeding groups, feeding rate remained the same: a ration of 2% BW per day with food divided between 2 equal portions per day at 08:30h (1% BW) and 18:30h (1% BW). For fasting groups, all fish were fasted for a week (7 days) before the experiment. In addition, these fish were divided into resting, low aerobic swimming and exhaustive swimming (U_{crit}) groups before sampling. The resting fish were sampled directly from the holding tank (without introduction into the swimming flume). Eight Blazka-style swimming flumes with a volume of 3.9 L were used for swimming tests. Selected fish were introduced into the swimming flumes 14 h prior to the experiment and allowed to orient themselves at a low velocity of 10 cm/s (approximately 1 BL/s) to acclimatise overnight to the swimming flumes and to reduce handling stress on the day of measurement. During overnight acclimatisation, all swimming flumes were continuously supplied with air-saturated water flow at 4 L/min to ensure sufficient oxygen supply and new water refreshment in the flume. Subsequently, low aerobic swimming fish were swum at ¼ of their U_{crit} at velocity of 10 cm/s (approximately 1 BL/s) (Liew et al., 2012) in the swimming flume for 3 h and sampled thereafter. Exhaustive swimming fish were swum up to U_{crit} with water velocity increments of 5 cm/s every 20 min until fish fatigued at 4.66±0.31BL/s for fasted and 4.23±0.29 BL/s for fed goldfish; 4.05±0.23 BL/s for fasted and 4.75±0.23 BL/s for fed common carp. Fatigue was determined as the point where the fish were

unable to swim against the current and swept to the mesh screen at the end of the tunnel. At this point, water velocity was reduced until fish recommenced swimming after which velocity was immediately increased to the last speed before fatigue occurred. Fish were considered totally fatigued when the fish were unable to swim against the current and swept twice to the mesh screen within a 20 min interval and fish were sampled immediately thereafter (De Boeck et al., 2006; Tudorache 2007b; Liew et al., 2012). Each sampling group consisted of eight fish.

2.3 Sampling

Fish were anaesthetized with Ethyl-3-aminobenzoate methanesulfonic acid (MS222) at 0.5 g/L (Acros Organics, Geel, Belgium) neutralized with NaOH. When fish became visibly disoriented, lost equilibrium, and no operculum movement was observed, blood was immediately drawn via caudal puncture using a heparinised syringe. Blood was instantly expelled into a heparinised tube and centrifuged (2 min; 13,200 rpm; 4 °C). Plasma was carefully transferred into cryogenic vials and immediately frozen in liquid N_2. Subsequently, gill samples were dissected and immediately frozen in liquid N_2 and stored at -80 °C for further analysis.

2.4 Plasma ions, cortisol and thyroid hormones

Plasma ions (Na^+, K^+, Cl^- and Ca^{2+}) were measured using an AVL 9180 Electrolyte Analyser (AVL, Roche Diagnostics, Belgium). Radioimmunoassay (RIA) was performed to measure plasma cortisol and thyroid levels. Plasma thyroid hormones (thyroxine, T_4 and triiodothyronine, T_3) were measured as described by Van der Geyten et al. (2001). Plasma cortisol levels were measured in duplicate using commercially available Cortisol ^{125}I RIA kits (ImmuChem^{TM}, MP Biomedicals LLC).

2.5 Gill NKA activity

Gill NKA activity was measured according to the method described by McCormick (1993). Gill samples were homogenized with ice cooled 4:1 SEI/SEID buffer solution (150 mM sucrose; 10 mM EDTA; 50 mM imidazole/SEI with 0.1% sodium deoxycholate; pH 7.5) and centrifuged (1 min at 5000 g, 4 °C). Duplicate 10 µl supernatant samples were pipetted into 96-well microplates in two series. Freshly made 200 µl mixture assay A (400 U lactate dehydrogenase; 500 U pyruvate kinase; 2.8 mM phosphoenolpyruvate; 0.7 mM ATP; 0.22 mM NADH; 50 mM imidazole) was added to the first series and 200 µl mixture assay B (mixture assay A with 0.4 mM ouabain) to the second series. The enzyme activities were measured kinetically with spectrophotometer ($ELX808_{IU}$ Bio-Tek Instruments Inc. VT, USA) at 340 nm for 10 min at 15 s intervals. A standard curve of ADP (Adenosine diphosphate) was used. ATPase activity was calculated by subtracting the oxidation rate of NADH to NAD in the presence of ouabain from the oxidation rate in the absence of ouabain. The crude homogenate protein was determined according to Bradford (1976) using a standard curve of bovine serum albumin (US Biochemical, Cleveland, OH, USA) and read at 430 nm.

2.6 Statistics analysis

Normality was verified by the Shapiro-Wilk test for all data sets. If conditions were fulfilled, statistical analysis was performed by analysis of variance (ANOVA). Homogeneity of variance was tested by the Hartley test. If condition was not fulfilled, data were log-transformed prior analysis. Differences between fasting and feeding effect were assessed by unpaired two-tail student *t*-test. One way ANOVA was performed to examine the significant effects of fasting and feeding under the different swimming conditions followed by multiple comparison of Tukey post-hoc test. The overall interaction effects between feeding and fasting, and different swimming regimes were performed by two-way ANOVA. The significance level for all tests was set at $P<0.05$. All values are given as mean value ± standard error (Mean±SEM; $n = 8$).

3. Results

3.1 Effects of feeding and swimming on plasma ions

Plasma ion levels and effects of feeding and swimming are presented in Fig. 1. In resting fish, fasting or feeding caused very little effect on plasma ion levels. In goldfish, plasma Na^+, Cl^- an Ca^{2+} tended to be lower in fasted fish (Fig. 1a, b, d), while the opposite was true in common carp (Fig. 1d, f, g), but none of these effects were significant. The only significant effect in resting fish was that starved goldfish showed higher plasma K^+ levels than fed goldfish (Fig. 1c).

Under low aerobic swimming, plasma Cl^- in fed goldfish was reduced, and this reduction persisted under exhaustive swimming (significant compared to starved and resting goldfish respectively, Fig. 1b). Overall, this resulted in a significant effect of feeding and of the interaction effect between feeding and swimming on plasma Cl^- in goldfish (Table 1). In common carp, the effect on plasma Cl^- was more irregular (Fig. 1f), with increases rather than decreases. Significant increases occurred in fed fish under low aerobic swimming (significant compared to resting and starved fish) and in starved fish under exhaustive swimming (significant compared to resting, low aerobic swimming and fed fish). Overall, this led to a significant effect of swimming and of the interaction effect between feeding and swimming (Table 1). Plasma Na^+ levels tended to follow the same trend as plasma Cl^- but this only resulted in a significant increase in fasting common carp under exhaustive swimming (Fig. 1e, significant compared to resting and low aerobic swimming carp). Overall, the effect of swimming on plasma Na^+ was significant in common carp and the interaction effect was significant in both species (Table 1).

Exhaustive swimming also caused changes in plasma K^+ in both species with an increase in fed goldfish (Fig. 1c, significant compared to resting fish) and in fasted common carp (Fig 1g, significant compared to resting, low aerobic swimming and fed carp). Overall, this resulted in a significant interaction effect in both species ($P=0.001$, Table 1). Also plasma Ca^{2+} tended to increase under swimming with a significant increase in fasting goldfish under low aerobic swimming (significant compared to resting fish, Fig. 1d) and in fed common carp under exhaustive swimming (significant compared to resting fish, Fig. 1h). This resulted in a significant interaction effect in both species (Table 1).

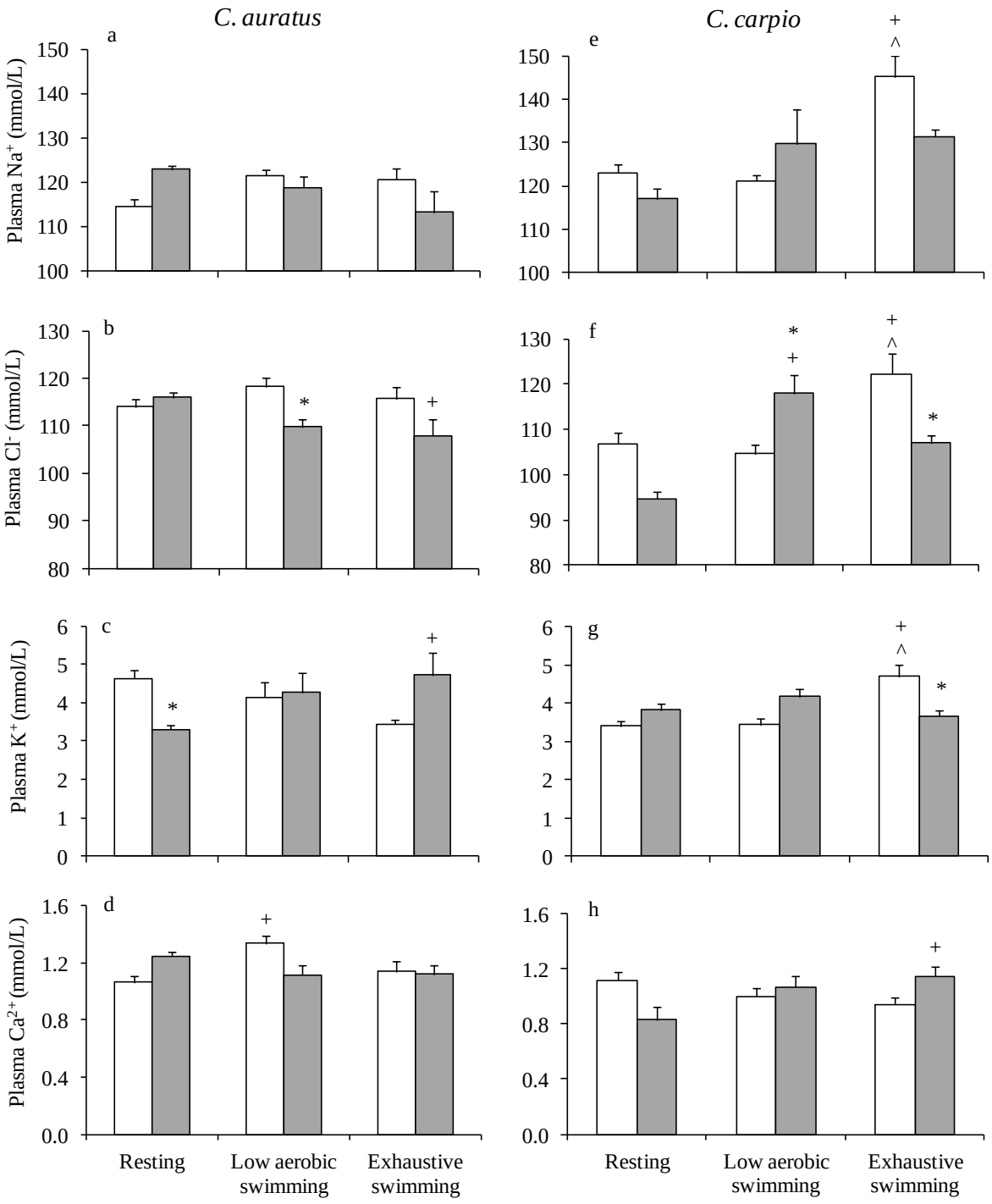

Fig. 1 Plasma Na^+, K^+, Cl^- and Ca^{2+} of *C. auratus* (a-d) and *C. carpio* (e-h) under different feeding regime and swimming conditions. Open bars indicate fasted fish, filled bars indicate fed fish. Significant differences at the $P<0.05$ ($^{*,+,\wedge}$). An asterisk (*) indicates a significant difference between fasted and fed fish. A plus ($^+$) indicates a significant difference corresponding to resting fish. A caret ($^\wedge$) indicates a significant difference corresponding to low aerobic swimming fish.

Table 1 Significance levels of the impact of feeding, swimming and the interaction effects on plasma ions, gill NKA activity, plasma cortisol and thyroid hormone status of *C. auratus* and *C. carpio*.

	Feeding		Swimming		Feeding * Swimming	
Goldfish	*F-value*	*P-value*	*F-value*	*P-value*	*F-value*	*P-value*
Plasma Na^+	0.083	0.775	0.893	0.418	5.217	0.010
Plasma K^+	0.002	0.967	0.332	0.721	9.028	0.001
Plasma Cl^-	8.571	0.006	1.615	0.214	5.410	0.009
Plasma Ca^{2+}	0.191	0.665	1.630	0.211	7.981	0.001
Gill NKA activity	9.450	0.004	4.592	0.016	9.665	0.000
Plasma cortisol	3.172	0.083	25.797	0.000	4.824	0.014
Plasma T_4	0.211	0.648	11.743	0.000	0.487	0.619
Plasma T_3	2.289	0.139	10.326	0.000	0.402	0.672
Plasma T_4/T_3 Ratio	2.126	0.154	15.294	0.000	0.867	0.129
Common carp						
Plasma Na^+	1.278	0.265	10.780	0.000	3.865	0.029
Plasma K^+	0.058	0.811	3.529	0.041	9.270	0.001
Plasma Cl^-	3.789	0.059	12.424	0.000	13.817	0.000
Plasma Ca^{2+}	0.013	0.910	0.624	0.541	6.920	0.003
Gill NKA activity	119.145	0.000	14.041	0.000	1.254	0.297
Plasma cortisol	0.372	0.545	1.759	0.185	0.367	0.695
Plasma T_4	2.534	0.121	1.326	0.280	2.026	0.148
Plasma T_3	8.986	0.005	2.062	0.145	2.311	0.115
Plasma T_4/T_3 Ratio	9.355	0.005	1.447	0.251	3.478	0.043

3.2. Effects of feeding and swimming on gill NKA activity

Gill NKA activity of goldfish and common carp is presented in Fig. 2. Overall, feeding significantly increased gill NKA activity in both species. For goldfish, feeding significantly up-regulated gill NKA activity when swum to exhaustion (Fig. 2a), whereas feeding significantly up-regulated gill NKA activity for common carp at all swimming conditions (Fig. 2b).

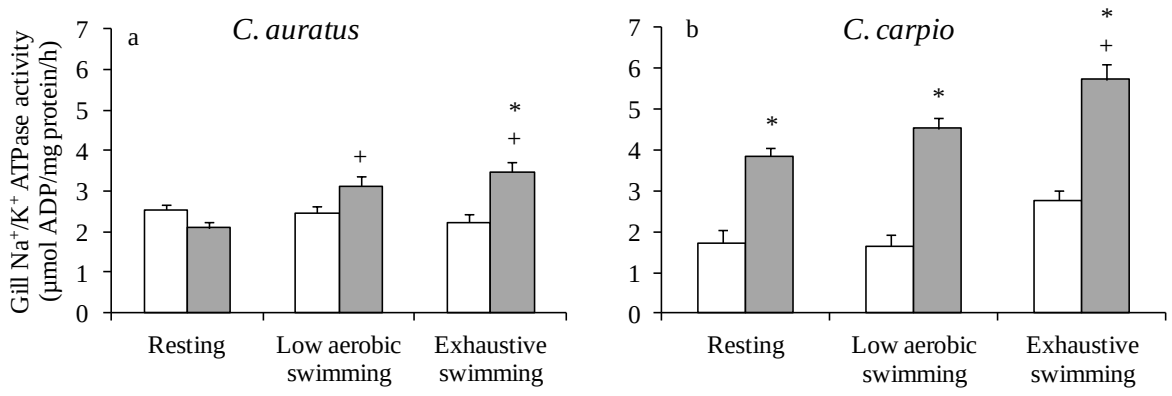

Fig. 2 Gill NKA activity of *C. auratus* (a) and *C. carpio* (b) under different feeding regime and swimming conditions. Open bars indicate fasted fish, filled bars indicate fed fish. Significant differences at the $P<0.05$ ($^{*,+}$). An asterisk (*) indicates a significant difference between fasted and fed fish. A plus ($^{+}$) indicates a significant difference corresponding to resting fish.

Swimming tended to increase gill NKA activity only in fed fish of both species. For goldfish, either low aerobic or exhaustive swimming significantly up-regulated gill NKA activity in fed fish (Fig. 2a) and resulted in a highly significant interaction effect (P=0.0004; Table 1). Exhaustive swimming significantly up-regulated gill NKA activity of common carp compared to resting fish (Fig. 2b). Overall the effects of feeding and the effect of swimming were highly significant for both species, although the interactive effect was only significant in goldfish (Table 1).

3.3 Effects of feeding and swimming on plasma cortisol

Plasma cortisol levels of goldfish and common carp in response to feeding and swimming are shown in Fig. 3 and Table 1. Swimming raised plasma cortisol levels in goldfish with progressively increasing levels as the swimming level intensified. In fed goldfish, elevation of plasma cortisol was significant in low aerobic and exhaustive swimming fish (Fig. 3a). In exhausted goldfish, this increase was larger in fasted goldfish compared to fed goldfish, which also resulted a significant interaction effect (Table 1). On the contrary, no effect of feeding, swimming or interaction effect was observed in common carp (Fig. 3b, Table 1). However, under resting conditions plasma cortisol level of common carp was approximately two-fold higher than in goldfish kept under the same conditions (Fig. 3).

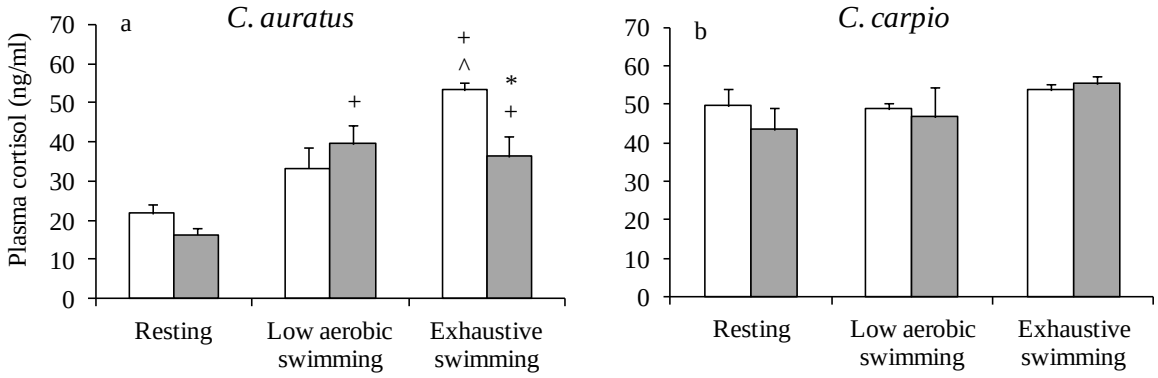

Fig. 3 Plasma cortisol levels of *C. auratus* (a) and *C. carpio* (b) under different feeding regime and swimming conditions. Open bars indicate fasted fish, filled bars indicate fed fish. Significant differences at the P<0.05 ($^{*,+,\wedge}$). An asterisk (*) indicates a significant difference between fasted and fed fish. A plus ($^{+}$) indicates a significant difference corresponding to resting fish. A caret ($^{\wedge}$) indicates a significant difference corresponding to low aerobic swimming fish.

3.4 Effects of feeding and swimming on plasma thyroxine, T_4 and triiodothyronine, T_3

Plasma thyroid hormones of goldfish and common carp are shown in Fig. 4 and showed clear species specific differences. Overall, feeding had no effect on plasma thyroid hormones in goldfish (Fig. 4a-c), but swimming induced clear effects. Plasma T_4 levels were significantly higher in both low aerobic and exhaustive swimming goldfish (significant compared to resting goldfish, Fig. 4a), and the same trend was observed in fasting fish. In both feeding regimes, a low plasma T_3 level was observed in exhaustively swimming goldfish, but the effect was only significant in fed fish (significant compared to low aerobic swimming goldfish, Fig. 4b). This resulted a significantly increased plasma

T_4/T_3 ratio in exhausted goldfish for both feeding regimes (significant compared to resting fish, Fig. 4c).

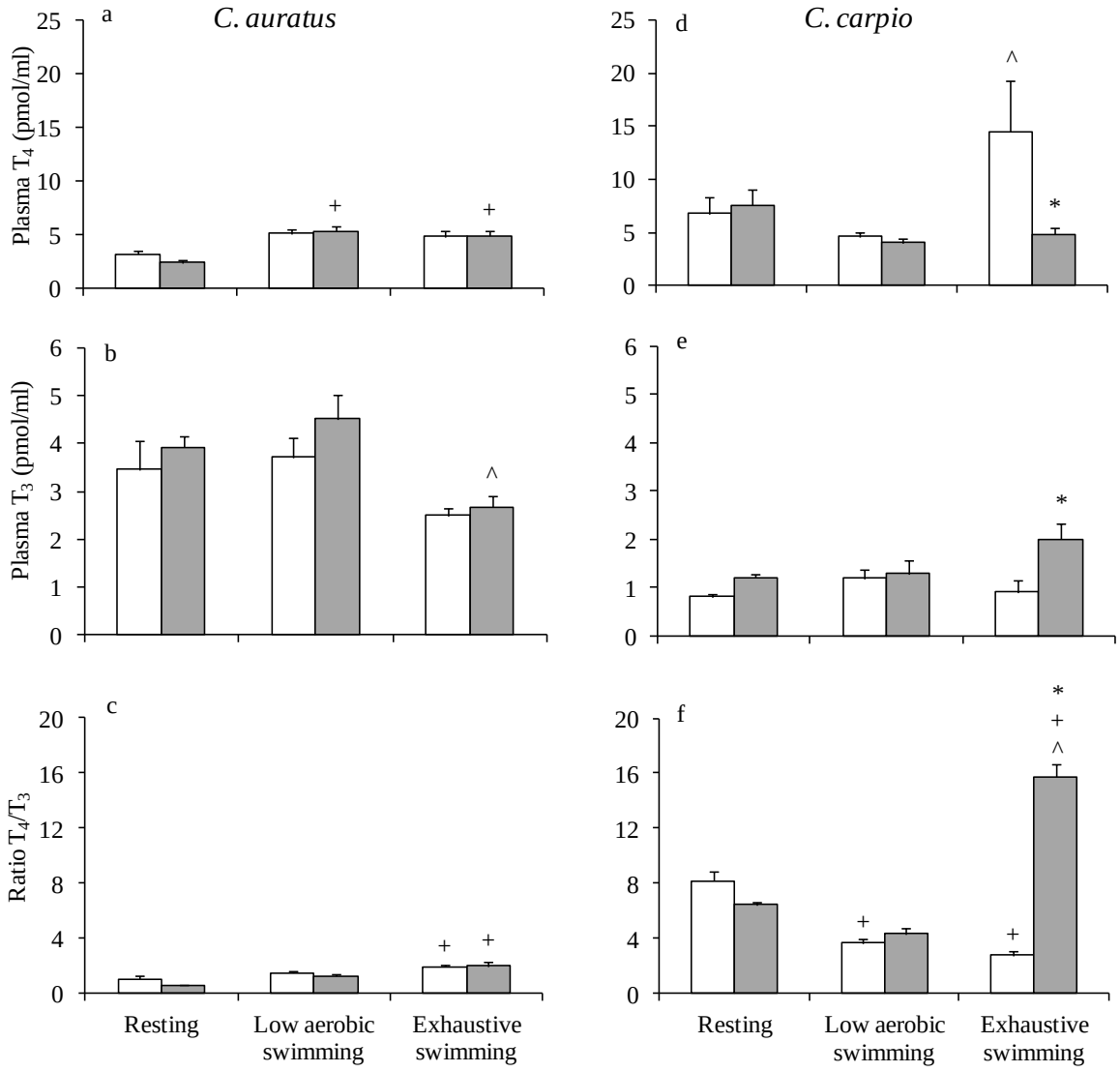

Fig. 4 Plasma thyroxine (T_4), triiodothyronine (T_3) and T_4/T_3 ratio of *C. auratus* (a-c) and *C. carpio* (d-f) under different feeding regime and swimming conditions. Open bars indicate fasted fish, filled bars indicate fed fish. Significant differences at the $P<0.05$ ($^{*,+,\wedge}$). An asterisk (*) indicates a significant difference between fasted and fed fish. A plus ($^{+}$) indicates a significant difference corresponding to resting fish. A caret ($^{\wedge}$) indicates a significant difference corresponding to low aerobic swimming fish.

For common carp, fasting fish swum to exhaustion showed higher T_4 levels (significant compared to low aerobic swimming fasted fish and to fed fish swum to exhaustion, Fig. 4d). Plasma T_3 levels tended to be higher in fed common carp and this effect was significant in exhausted fed fish (Fig. 4e). High plasma T_4 and low T_3 levels in fasted fish under exhaustive swimming resulted a significant decrease in plasma T_4/T_3 ratio (significant compared to resting and fed fish swum to exhaustion, Fig. 4f), while this ratio was significantly increased in fed carp compared to all other groups.

4. Discussion

Both species seem to deal relatively well with the osmorespiratory compromise as fairly stable plasma Na^+ levels were observed. Our original hypothesis that ion balance would be compromised in fasted fish during swimming was not confirmed. Even fasted goldfish, which increased oxygen consumption rates at exhaustive swimming to the same extend as fed fish (Liew et al., 2012) were able to maintain their ions at a relatively stable level.

4.1. Iono-and-hormonal regulation in goldfish

In goldfish, previous research showed that metabolic rate went up considerably during active swimming both in fed and fasted fish (Liew et al., 2012). Yet, neither fasting nor swimming impacted plasma ion levels in a substantial way in the present study. A stable level of plasma Na^+ in fed fish was expected as fed fish would obtain sufficient Na^+ ion through dietary intake. However, it was in fed fish rather than fasted fish that NKA levels increased with increasing swimming speeds. Evidently, feeding granted sufficient energy supply to sustain high gill NKA activity and increase Na^+ uptake, but more importantly this would also facilitate ammonia excretion (Wilkie, 1997). Increased protein breakdown during aerobic swimming occurs to a much larger extent in fed goldfish leading to higher ammonia excretion rates and higher ammonia quotients (AQ = 0.31) (Liew et al., 2012), where the theoretical maximum AQ in teleost is 0.33 known as the maximum protein contribution during aerobic metabolism (Kutty, 1978). This extra ammonia excretion through gills is facilitated via the Na^+/NH_4^+ exchanger and the $Na^+/NH_4^+/2Cl^-$ co-transporter both driven by the Na^+ gradient created by NKA and increases in NH_3 excretion concomitant with altered gill NKA have been observed previously in other species (Salama et al., 1999; Shrimpton and McCormick, 1999; Randall and Tsui, 2002; Alam and Frankel, 2006; Yang et al., 2010). Moreover, an elevation of endogenous ammonia might stimulate Rhesus glycoproteins expression as was proven by recent evidence discovering that the presence of Rhesus glycoproteins in fish challenged with high environmental ammonia significantly improved NH_3 excretion in teleosts such as rainbow trout (Nawata et al., 2007; Zimmer et al., 2010), killifish (Hung et al., 2007), zebrafish (Nakada et al., 2007a) and pufferfish (Nakada et al., 2007b; Nawata et al., 2010). Additionally, as a consequence of swimming, increased CO_2 production rates lead to CO_2 hydration to form H^+ and HCO_3^-. Following active H^+ transport via apical H^+ ATPase creates acidification of the gill water boundary layers which facilitates NH_3 diffusion due to NH_4^+ formation in the external boundary layer (Wilkie, 1997; Weihrauch et al., 2009). Subsequently, this creates a potential electrogradient and facilitates the uptake of Na^+ via the Na^+/H^+ exchanger (Salama et al., 1999) and Na^+ channels energized by H^+ ATPase (Zimmer et al., 2010), which could explain the constant plasma Na^+ level. Furthermore, swimming also increased lactate accumulation by the breakdown of glycogen as an immediate energy source via glycolysis pathway (Liew et al., 2012) that might decrease intracellular pH level as well and induce extra H^+ secretion. Due to excessive recruitment of red and white muscle to support increased swimming capacity, both aerobic and anaerobic metabolisms were activated simultaneously during exhaustive swimming (high AQ and plasma lactate levels) (Liew et al., 2012). Subsequently this might lead to K^+ losses from muscle tissue into the blood stream when swum to exhaustion. Release of K^+ from working muscle into blood stream was found in rainbow trout swum at level above 2.0 BL/s (Holk and Lykkeboe, 1998).

In contrast, fasting seemed to lower the gill NKA activity in goldfish to a minimal steady level at all swimming conditions. This partially allowed fasted fish to conserve energy expenditure for basal metabolism while maintaining homeostasis with a relative stable plasma Na^+ level as a survival strategy when food was absent. According to our previous study, active swimming goldfish consumed approximately 2-fold more oxygen than low aerobic swimming fish (Liew et al., 2012). Despite this increase in gas exchange during swimming, possibly coinciding with increased branchial functional surface area and branchial blood and water flow, fasting goldfish seem able to maintain basal Na^+, Cl^- and K^+ at a fairly stable level, possibly by decreasing epithelium permeability to minimize diffusive ion losses. Such an increase of functional surface area has been reported previously in swimming rainbow trout (Wood and Randall, 1973a; 1973b; Gonzalez and McDonald, 1992), crucian carp and goldfish (Sollid et al., 2005; Nilsson 2007) and Amazonian Oscar (Wood et al., 2009). Water gain and Na^+ loss is well-documented in the early stage of active swimming followed by activation of compensatory mechanisms to restore ion balance partially or completely afterwards (Wood and Randall, 1973a), allowing gill permeability for efficient gas transfer but reducing epithelium permeability to minimise ion losses and water gain (Wood and Randall, 1973b; Gonzalez and McDonald, 1994; Postlethwaite and McDonald, 1995; Wood et al., 2009). Fasting seemed to increase rather than decrease plasma K^+ level in resting goldfish. The ability of fasted goldfish to maintain plasma K^+ level could be facilitated by the active K^+ uptake via K^+ channels in gills and/or reabsorption in kidney. According to Eddy (1985) fasting rainbow trout were able to regulate K^+ independently from food under dilute (2/3 salinity) seawater or in freshwater, mainly through the gill (drinking contributed only 3% in seawater trout).

As expected, swimming elevated plasma cortisol levels in goldfish as it progressed to exhaustion. This swimming effect became highly significant when feeding was absent. The plasma cortisol level of exhausted fasted goldfish was approximately three-fold and two-fold higher as compared to resting and low aerobic swimming fish, respectively. Our results are in line with those on sockeye salmon (Fagerlund, 1967), striped bass (Young and Cech, 1993) and rainbow trout (Gregory and Wood, 1998) with higher plasma cortisol levels detected in swimming fish. Elevation of circulating cortisol levels is believed to be involved in modulating hydromineral-balance. A rapid increase of cortisol induced gill NKA up-regulation also was observed in rainbow trout (Laurent et al., 1994) and tilapia (Dang et al., 2000). A recent study by Chasiotis and Kelly (2012) showed that goldfish implanted with cortisol up-regulated gill NKA and activity. This up-regulation likely indicates that cortisol alters transcellular transport processes to enhance active ion acquisition. Further, cortisol also up-regulates kidney NKA activity to enhance renal Na^+ reabsorption in catfish (Babitha and Peter, 2010). Additionally, elevation of basal cortisol in swimming fish might promote ammonia excretion as reported in our previous study (Liew et al., 2012) by increasing Na^+ uptake via the apical NH_4^+/Na^+ exchanger to compensate for Na^+ loss (Walsh, 1998; Wu et al., 2010).

Overall, plasma thyroid status of goldfish in present study was relatively stable. No feeding effect was observed and only swimming seemed to play a role in thyroid hormone metabolism. Plasma T_4 was low in resting fish and increased slightly with swimming while low plasma T_3 was found in fish swum to exhaustion. The depression of T_3 could be due to a decrease of T_4 secretion and/or change of peripheral thyroid hormone metabolism (Walpita et al., 2007). Since plasma T_4 was not reduced, this suggests that the conversion of plasma T_4 to T_3 under these circumstances was somehow compromised. Indeed a high T_4/T_3 ratio was recorded in both fasted and fed fish at U_{crit}. Previous studies reported that elevation of cortisol levels increased metabolic T_3 clearance rate

(Vijayan et al., 1997) and reduced plasma T_3 (Mommsen et al., 1999). This seems to support our findings in goldfish swum to exhaustion (high plasma cortisol and low plasma T_3 level).

4.2. Iono-and-hormonal regulation in common carp

Similar to goldfish, feeding remarkably altered gill NKA activity in carp at all swimming conditions, even when resting, while fasted common carp seemed to maintain a relatively constant lower level of NKA activity at all swimming levels. An increase of NKA activity and NKA expression to maintain plasma Na^+ and Cl^- levels was observed in carp swum to maximum velocity before (Metz et al., 2003). This is in agreement with our result showing no significant reduction of plasma Na^+ and Cl^- levels when carp were swum to U_{crit}. There are two possibilities to explain why the increase of NKA activity only occurs in fed fish, while ion levels were stable in both fed and fasted fish; one is likely in associated with ammonia excretion and the other one with feeding itself. As observed in goldfish this NKA increment might have helped in the ammonia excretion in fed fish, especially at high swimming speeds. The fact that the increment of NKA occurs only in fed fish, which could easily maintain stable ions levels through dietary intake, also suggests a different contribution from the feeding process. According to Perry et al. (2007), high dietary Na^+ and Cl^- level (11% NaCl) intake would trigger seawater gill phenotype transformation in freshwater rainbow trout suggested internal salt loading alone was able to induce various elements of seawater gill phenotype with up-regulation of three essential ion transport genes, cystic fibrosis transmembrane conductance regulator (CFTR), $Na^+/K^+/2Cl^-$ co-transporter (NKCC) and NKA (α1b isoform). The increase in plasma Cl^- in fasted common carp at exhaustive swimming might have been facilitated by a Cl^-/HCO_3^- exchanger in gills caused by an increasing CO_2 production by swimming.

A completely different explanation for the preserved plasma Na^+ and Cl^- in exhaustive carp might be a drop of blood volume. The decrease of blood volume during active swimming has been well documented in rainbow trout by Wood and Randall (1973b). They demonstrated that an elevation of plasma Na^+ level during active swimming was attributed to a reduction of plasma volume due to net water loss (Wood and Randall, 1973a). In addition, Roa (1969) also noted an increase of plasma osmolarity and a slight increase in plasma Cl^- level associated with a reduction of plasma water content. The raise of plasma K^+ in fasted carp could be again due to a release of tissue K^+ into blood stream by exhaustive swimming. This phenomenon was reported previously in carp swum to exhaustion by Knudsen and Jensen (1998). Feeding seemed to have a minimal effect on plasma K^+ level in carp, as was also observed in rainbow trout (Bucking and Wood, 2006).

In contrast to goldfish, neither feeding nor swimming affected plasma cortisol in carp. Carp appeared to maintain their plasma cortisol at a considerably higher basal level as goldfish under the same conditions. These cannot be considered as 'stress-free' levels as they are similar (at approximately 50 ng/ml) to those reported previously in common carp that were stressed by angler keepnetting (Pottinger, 1998), cold-shock (Tanck et al., 2000) and deep-netting (Ruane et al., 2001). Previous work in our lab showed that control cortisol levels for common carp were between 9 to 15 ng/ml (De Boeck et al., 2001), however, extreme precautions were taken in this experiment to avoid any visual disturbance (e.g. even at feeding times). This does not explain the lack of response as the same study showed that common carp can maintain levels between 100 and 300 ng/ml for days when stressed (De Boeck et al., 2001). It is known that elevated plasma cortisol levels in acclimatised common carp stimulate Na^+ uptake and upregulate gill NKA activity (Abo Hegab and Hanke, 1984). A positive correlation between plasma cortisol and gill NKA activity was also detected in carp

acclimatised to different temperatures (Metz et al., 2003). Possibly, the cortisol levels in the present study were sufficient to stimulate adequate ion uptake under all conditions.

Our results showed that fasted carp had higher plasma T_4 levels than low aerobic swimming carp and fed carp at U_{crit}. In contrast, plasma T_3 levels in fed carp were higher than in fasted fish, thus it seems that feeding enhances T_4 metabolisation to T_3 (Eales, 1988). It was seen before that a depression of plasma T_4 in fed fish was caused by reduction of thyroid stimulating hormone (Leatherland, 1982; Eales, 1988) and a faster plasma T_4 clearance phase resulting in reduced T_4 secretion in teleosts (Eales, 1985). In contrast with fed fish, the availability of T_4 in fasted carp and the decrease of plasma T_3 might be explained due to the depression of deiodination from T_4 to T_3 resulting in reduced T_3 (Eales, 1988). Long-term fasting has been reported to decrease both plasma T_4 and T_3 in salmonids (Milne et al., 1979; Eales, 1988). Another explanation of low T_3 levels in fasted fish might be a reduction of thyroid sensitivity which deactivated the thyroid stimulating hormone. In fed rainbow trout this was supported by the observation that 'temporary stressor-related changes' were seen in the plasma T_4 level (Leatherland and Farbridge, 1992). These authors hypothesized that if the stressor-related response is dependent on thyroid stimulating hormone, the thyroid tissue in fasted fish might be insensitive to stimulation of endogenous thyroid stimulating hormone. As supported by other studies it was also suggested that T_4 level was depended on T_3 clearance phase (Vijayan et al., 1988), thyroid stimulating hormone status and that T_3 was regulated by peripheral T_3 deiodination (Van der Geyten et al., 2005; Walpita et al., 2007).

5. Conclusion

Overall, we can conclude that both goldfish and common carp were able to maintain plasma ion levels very well at two levels of swimming, independent of the feeding regime. In fasted fish this occurred without a concomitant increase in gill NKA activity which remained at a low but stable level. In fed fish however, feeding and swimming induced increases in gill NKA activity in both species. We hypothesize that this increase in gill NKA activity is related to the increased levels of ammonia excretion, rather than to ion uptake as feeding granted dietary ion uptake. In goldfish, but not common carp, this increased NKA activity coincided with elevations in plasma cortisol. Thyroid hormones seem not directly related to iono-osmoregulation but rather to energy metabolism in both species. In goldfish T_4/T_3 ration were low, but increased slightly with swimming, while in common carp responses seemed to correlate with fasting.

Chapter 3

Cortisol emphasizes the metabolic strategies employed by common carp, *Cyprinus carpio* at different feeding and swimming regimes

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Abstract

The objective of this study was to investigate the interaction between feeding, exercise and cortisol on metabolic strategies of common carp over a 168h post-implant period. Feeding provided readily available energy and clearly increased muscle and liver protein and glycogen stores. Swimming, feeding and cortisol all induced aerobic metabolism by increasing oxygen consumption, and stimulated protein metabolism as demonstrated by the increased ammonia and urea excretion and ammonia quotient. Hypercortisol stimulated ammonia self-detoxifying mechanisms by enhancing ammonia and urea excretion, especially during severe exercise. At high swimming level, higher branchial clearance rates in cortisol treated fish succeeded in eliminating the elevation of endogenous ammonia, resulting in reduced plasma T_{amm} levels compared to control and sham implanted fish. Carp easily induced anaerobic metabolism, both during routine and active swimming, with elevated lactate levels as a consequence. Both feeding and cortisol treatment increased this dependence on anaerobic metabolism. Hypercortisol induced both glycogenesis and gluconeogenesis resulting in hyperglycemia and muscle and liver glycogen deposition, most likely as a protective mechanism for prolonged stress situations and primarily fuelled by protein mobilisation.

Keywords: Aerobic metabolism, ammonia excretion, anaerobic metabolism, critical swimming speed, energy budget, respirometry, stress, urea

1. Introduction

Feeding is the key to optimise growth and survival either in nature or captivity. Feeding is physiologically well known to be followed by oxygen expenditure for nutritive metabolism known as the specific dynamic action (SDA) which involves processes such as ingestion, digestion, nutrient absorption and transformation which all require oxygen (Lyndon et al., 1992). Swimming on the other hand competes with the need for oxygen for SDA and requires extra oxygen supply simultaneously. Obviously, swimming is another important key for growth and survival. The effect of feeding on swimming performance depends on feeding rate, types of food and environmental conditions (Fu et al., 2009). Whether feeding or swimming metabolism is either compromised or prioritized depends on species specific metabolic strategies (Liew et al., 2012). These strategies have recently been examined with a focus on increases of aerobic metabolism and the changes from aerobic to anaerobic metabolism (Alsop and Wood, 1997; Liew et al., 2012), changes in nitrogen waste and/or ammonia excretion efficiency (Nawata et al., 2007; Bucking and Wood, 2008; Zimmer et al., 2010), energy budgets (De Boeck et al., 2001) and swimming performance (Li et al., 2010; Pang et al., 2010; Zhang et al., 2010). In reality, food accessibility might differ between or within populations and can be associated with dominance hierarchies resulting in feed limitations for subordinate individuals. Such competition may cause stress and subsequently alter endocrine responses such as catecholamine and cortisol levels, and subsequently increase oxygen consumption rates (Morgan and Iwana, 1996; De Boeck et al., 2001).

Cortisol is a stress hormone and has long been recognised for its wide range of effects in stressed fish (Wendelaar Bonga, 1997; Mommsen et al, 1999). Research has shown that cortisol affects teleost spontaneous locomotion, behavior, feeding and growth (Gregory and Wood, 1999), intermediary energy metabolism (Mommsen et al., 1999; De Boeck et al., 2001), hematological properties (Wojtaszek et al., 2002), and nitrogen metabolism (McDonald and Wood, 2004), and it

induces gluconeogenesis and hyperglycemia to provide easily accessible energy for metabolism during stress (Vijayan et al., 1991; 1997). Changes in swimming and feeding conditions can induce cortisol secretion as a stress response as well (Barcellos et al., 2010). Basal cortisol values in wild teleosts are typically <10 ng/ml, but a number of species show values orders of magnitude higher in unstressed fish (Pottinger, 2010; Pankhurst, 2011). For example, unstressed cortisol levels for common carp have been reported to be about ≈10-150 ng/ml and under stressed conditions in the range of ≈300-450 ng/ml (Dabrowska et al., 1991; Van Dijk et al., 1993; Pottinger, 1998; Pottinger et al., 2000; De Boeck et al., 2001; Liew et al., 2013b). Our previous investigation found that carp fed at 2% of body weight (BW) are able to perform both digestion and swimming independently and sustain swimming performance by increasing oxygen consumption ($\dot{M}O_2$) and ammonia excretion (J_{amm}) rates (Liew et al., 2012), while plasma cortisol level remained unaltered (Liew et al., 2013).

In this study our first aim was to investigate how common carp prioritize their metabolic needs during cortisol elevation. Does cortisol alter their physiological capacities to maintain feeding and swimming metabolism simultaneously and will it affect their swimming capacity? Together with our previous study (Liew et al., 2012), we hypothesized that hypercortisol may lead to extra energy expenditure, increased $\dot{M}O_2$ and hyperglycemia, and that it stimulates endogenous ammonia production as a stress response, and therefore impairs swimming performance. Additionally, we speculated that these effects were more harmful under low feeding regimes due to the limited energy supply and reduced ammonia excreting capacities. To address these hypotheses, common carp were conditioned to different feeding regimes (high ration - 3% BW and low ration - 0.5% BW) for three weeks. Thereafter, those carp received no implant, a sham implant or a cortisol implant (250 mg cortisol/kg BW) and were monitored up to 168 hours post-implant (h-PI). In addition, carp were submitted to different swimming conditions (resting, low aerobic, high aerobic and exhaustive swimming). Besides measuring oxygen consumption ($\dot{M}O_2$) to examine aerobic metabolism, plasma and tissue metabolites were evaluated to investigate energy mobilization and the involvement of anaerobic metabolism. Plasma ammonia (T_{amm}), urea excretion (J_{urea}), ammonia excretion (J_{amm}) and branchial ammonia clearance rates were assessed in order to evaluate any changes in excretory capacity as well.

2. Materials and methods

2.1 Fish maintenance and experimental set-up

Common carp, *Cyprinus carpio* were obtained from a commercial fish farm (Viskweekcentrum Valkenswaard, Leende, The Netherlands) and kept in the aquarium facilities of the Systemic Physiological and Ecotoxicological Research group (SPHERE) at the University of Antwerp, Belgium in Antwerp City tap water. A total of 800 fish were selected for equal size (22.8±0.8 g BW and 12.9±0.9 cm body length (BL) (Mean±SEM)) and distributed equally into two 500 L aquaria. Fish were acclimated for 6 weeks and fed at 1.5% BW per day with commercial Koi pellet (Hikari Staple, Kyorin Food Ind. Ltd., Japan).

Three weeks prior to the experiment, 32 fish were selected randomly per series, weighed and distributed equally into four 50 L holding aquaria filled with Antwerp city tap water (≈ pH 8.2±0.4). Each aquarium was equipped with gentle aeration and a 6 L external filter consist of fine sponges, activated carbon and ≈16 mm lava stone at a flow rate of 5 L/min. Aquaria were covered with a black plastic sheet to avoid external disturbance. Room temperature was set at 17 °C with constant

photoperiod of 12L:12D. These relatively high pH values and low temperatures were similar with the outdoor conditions fish were raised in before. About 80% of water was refreshed every 3 days with a semi-auto-siphoning procedure to avoid handling disturbance. Water samples were taken every 2 days for water quality control. Throughout the entire experiment, water temperature, NH_3/NH_4^+, NO_2^-, and NO_3^- were maintained at 16±0.8 °C, <0.1 mg/L, <0.03 mg/L and <25 mg/L respectively. During this three weeks acclimation period, feeding was kept according to the high and low feeding ratio (0.5 and 3.0% BW). Pellets were divided over 2 equal portions per day fed at 08:30 h and 18:30 h daily by slowly introduced pellets at the same corner of aquaria over a 20 min period. All experimental procedures applied in this study were approved by the University of Antwerp Animal Care Committee, and conducted according to the guidelines of the Federation of European Laboratory Animal Science Associations.

2.2 Treatment groups and implantation

Fish from both feeding regimes were given a sham or cortisol implant. Selected fish were anaesthetized individually in 0.1 g/L Ehty-3-aminobenzoate methanesulfonic acid (MS222) (Acros Organics, Geel, Belgium) neutralized with 1N NaOH to pH 8.0 (Antwerp City tap water pH) and weighed. Implantation with a cortisol or coconut oil implant was conducted intraperitoneally at a ratio of 10 µl/g BW as described by Gregory and Wood (1999); De Boeck et al. (2001) and McDonald and Wood (2004). Implantation was conducted 12h post feeding. Fish were implanted with a mixture of 25 mg cortisol (Hydrocortisone 21-hemisuccinate; Sigma Diagnostics) in 1 ml of melted coconut oil (≈27^{o} C; Sigma Diagnostics) resulting in an overall dose of 250 mg cortisol per kg of fish (cortisol group). This dose was selected based on our preliminary test showing that this dose significantly elevated baseline cortisol levels and allowed them to recover to control levels within a week. Fish implanted with only melted coconut oil served as sham group, whereas control fish receive no implant. All implanted fish were briefly placed on ice for about 30 s to facilitate solidification of the implant and immediately placed in well oxygenated water to recover from unconsciousness. All groups (control, sham and cortisol) were monitored at 12h, 24h, 72h and 168h-PI intervals. Each group consisted of eight fish.

2.3 Experimental series and swimming performance

Each treatment group was subjected to four swimming conditions: resting, low aerobic swimming, high aerobic swimming and exhaustive swimming (swum up to fatigue). For resting series, fish were directly sampled from the holding tank and they served as control for the handling stress induced by transferring the fish to the respirometers. For low aerobic, high aerobic and exhaustive swimming series, fish were introduced individually into eight respirometers which were Blazka-style swimming tunnels with a volume of 3.9 L for 12 h prior to measurement to acclimatise to the swimming tunnel to reduce handling stress on the day of measurement. Water speed was set at a velocity of 10 cm/s (approximately 1BL/s) and water was continuously refreshed with well oxygenated water at 4 L/min in a flow-through system (Liew et al., 2012). Exhaustive swimming (U_{crit}) was performed by increasing water velocity at 5 cm/s every 20 min interval until fish fatigued. The point of fatigue was determined when the fish were no longer able to swim any further against the water current and were swept downstream against the mesh screen at the end of tunnel. Thereafter, water velocity was reduced until the fish recommenced swimming. Fish were considered

totally fatigued when the fish were incapable to swim against the water current and were swept downstream to the mesh screen twice within a 20 min interval (De Boeck et al., 2006; Tudorache et al., 2007; Liew et al., 2012). The performance test was then terminated. U_{crit} was calculated as $U_{crit} = U_i + [U_{ii}(T_i/T_{ii})]$, where U_i referred to highest velocity sustained for the whole interval, U_{ii} is the velocity increment (5 cm/s), T_i is the time elapsed at fatigue and T_{ii} is the interval time (20 min) (Brett, 1964; Tudorache et al., 2007; Farrell, 2008). The absolute values (cm/s) were converted to relative swimming speeds in body lengths per second (BL/s) by factoring the absolute values with the fish body length. U_{crit} values obtained from the exhaustive swimming test were then used to determine low aerobic swimming (swum at 1/4 U_{crit}; 1.0 BL/s) and high aerobic swimming (swum at 3/4 U_{crit}; 3.7 BL/s) speeds.

2.4 Respirometery

MO_2 was performed for low aerobic and high aerobic swimming series but not in U_{crit} series to avoid any effect of O_2 depletion. For low aerobic swimming series, fish were swum for 3 h at 1.0 BL/s ($MO_{2\text{-low aerobic}}$) to ensure measureable MO_2 and ammonia excretion rate, whereas high aerobic swimming fish were swum for 1 h at 3.7 BL/s ($MO_{2\text{-high aerobic}}$) to avoid oxygen depletion. U_{crit} was performed in the same swimming tunnel without MO_2 measurement by increasing water velocity until fish fatigued (U_{crit}) as described above (2.3). MO_2 measurement was conducted by closed respirometry using WTW- oxygen electrodes (Oximeter Oxy-340, Wissenschaftlich-Technische Werkstatten & Co KG, Weilheim, Germany). MO_2 was recorded using Windmill Logger (Windmill Software Limited, Manchester, UK) with a reading frequency of 26 Hz. MO_2 was calculated according to Ultsch et al. (1980) method as $MO_2 = (\Delta O_{2i} - O_{2f}) \times V \times 1000 \times (1/O_{2MW}) \times (1/BW) \times T$, where O_{2i} is initial reading of oxygen concentration (mg/L) and O_{2f} is final reading of oxygen concentration (mg/L); V is total water volume of the respirometer (3.9 L); O_{2MW} is molecular weight of oxygen (32); BW is body weight (g) and T is time (h); and was expressed as µmol/g/h (De Boeck et al., 2006; Liew et al., 2012).

A 2 ml water sample from the low aerobic and high aerobic swimming series prior and post MO_2 measurement were taken from each respirometer to measure the total ammonia (J_{amm}) and urea (J_{urea}; total branchial excretion plus urine) excretion rate. Ammonia was determined using the salicylate-hypochlorite assay (Verdouw et al., 1978). Urea was determined with the diacetyl-monoxime assay (Rahmatullah and Boyde, 1980). Excretion rate was calculated as change in molar concentration of the substances (J_{amm} and J_{urea}) in the water factored by molecular weight, body weight, volume of repirometer and time: $J_{substance} = (\Delta\ substance_{f} - _{i}) \times V \times (1/substance_{MW}) \times (1/BW) \times (1/T)$, where substance $_i$ is initial concentration (µg/L); substance $_f$ is final concentration (µg/L); V is volume of respirometer (3.9 L); substances $_{MW}$ is the molecular weight of substances; BW is body weight (g); T is time (h) and was expressed as µmol/g/h (De Boeck et al., 2001). Ammonia quotient (AQ) of each fish was determined as $AQ = J_{amm} / MO_2$. The theoretical maximum AQ in teleost is 0.33 and this theoretical maximum AQ is known as maximum protein contribution during aerobic metabolism (Kutty, 1978; De Boeck et al., 1995). Branchial ammonia clearance rate was calculated according to McDonald et al., (2002) with the standard equation = $ammonia_w \times V \times (1/BW) \times (1/ammonia_p) \times (1/T)$, where $ammonia_w$ is ammonia concentration in respirometer water (µmol/L); V is volume of respirometer (3.9 L); BW is body weight (g); $ammonia_p$ is ammonia concentration in plasma (µmol/ml); T is time (h) and the unit was ml/g/h.

2.5 Plasma and tissue sampling

All fish from resting, low aerobic swimming and exhaustive swimming series were sampled. Fish from high aerobic swimming series were only used for respirometry and were not sampled. Sampling was conducted 12h post feeding for resting fish, while low aerobic swimming and exhausted fish were sampled after respiration measurements (for low aerobic swimming) and U_{crit} measurements (exhaustive). Fish were carefully removed from the respirometers, anaesthetised (neutralized MS22; 0.5 g/L), quickly blotted dry and weighed and measured followed by sampling. Blood was drawn from the caudal vessels using a heparinised (2500 units/ml lithium heparin, Sigma, Munich, Germany) needle and syringe within 1 min of sedation. The blood was immediately centrifuged to 13,200 rpm at 4 ^{o}C for 1.5 min. Plasma was pipetted into three 500 µl eppendorf tubes and immediately frozen in liquid N_2. Gill and kidney samples were wrapped in aluminium foil, while muscle tissue and whole liver were stored in cryogenic vials. All samples were immediately frozen in liquid N_2 and stored at -80 ^{o}C for further analysis.

2.6 Biochemical analysis

All parameters were measured in triplicate. Radioimmunoassay was performed to measure plasma cortisol levels using a commercial Cortisol ^{125}I RIA kit according to the kit instruction (ImmuChem^{TM}, MP Biomedicals LLC, Orangeburg, NY). Plasma glucose, lactate and ammonia were determined according to the commercial enzymatic kit instructions (R-Biopharm AG, Darmstadt, Germany). Plasma urea was determined using the salicylate-hypochlorite assay (Rahmatullah and Boyde, 1980). Tissue energy (protein, glycogen and lipid) content was determined in both liver and muscle tissues. Glycogen was measured using Anthron reagent and a glycogen standard curve (Roe and Dailey, 1966). Protein was determined according to Bradford (1976) using a standard curve of bovine serum albumin. Total lipid was extracted by methanol-chloroform and measured with a tripalmitin standard curve (Blingh and Dyer, 1959).

2.7 Statistics analysis

Normality was checked prior to the analysis by Shapiro-Wilk test. Homogeneity of variance was verified using Hartley test. Log-transformed data were applied if the requirements for ANOVA were not fulfilled. Effect of feeding regime was assessed by unpaired two-tail student *t*-test. One way ANOVA was performed to examine significant effects of implantation within and between treatments at different time points and swimming conditions followed by multiple comparison of Tukey post-hoc test. Multifactor analysis of variance (MANOVA) was performed with multiple factorial cross design to examine overall interaction effects of feeding, swimming, implantation and time. Level of significance was set at $P<0.05$. All data were expressed as mean values with standard error (Mean ± SEM; $n = 8$).

3. Results

3.1 Plasma cortisol

Effects of feeding, swimming and sham or cortisol implant on plasma cortisol levels are shown in Fig. 1. Neither feeding nor swimming had an effect on plasma cortisol level. Overall, plasma cortisol levels of both control and sham implanted fish were relatively constant throughout the experimental period and were not significantly different (Fig. 1). However, carp implanted with cortisol had significantly elevated plasma cortisol levels when compared to control and sham fish. Cortisol levels peaked at 12h-PI to levels that were about 7-fold higher than control levels. This level started to decrease at 24h-PI, but remained elevated up to 72h-PI in both feeding groups. At 168h-PI, cortisol levels in cortisol-implanted carp returned to levels similar to control and sham groups. This resulted in a significant interaction between cortisol implant and time interval.

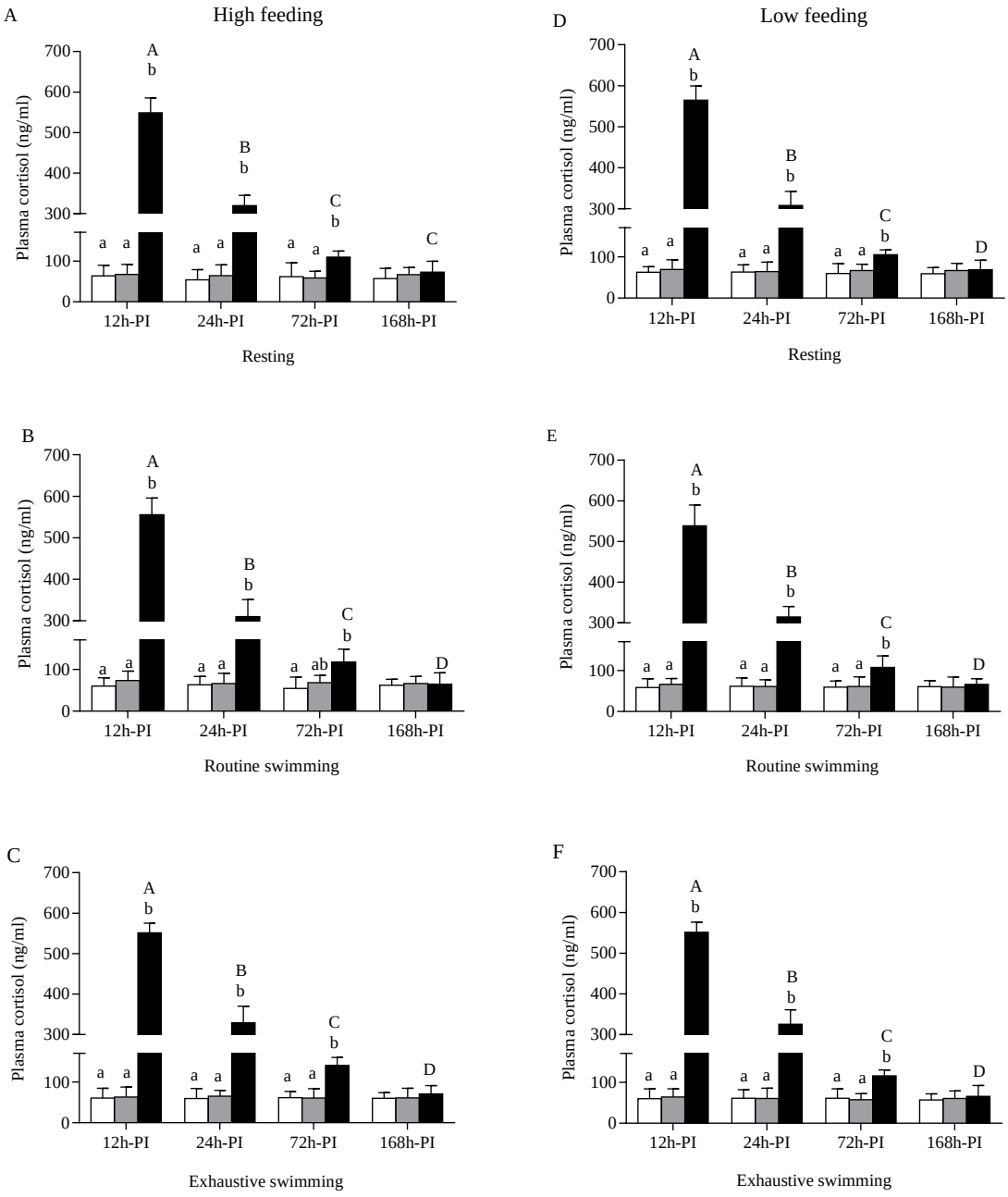

Fig. 1 Plasma cortisol levels of control carp without implants (open bars), sham implanted carp (light grey bars) and cortisol implanted carp (dark grey bars) at different feeding and swimming regimes at different post-implant intervals (hours post-implant, h-PI). Small letters indicate significant differences between cortisol treatments. Capital letters indicate significant differences between time intervals.

3.2 Swimming performance

Overall, swimming performance (U_{crit}) in all groups from both feeding regime was relatively constant. Neither feeding nor cortisol implantation had an effect on swimming performance of carp (Table 1). On average high feeding groups exhibited a slightly lower swimming capacity than low feeding groups, but this variation was not significant. Consequently, no interactive effect was observed in swimming performance.

Table 1 Swimming performance of common carp without implants (control), and sham and cortisol implanted carp at different feeding regimes were monitored at different post-implant intervals (hours post-implant, h-PI).

	U_{crit} (BL/s)							
	High feeding (3.0% BW)				Low feeding (0.5% BW)			
	12h-PI	24h-PI	72h-PI	168h-PI	12h-PI	24h-PI	72h-PI	168h-PI
Control	4.43±0.13	4.74±0.21	4.28±0.25	4.32±0.18	4.91±0.18	5.24±0.22	5.41±0.23	5.20±0.16
Sham	4.09±0.10	4.34±0.18	4.66±0.29	4.95±0.25	4.87±0.22	4.93±0.17	4.98±0.16	5.07±0.27
Cortisol	4.47±0.28	4.53±0.19	4.71±0.32	4.58±0.12	5.25±0.21	5.05±0.16	5.16±0.22	5.02±0.10

3.3 Oxygen consumption

As expected, a high level of swimming induced higher oxygen consumption rates (MO_2) and increased $MO_{2\text{-high aerobic}}$ over the corresponding $MO_{2\text{-low aerobic}}$ were observed in all treatment groups at both feeding regimes (Fig. 2). Feeding significantly increased MO_2 when fish swum at high aerobic speeds (Fig. 2a and 2c). In low aerobic swimming fish, carp on the high feeding regime only showed higher $MO_{2\text{-low aerobic}}$ compared to the low feeding regime during the first 72h-PI implanted with cortisol (Fig. 2b). Sham implants did not increase MO_2 but cortisol implants induced a higher oxygen consumption at the first 24h-PI for the high feeding fish (Fig. 2a and 2b) and at the first 12h-PI for the low feeding groups under both low aerobic and high aerobic swimming conditions (Fig. 2c and 2d). Therefore, the interaction between feeding and cortisol implant was found to be almost significant (P = 0.056).

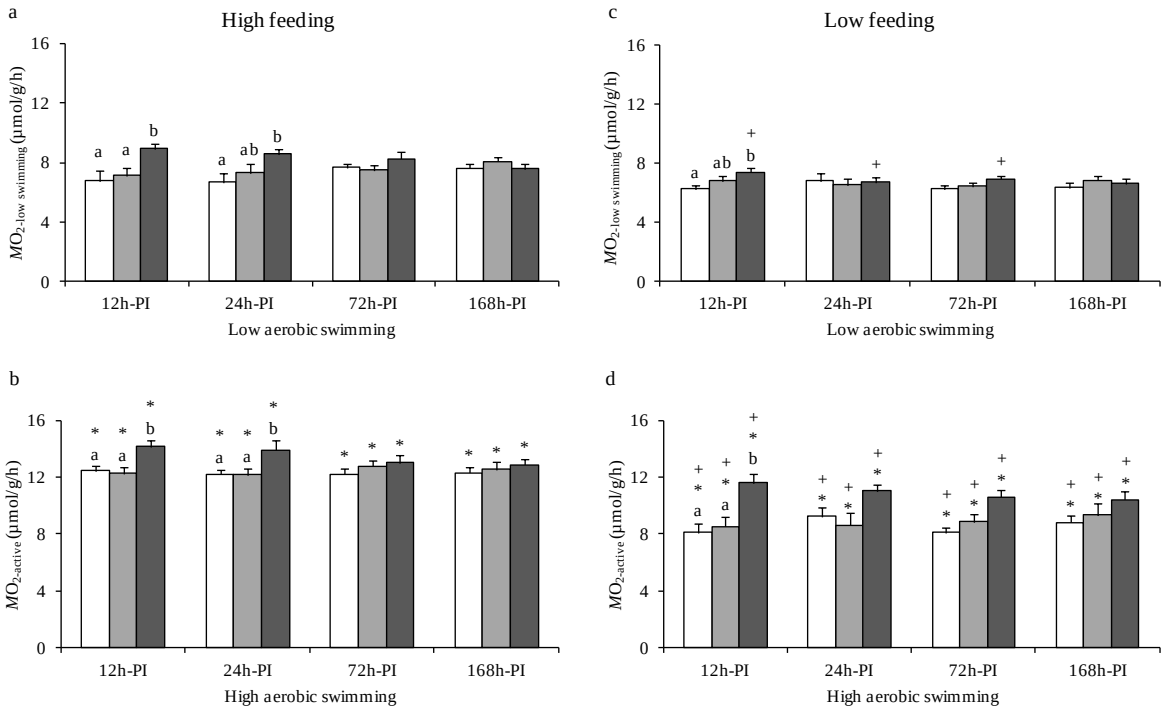

Fig. 2 Oxygen consumption of control carp without implants (open bars), sham implanted carp (light grey bars) and cortisol implanted carp (dark grey bars) at different feeding and swimming regimes at different post-implant intervals (hours post-implant, h-PI). A plus ($^{+}$) indicates significant differences between feeding regimes. An asterisk (*) indicates significant differences between swimming regimes. Small letters indicate significant differences between cortisol treatments.

3.4 Ammonia and urea excretion

As for MO_2, swimming also induced a higher ammonia excretion rate (J_{amm}) throughout the 168h-PI in carp fed a high ration (Fig. 3b). However, this increase of J_{amm} due to swimming was absent in carp fed a low ration (Fig. 3d). As a result, J_{amm} in high aerobic swimming fish that were fed a high ration was significantly higher than those in fish fed a low ration (Fig. 3). Inversely, no feeding effect was observed when fish were swum at low aerobic speeds (P = 0.084; Fig. 3a and 3c). High aerobic swimming significantly increased J_{amm} by about 2-fold for control and sham groups and about 2.5-fold for cortisol implanted groups compared to low aerobic swimming fish over the entire period in the high feeding regime as well as compared to low and high aerobic swimming fish in the low feeding regime (Fig. 3). This led to a significant interaction between feeding and swimming regimes; and also between swimming and time interval. Elevated circulating cortisol levels induced higher J_{amm} in all high feeding fish and also in low feeding fish swum to high aerobic speeds. The J_{amm} was significantly greater compared to control fish during the first 72h-PI in high aerobic swimming fish fed a high ration (Fig. 3b) and only during the first 24h-PI in low aerobic swimming fish and low feeding fish swum to high aerobic speeds (Fig. 3a and 3d). Although, J_{amm} tended to be higher in sham implanted fish fed at high ratio swum at low aerobic speeds, this effect was not significant when averaged over the entire experiment period (e.g. 24h-PI; Fig. 3a). With this, the interaction between feeding and cortisol implant (P = 0.036) and between implant and time interval was significantly.

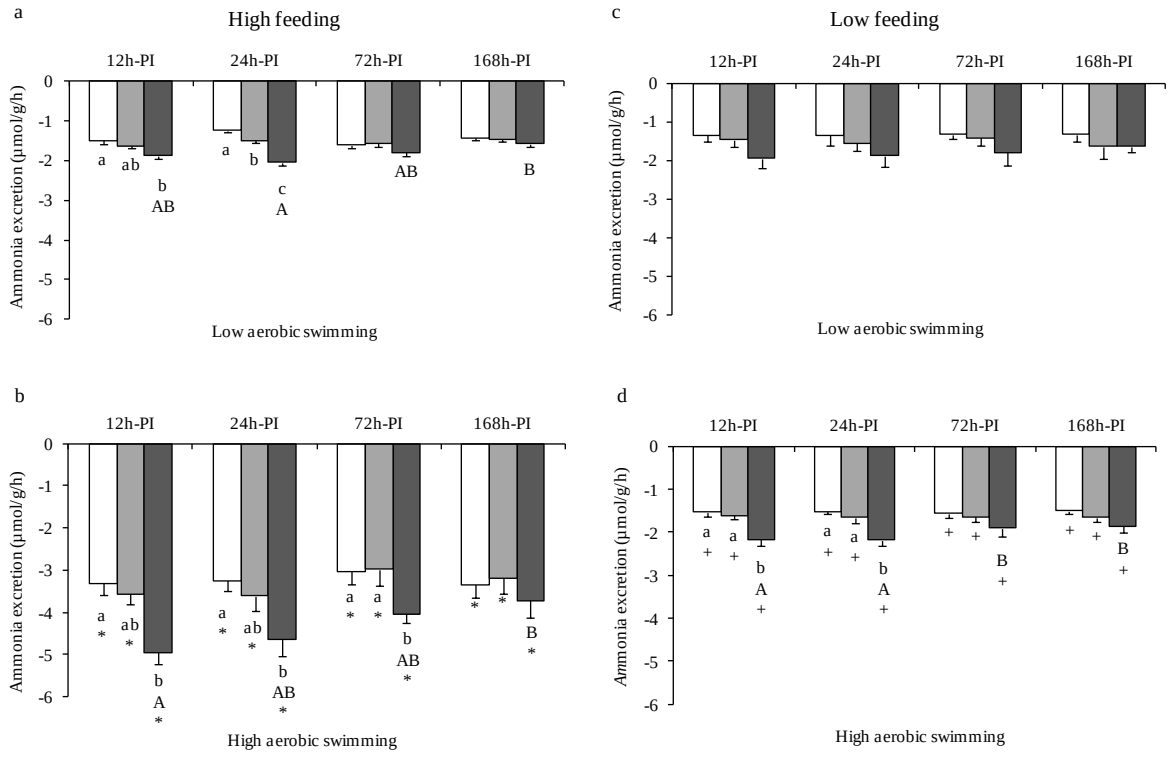

Fig. 3 Branchial ammonia excretion of control carp without implants (open bars), sham implanted carp (light grey bars) and cortisol implanted carp (dark grey bars) at different feeding and swimming regimes at different post-implant intervals (hours post-implant, h-PI). A plus ($^{+}$) indicates significant differences between feeding regimes. An asterisk (*) indicates significant differences between swimming regimes. Small letters indicate significant differences between cortisol treatments. Capital letters indicate significant differences between time intervals.

When calculating the branchial ammonia clearance rate, the effect of cortisol was totally abolished in low aerobic swimming fish, but reinforced in high aerobic swimming fish (Fig. 4). Swimming enhanced the branchial clearance rate in low feeding fish over the entire experimental period in all groups (Fig. 4c and 4d), but only in cortisol implanted fish in the high feeding groups (Fig 4a and 4b). In control fish at low aerobic swimming, high feeding fish displayed a significant greater branchial clearance rate than low feeding fish throughout experiment period (Fig. 4b and 4d). This effect of feeding was only notable at 24h-PI for sham and cortisol implanted fish. Figure 4b and 4d clearly demonstrated that cortisol implant remarkably enhanced branchial ammonia clearance rate when fish swum at high aerobic speeds in both high and low feeding. For the first 12h-PI, cortisol significantly induced branchial ammonia clearance rate 3-fold and 2-fold compared to control and sham implanted fish. The effect persisted for the first 72h-PI and returned to normal level at 168h-PI (Fig. 4b and 4d). Overall, these changes led to a significant interaction between feeding, swimming, implantation and time interval. For two way interaction, significant interactions occurred between swimming and implantation; implantation and time interval. For three way interactions feeding, swimming and implantation; swimming, implantation and time interval, were significant respectively.

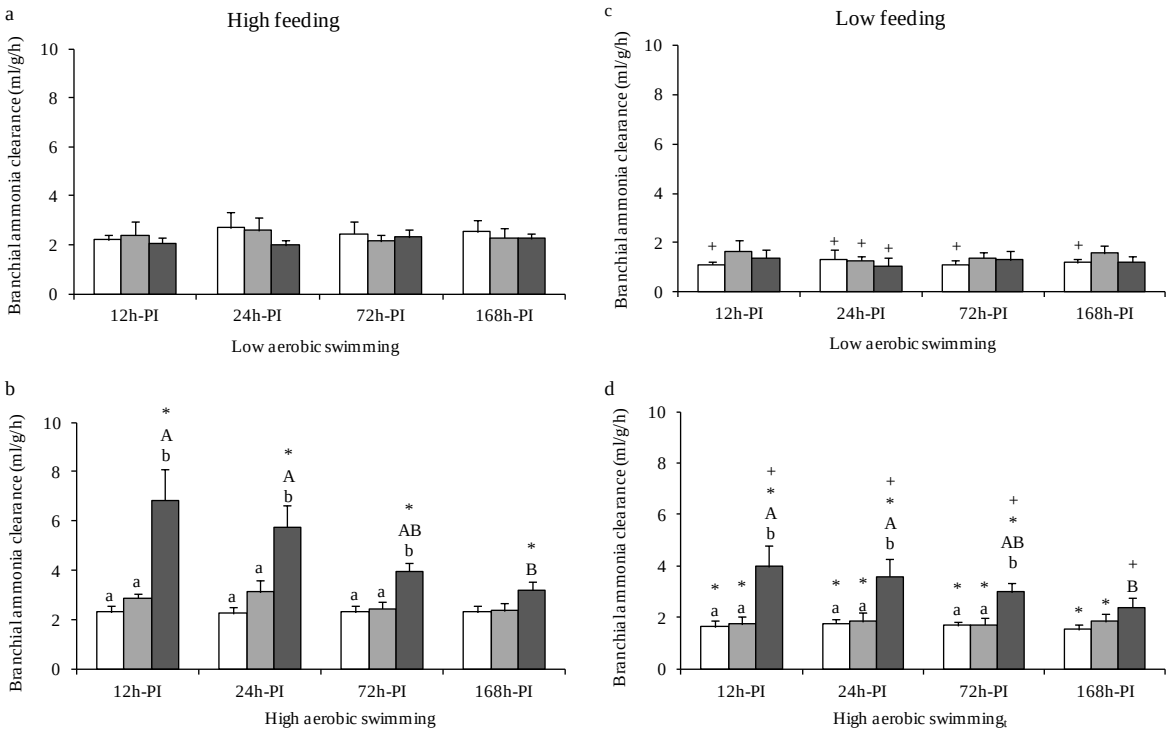

Fig. 4 Branchial ammonia clearance rate of control carp without implants (open bars), sham implanted carp (light grey bars) and cortisol implanted carp (dark grey bars) at different feeding and swimming regimes different post-implant intervals (hours post-implant, h-PI). A plus ($^{+}$) indicates significant differences between feeding regimes. An asterisk (*) indicates significant differences between swimming regimes. Small letters indicate significant differences between cortisol treatments. Capital letters indicate significant differences between time intervals.

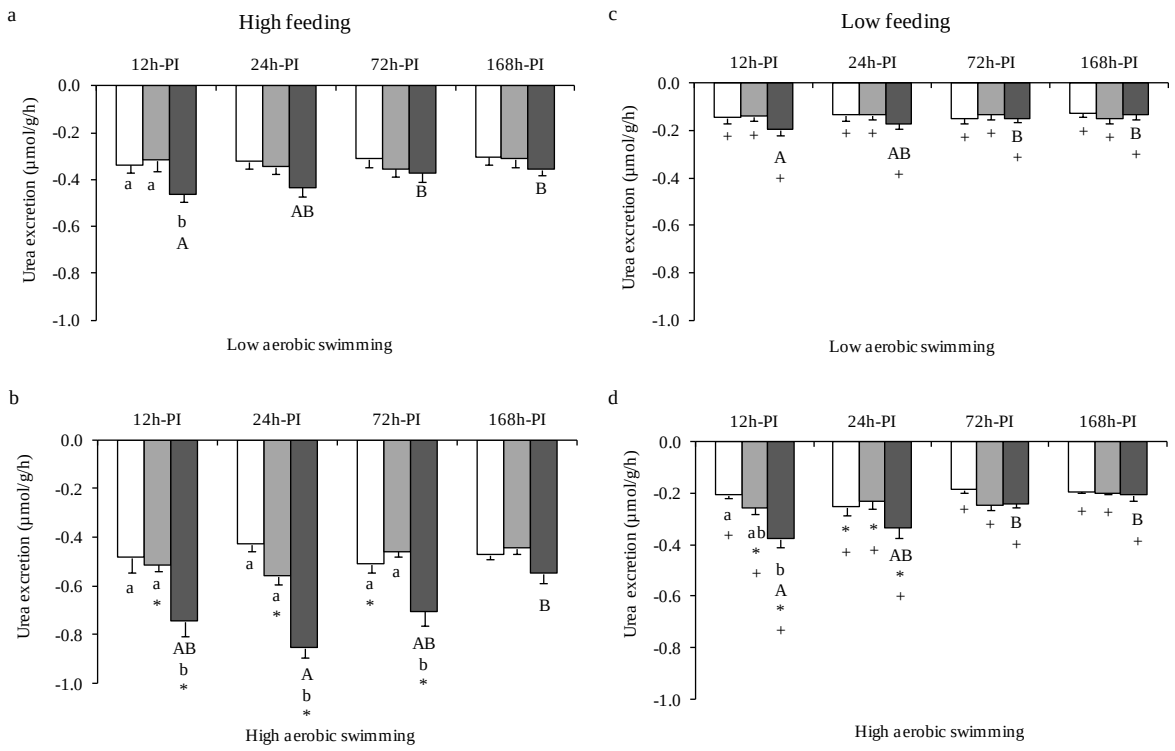

Fig. 5 Urea excretion of control carp without implants (open bars), sham implanted carp (light grey bars) and cortisol implanted carp (dark grey bars) at different feeding and swimming regimes at different post-implant intervals (hours post-implant, h-PI). A plus ($^{+}$) indicates significant differences between feeding regimes. An asterisk (*) indicates significant differences between swimming regimes. Small letters indicate significant differences between cortisol treatments. Capital letters indicate significant differences between time intervals.

Feeding greatly induced urea excretion (J_{urea}) resulting in about 2-fold higher values in high feeding compared to low feeding fish in all groups that were either swum at low aerobic speeds or high aerobic speeds. Thus, both feeding and swimming significantly interacted (Fig. 5). High aerobic swimming on the other hand also significantly induced J_{urea} during the first 24h-PI in low feeding fish and the first 72h-PI in high feeding fish (Fig. 5b and 5d). Highest J_{urea} rates were seen in cortisol implanted fish fed a high ration at high aerobic swimming, and their J_{urea} rate remained significantly elevated up to 72h-PI (Fig. 5b). However, this effect only lasted for the first 12h-PI in low feeding fish when swum at high aerobic speeds and in high feeding fish swum at low aerobic speeds (Fig. 5a and 5c), and was absent in low feeding fish swum at low aerobic speeds (Fig. 5d). The J_{urea} rate for cortisol implanted fish in all groups returned to control levels at 168h-PI (Fig. 5).This contributed to a significant interaction between feeding and implantation, swimming and implantation, implantation and time interval, as well as interaction between feeding, swimming and implantation.

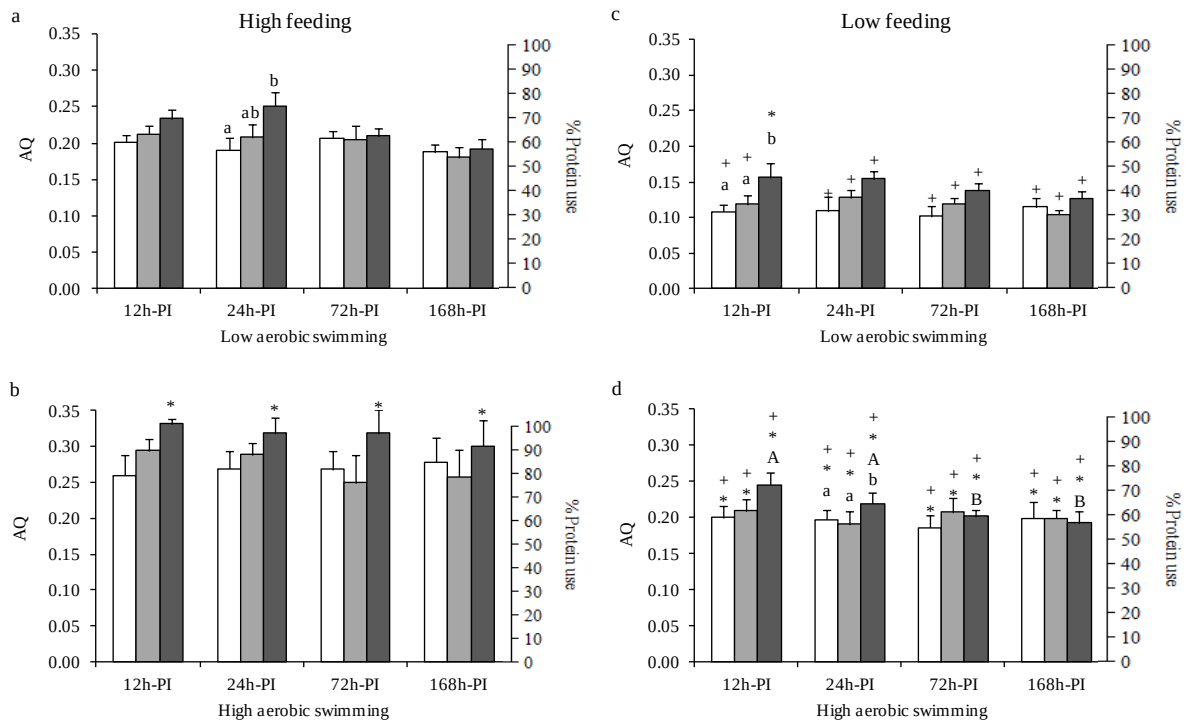

Fig. 6 Ammonia quotient of control carp without implants (open bars), sham implanted carp (light grey bars) and cortisol implanted carp (dark grey bars) at different feeding and swimming regimes at different post-implant intervals (hours post-implant, h-PI). A plus (+) indicates significant differences between feeding regimes. An asterisk (*) indicates significant differences between swimming regimes. Small letters indicate significant differences between cortisol treatments. Capital letters indicate significant differences between time intervals.

The high feeding regime significantly increased the AQ level over the AQ level obtained in low feeding fish that were either swum at low or high aerobic speeds (Fig. 6). At low aerobic swimming, AQ in high feeding fish was about 2-fold higher than in low feeding groups (Fig. 6a and 6c) and the increase was slightly lower in high aerobic swimming fish (Fig. 6b and 6d) and this resulted in a significant interaction effect between feeding and swimming. At low aerobic swimming, some transient effects of cortisol on AQ were noted in the high feeding groups at 24h-PI and in the low feeding groups at 12h-PI (Fig. 6a and 6c). In the high feeding regime, the effect of swimming on AQ was only observed in cortisol implanted fish (at all time points) (Fig. 6b), but in the low feeding regime, swimming increased AQ in all treatments including controls fish (Fig. 6c and 6d). Therefore, feeding, swimming and implantation interacted significantly.

3.5 Plasma metabolites

3.5.1 Plasma glucose

Feeding significantly elevated plasma glucose, while swimming only had an effect in sham and cortisol implanted fish at 24h-PI and control fish at 168h-PI (Fig. 7). On average, carp with sham implants had plasma glucose levels that were not significantly different from control fish over the 168h-PI experimental period. However, the effect between feeding and swimming was significant. Elevation of plasma glucose in cortisol implanted carp was highly significant compared to both

control and sham fish. A peak level of plasma glucose was observed at 24h-PI in cortisol implanted carp and the level was decreased subsequently, although it still remained significantly higher than in the control and sham fish until the end of the experimental period (Fig. 7). Therefore, the interaction effect between implantation and time interval was significant.

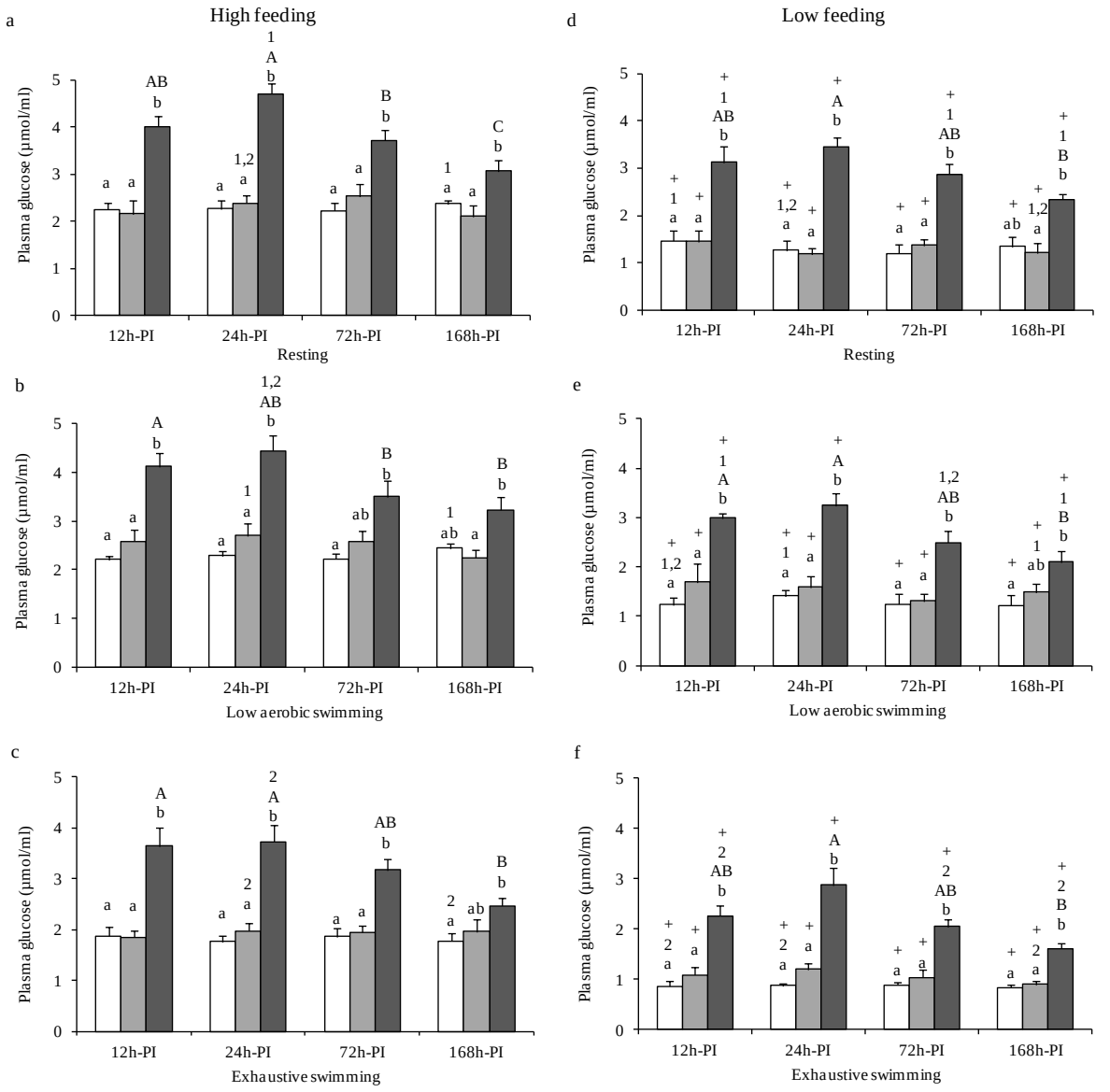

Fig. 7 Plasma glucose levels of control carp without implants (open bars), sham implanted carp (light grey bars) and cortisol implanted carp (dark grey bars) at different feeding and swimming regimes at different post-implant intervals (hours post-implant, h-PI). A plus ($^{+}$) indicates significant differences between feeding regimes. Numeric numbers indicate significant differences between swimming regimes. Small letters indicate significant differences between cortisol treatments. Capital letters indicate significant differences between time intervals.

3.5.2 Plasma lactate

For plasma lactate, a remarkable feeding effect was observed at resting and low aerobic swimming fish where plasma lactate accumulated to a larger extent in all high feeding fish compared to low feeding fish (Fig. 8a-b and 8d-e). At exhaustive swimming, the feeding effect was mainly observed in cortisol implanted fish, and lactate levels were also increased in fish fed a low ration (Fig. 8f). Not surprisingly, swimming significantly elevated plasma lactate levels as compared to resting fish in both feeding regimes (Fig. 8c and 8f) with a significant interaction between feeding and swimming. Clearly, cortisol implanted fish had lactate levels that were significantly higher than in control and sham fish in all experimental groups. This trend was similar as seen for plasma cortisol and glucose levels with a peak at 24h-PI and a decrease to basal levels at 168h-PI (Fig. 8). Therefore, the interaction between implant and time interval was significant.

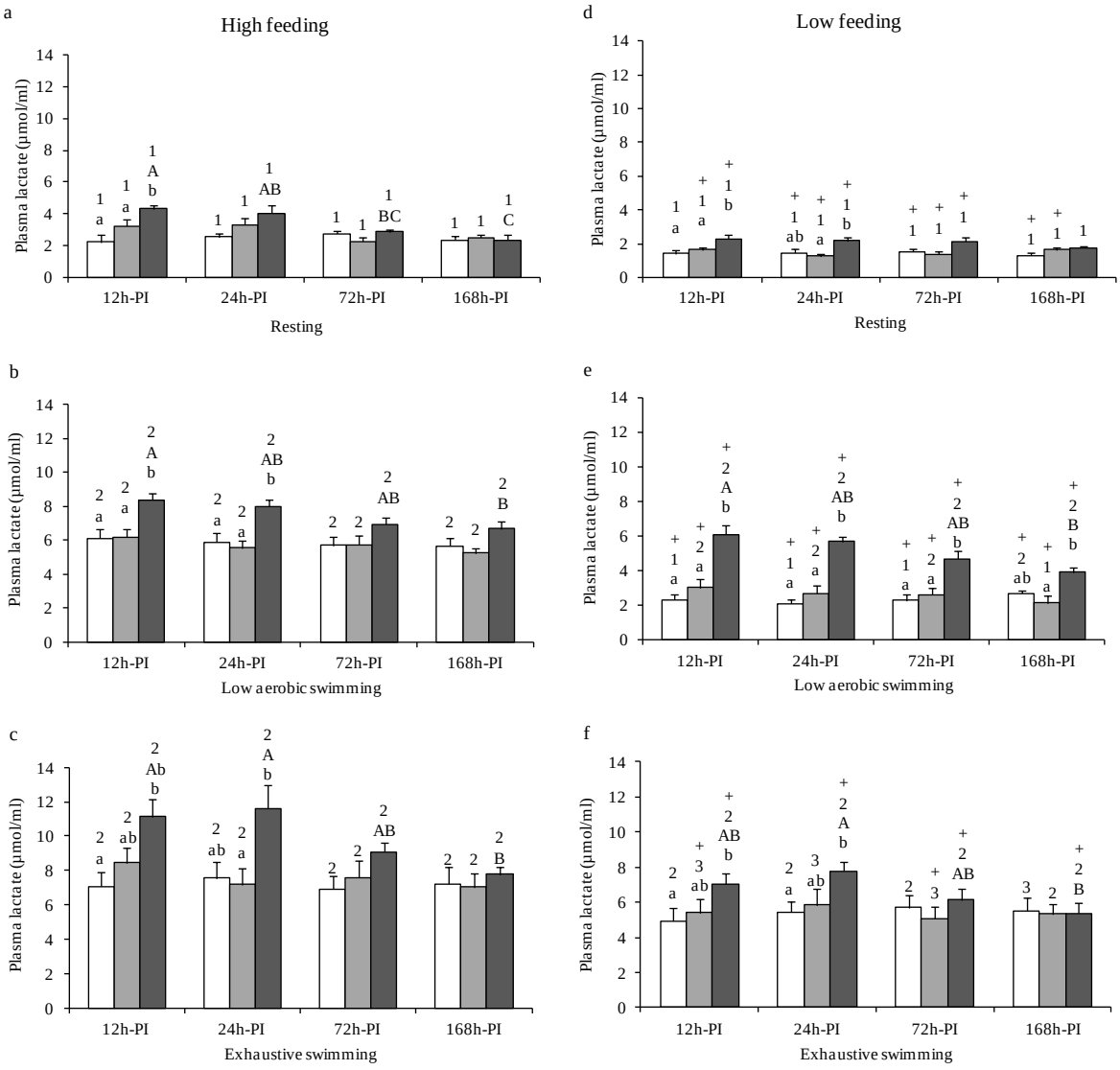

Fig. 8 Plasma lactate levels of control carp without implants (open bars), sham implanted carp (light grey bars) and cortisol implanted carp (dark grey bars) at different feeding and swimming regimes at different post-implant intervals (hours post-implant, h-PI). A plus ($^{+}$) indicates significant differences between feeding regimes. Numeric numbers indicate significant differences between swimming regimes. Small letters indicate significant differences between cortisol treatments. Capital letters indicate significant differences between time intervals.

3.5.3 Plasma total ammonia

Both feeding and swimming played a major role in increasing plasma ammonia (T_{amm}) levels. Feeding led to high plasma T_{amm} accumulation in the high feeding fish either swum at low aerobic speeds or swum to exhaustion (Fig. 9), except in sham implanted fish swum to exhaustion (Fig. 9f). A significant interaction was detected between feeding and swimming. In resting fish, the feeding effect was only detected in cortisol implanted fish (Fig. 9a and 9d). Exhaustive swimming highly induced plasma T_{amm} in all groups of both feeding regimes compared to resting and low aerobic swimming fish (Fig. 9). On the other hand, cortisol implants clearly reduced plasma T_{amm} accumulation compared to control and sham implanted fish when fish were swum to exhaustion in both feeding regimes (Fig. 9c and 9f). A significant interaction between swimming and cortisol implant was recorded. The effect of cortisol promoting ammonia efflux (Fig. 2) leading to lower plasma ammonia accumulation (Fig 9c and 9f) began at 12h-PI and continued to 72h-PI for high feeding fish and 24h-PI for low feeding fish, similar to the trend seen for cortisol (Fig. 1). Conversely, high plasma T_{amm} accumulation was observed in cortisol implanted fish swum at low aerobic speeds for the first 72h-PI (Fig. 9e) and a similar effect was observed in resting high feeding fish for the first 24h-PI (Fig. 9c). No cortisol effect was noticed for the high feeding fish swum at low aerobic speeds (Fig. 9b) and resting low feeding fish (Fig. 9d). Overall, feeding, swimming and implantation highly interacted.

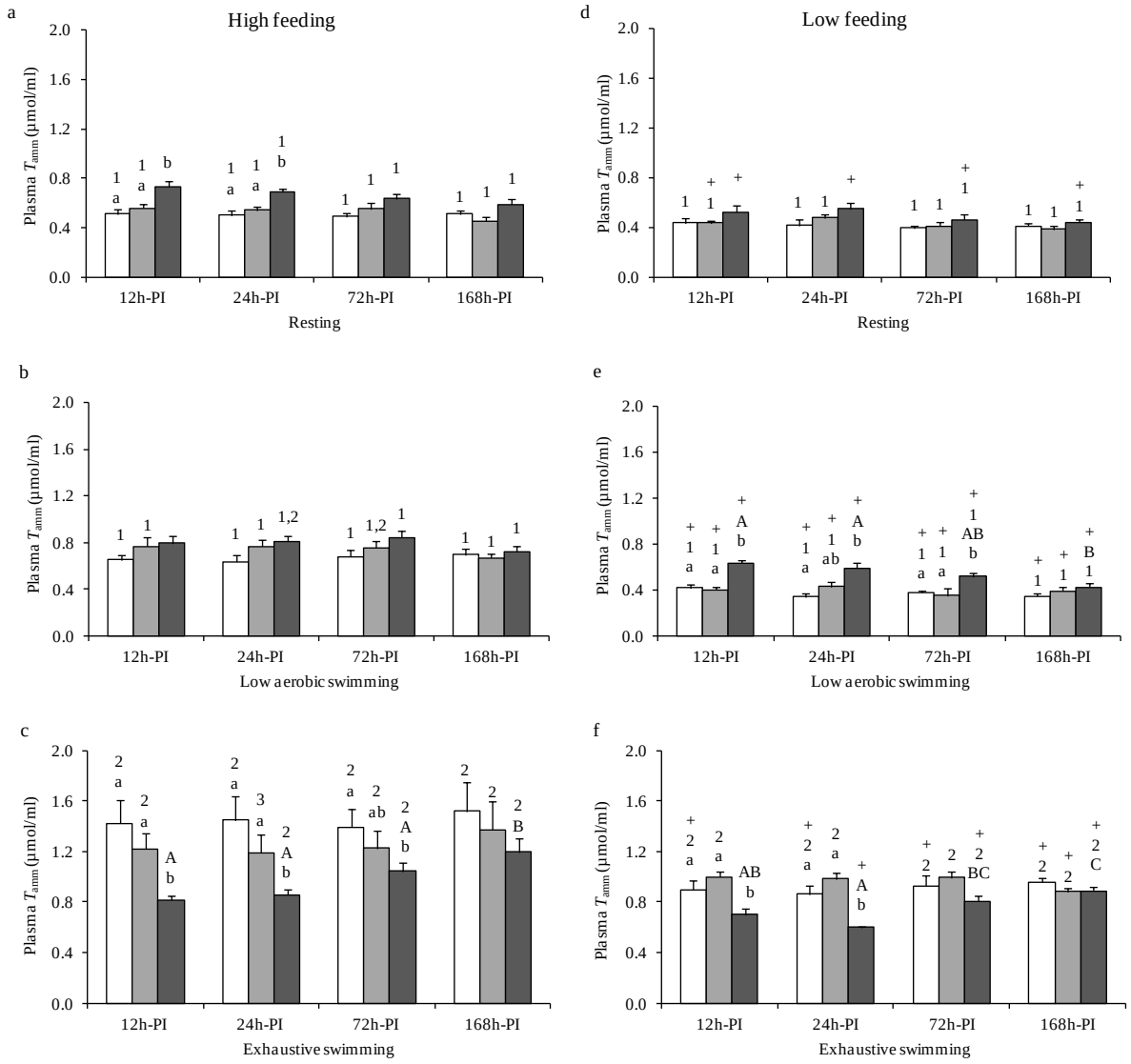

Fig. 9 Plasma total ammonia accumulation of control carp without implants (open bars), sham implanted carp (light grey bars) and cortisol implanted carp (dark grey bars) at different feeding and swimming regimes at different post-implant intervals (hours post-implant, h-PI). A plus ($^{+}$) indicates significant differences between feeding regimes. Numbers indicate significant differences between swimming regimes. Small letters indicate significant differences between cortisol treatments. Capital letters indicate significant differences between time intervals.

3.5.4 Plasma Urea

Overall, feeding stimulated urea production in all high feeding groups (Fig. 10a-c) and in some exhaustive swimming fish where low feeding fish tended to show an increase in plasma urea level (Fig. 10f). Others effect were much smaller. Swimming introduced a few significant but inconsistent changes and the effect of cortisol was only observed at 12h-PI of low feeding resting and low aerobic swimming fish (Fig. 10d and 10e). Nevertheless, a significant interaction was noticed between feeding and swimming; and between feeding and cortisol implantation.

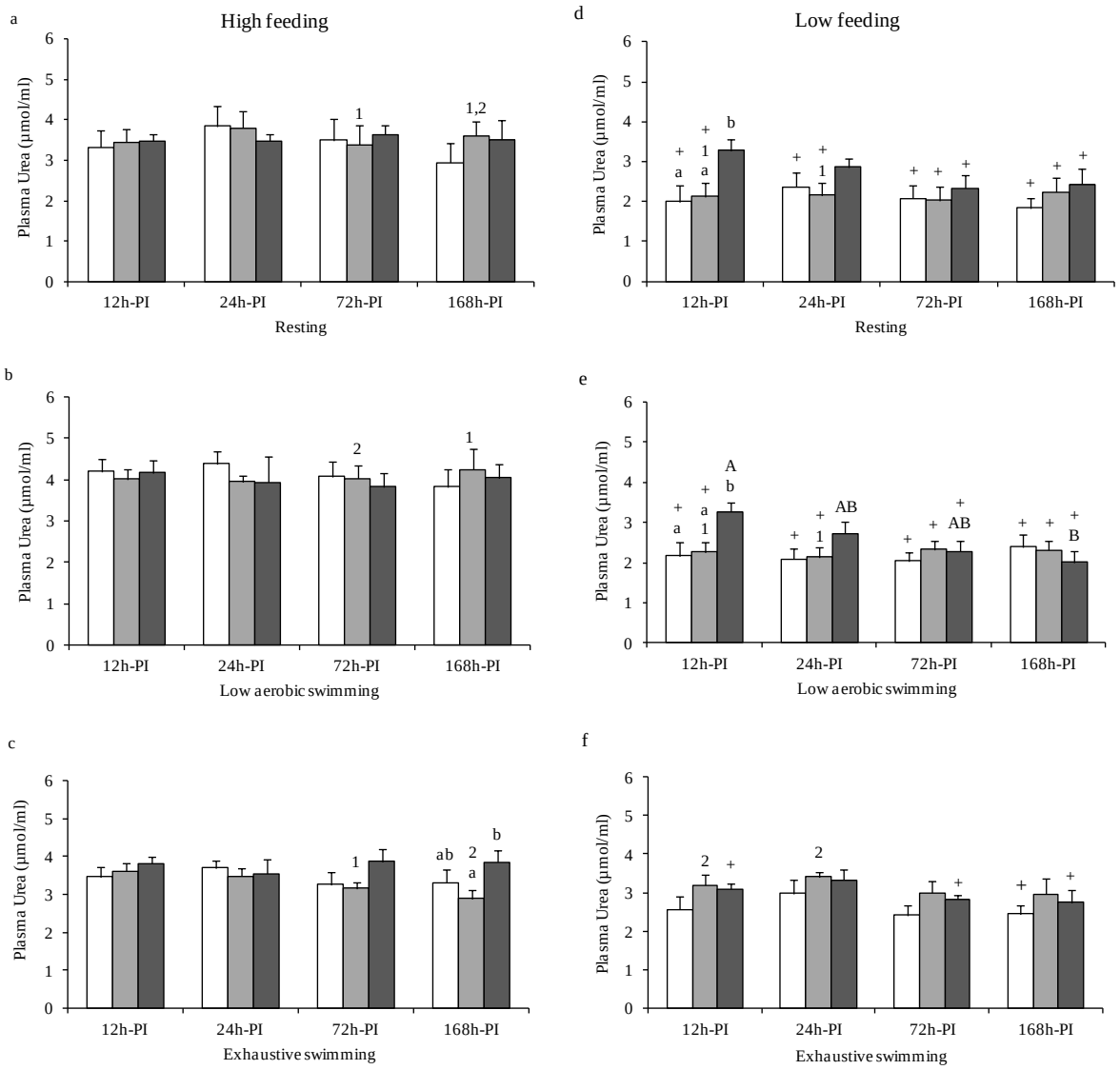

Fig. 10 Plasma urea levels of control carp without implants (open bars), sham implanted carp (light grey bars) and cortisol implanted carp (dark grey bars) at different feeding and swimming regimes at different post-implant intervals (hours post-implant, h-PI). A plus ($^{+}$) indicates significant differences between feeding regimes. Numbers indicate significant differences between swimming regimes. Small letters indicate significant differences between cortisol treatments. Capital letters indicate significant differences between time intervals.

3.6 Energy budgets

3.6.1 Liver and muscle protein

Feeding influenced liver protein content and the liver protein levels were about 2-fold lower in all groups of the low feeding regimes (Fig.11). No swimming effect was observed for the high feeding regime (Fig. 11c). For the low feeding, a significant mobilization of protein was noticed in the fish swimming to exhaustion (Fig. 11f). With this, the interaction between feeding and swimming was significant (P = 0.031). Clearly, fish implanted with cortisol mobilized liver protein to a much larger extent compared to control and sham implanted fish for all groups throughout the entire experiment period. However, this effect started to wear off in 168h-PI high feeding fish at exhaustive

swimming (Fig. 11c) and all low feeding fish at 168h-PI (Fig. 11d-11f). A significant interaction effect was detected between swimming and implantation; and between implantation and time interval.

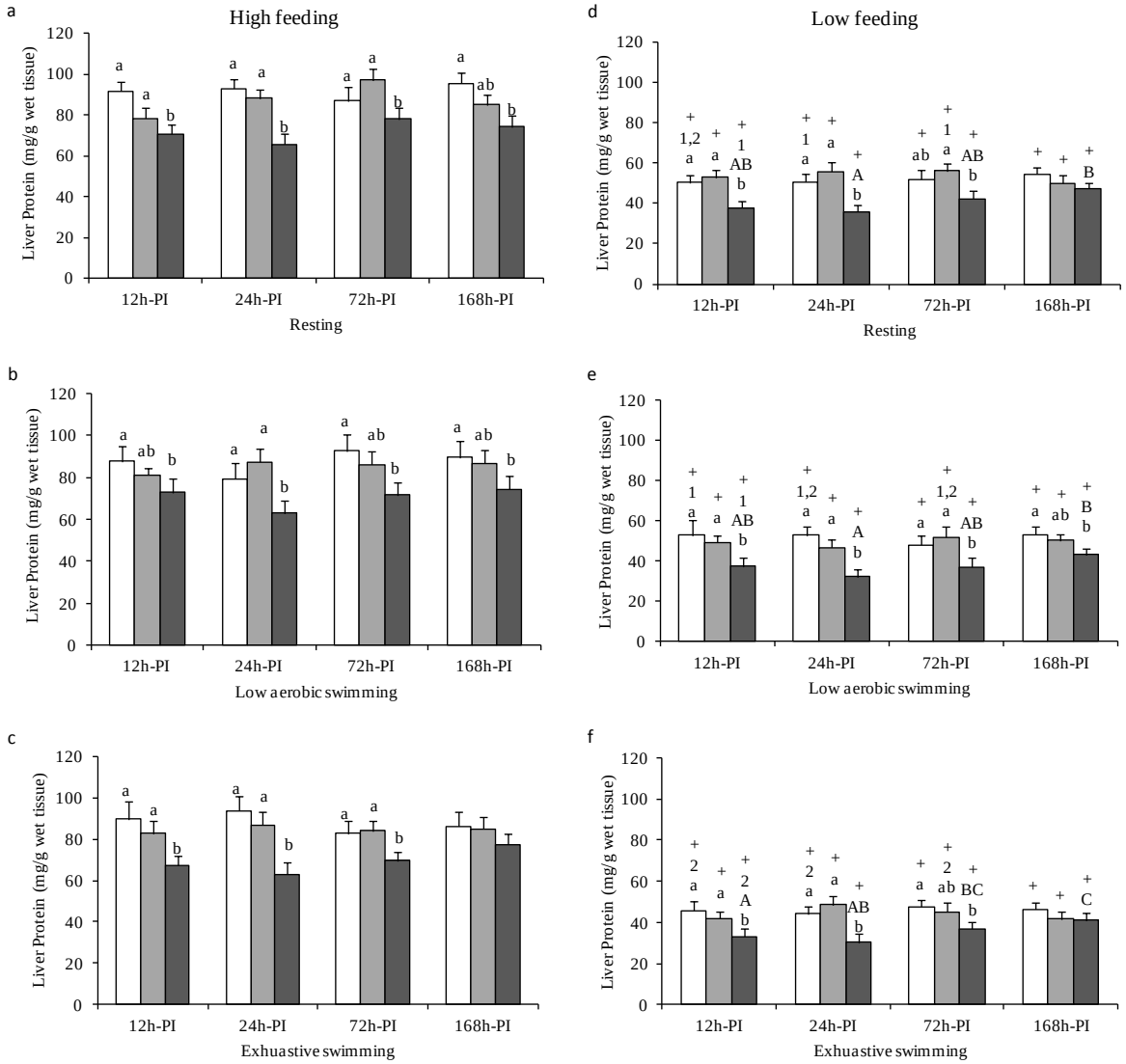

Fig. 11 Liver protein of control carp without implants (open bars), sham implanted carp (light grey bars) and cortisol implanted carp (dark grey bars) at different feeding and swimming regimes at different post-implant intervals (hours post-implant, h-PI). A plus (+) indicates significant differences between feeding regimes. Numbers indicate significant differences between swimming regimes. Small letters indicate significant differences between cortisol treatments. Capital letters indicate significant differences between time intervals.

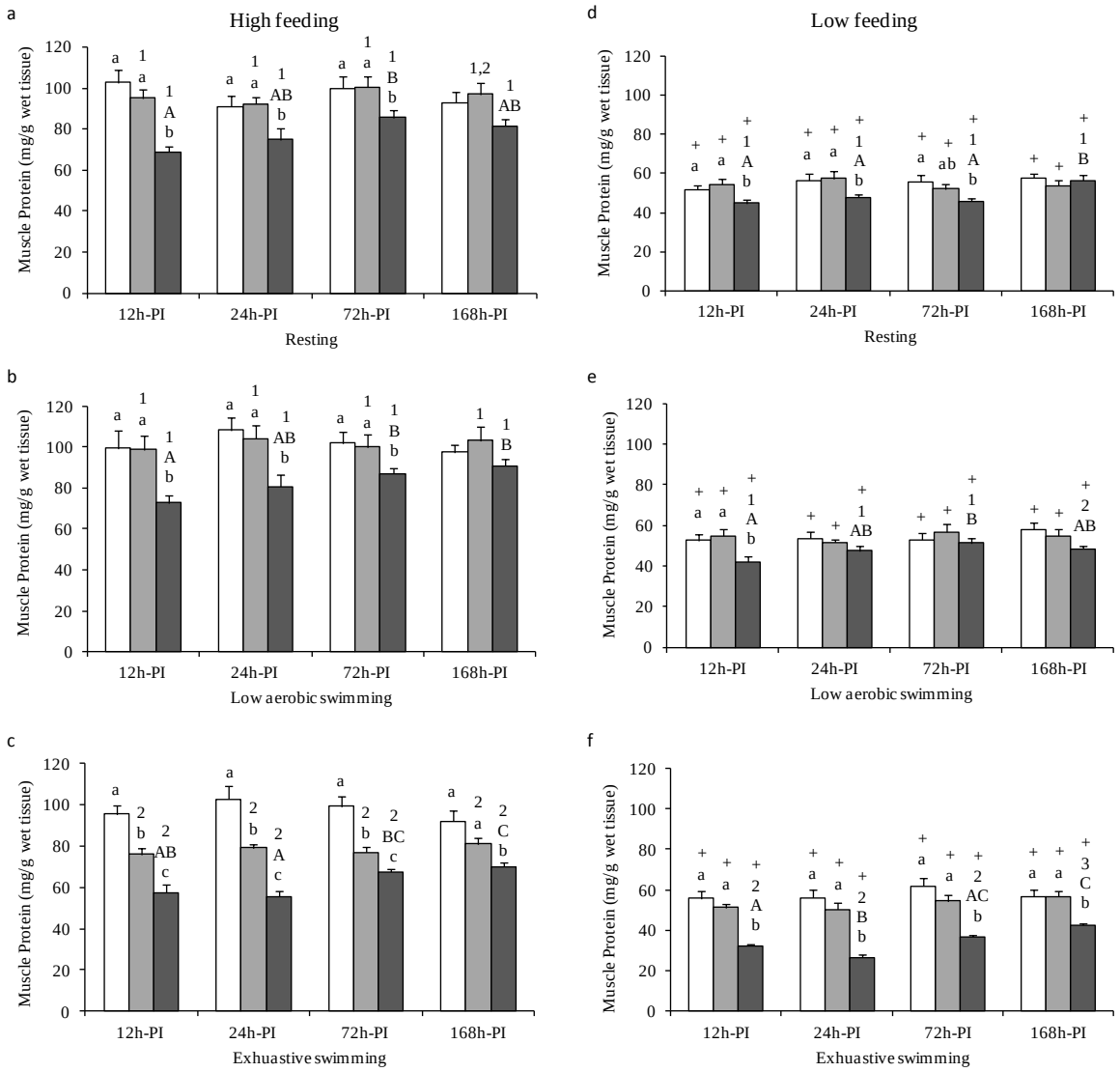

Fig. 12 Muscle protein of control carp without implants (open bars), sham implanted carp (light grey bars) and cortisol implanted carp (dark grey bars) at different feeding and swimming regimes at different post-implant intervals (hours post-implant, h-PI). A plus ($^{+}$) indicates significant differences between feeding regimes. Numbers indicate significant differences between swimming regimes. Small letters indicate significant differences between cortisol treatments. Capital letters indicate significant differences between time intervals.

Similar with the liver protein, feeding played a significant role in muscle protein content which was about 2-folds higher in the high feeding fish compared to the low feeding fish (Fig. 12). Mobilization was highly significant when fish were implanted with cortisol. For high feeding fish, the swimming effect was absent for the control fish, however a swimming effect was observed in sham and cortisol implanted fish with a large muscle protein mobilization at exhaustive swimming throughout the whole experiment period (Fig. 12c). For low feeding fish, the swimming effect was only found in cortisol implanted fish swum to exhaustion (Fig. 12f). In general, cortisol reduced muscle protein content independent of the feeding and/or swimming regimes with significant interactions between feeding, swimming and implantation (and between feeding and swimming; swimming and implantation; implantation and time interval; feeding, swimming and implantation).

3.6.2 Liver and muscle glycogen

As demonstrated in Fig.13, feeding increased liver glycogen content and all high feeding groups displayed a significant 2-fold higher glycogen level than low feeding groups. In addition, liver glycogen mobilization was shown in all groups of fish at exhaustive swimming compared to resting and low aerobic swimming fish (feeding and swimming significantly interacted; Fig. 13c and 13f). In contrast to liver protein (Fig. 11), cortisol implantation enhanced liver glycogen deposition both in the high and low feeding regime (Fig. 13). Overall, higher glycogen levels were noticed at the first 72h-PI in both feeding regimes. This led to a highly significant interaction between feeding, swimming and implantation (and between feeding, swimming and implantation; swimming, implantation and time interval).

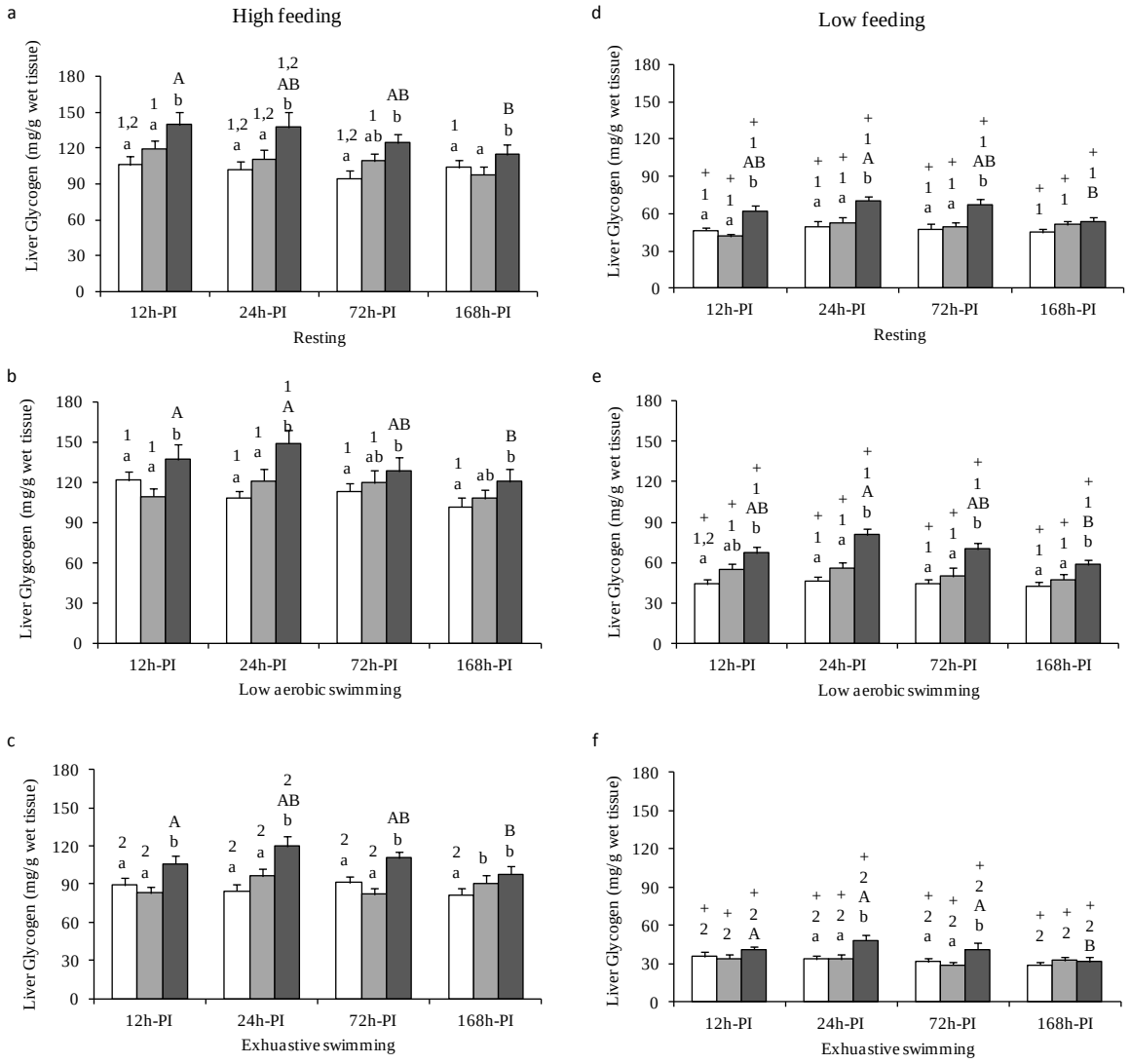

Fig. 13 Liver glycogen of control carp without implants (open bars), sham implanted carp (light grey bars) and cortisol implanted carp (dark grey bars) at different feeding and swimming regimes at different post-implant intervals (hours post-implant, h-PI). A plus ($^{+}$) indicates significant differences between feeding regimes. Numbers indicate significant differences between swimming regimes. Small letters indicate significant differences between cortisol treatments. Capital letters indicate significant differences between time intervals.

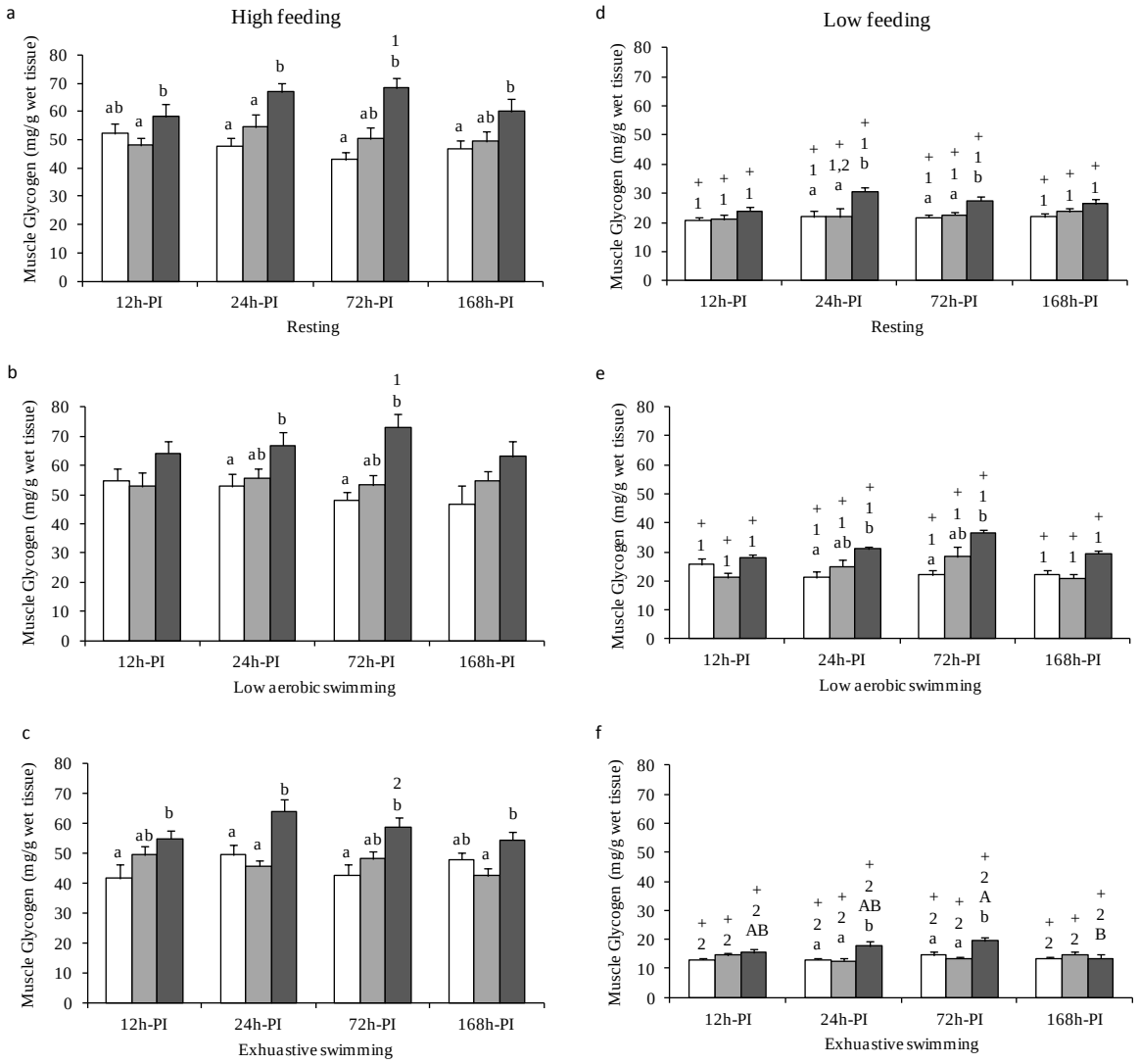

Fig. 14 Muscle glycogen of control carp without implants (open bars), sham implanted carp (light grey bars) and cortisol implanted carp (dark grey bars) at different feeding and swimming regimes at different post-implant intervals (hours post-implant, h-PI). A plus ($^{+}$) indicates significant differences between feeding regimes. Numbers indicate significant differences between swimming regimes. Small letters indicate significant differences between cortisol treatments. Capital letters indicate significant differences between time intervals.

A similar feeding effect as in liver glycogen was also noticed in muscle glycogen content (Fig. 14). Overall, the high feeding fish maintained muscle glycogen content about 3 to 4-fold higher than the low feeding fish. No obvious swimming effect was discovered for high feeding regime, only 72h-PI cortisol fish swum to exhaustion have lower muscle glycogen levels than the resting or low aerobic swimming fish (Fig. 14a-14c). Nevertheless, swimming played a significant role in the low feeding regime, where exhaustive swimming significantly mobilized muscle glycogen in all groups compared to resting and low aerobic swimming fish (Fig. 14f). Interaction effect was significant between feeding and swimming. Like for liver glycogen, cortisol implants led to a significant build-up of muscle glycogen for both feeding regimes, especially at 24h-PI and 72h-PI under resting (Fig. 14a-14b) and low aerobic swimming conditions (Fig. 14c-14d). Therefore, there was only an interaction effect between implantation and time interval.

3.6.3 Liver and muscle lipid

Liver lipid content was relatively constant without any influences by either swimming or cortisol implantation (Fig. 15). The only effect detected was a significant liver lipid mobilization in low feeding fish implanted with cortisol for all swimming conditions (interaction between feeding and implantation was significant; Fig. 15d-15f). Additionally, low liver lipid levels were also noticed in sham implanted fish at 72h-PI swum to exhaustion (Fig. 15f); and at 24h-PI and 72h-PI of resting fish (Fig. 15d); and 72h of control fish for all swimming conditions in low feeding fish (Fig. 15d-15f).

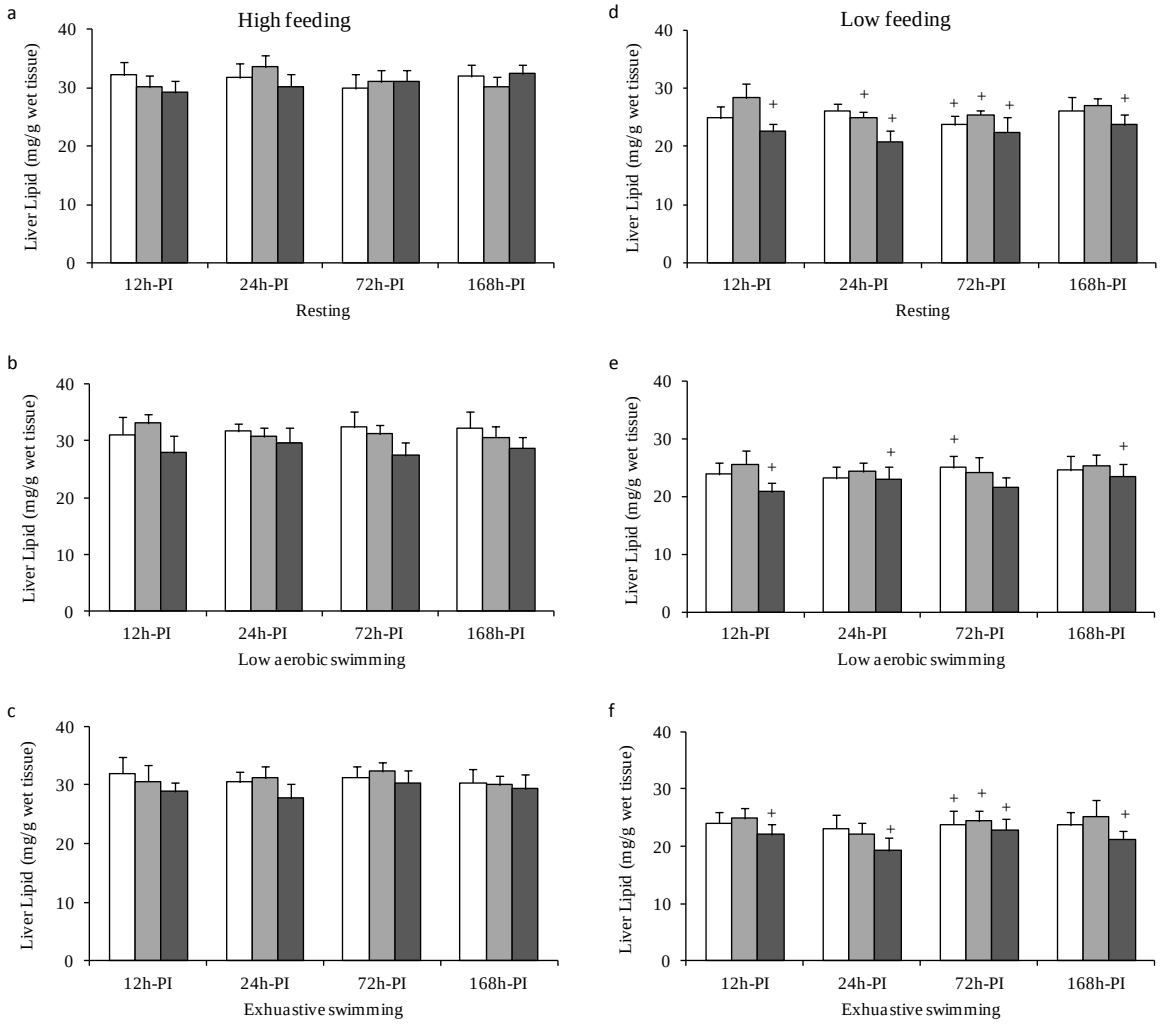

Fig. 15 Liver lipid of control carp without implants (open bars), sham implanted carp (light grey bars) and cortisol implanted carp (dark grey bars) at different feeding and swimming regimes at different post-implant intervals (hours post-implant, h-PI). A plus ($^{+}$) indicates significant differences between feeding regimes.

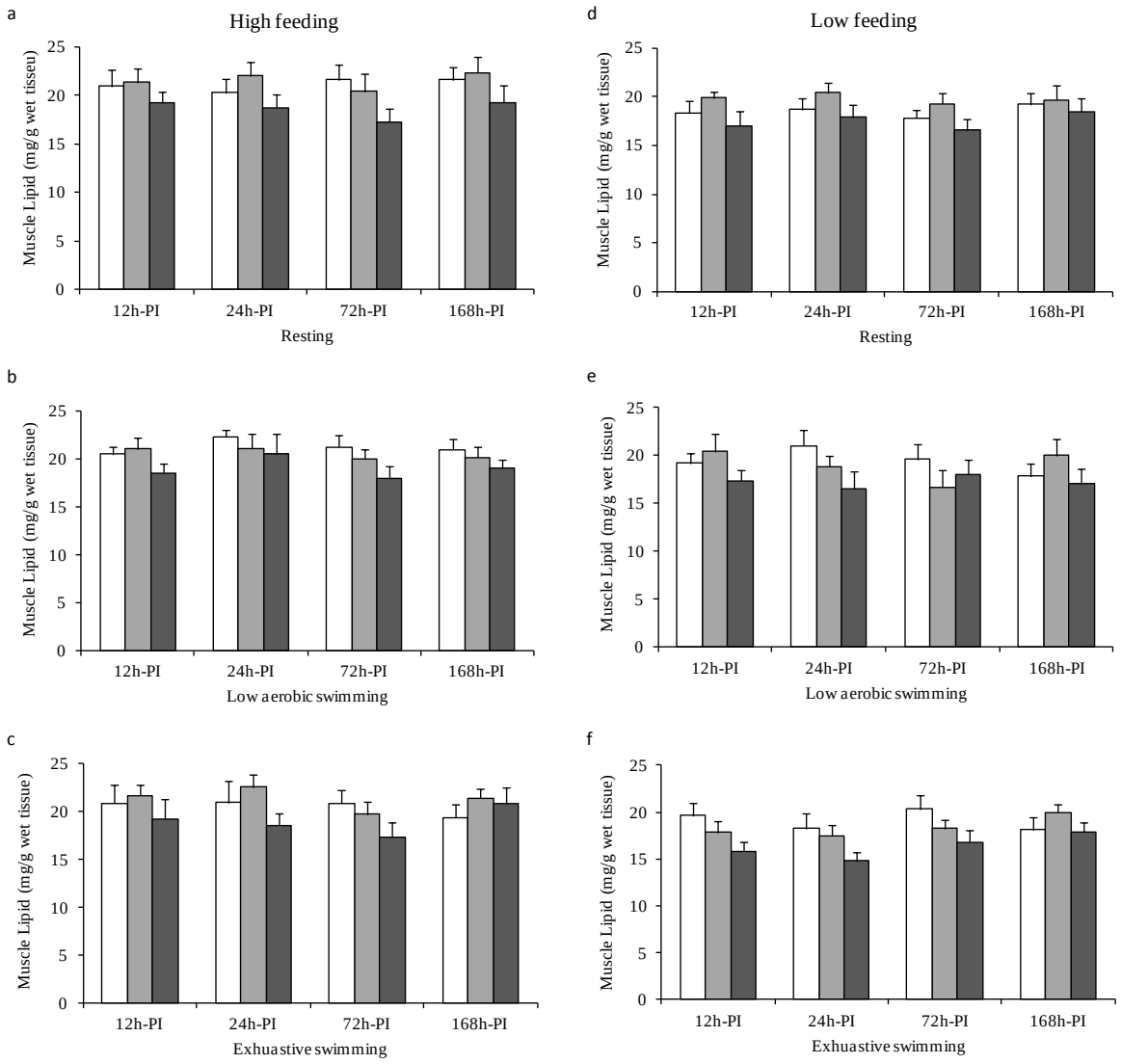

Fig. 16 Muscle lipid of control carp without implants (open bars), sham implanted carp (light grey bars) and cortisol implanted carp (dark grey bars) at different feeding and swimming regimes at different post-implant intervals (hours post-implant, h-PI).

Muscle lipid content remained relatively stable and was not affected by either feeding, swimming or cortisol effects (Fig. 16). Overall, muscle lipid content in the low feeding regime was slightly lower in fish implanted with cortisol, but this mobilization was not significantly different (Fig. 16). Therefore, no interaction was observed.

4. Discussion

4.1 Swimming and respiration

Once again, our results showed that feeding alone had no effect on swimming performance of common carp either fed at 0.5% or 3.0% BW. This is in agreement with our previous study which showed that swimming performance of common carp was not affected in fish that were either starved for a week or were fed at 2% BW (Liew et al., 2012). In fact, both digestion and swimming were performed independently in fed carp by increasing $\dot{M}O_2$ to sustain both metabolisms (Liew et al., 2012). Also the present results showed that all fish that were subjected to high swimming levels experienced an increase in $\dot{M}O_2$ and the oxygen consumption rates were greater in high feeding fish compared to the low feeding fish. This illustrated that extra oxygen was used in high feeding fish when swimming to sustain both SDA and swimming metabolism simultaneously. A similar response was reported in rainbow trout fed at different rations (Gregory and Wood, 1998); in darkbarbel catfish and also another closely related family member the grass carp (Fu et al., 2009), where SDA metabolism was maintained while extra oxygen was consumed for swimming.

Since swimming is a key trait for survival and is a predominant behavioural reaction to stress or predators, the elevation of cortisol as a primary stress response should not impair swimming capacity in fish (Peake et al., 1997). Despite the fact that cortisol elevation had deleterious effects on feeding behaviour and growth, chronic cortisol elevation had no effect on the swimming performance of rainbow trout (Gregory and Wood, 1999). Peake et al. (1997) also reported that handling stress induced cortisol elevation, but had no effect on the U_{crit} value of juvenile rainbow trout. This supports our results that elevated cortisol level did not impair swimming performance of common carp. In addition to feeding and swimming, cortisol also induced increases in $\dot{M}O_2$. This increase in $\dot{M}O_2$ was therefore not used to sustain aerobic metabolism for digestion and swimming but a consequence of the cortisol response. However, this induction was just a short lived, transient effect for the first 24h-PI in high feeding and 12h-PI in low feeding fish. It is known that stress induces hyperventilation and simultaneous increases in $\dot{M}O_2$ (Alvaenga and Volpato, 1995; Morgan and Iwana, 1996; De Boeck et al, 2001; Brown et al., 2005).

4.2 Metabolic trade-off

Overall, high feeding fish excreted a greater volume of ammonia and urea, and had greater AQ values than low feeding fish. This effect was strengthened by high aerobic swimming, undoubtedly demonstrating the importance of the protein catabolism. Feeding induces the use of protein during aerobic metabolism which was also observed in rainbow trout (Kajimura et al., 2004). Additionally, hypercortisol augmented the use of protein showing stress induced protein oxidation in carp. This was consistent with the remarkable liver and muscle protein mobilisation indicating the use of protein from dietary breakdown in high feeding fish and protein catabolism in low feeding fish during swimming. Chronic cortisol elevation leads to peripheral protein catabolism, altered plasma free amino acid levels and increased amino acid metabolism via muscle proteolysis (Anderson et al., 1991; van der Boon et al., 1991; Vijayan et al., 1991; Milligan, 1997). Cadmium exposure accelerated amino acid catabolism in common carp by increased liver transaminase activity (de la Torre et al., 1999) and liver protease activity to generate free amino acids (De Smet and Blust, 2001), which were broken down via deamination and converted into energetic compounds (Stone et al., 2003). Davison

and Goldspink (1978) also found that muscle protein in brown trout dropped up to 30% of its initial content when swum at 4.5 BL/s. This protein breakdown would lead to an increase of plasma ammonia (Kaushik and Teles, 1985) and subsequently increased ammonia excretion rate in teleosts (Leung et al., 1999).

Parallel with endogenous ammonia elevation, cortisol seemed to enhance branchial ammonia clearance rates in both feeding regimes during high aerobic swimming leading to an improved ammonia excretion capacity. This branchial ammonia clearance and excretion rate sufficiently reduced plasma T_{amm} below the level observed in control or sham implanted fish. This increased excretion capacity was more pronounced in carp fed a high ration and swimming at high speeds. A combination of hyperventilation and synergistic induction of ammonia transporter Rhesus glycoproteins by both feeding and cortisol elevation could have played an important role here to enhance branchial ammonia excretion rate (Wright and Wood, 2009; Zimmer et al, 2010). Chan and Woo (1978) also recorded an increment of 48% in branchial ammonia excretion in eel implanted with cortisol; and McDonald and Wood (2004) found that ammonia excretion was significantly increased in cortisol implanted rainbow trout after the first 12h-PI. Nevertheless, this improvement in excretion capacity was not only limited to ammonia but also induced urea excretion was improved in the present study. Even though overall high feeding carp excreted a greater volume of urea than low feeding carp, the level of urea in plasma of high feeding fish still remained higher. The increase of plasma urea level and excretion provides additional evidence for the increased use of protein during stress in common carp. Cortisol plays a significant role in regulating urea production (Vijayan et al., 1996; Mommsen et al., 1999; McDonald and Wood, 2004), and rainbow trout treated with cortisol had plasma urea levels that were 3-folds higher than in sham implanted fish, while accomplishing a 2 to 3-fold greater branchial and urinary urea excretion rate (McDonald and Wood, 2004).

Cortisol induced hyperglycemia is the most pronounced effect throughout the entire experimental period. Plasma glucose elevation is believed to be due to increase of gluconeogenesis from liver and muscle protein. Plasma cortisol level elevation caused by both acute and chronic stress is commonly accompanied with plasma glucose level elevation in teleosts (Kubokawa et al., 1999; Correa et al., 2003). Stress induced hyperglycemia in carp has been reported due to prolonged overcrowding (Yin et al., 1995) and hypoxia (van Raaij et al., 1996; Pottinger, 1998). Another possible cause of plasma glucose elevation during stress could be due to decrease of glucose utilisation by tissues in cortisol implanted fish (De Boeck et al., 2001), however this seems unlikely as both aerobic and anaerobic energy metabolism were increased. The present study shows that hypercortisol also induced anaerobic metabolism with higher plasma lactate levels detected in fish swum to exhaustion. This illustrates that carp were able switch to anaerobic metabolism to access extra fuel from glycogen to sustain the increasing energy demand for swimming. This was proven by liver glycogen mobilisation rather than muscle glycogen in low feeding fish, whereas high feeding fish seemed to obtain sufficient carbohydrate supplied from dietary intake.

4.3 Tissue metabolites

The present results clearly showed that hypercortisol induced liver and muscle protein mobilisation to fuel gluconeogenesis, even when fish were resting. Together with feeding, swimming and hypercortisol all induced MO_2 and ammonia excretion, resulting in increases of AQ, which directly reflects the contribution of protein as a priority energy source. The use of protein in carp as

a major fuel during aerobic metabolism and food deprivation was recently noted (Liew et al., 2012). Protein is well known to play a central role in the energy production during stress and aerobic and/or endurance swimming to reallocate energy use from growth to other priority metabolic needs (Mommsen et al., 1980; Sumpter, 1992; Milligan, 1997; Ferrari et al., 2011). Other species also demonstrated a rapid muscle amino acid metabolism following exhaustive swimming altered by cortisol elevation in sockeye salmon (Mommsen et al., 1980); Arctic char (Barton et al., 1995); and rainbow trout (Milligan, 1997). Van den Thillart (1986) found that rainbow trout swum at 80% of their U_{crit} for 3 h acquired approximately 90% of their energy from amino acid oxidation. However, cortisol induced protein catabolism is highly variable either inter-or-intra species-specific (Foster and Moon, 1986; Mommsen et al., 1999) and determined by nutritional availability of amino acids for gluconeogenesis (Vijayan and Leatherland, 1989; Wood, 2001). This was confirmed with the increase of hepatic alanine gluconeogenesis in rainbow trout (Vijayan et al., 1993) and amino acid catabolism in sea raven (Vijayan et al., 1996) during elevated cortisol levels. Increase of protein mobilization coupled with high levels of free amino acids in plasma as a result of an increase of plasma cortisol level was observed in rainbow trout swum to exhaustion (Milligan, 1997).

On the other hand, feeding also determines glycogen availability with large glycogen depositions in both liver and muscle in high feeding fish. It seems that excess protein from dietary intake and protein catabolism induced by hypercortisol was also channelled towards glycogenesis. Although, carp were utilized protein as primary fuel during aerobic metabolism, glycogen was also being mobilised simultaneously when fish were swum to exhaustion. The use of glycogen was confirmed by an elevation of plasma lactate and the depletion of glycogen reserves even under low aerobic swimming. However, this easy switch to anaerobic metabolism did not cause a severe depletion of glycogen reserves. Only low feeding fish swum to exhaustion seemed to mobilise liver glycogen as a readily accessible fuel to sustain swimming metabolism in a substantial way. The use of glycogen in carp has been reported previously when challenged with exhaustive swimming (Liew et al., 2012), salinity (De Boeck et al., 2001) and copper exposure in gibel carp (De Boeck et al., 2010).

Nevertheless, the effect of cortisol on glycogen metabolism is also species-specific and depends on the degree of stress (van der Boon et al., 1991; Mommsen et al., 1999; Laiz-Carrion et al., 2003). Our study showed that liver glycogenesis in carp implanted with cortisol is likely associated with a preparation for any prolonged stress to ensure that accessible energy is available. This strategy was recorded in tilapia implanted with cortisol, which induced increased amino acid mobilization and enhanced hepatic gluconeogenesis capacity for hepatic glycogen synthesis (Vijayan et al., 1997). A similar hepatic glycogenesis induced by cortisol implants was observed previously in rainbow trout (Hill and Fromm, 1968) and eel 9h-PI (Chan and Wood, 1978). Moreover plasma lactate elevation could be an additional contributor to the glycogen deposition via glyconeogenesis stimulated by hypercortisol. Lactate had been reported as an important substrate for gluconeogenesis under high cortisol (Suarez and Mommsen, 1987; Mommsen et al., 1992), and subsequently induced hyperglycemia (De Boeck et al., 2001). Fasted killifish receiving cortisol injections 5 times daily at 20 μg cortisol/g BW showed a serum glucose elevation that subsequently promoted liver glycogenesis (Leach and Taylor, 1982). Active gluconeogenesis from amino acid and lactate induced hyperglycemia contributed to the adenosine triphosphate and phosphocreatine restoration; and muscle glycogenesis in carp after exhaustive swimming (Sugita et al., 2000).

Furthermore, hyperglycemia in cortisol implanted fish could also be due to breakdown of non-protein sources such as triglycerides. The use of glucose and free fatty acids as major energy during confinement has been shown in common carp (Ruane et al., 2001) and depended on food intake

(Ruane et al., 2002). Cortisol induced hepatic glycerol kinase and fructose biphosphatase activity to enhance the gluconeogenic potential from glycerol was previously discovered in brook charr implanted with cortisol (Vijayan and Leatherland, 1989; Vijayan et al., 1991; Vijayan et al., 1993). Although, lipid mobilisation in the present study was not obvious, the use of lipid in this scenario could be fuelled by a contribution from dietary intake for high feeding fish and some contribution from liver in the low feeding fish. A similar response has been observed in our previous study which showed that carp fasted for a week had a lower lipid level than fed fish (Liew et al., 2012).

5. Conclusion

As a conclusion, this study answered our first question and showed that neither feeding nor cortisol elevation have an affect on swimming performance of common carp; while both digestion and swimming metabolisms were performed independently. Feeding, swimming and cortisol elevation not only promoted aerobic metabolism and induced protein mobilisation but also induced anaerobic metabolism with lactate accumulation. Hypercortisol clearly enhanced branchial ammonia clearance rate when swimming, which resulted in lower plasma ammonia accumulation in cortisol implanted fish when swum to exhaustion. Our second hypothesis proved only partly true, high cortisol levels did lead to extra energy expenditure, increased MO_2 and hyperglycemia, but due to the increased ammonia clearance rates, plasma ammonia levels actually decreased and therefore swimming performance was not impaired. Hypercortisol induced protein mobilization for gluconeogenesis and hyperglycemia to promote glycogenesis. Protein remained the primary energy source mobilised during aerobic metabolism and hypercortisol. Glycogen was mainly being used at exhaustive swimming to sustain anaerobic metabolism especially when dietary intake was limited. Our last hypothesis, that fish on a lower feeding ration would be more affected, was only partly confirmed, with lower lover and muscle protein and glycogen levels in most treatments and lower liver lipid levels in cortisol treated fish on a low feeding level.

Chapter 4

Ion homeostasis in common carp, *Cyprinus carpio*: interactive effects of cortisol, feeding and swimming

Comparative Biochemistry and Physiology, Part A
(*In Preparation*)

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Abstract

The interactive effects of cortisol, feeding and exercise on ionoregulation in common carp were investigated. Fish subjected to different feeding regimes (0.5 or 3.0% body weight (BW) daily) received no implant or an intraperitoneal sham-or-cortisol implant (250 mg/kg BW) and were monitored over a 168h post-implant period under resting, low aerobic swimming or exhaustive swimming conditions. Plasma osmolality was maintained at relatively stable levels without much influence of feeding, swimming or cortisol, especially in low feeding groups. A transient hyponatremia was observed in all low feeding fish implanted with cortisol. This was more pronounced in fish swum to exhaustion but even in this group Na^+ levels returned to control levels as cortisol levels recovered (168h-PI). Cortisol implanted fish also had lower plasma Cl^- level, and the loss of plasma Cl^- was more prominent in exhaustive swimming fish fed a high ration (recovered at 168h-PI). Cortisol provoked gill Na^+/K^+ ATPase (NKA) and H^+ ATPase activities in high feeding fish, probably associated with ammonia efflux. In contrast, low feeding fish upregulated kidney NKA and H^+ ATPase activities when experiencing hypercortisol.

Keywords: Fatigue, feeding, fasting, ionoregulation, energy metabolism, plasma ion, gill, kidney, swimming, U_{crit}

1. Introduction

Feeding and swimming are both keys for growth and survival in the aquatic animal kingdom. The impact of feeding on post-prandial ionic and water dynamics has received considerable attention in today's research (Bucking and Wood, 2006a; 2006b; Bucking et al., 2010; Bucking et al., 2011; Bucking and Wood, 2012). These authors found that feeding granted clear-cut benefits in maintaining ion homeostasis that were absent in fasting animals (Bucking and Wood, 2012). Swimming on the other hand induces increased ventilation rates resulting in an increased volume of fast flowing water over the gill lamellae to enhance gas exchange and excretion of endogenous nitrogen waste (Jones and Randall, 1978). Additionally, enhanced functional surface area of gills facilitates both gas and ion exchange (Gallaugher et al., 2001). The increased contact between gill lamellae and the hypoosmotic environment, possibly concurrent with an increment of gill permeability, may induce additional ion losses. In order to maintain ion homeostasis, freshwater fish developed a strategy called the 'osmorespiratory compromise' which includes increasing the gill functional respiratory surface area for gas exchange but limiting ion loss simultaneously during swimming or under hypoxia (Gonzalez and McDonald, 1992; Gonzalez and McDonald, 1994; Postlethwaite and McDonald, 1995; Nilsson, 2007; Wood et al., 2009). Previous work demonstrated that common carp successfully compromise osmorespiration during exhaustive swimming and maintain their plasma Na^+ at a fairly stable level in either fed and unfed fish (Liew et al., 2013a), whilst increasing oxygen consumption and ammonia excretion rates (Liew et al., 2012). It was suggested that the increase of gill Na^+/K^+ ATPase (NKA) activity in fed common carp was associated with ammonia excretion rather than ion uptake per se. In parallel, swimming also induced branchial ammonia clearance in common carp treated with cortisol, but we have no information on the link with gill NKA (Liew et al., 2013b). In unfed carp gill NKA activity remained low, even when carp were swum to exhaustion, and plasma cortisol was maintained at a level similar to carp that were resting or swum at low aerobically sustainable speeds (Liew et al., 2013a). Thus, there seem to be

contrasting strategies between fed and fasted fish when looking at cortisol induction and gill NKA activities. How these unfed fish keep their ion levels in balance without access to ions from food and without increasing gill NKA levels remains to be elucidated.

Moreover, cortisol has been well accepted as an important factor in regulating hydromineral balance in stenohaline or euryhaline teleosts adapted to seawater (Eckert et al., 2001; Chasiotis and Kelly, 2012). There is also a growing evidence that cortisol also plays a significant role in ionoregulation of freshwater fish (Marshall and Grosell, 2005; Babitha and Peter, 2010) and that it affects ion homeostasis (Overli et al., 2002). Cortisol plays a direct role in ionoregulation by upregulating branchial NKA expression and activity (McCormick et al., 2008). This is well characterized in gills of salmonid species, and an elevation of cortisol occurs synchronously with the development of hypo-osmoregulatory mechanisms (Madsen, 1990; Seidelin et al., 1999; Sloman et al., 2001; Zhou et al., 2003) such as chloride cell proliferation and increased branchial NKA activity in tilapia (Dang et al., 2000), channel catfish (Eckert et al., 2001) and North African catfish (Babitha and Peter, 2010). Additionally, the direct regulation of cortisol on hydromineral balance in the intestine has been well examined *in vitro* and *in vivo* in sockeye salmon (Veillette and Young, 2005) and Mozambique tilapia (Takahashi et al., 2006). Nevertheless, the effect of cortisol on kidney ionoregulation still remains to be documented (McCormick et al., 2008; Babitha and Peter, 2010). Furthermore, inconsistencies remain as far as plasma osmolality and Na^+ status is concerned when looking at stenohaline freshwater teleosts treated with cortisol, and studies have shown either an increase, decrease or no change (De Boeck et al., 2001a; Eckert et al., 2001; Babitha and Peter, 2010; Bui et al., 2010; Chasiotis and Kelly, 2012).

With this background information, our present study aimed to investigate the effect of cortisol on ionoregulatory strategies in both gills and kidney of common carp subjected to different feeding and swimming regimes. In order to achieve our aim, carp were conditioned to feeding rates of 3.0% body weight (BW) and 0.5% BW for three weeks, and subsequently implanted with a sham or 250 mg cortisol/kg BW implant and monitored over a168 hours post-implant period (168h-PI). In addition, carp were sampled after resting or after low aerobic or exhaustive swimming. The gill and kidney NKA and H^+ ATPase activities as well as plasma osmolality and electrolytes were measured. A previous study found that carp were able sufficiently adapt their osmorespiratory strategy by increasing gas exchange during active swimming while minimizing branchial ions losses. We concluded that the increase of NKA activity was related to ammonia excretion rather than ion uptake per se, because feeding granted sufficient dietary ion intake (Liew et al., 2013a). Additionally, cortisol induced protein mobilization and remarkably increased branchial ammonia excretion especially when carp were swum exhaustively (Liew et al., 2013b). Therefore we hypothesize that high feeding fish will have higher gill NKA and H^+ ATPase activities. The activity would be greater in cortisol implanted fish due to higher ammonia excretion rates especially in exhausted fish. Since under limited food accessibility, earlier work did not show an induction of gill NKA, we hypothesized that low feeding fish would amplify their kidney NKA and H^+ ATPase activities to a greater extend to allow ion reabsorption and that this activity would intensify when subjected to high cortisol levels.

2. Materials and methods

2.1 Experimental animals

A total of 600 common carp, *Cyprinus carpio* (Viskweekcentrum Valkenswaard, Leende, The Netherlands) average body weight 22.8±0.8 g (BW) and body length 12.9±0.9 cm (BL) were kept in the aquarium facilities of the Systemic Physiological and Ecotoxicological Research at the University of Antwerp, Belgium with Antwerp City tap water in two 1000 L polyethylene tanks for 6 weeks of acclimation. Fish were fed at 1.5% BW with commercial Koi pellets daily (Kyorin Food Ind. Ltd., Japan with nutrition values of ≥35% protein, ≥3% lipid, 5% fibre, 13% ash, 1% phosphorus and 10% mixture of vitamins and minerals) and 80% of the water was renewed weekly. After acclimation, 96 fish were selected, weighed and distributed into 12 aquaria filled with 50 L Antwerp city tap water (≈pH 8.2±0.4) equipped with 6 L external filters for another 3 weeks. Fish were fed either 3.0% BW (high feeding ratio) or 0.5% BW (low feeding ratio). Food was divided equal 2 portions and fish were fed at 08:30 h and 18:30 h daily. Food was introduced slowly at the same corner of aquaria until food was finished within 20 min. About 80% of water was renewed every 3 days with a semi-auto-siphoning procedure to avoid disturbance. Water temperature, NH_3/NH_4^+, NO_2^- and NO_3^- were maintained at 16±0.8° C, <0.1 mg/L, <0.03 mg/L and <25 mg/L with constant photoperiod of 12L:12D, respectively. Aquaria were covered with a black plastic sheet to avoid external visual disturbance. All experimental procedures were approved by the University of Antwerp Animal Care Committee according to the guidelines of the Federation of European Laboratory Animal Science Associations.

2.2 Cortisol implantation

Fish were implanted intraperitoneally with sham coconut oil or with cortisol containing implants 12h post feeding at a ratio of 10 µl/g BW as described by Gregory and Wood (1999); De Boeck et al. (2001a) and McDonald and Wood (2004). Fish were anaesthetized individually in 0.1 g/L Ehty-3-aminobenzoate methanesulfonic acid (MS222) (Acros Organics, Geel, Belgium) neutralized with 1N NaOH to pH 8.0 (Antwerp City tap water pH) and weighed. The cortisol group were implanted with mixture of 25 mg cortisol (Hydrocortisone 21-hemisuccinate; Sigma Diagnostics) in 1 ml of melted coconut oil (≈27^o C; Sigma Diagnostics) resulting an overall dose of 250 mg cortisol per kg of fish. This dose was selected based on our preliminary test showing that this dose significantly elevated baseline cortisol levels and allowed a recovery to baseline levels within a week. Fish implanted with melted coconut oil only served as sham group. All implanted fish were briefly placed on ice for about 30 s to solidify the implant and immediately introduced into well oxygenated water to recover from unconsciousness. A similar procedure was applied to control fish but they received no implant. All groups (control, sham and cortisol) were monitored at 12h, 24h, 72h and 168h-PI with eight fish for each time point.

2.3 Experimental series

All groups were subjected to three swimming conditions: resting, low aerobic swimming (swum at ±¼ U_{crit}; 1.0 BL/s) and exhaustive swimming (U_{crit}). Resting fish were directly sampled from the holding tank served as control without the handling stress of introducing them into the swimming tunnel. For low aerobic and exhaustive swimming fish were introduced into swimming

tunnels 12 h prior measurement allowing them to acclimatize to this environment overnight and to reduce handling stress on the day of measurement. During this acclimatization period, fish were oriented using a water velocity of 10 cm/s with a continuous supply of well oxygenated water at a rate of at 4 L/min. Exhaustive swimming was performed by increasing water velocity at 5 cm/s every 20 min interval until fish fatigued. Fatigue was determined when fish was swept downstream against the mesh screen at the end of tunnel twice within 20 min (Tudorache et al., 2007). For low aerobic swimming, fish were swum at 1.0 BL/s (± ¼ U_{crit} according to the U_{crit} obtained from exhaustive swimming test) value for 3 h.

2.4 Sampling

Fish were carefully removed from the swimming tunnel or holding tank and immediately anaesthetised (neutralized MS222; 0.5 g/L), blotted dry followed by biometric measurement and sampling. Blood was drawn via the caudal puncture using a heparinised syringe (2500 units/ml lithium heparin from Sigma, Munich, Germany) within 1 min. The blood was immediately centrifuged in a heparinised bullet tube (13,200 rpm; 4^{o} C; 1.5 min). Fresh plasma was used for osmolality measurement and the remaining plasma was immediately frozen in liquid N_2 for electrolytes and plasma cortisol measurement. Gill and kidney samples were enveloped in aluminium foil and immediately frozen in liquid N_2 and stored in -80^{o} C for further analysis.

2.5 Plasma metabolites

Plasma osmolality was determined by using The Advanced^{TM} Micro Osmometer (Model 3300, Advanced Instruments, USA). Plasma electrolytes (Na^+, K^+ and Cl^-) were measured by using an AVL 9180 Electrolyte Analyser (AVL, Roche Diagnostics, Belgium). Radioimmunoassay was performed to measure plasma cortisol levels by using a commercial Cortisol ^{125}I RIA kit according to the kit instruction (ImmuChem^{TM}, MP Biomedicals LLC, Orangeburg, NY).

2.6 Gill and kidney ATPase activity

NKA was measured according to the method described by McCormick (1993) and H^+ ATPase activity was measured as described by Lin and Randall (1993) modified by Nawata et al. (2007). Tissues were homogenized with ice cooled 4:1 SEI/SEID buffer solution (150mM sucrose; 10mM EDTA; 50mM imidazole/SEI with 0.1% sodium deoxycholate) and centrifuged (5000 g; 4° C; 1 min). Duplicate 10 µl homogenates were pipetted into a 96-well microplate in four series. A 200 µl mixture assay-A was added to the first series (400U lactate dehydrogenase; 500U pyruvate kinase; 2.8 mM phosphoenolpyruvate; 0.7 mM ATP; 0.22 mM NADH; 50 mM imidazole) and 200 µl mixture assay-B to the second series (mixture assay-A with 0.4 mM ouabain) for NKA measurement. 200 µl of mixture assay-C was added to the third series (mixture assay-B with 500 mM NaN_3) and 200 µl of mixture assay-D to the fourth series (mixture assay-C with 100 mM NEN) for measurement of H^+ ATPase activity. The enzyme activities were measured kinetically with a plate reader ($ELX808_{IU}$ Bio-Tek Instruments Inc. VT, USA) at 340 nm for 30 min at 30 s intervals. An ADP (Adenosine diphosphate) standard curve was used to calculate ATPase activity by subtracting oxidation rate of NADH to NAD in the absence to the presence of ouabain for NKA and NEN for H^+ ATPase. Crude

homogenate protein was determined according to Bradford (1976) using bovine serum albumin as standard curve (US Biochemical, Cleveland, OH, USA).

2.7 Statistical analysis

Normality was checked prior to the analysis by Shapiro-Wilk test. Homogeneity of variance was verified using Hartley test. Log-transformed data were applied if the ANOVA requirements were not fulfilled. Effect of feeding regime was assessed by unpaired two-tail student *t*-test. One way ANOVA were performed to examine significant level of implantation within and between treatments at different time points and swimming conditions followed by multiple comparison of Tukey post-hoc test. Overall, the interactive effects between feeding, swimming and implantation over 168h-PI time point intervals were examined with multifactor analysis of variance (MANOVA) with multiple factorial cross design. The significance level for all tests was set at $P<0.05$. All values are given as mean values with standard error (Mean±SEM; $n=8$).

3. Results

Overall feeding, swimming or sham implants had no significant effect on plasma cortisol levels throughout the entire experiment. Cortisol implants significantly elevated plasma cortisol (551±45 ng/ml) to about 8-fold higher levels than in the control (60±21 ng/ml) and sham implanted (67±21 ng/ml) fish at 12h-PI. The level started to deplete again to about 5-fold higher (317±33 ng/ml) at 24h-PI and continued to drop to levels that were slightly but still significantly higher at 72h-PI (115±20 ng/ml in cortisol implanted fish. At 168h-PI, no significant difference was observed in cortisol implanted fish compared to control or sham implanted fish (68±23 ng/ml compared to 59±16 ng/ml and 63±19 ng/ml, respectively) (Liew et al., 2013b).

3.1 Plasma osmolality

Overall, there were few consistent trends in plasma osmolality (Fig. 1). fish fed a high food ration plasma osmolality was a bit more variable (Fig.1a-c), while in fish fed at the low food ration showed a relative stable plasma osmolality level (Fig. 1c-f). Accordingly, swimming had no effect on plasma osmolality in low feeding fish, but some trends were seen in high feeding fish. In general, exhaustive swimming fish had a plasma osmolality that was higher than the low aerobic swimming and resting fish (Fig. 1c). Control fish swum to exhaustion showed higher osmolality levels compared to fish swum at low aerobic swimming at 24h and 72h. Sham implanted fish showed lower osmolality levels at resting compared to low aerobic and exhaustive swimming fish (72h and 168h-PI) (Fig. 1c). When cortisol implanted fish swum to exhaustion, a high plasma osmolality level was detected compared to low aerobic swimming and resting fish at 24h and 168h-PI (Fig. 1c). A cortisol effect was seen in resting fish only for the first 24h-PI after which levels returned to basal level at 72h-PI onward (Fig. 1a).

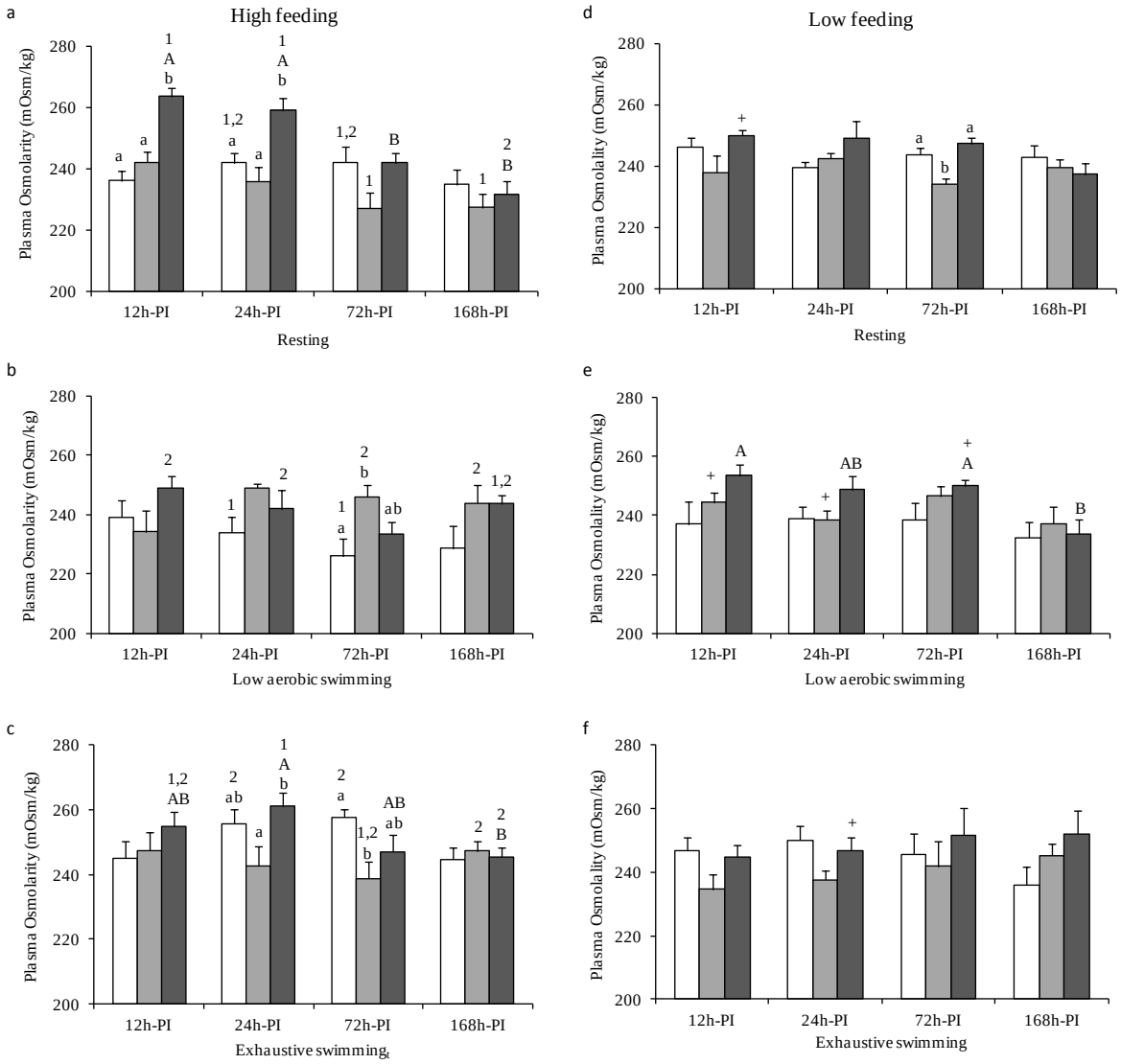

Fig. 1 Plasma osmolality levels of control carp without implants (open bars), sham implanted carp (light grey bars) and cortisol implanted carp (dark grey bars) at different feeding and swimming regimes. A plus ($^{+}$) indicates significant different between feeding regimes. Numeric number indicates significant difference between swimming regimes. Small letters indicates significant different between cortisol treatments. Capital letters indicates significant difference between time intervals. Data are expressed as mean ± SEM, *n* = 8.

Feeding had a small effect on plasma osmolality. A significant greater plasma osmolality level was detected in resting fish implanted with cortisol and fed a high ration at 12h-PI compared to the same group fed a low ration (Fig.1a and 1d). At low aerobic swimming, a slightly higher osmolality level was detected in sham implanted fish fed a low ration at 12h-PI (Fig. 1e). This level became lower at 24h-PI comparable to high feeding fish thereafter. The only difference at exhaustive swimming was observed in cortisol implanted fish at 24h-PI with higher osmolality levels in high feeding fish compared to low feeding fish (Fig. 1c and 1f). Overall this led to a significant interaction between feeding, swimming, implantation and time (*P*=0.031). Significant interactions between two factors were observed between feeding and swimming, swimming and implantation, and implantation and time. Three factor interactions were observed between feeding, swimming and implantation; swimming, implantation and time, while the interaction between feeding, implantation and time was almost significant (*P*=0.052).

3.2 Plasma sodium, Na^+

Feeding had no effect on plasma Na^+ levels in any group of fish (Fig. 2). Overall, cortisol profoundly reduced plasma Na^+ levels in fish swum to exhaustion. For high feeding fish, Na^+ was significantly decreased at 12h-PI for fish implanted with cortisol and it recovered at 24h-PI (Fig. 2c). This effect was more pronounced in low feeding fish, with significant reductions compared to control and sham implanted fish at 12h-PI at all swimming regimes, whilst at 24h-PI it still existed in resting (Fig. 2c) and exhaustive swimming fish, and Na^+ losses continued up to 72h-PI in fish swum to exhaustion (Fig. 2f).

An effect of swimming was observed with increased Na^+ levels in control fish fed at high ration and swum to exhaustion (at 12h-PI and 168h-PI compared to resting fish) (Fig. 2c). Whereas at low feeding, sham implanted fish exhibited greater Na^+ levels in low aerobic and exhaustive swimming fish compared to resting fish at 72h-PI (Fig. 2d-f). Overall, interaction between feeding, swimming, implantation and time was significant. Significant two way interactions existed between feeding and implantation, swimming and implantation as well as implantation and time. Whilst, feeding, implantation and time; and swimming, implantation and time had significant interaction when looking at three factors.

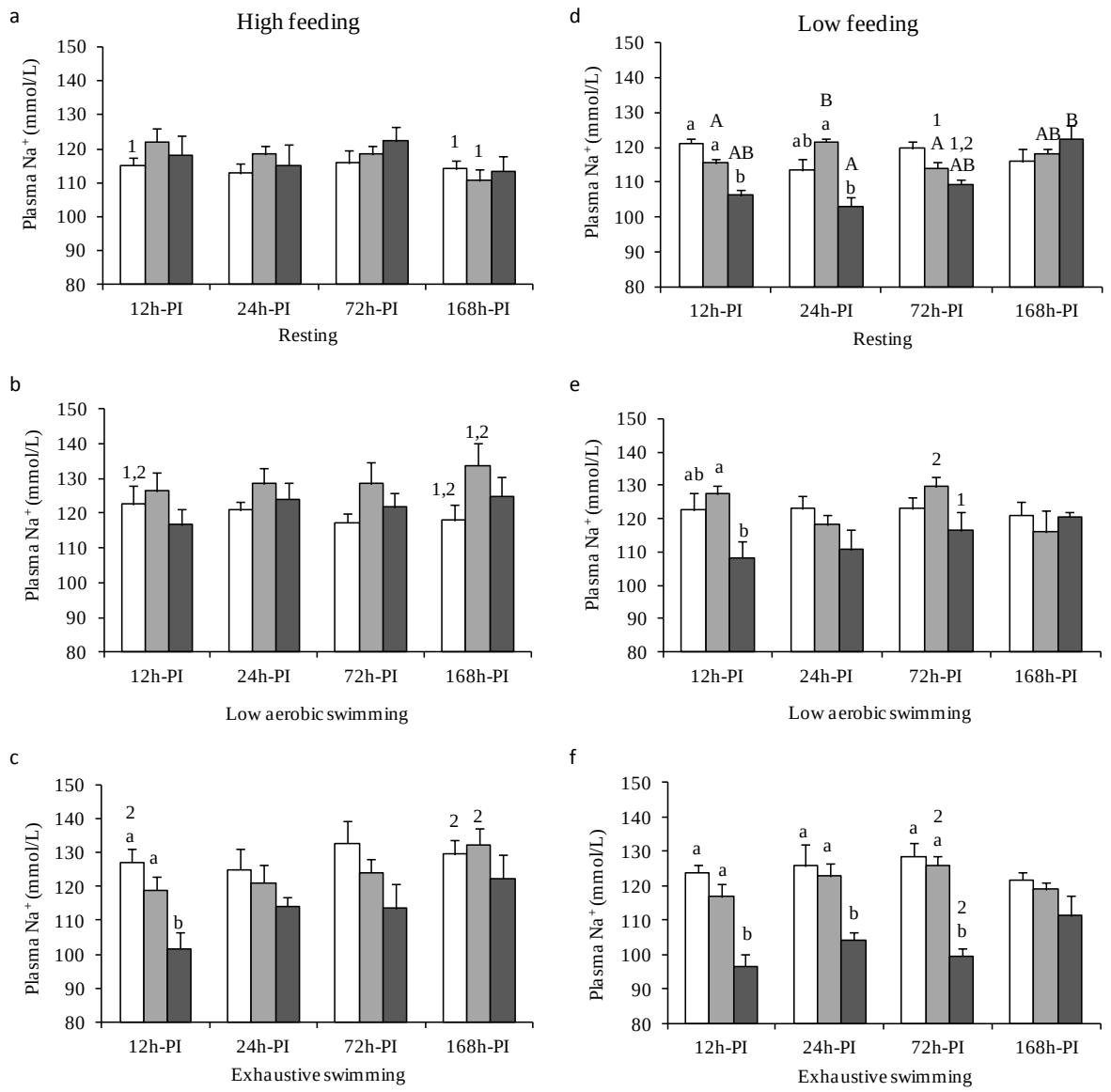

Fig. 2 The plasma Na^+ levels of control carp without implants (open bars), sham implanted carp (light grey bars) and cortisol implanted carp (dark grey bars) at different feeding and swimming regimes. Numeric number indicates significant difference between swimming regimes. Small letters indicates significant different between cortisol treatments. Capital letters indicates significant difference between time intervals. Data are expressed as mean ± SEM, *n* = 8.

3.3 Plasma chloride, Cl^-

Again, the effect of cortisol implants is more pronounced in high feeding fish than in low feeding fish (Fig. 3). Overall, cortisol implanted fish had lower plasma Cl^- levels compared to control and sham implanted fish (first 24h-PI at rest and first 12h-PI at low aerobic swimming). The loss of plasma Cl^- was more prominent in exhausted fish where the effect was observed until 72h-PI and recovered to baseline levels at 168h-PI (Fig. 3c). For low feeding fish, the effect of cortisol was only observed at 12h-PI in resting and low aerobic swimming fish (Fig. 3d and 3e).

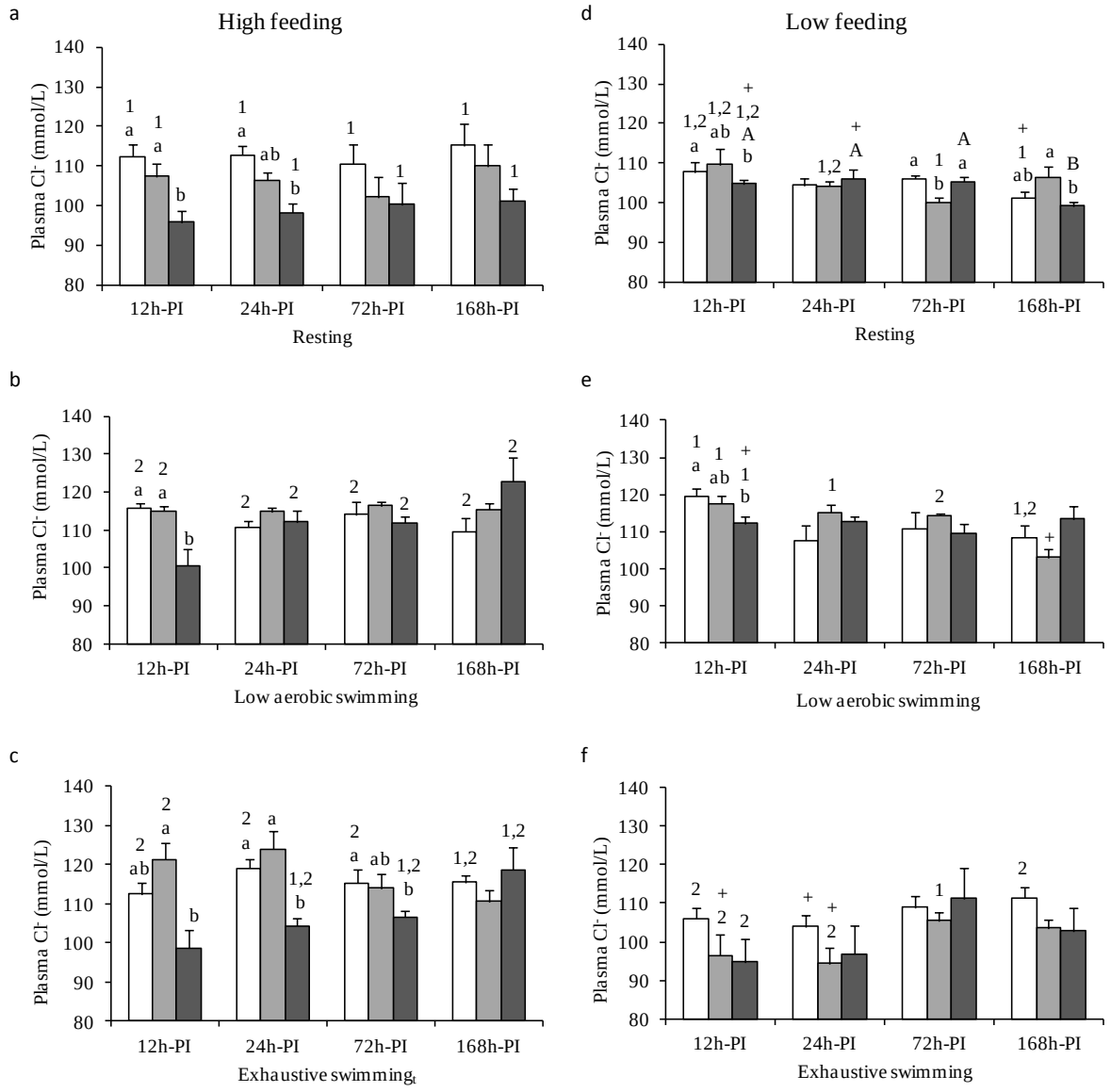

Fig. 3 The plasma Cl^- levels of control carp without implants (open bars), sham implanted carp (light grey bars) and cortisol implanted carp (dark grey bars) at different feeding and swimming regimes. A plus ($^+$) indicates significant different between feeding regimes. Numeric number indicates significant difference between swimming regimes. Small letters indicates significant different between cortisol treatments. Capital letters indicates significant difference between time intervals. Data are expressed as mean ± SEM, *n* = 8.

Exhaustive swimming caused low plasma Cl^- levels in control and sham implanted fish fed a low ration at the first 24h-PI (Fig. 3c and 3f). Overall, low aerobic swimming fish have higher plasma Cl^- levels in both feeding regimes (Fig. 3b and 3e). This was obvious in both control and cortisol implanted fish fed a high ration which showed higher plasma Cl^- throughout the 168h-PI period, except for 12h-PI in cortisol implanted fish, compared to resting and/or exhaustive swimming fish (Fig. 3b). For low feeding fish, this effect was prominent in all groups at 12h-PI and the effect remained in sham implanted fish until 72h-PI compared to exhaustive swimming and resting fish (Fig. 3e). At 168h-PI, a high plasma Cl^- level was recorded in control fish swum to exhaustion compared to resting fish (Fig. 3f).

Feeding had inconsistent effects on plasma Cl^- (Table 3). At rest, high feeding fish implanted with cortisol had low plasma Cl^- levels at 12h-PI and 24h-PI, and control resting fish had higher plasma Cl^- level at 168h-PI compared to low ration feeding fish (Fig.3a and 3d). Similarly, high feeding fish implanted with cortisol swum at low aerobic speed, exhibited low plasma Cl^- level at 12h-PI compared to low feeding fish (Fig.3b and 3e). Contrary high plasma Cl^- was recorded in sham implanted fish at 168h-PI. At exhaustive swimming, sham implanted fish had greater plasma Cl^- level at 12h and 24h-PI; and control fish at 24h-PI in high feeding fish compared to low feeding fish(Fig.3c and 3f).

Therefore, overall interaction effect between feeding, swimming, implantation and time was significant. Significant interaction between feeding and implantation; and swimming and implantation existed for two factor interaction. Whilst, feeding, swimming and implantation; feeding, implantation and time; swimming, implantation and time were significant for three factor interaction.

3.4 Plasma potassium, K^+

Feeding had no effect on plasma K^+ levels in any group of fish and overall fish swum at low aerobic speeds had lower plasma K^+ levels compared to resting and exhaustive swimming fish in both feeding regimes (Fig. 4). Cortisol implantation induced transient plasma K^+ elevation at 12h-PI compared to control and sham implanted fish at resting (Fig. 4a and 4d). A high plasma K^+ level was also observed in sham and cortisol implanted fish when swum to exhaustion at 168h-PI (Fig. 4c). An opposite trend was observed in low feeding fish, where control fish exhibited higher plasma K^+ levels compared to sham and cortisol implanted fish at 24h and 72h-PI (Fig. 4f). At rest, this effect was detected at 24h-PI (Fig. 4d), and this effect was extended to 72h-PI when fish were swum to exhaustion (Fig. 4f) although no effect was observed in the first 12h-PI. Overall interaction was not significant, but there was an interaction effect between feeding and implantation; and feeding, swimming and implantation.

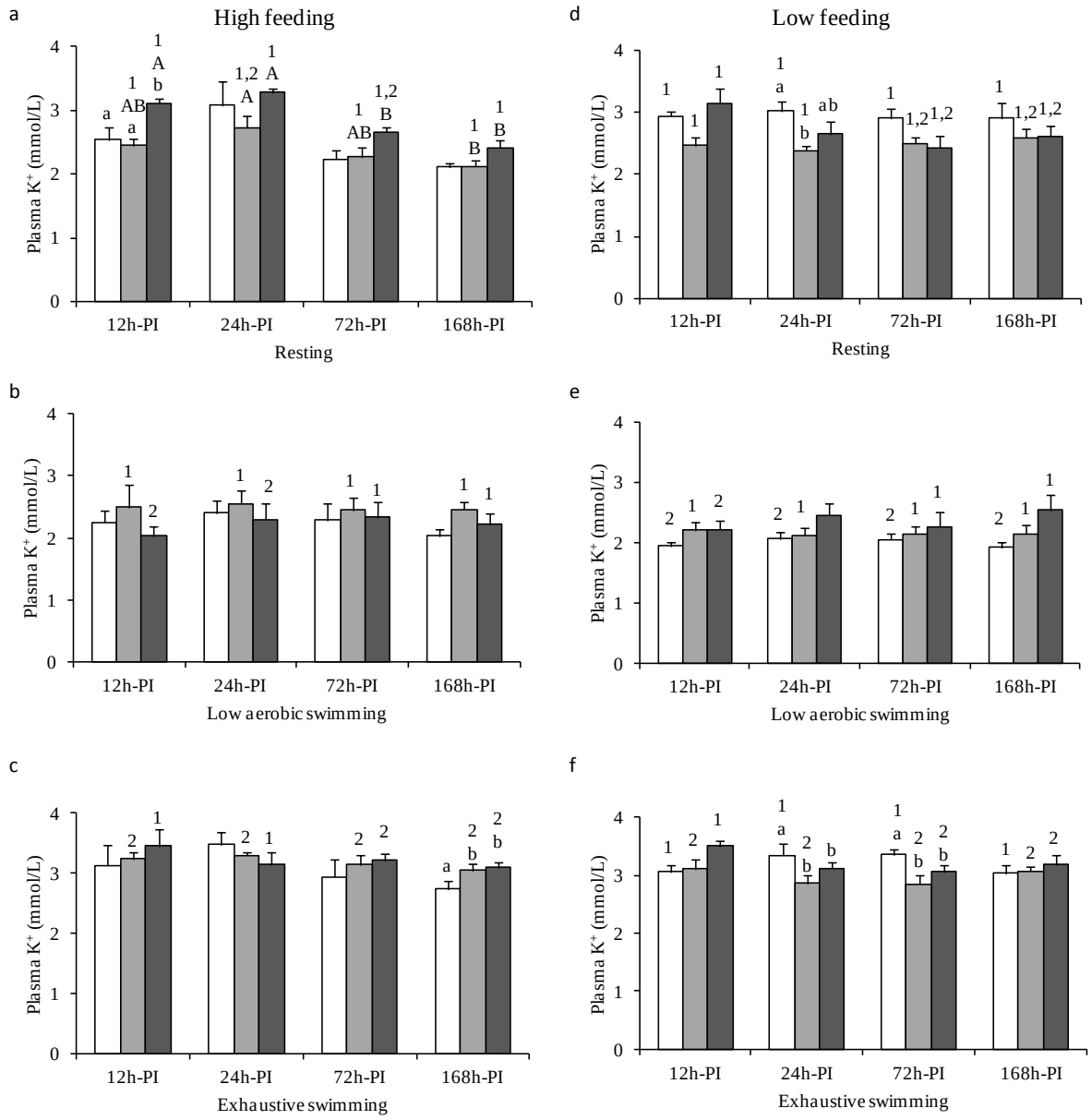

Fig. 4 The plasma K^+ levels of control carp without implants (open bars), sham implanted carp (light grey bars) and cortisol implanted carp (dark grey bars) at different feeding and swimming regimes. Numeric number indicates significant difference between swimming regimes. Small letters indicates significant different between cortisol treatments. Capital letters indicates significant difference between time intervals. Data are expressed as mean ± SEM, *n* = 8.

3.5 Gill and kidney Na^+/K^+ ATPase (NKA) activity

Feeding had no significant effect on gill NKA activity in any group of fish, although on average high feeding fish had higher NKA activity than low feeding fish (Fig. 5). An effect of swimming was only noticed in high feeding control fish swum to exhaustion that had a greater NKA activity than low aerobic swimming fish (Fig. 5a-c). However, cortisol caused a significant upregulation of gill NKA activity, especially in fish fed a high ration. At rest, cortisol significantly induced gill NKA activity for the first 24h-PI as compared to the sham implanted fish (Fig. 5a). The largest increase was observed in high feeding fish implanted with cortisol swum at low aerobically speeds, where the elevation of

NKA lasted for 72h-PI and returned to basal level at 168h-PI (Fig. 5b). In fish swum to exhaustion, cortisol implantation significantly induced gill NKA at 24h-PI and 72h-PI compared to control fish fed to high ration (Fig. 5c). For the low feeding fish, a significant cortisol effect was only observed at 24h-PI for resting fish and first 24h-PI for the fish swum to exhaustion (Fig. 5d and 5f). There was no overall interaction, but there was a significant three factor interaction (feeding, swimming and implantation) and two factor interaction (feeding and swimming, *P*=0.048; swimming and implantation; and implantation and time interval).

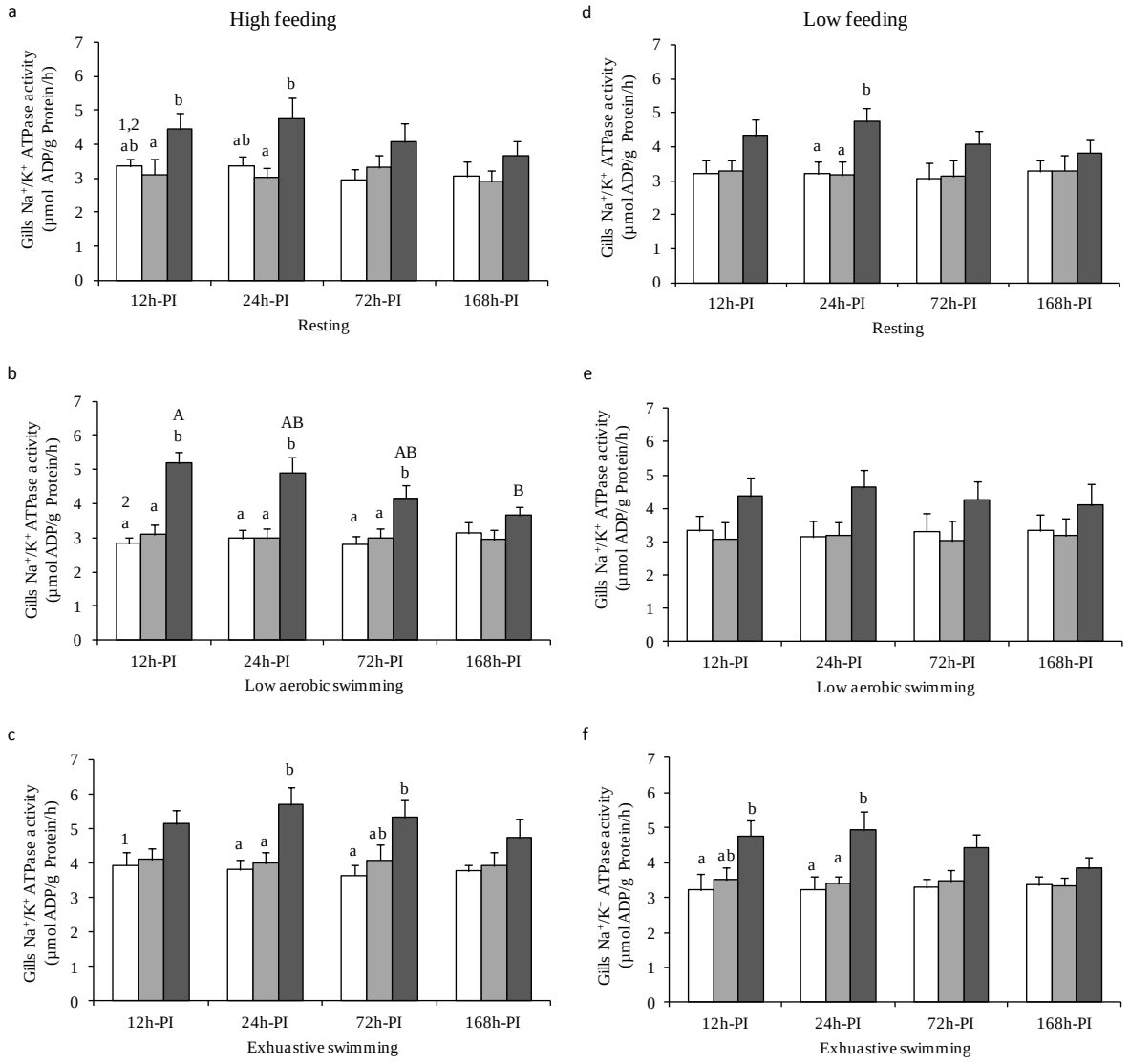

Fig. 5 Gills Na^+/K^+ ATPase activity of control carp without implants (open bars), sham implanted carp (light grey bars) and cortisol implanted carp (dark grey bars) at different feeding and swimming regimes. Small letters indicates significant different between cortisol treatments. Capital letters indicates significant difference between time intervals. Data are expressed as mean ± SEM, *n* = 8.

Feeding reduced kidney NKA activity and all low feeding fish expressed significantly greater kidney NKA activity than the high feeding fish at all swimming conditions (Fig. 6). Kidney NKA activity for the high feeding fish was maintained at a relatively stable level without any influence of either swimming or implants (Fig. 6a-c). Swimming had no effect on kidney NKA activity of low feeding fish

(Fig. 6d-f), but cortisol increased NKA activity. For low feeding fish, cortisol implants significantly upregulated kidney NKA activity at 24h-PI and 72h-PI in resting fish (Fig. 6f), whilst this effect was only noticed at 24h-PI in low aerobic swimming fish (Fig. 6e) and at the first 24h-PI when fish swum to exhaustion (Fig. 6f). Therefore, the only significant interaction effects were observed between feeding, swimming and implantation for three factors (P=0.046); and between feeding and implantation for two factors.

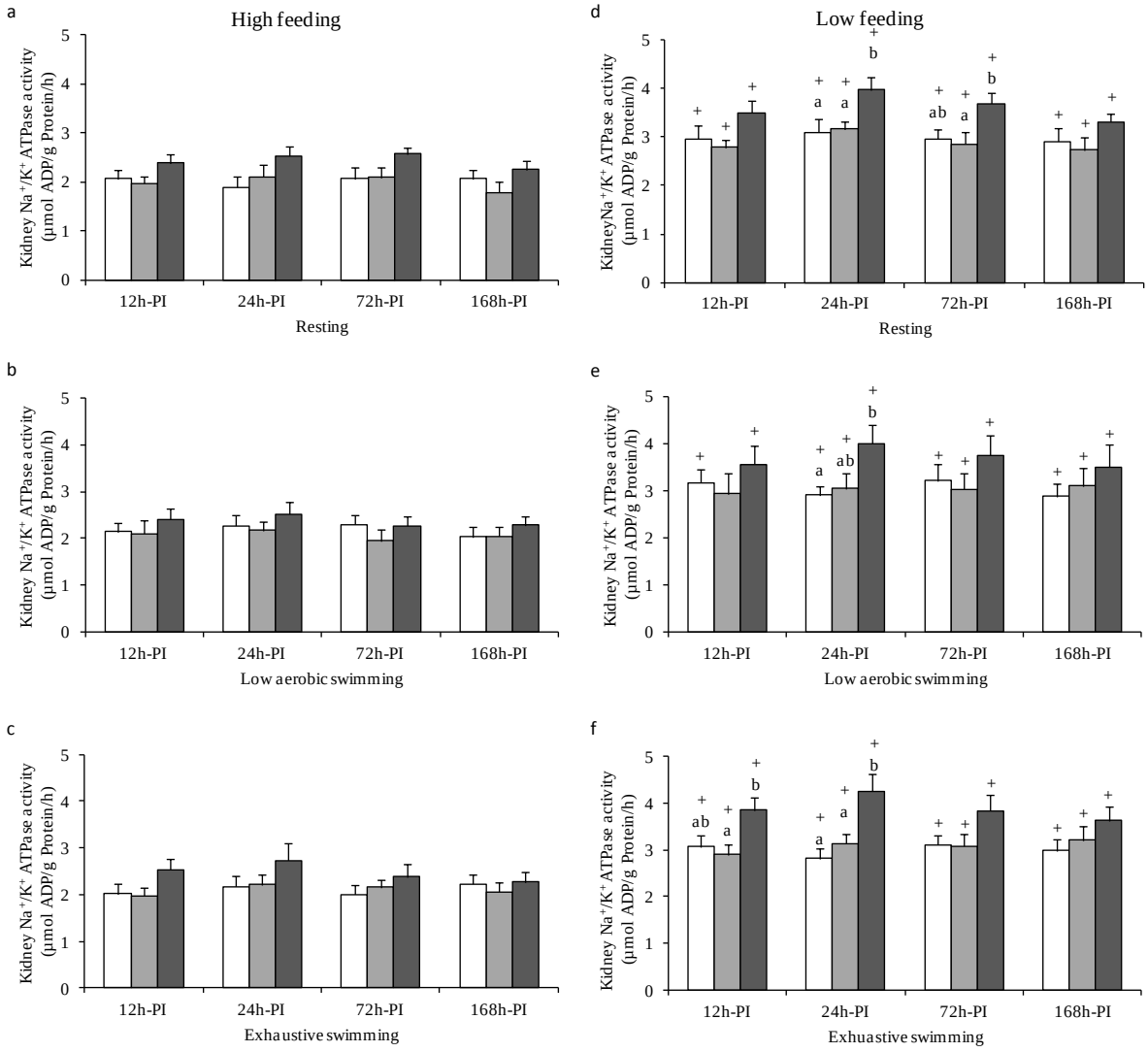

Fig. 6 Kidney Na^+/K^+ ATPase activity of control carp without implants (open bars), sham implanted carp (light grey bars) and cortisol implanted carp (dark grey bars) at different feeding and swimming regimes. A plus ($^+$) indicates significant different between feeding regimes. Small letters indicates significant different between cortisol treatments. Data are expressed as mean ± SEM, n = 8.

3.6 Gill and kidney H^+ ATPase activity

An almost identical trend as for gill NKA activity was also observed for gill H^+ ATPase activity with no significant effect of feeding and swimming in any group (Fig. 7). Fish implanted with cortisol significantly upregulated gill H^+ ATPase activity for the first 72h-PI and activity returned to baseline levels at 168h-PI in high feeding fish (Fig. 7a-c). This upregulation only lasted until 24h-PI and

returned to baseline levels at 72h-PI onward for low feeding fish (Fig. 7d-f). Therefore, a significant interaction effect was only detected between swimming and implantation, as well as implantation and time.

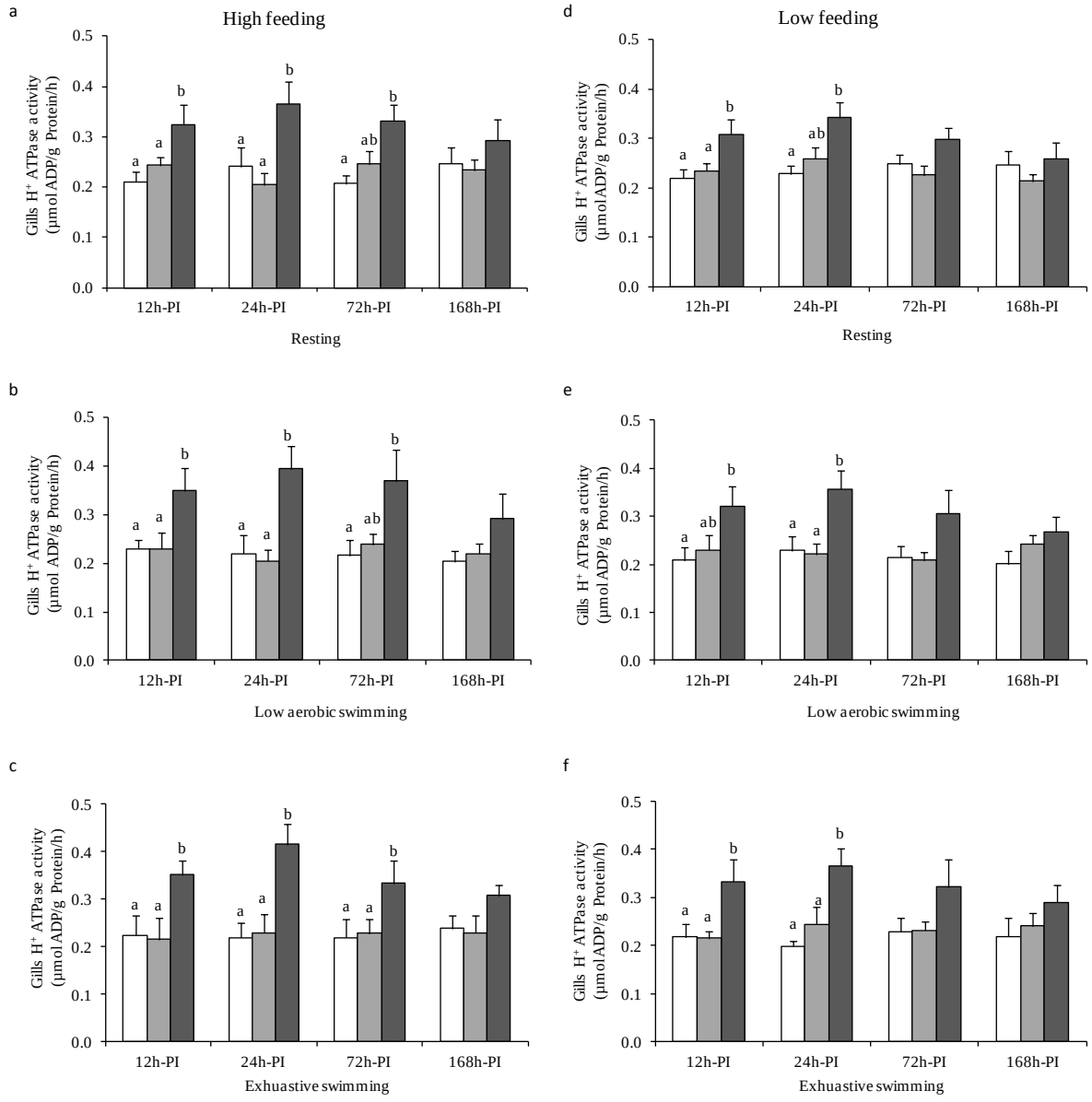

Fig. 7 Gill H^+ ATPase activity of control carp without implants (open bars), sham implanted carp (light grey bars) and cortisol implanted carp (dark grey bars) at different feeding and swimming regimes. Small letters indicates significant different between cortisol treatments. Data are expressed as mean ± SEM, *n* = 8.

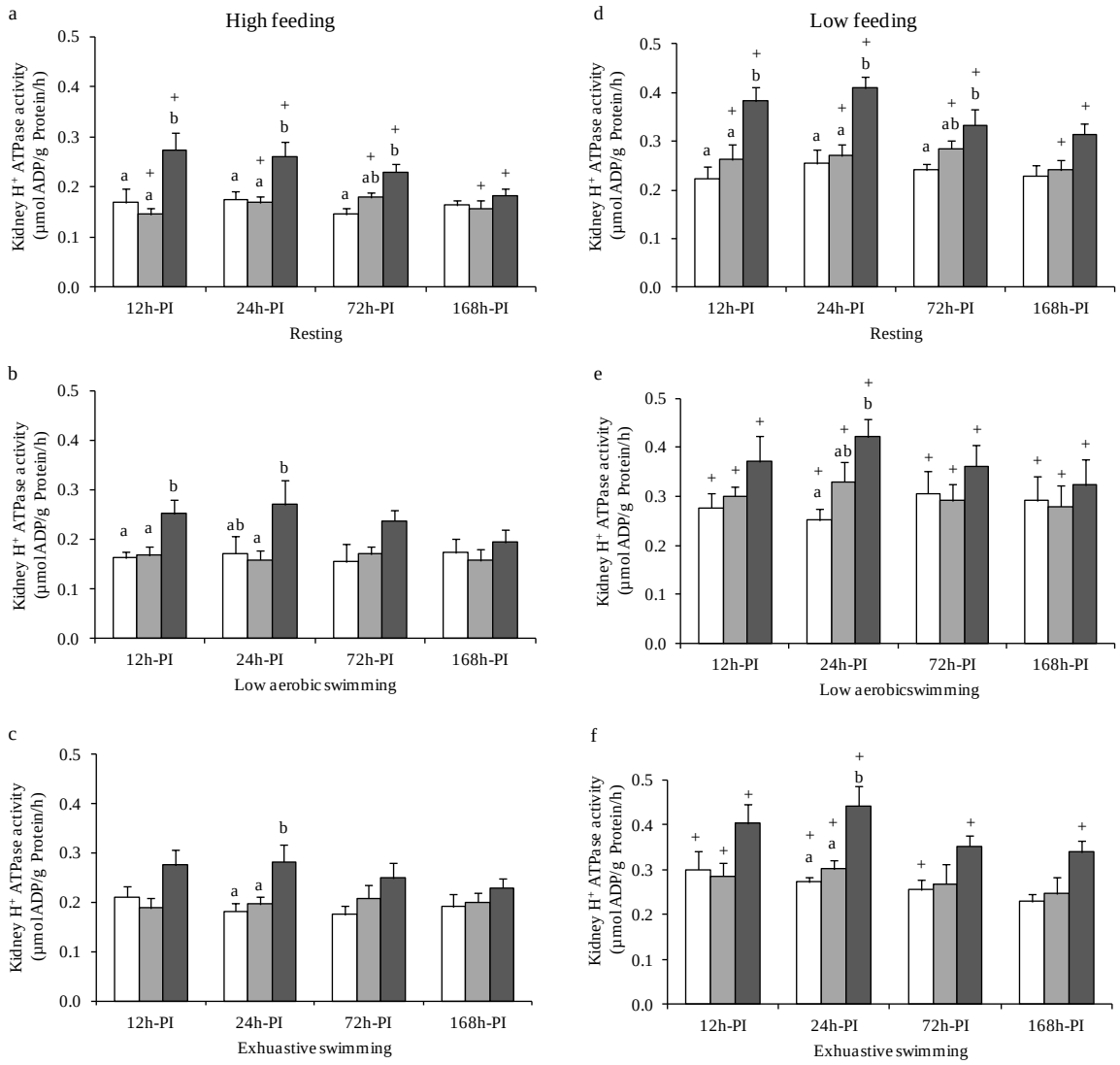

Fig. 8 Kidney H^+ ATPase activity of control carp without implants (open bars), sham implanted carp (light grey bars) and cortisol implanted carp (dark grey bars) at different feeding and swimming regimes. A plus ($^+$) indicates significant different between feeding regimes. Small letters indicates significant different between cortisol treatments. Data are expressed as mean ± SEM, *n* = 8.

In kidney, feeding played a significant role in H^+ ATPase activity with greater H^+ ATPase activity in low feeding fish than in high feeding fish (Fig. 8), except for control fish at rest (Fig. 8a and 8d). Swimming had no effect at all on kidney H^+ ATPase activity. Interestingly, and in contrast to kidney NKA, both feeding regimes demonstrated an identical expression trend in H^+ ATPase activity when implanted with cortisol. At rest, cortisol implanted fish showed a significant greater activity for the first 72h-PI in both feeding regimes (Fig. 8a and 8d). At low aerobic swimming, cortisol significantly upregulated kidney H^+ ATPase activity at 12h-PI compared to control and sham implant fish; and at 24h-PI compared to sham implanted fish fed to high feeding ratio (Fig. 8b). For low feeding fish, this effect was only observed at 24h-PI compared to control fish (Fig. 8e). When swum to exhaustion, cortisol significantly upregulated kidney H^+ ATPase activity at 24h-PI in both feeding regimes (Fig. 8c and 8f). As a result, feeding, implantation and time significantly interacted. Also, feeding and implantation; and implantation and time significantly interacted.

4. Discussion

Direct measurements of osmolality showed that feeding, swimming and cortisol administration had not much effect on plasma osmolality in common carp. Stable osmolality levels in the present study were similar with cortisol implanted tilapia (Abo Hegab and Hanke, 1984), channel catfish (Eckert et al., 2001), goldfish (Chasiotis and Kelly, 2012) and rainbow trout (Teles et al., 2013). Previous studies noted different responses in plasma osmolality and ion levels following cortisol treatment in goldfish (Umminger and Gist, 1973); common carp (De Boeck et al., 2001a); channel catfish (Eckert et al., 2001); stinging catfish (Sherwani and Parwez, 2008) and North African catfish (Babitha and Peter, 2010).

Plasma osmolality remained constant even at exhaustive swimming which accelerated nitrogen waste excretion and increased oxygen consumption (Liew et al., 2013b). Only a transient hyponatremia was noticed in the high feeding - high cortisol group at 12h-PI, but the Na^+ level recovered from 24-PI onward. The absence of disturbance during severe exercise suggests that carp are extremely successful in dealing with the osmorespiratory compromise and can successfully increase gill functional surface area or promote increased blood and water flow over gill filaments for gas exchange and ammonia excretion without excessive ion losses, at least when they have access to food. This capacity is slightly compromised by high levels of cortisol, but homeostasis is quickly restored. This was also observed in rainbow trout where the loss of Na^+ and water gain in the early active swimming stage was partially or completely restored in a later swimming stage and an increased efficiency of gill permeability for gas exchange occurred whilst ion losses and water gain were minimized (Wood and Randall, 1973a; 1973b; Gonzales and McDonald, 1992).

Dietary Na^+ intake via the gastrointestinal system might have contributed to maintain basal Na^+ levels, as losses were less severe in the high feeding group compared to the low feeding group. This was shown by gill NKA activity being unaffected by swimming or feeding (Fig. 1). In parallel with the reduction in Na^+ levels in carp experiencing hypercortisol, gill NKA activity was upregulated. Also other freshwater species implanted with cortisol and/or challenged with other environmental changes such as common carp stressed with Cu^{2+} (De Boeck et al., 2001b) or temperature (Metz et al., 2003); channel catfish implanted with cortisol and prolactin (Eckert et al., 2001); Asian stinging catfish (Sherwani and Parwez, 2008); African catfish (Babitha and Peter, 2010); Mozambique tilapia in freshwater (McGuire et al., 2010); and goldfish (Chasiotis and Kelly, 2012) implanted with cortisol showed this increased NKA activity. In addition, the upregulation of NKA activity during hypercortisol might also be associated with branchial ammonia excretion as protein mobilization during stress and aerobic metabolism led to endogenous ammonia elevation (Liew et al, 2013b). Hypercortisolism altered branchial ammonia excretion and clearance capacity which appears as an additive strategy to enhance ammonia self-detoxification (Liew et al., 2013a; 2013b), and carp induced gill NKA activity and ammonia excretion simultaneously when swimming actively. This potential link between Na^+ uptake mechanisms and ammonia excretion rates is suggested to be part of the ammonia excretion metabolon which consists of different elements such as Rhesus glycoproteins, and NH_4^+/Na^+ and/or Na^+/H^+ exchangers at the apical membrane as well as NKA or Na^+ channels at the basolateral membrane (Walsh, 1998; Salama et al., 1999; Nawata et al., 2007; 2010; Wu et al., 2010; Zimmer et al., 2010). Also other studies have shown that active swimming carp induced gill NKA activity and NKA protein expression to facilitated Na^+ and Cl^- uptake (Metz et al., 2003).

On the other hand, when food availability was reduced, hyponatremia was more prominent. Na^+ levels were significantly reduced in low feeding fish subjected to hypercortisol especially when

fish swum to exhaustion. Nevertheless, the level recovered at 168h-PI concurrent with the plasma cortisol. This showed that Na^+ losses in low feeding fish were more severe due to the limited dietary Na^+ intake but were also related to high cortisol levels resulting in Na^+ losses. Due to limited Na^+ accessibility from dietary intake, low feeding fish upregulated kidney NKA activities to reabsorb Na^+. Cortisol seemed to induce increased kidney NKA activity at the early period of implantation at all swimming conditions in the present study. However, this kidney NKA activity was not sufficient to restore Na^+ level in hypercortisol fish, especially when fish were swum to exhaustion. Cortisol inducing the increase of branchial and kidney NKA activity was also observed in freshwater tilapia (Dang et al., 2000), and African catfish (Babitha and Peter, 2010).

Similarly, branchial H^+ ATPase activity was maintained at relatively stable levels without being influenced by either feeding or swimming, but was accelerated by hypercortisol (Fig. 3). This alteration again is believed to be associated with the increased NH_3 excretion as H^+ excretion and CO_2 hydration coupled with carbonic anhydrase create acidification at the gill boundary layer helping NH_3 and CO_2 diffusion. They subsequently create an electro gradient for Na^+ uptake, and as a result plasma Na^+ was maintained at a fairly stable level at least in high feeding fish. Upregulation of H^+ ATPase activity in hypercortisol carp in the present study was in line with Lin and Randall (1993) who demonstrated that cortisol stimulated 30% increments of gill H^+ ATPase activity in trout. Together with the presence of cortisol receptor gene expression in pavement cells (Uchida et al., 1998), this suggests that cortisol contributes to Na^+ homeostasis in freshwater fishes (Mommsen et al., 1999). Hypercortisolism not only upregulated NKA and H^+ ATPase activities in the gill, but also upregulated kidney NKA and H^+ ATPase activities in low feeding fish. These ionoregulatory upregulation was also seen in previous research in other species such as channel catfish (Eckert et al., 2001), common carp (Metz et al., 2003), stinging catfish (Sherwani and Parwez, 2008) and North African catfish (Babitha and Peter, 2010).

Feeding had no obvious effect on Cl^- balance. Cortisol caused hypochloremia more prominently in fish fed a high ration, whilst a transient hypochloremia was found in fish fed a low ration in the present study. Cortisol also caused hypochloremia in goldfish (Umminger and Gist, 1973; Chasiotis and Kelly, 2012). Nevertheless, the hypochloremia occurring in high feeding fish is probably due to the increase of Na^+ uptake via the Na^+/Cl^- exchanger. As in rainbow trout, feeding provided excessive base which consequently led to uptake of Cl^- via branchial Cl^-/HCO_3 exchanger (Bucking and Wood, 2006a; 2008). According to Perry and Fryer (1997) the unidirectional influx and efflux of Na^+ and Cl^- might be adjusted to a net flux by stimulating Cl^- uptake via gills Cl^-/HCO_3^- exchanger during alkalosis (Perry and Goss, 1994).The upregulation of kidney H^+ ATPase activity enabled the reabsorption of additional HCO_3^- to ensure plasma HCO_3^- remained elevated to compensate respiratory acidosis, which promoted Cl^-/HCO_3^- exchanger (Perry and Fryer, 1997).

On the other hand, swimming seems to increase plasma K^+, rather than feeding and cortisol. Similarly, feeding had no effect on plasma K^+ in rainbow trout (Bucking and Wood, 2006b). Interestingly, the present result showed that carp swum exhaustion have higher plasma K^+ level. The increase of K^+ could probably be due to the release of tissue K^+ into the blood stream to neutralize intracellular pH while preventing tissue depolarization (Van Djik et al., 1993), especially when endogenous ammonia was increased at high swimming level. The overall balance of K^+ during exhaustive swimming in both feeding regimes could also be due to active K^+ recycling through K^+ channels to sustain NKA where high NKA activity was observed concurrently. This is in support by a statement mentioned by Culter et al. (2009) that the K^+ level over the paracellular pathway would

need to be maintained at a sufficient level to allow continued NKA activity and in addition the tight junction permeability for K^+ could contribute to K^+ balancing.

5. Conclusion

Our study shows that feeding ration can induce different ionoregulatory strategies in fish facing additional challenges such as exhaustive swimming or cortisol elevations. Whereas fish on a high feeding regime stimulated primarily branchial NKA and H^+ ATPase activities in response to high cortisol levels, fish on a low feeding regime relied more on an upregulation of kidney NKA and H^+ ATPase for ion reabsorption. Hypercortisol appeared to have a negative effect on the hydromineral status as the plasma Na^+ that was lost at high swimming speeds recovered to control levels only when cortisol levels decreased. Therefore, we could only partially confirm our first hypothesis as feeding had no effect on gill NKA and H^+ ATPase activities per se, but when challenged with high cortisol levels, high feeding fish did respond with greater gill ATPase activities. Our second hypothesis was accepted as low feeding fish amplified their kidney ATPase activities to promote ion reabsorption, especially in cortisol implanted fish.

Chapter 5

Cortisol signaling induces non-genomic metabolic and ionoregulation responses before genomic changes occur in common carp, *Cyprinus carpio* fed at different rations

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Abstract

This study investigated non-genomic and genomic responses in the energy metabolism and ionoregulation of common carp fed at 0.5% or 3.0% body weight (BW) which received either no implant, a sham implant or a cortisol implant (250 mg/kg BW) throughout a 168 hour post-implant period (168h-PI). Immediately, cortisol implant induced a plasma cortisol, glucose and lactate elevation. Plasma osmolality and ion levels remained largely unchanged, but cortisol induced increased gill and kidney Na^+/K^+ ATPase (NKA) and H^+ ATPase activities as a non-genomic response. Gill ionoregulatory activities were greater in carp fed a high ration, and whereas kidney NKA was upregulated to a comparable level in both feeding regimes, kidney H^+ ATPase activity was greater in low feeding fish. No significant effect was observed at mRNA level for these transporters, but an increasing trend was noted. Cortisol induced liver protein mobilization and repartitioned liver and muscle glycogen. In fish fed on the 3.0% BW ration, increased protein mobilization did not increase plasma ammonia and urea levels, reflecting an improvement of excretion efficiency with a significant Rhcg-1 upregulation. Cortisol induced glucocorticoid receptors (GR1 and GR2) and the mineralocorticoid receptor (MR) upregulation from 24h to72h-PI in both feeding groups. GR1 remained upregulated at 168h-PI in low feeding carp, whereas GR2 and MR were upregulated in high feeding carp. In the liver, only GR2 and MR were upregulated at 24h-PI in low feeding carp, whilst all three receptor were upregulated in high feeding carp. Despite an increasing trend in the gill, only GR2 was upregulated at 72h-PI in low feeding carp. In the high feeding fish, GR2 and MR were upregulated at 72h-PI, whereas GR1 was upregulated at 168h-PI. We demonstrated that cortisol significantly induced *in situ* metabolic responses to compensate acute stress (12h-PI) as non-genomic actions, followed by genomic responses with mRNA upregulation of ionoregulatory and corticosteroid receptors (24h-PI onward).

Keywords: Cyprinid, corticoid receptors, hydromineral balance, energy metabolism, Rhesus glycoprotein, stress.

1. Introduction

Cortisol, the main corticosteroid is well known to play a pivotal role in stress responses and it is synthesized in the inter-renal cells of the head kidney. It regulates both glucocorticoid and mineralocorticoid responses which are ultimately determined by binding to corticoid receptors which act as ligand-inducible transcription factors; and subsequently target gene expression is achieved (Bury et al., 2003; Bury and Sturm, 2007; Stolte et al., 2008a; Kiilerich et al., 2011). To date, four corticoid receptors have been identified in teleosts, glucocorticoid receptor 1 (GR1a and GR1b); glucocorticoid receptor 2 (GR2) and a mineralocorticoid receptor (MR) (Bury et al., 2003; Bury and Sturm, 2007; Stolte et al., 2008a; 2009; Aruna et al., 2012a). These corticoid receptors respond with a different sensitivity to cortisol in transactivation and transrepression via high affinity and low capacity receptors in the specific tissue or species (Bury and Sturm, 2007; Stolte et al., 2008a). This subsequently induces and regulates a wide range of physiological compensations and/or adaptations such as in intermediary metabolism, immune capacity, osmoregulation, growth and reproduction (Vijayan et al., 1997; Mommsen et al., 1999; De Boeck et al., 2001; Bury et al., 2003; Flik et al., 2006; Kiilerich et al., 2007; Stolte et al., 2008a; Chasiotis and Kelly, 2012; Teles et al., 2013).

Emerging *in vivo* and *in vitro* evidence shows that cortisol plays a significant role in ion homeostasis in freshwater fish (Babitha and Peter, 2010; Hwang et al., 2011; Chasiotis and Kelly, 2012; Kumai et al., 2012). Cortisol induces upregulation of Na^+/K^+ ATPase (NKA) activity which has been documented in several freshwater fish such as tilapia (Dang et al., 2000), channel catfish (Eckert et al., 2001); rainbow trout (Sloman et al., 2001); North African catfish (Babitha and Peter, 2010); goldfish (Chasiotis and Kelly, 2012) and zebrafish (Kumai et al., 2012). Recently McCormick (2011) stated that cortisol induces increased gill chloride cells surface area and influences both branchial and renal function in freshwater teleosts, which subsequently affects the influx of Na^+ and Cl^-. The overall regulation behind this mechanism is believed to be modulated by the mineralocorticoid receptor (MR) which is responsible for maintaining ion homeostasis in teleosts (Gilmour, 2005; Bury and Sturm, 2007; Stolte et al., 2008a). Moreover, our own investigations showed that cortisol not only upregulated gill NKA and H^+ ATPase activities in fish fed a high ration but also induced kidney NKA and H^+ ATPase activities in fish fed a low ration (Chapter 4).

In order to sustain homeostasis and survive under stress, extra energy expense is expected to fuel basal metabolism (Vijayan et al., 1997; Gregory and Wood, 1999; De Boeck et al., 2001). Subsequently, energy channelled towards somatic growth is limited, and a low growth and a 'high living cost' has been classified as a common secondary stress indication (Gregory and Wood, 1999). Hyperglycemia during hypercortisol is regulated by glucocorticoid receptors and provides easily accessible energy substrates (Bury et al., 2003). Furthermore, a similar increase in lactate level during cortisol elevation suggests the involvement of anaerobic metabolism (Pottinger, 1998). Health (1995) and De Boeck et al., (2001) stated that stress might increase aerobic or anaerobic metabolism or both. Our previous study showed that cortisol induced both aerobic and anaerobic metabolism simultaneously (Liew et al., 2013a). This energy availability is also determined by the quantity of dietary food intake (Vijayan and Moon, 1992; Reddy et al., 1995).

Studies on the glucocorticoid receptors (GR1 and GR2), the mineralocorticoid receptor (MR) and the ion transporters (NKA and H^+ ATPase) mRNA expression in response to cortisol elevation have been performed by either challenging the fish with cortisol implants, salinity, husbandry stress or pollutants (Pottinger et al., 2000; Greenwood et al., 2003; Sturm et al., 2005; Nawata et al., 2007; McCormick et al., 2008; Roy and Rai, 2009; Stolte et al., 2009; Kiilerich et al., 2011; Aruna et al., 2012a; Teles, 2013). In teleosts, cortisol is well known for its dual role: ion retention in freshwater fish and ion secretion in seawater fish (McCormick et al., 2008; Tipsmark and Madsen, 2009). However, the underlying molecular mechanisms and the signaling pathways still remain uncertain and may be species-specific, dose dependent and vary with condition (Kiilerich et al., 2011). Furthermore, there are controversial discussions about the involvement of GR in ionoregulation, and MR has recently been identified to be more sensitive to cortisol than GR in the process of controlling hydromineral balance (Greenwood et al., 2003; Sturm et al., 2004; Takahashi et al., 2006; Takahashi and Sakamoto, 2013). Feeding also plays a role in both energy metabolism and ionoregulation but information on the interaction between different feeding regimes and hypercortisol on corticoid and ionoregulatory mRNA expression is scarce. Therefore, the objective of the present study was to investigate the effect of cortisol on the expression of corticoids receptors, Rhesus glycoproteins and ionoregulatory genes (NKA and H^+ ATPase) in the gill, kidney and liver of common carp over a period of 168 hours-post cortisol implant (168h-PI) in common carp fed a low (0.5% BW) or high (3.0% BW) feeding regime. In our previous studies, we found that cortisol induced ionoregulatory responses in kidney rather than gill to enhance ion reabsorption in fish fed a low ration, whilst cortisol improved branchial ammonia excretion and clearance rate in fish fed a high ration (Liew et al., 2013b).

Therefore in this study, we hypothesize that (i) hypercortisol will stimulate NKA and H^+ ATPase activities in the gill to improve ammonia excretion in high feeding fish and promote renal ion reabsorption capacity in low feeding fish. (ii) expression of gill Rhesus glycoproteins will be greater in high feeding fish to facilitate ammonia excretion, and therefore plasma ammonia level is maintained either below or at the same level as in low feeding fish. In order to address these hypotheses, plasma metabolites, plasma osmolality and ion (Na^+, Cl^-, K^+) as well as NKA and H^+ ATPase activities in both gill and kidney were measured. And lastly, we hypothesize that (iii) hypercortisol will induce GR1, GR2 and MR expression and that this effect will be more prominent in liver and kidney of low feeding fish to induce glycogenolysis and/or gluconeogenic actions. To address this hypothesis, liver and muscle metabolites were also measured.

2. Materials and Methods

2.1 Fish maintenance and experimental set-up

A total of 200 common carp, *Cyprinus carpio* (23.9±1.2 g BW and 13.3±0.3 cm body length (BL) (Mean±SEM)) purchased from commercial fish farm (Intratuin, Halsteren, The Netherlands) were pre-acclimatized and distributed equally into two 500 l rectangular fiberglass tanks (50×30×50 in.) in the aquarium facilities of the Systemic Physiological and Ecotoxicological Research group at the University of Antwerp, Belgium in Antwerp City tap water. Fish were acclimated for 8 weeks and fed at 1.5% BW per day with commercial Koi pellet (Hikari Staple, Kyorin Food Ind. Ltd., Japan). After pre-acclimatization, per series (control, sham and cortisol implanted) and feeding regime (low and high) 32 fish were randomly selected, weighed and distributed equally into four 50 l experiment aquaria filled with Antwerp city tap water (pH 8.2±0.4) for 6 weeks prior the start of the experiment. In this period, feeding regimes were adjusted to either a low (0.5% BW) or high (3.0% BW) feeding ration. Pellets were divided over 2 equal portions per day fed at 08:30 h and 18:30 h daily by slowly introduced pellets at the same corner of aquaria over a 20 min. All pellets were consumed throughout the experiment. Each aquarium was equipped with gentle aeration and a 6 l external filter consist of fine sponges, activated carbon and ≈16 mm lava stone at a flow rate of 5 l/min. Aquaria were covered with a black plastic sheet to reduce visual disturbance. Room temperature was set at 17 °C with constant photoperiod of 12L:12D. About 80% of water was refreshed every 3 days with a semi-auto-siphoning procedure to avoid handling disturbance. Water temperature, NH_3/NH_4^+, NO_2^-, NO_3^-, pH were maintained at 16±0.8 °C, <0.1 mg/l, <0.03 mg/l, <25 mg/l and pH 8.2±0.5 (7.8 – 8.6) respectively throughout the experiment.

2.2 Treatment groups and implantation

After acclimation, fish were subjected to control (no implant) and sham or cortisol implantation. Implanted fish were anaesthetized individually in 0.1 g/l Ehty-3-aminobenzoate methanesulfonic acid (MS222) (Acros Organics, Geel, Belgium) neutralized with 1N NaOH to the holding pH ±8.2 (Antwerp City tap water pH) and weighed. Implantation was performed intraperitoneally with coconut oil and/or cortisol at 10 µl/g of BW at 12h post feeding (Gregory and Wood, 1999; De Boeck et al., 2001; McDonald and Wood, 2004; Liew et al., 2013b). Fish implanted with melted coconut oil only (≈27 ^oC; Sigma Diagnostics) served as sham group. Fish implanted with a mixture of 25 mg cortisol (Hydrocortisone 21-hemisuccinate; Sigma Diagnostics) in 1 ml of melted

coconut oil served as cortisol group. This dose was selected based on our preliminary test resulting in an overall dose of 250 mg cortisol per kg of fish. This significantly elevated cortisol levels and allowed recovery to control levels within a 168 hour post injection period (168h-PI). All implanted fish were briefly placed on ice for approximately 30 s to facilitate solidification of the implant and immediately placed in well oxygenated water to recover from unconsciousness. All groups (control, sham and cortisol) were monitored at 12h, 24h, 72h and 168h-PI intervals with eight fish in each group.

2.3 Sampling

Fish were carefully removed from the aquaria, anaesthetised (0.5 g/l, neutralized MS222; Acros Organics, Geel, Belgium), quickly blotted dry and weighed followed by sampling. Blood was drawn from the caudal vessels using a heparinised needle and syringe within 1 min of sedation (2500 unit/ml lithium heparin, Sigma, Munich, Germany). The blood was immediately centrifuged to 13,200 rpm at 4^{o} C for 1.5 min. Plasma was pipetted into three 500 µl Eppendorf bullet tubes. Gills, kidney, liver and muscle tissues were wrapped in aluminium foil. All samples were immediately frozen in liquid N_2 and stored at -80^{o} C for further analysis.

2.4 Biochemical analysis

Osmolality was measured on fresh plasma using The Advanced™ Micro Osmometer (Model 3300, Advanced Instruments, USA). Radioimmunoassay was performed to measure plasma cortisol levels by using a commercial Cortisol ^{125}I RIA kits (ImmuChem™, MP Biomedicals LLC). Plasma glucose, lactate and ammonia were determined with commercial Enzymatic Kits (R-Biopharm AG, Darmstadt, Germany). Plasma urea was determined using the salicylate-hypochlorite assay (Rahmatullah and Boyde, 1980). Tissue energy (protein, glycogen and lipid) content was determined in both liver and muscle tissues. Protein was determined according to Bradford (1976) using a standard curve of bovine serum albumin. Glycogen was measured using Anthron reagent and a glycogen standard curve (Roe and Dailey, 1966). Total lipid was extracted by methanol-chloroform and measured with a tripalmitin standard curve (Blingh and Dyer, 1959). All samples were read with Ultra microplate spectrophoto reader (ELx 808, Bio-Tek Instrument Inc., Winooski VT, USA).

2.5 Gill and kidney ATPase activity

NKA was measured according to the method described by McCormick (1993) and H^+ ATPase activity was measured as described by Lin and Randall (1993) modified by Nawata et al. (2007). Tissues were homogenized with ice cooled 4:1 SEI/SEID buffer solution (150mM sucrose; 10mM EDTA; 50mM imidazole/SEI with 0.1% sodium deoxycholate) and centrifuged (1 min at 5000 g, 4° C). Duplicate homogenates of 10 µl were pipetted into 96-wells microplate in four series. For NKA measurement, a first series of homogenate was mixed with with 200 µl assay solution A (400U lactate dehydrogenase; 500U pyruvate kinase; 2.8 mM phosphoenolpyruvate; 0.7 mM ATP; 0.22 mM NADH; 50 mM imidazole) and a second series with 200 µl assay solution B (mixture assay A with 0.4 mM ouabain). For H^+ ATPase measurement, a third series of homogenate was mixed with 200 µl of assay solution C (mixture assay B with 500 mM NaN_3) and a fourth series with 200 µl of assay solution D (mixture assay C with 100 mM NEN). The enzyme activities were measured kinetically at

30 s intervals for 30 min at wavelength of 340 nm with spectrophotometer ($ELX808_{IU}$ Bio-Tek Instruments Inc. VT, USA). Calculation was performed with standard curve of ADP (Adenosine diphosphate). ATPase activities were calculated by subtracting oxidation rate in the absence to the presence of ouabain for NKA and NEN for H^+ ATPase. Unit of ATPase activity was expressed as µmol ADP/mg protein/h.

2.6 RNA extraction and real time PCR

Total RNA from gill, kidney and liver tissues were individually extracted by using Trizol (Invitrogen, Merelbeke, Belgium) following the manufacturer's instructions. DNase treatment was performed to avoid genomic DNA contamination by precipitating extracted total RNA in ethanol, and the RNA pellets were dissolved in RNAase-free water. RNA quantification was assessed with Nano-Drop spectrophotometry (NanoDrop Technologies, Montchanin, DE, USA) and samples were used if purity was >1.75 ratio (OD_{260}/OD_{280} nm absorption). RNA integrity was checked by denaturing gel electrophoresis through 1% agarose gel. An initial amount of 1µg RNA was synthesized to First strand complementary DNA (cDNA) according to Revert Aid H minus First strand cDNA synthesis kit instructions (Thermo Fisher Scientific, Zellik, Belgium). cDNA was used as a template for quantitative PCR (qPCR) analysis for NKA, H^+ ATPase, Rhesus glycoproteins (Rhbg, Rhcg-A, Rhcg-B), GR1, GR2 and MR quantification. Amplification were performed in 20 µl mixture reaction volume containing 5 µl cDNA, 4 pmol each of forward and reverse primers, 0.8 µl calibration ROX dye (500× dilution) and 10 µl Platinum SYBR Green qPCR SuperMix-UDG (Brilliant® II SYBR® Green QPCR mastermix, Agilent Technologies, Santa Clara, CA) on specific PCR plates (BIOplastics BV, Landgraaf, The Netherlands). The PCR conditions were set as follows: amplification at 50 °C (2 min) and 95 °C (2 min); followed by 40 cycles initial denaturation at 95°C (15 s), annealing at 60 °C (30 s) and extension at 75 °C (30 s). Melt curve analyses of the reference genes and target genes were performed to obtain specific melting temperatures. Real-time quantitative PCR primer sets used for NKA, H^+ ATPase, Rhbg, Rhcg-1 and Rhcg-2 were the same used by Sinha et al. (2013) and corticoid receptors were the same used by Stolte et al. (2008a) (Table 1 and 2).

Table 1 Sequences of ionoregulatory primers used in the qPCR assay.

Genes	**Primers sequence**	**Genbank Acc. No.**
β-actin	FW: 5'-CGT-GAT-GGA-CTC-TGG-TGA-TG-3' RV: 5'-TCA-CGG-ACA-ATT-TCC-CTC-TC-3'	M24113
NKA	FW: 5'-AGG-TGG-ACA-ACT-CCT-CCC-TG-3' RV: 5'-ATA-CGA-CCC-ATG-ACA-GTA-CG-3'	JX570881
H^+ ATPase	FW: 5'-CTA-TGG-GGG-TCA-ACA-TGG-AG-3' RV: 5'-CCA-ACA-CGT-GCT-TCT-CAC-AC-3'	JX570880
Rhbg	FW: 5'- TCC-CAG-TTT-CCA-GGA-TGT-TC-3' RV: 5'-TGG-AAA-AAG-CCC-TGC-ATA-AG-3'	JX570877
Rhcg-1	FW: 5'-ATC-CTG-AAC-ATC-CTC-CAT-GC-3' RV: 5'-AAC-TTG-GCC-AGA-ACA-TCC-AC-3'	JX570878
Rhcg-2	FW: 5'-CAC-AAA-GCC-ACA-CAC-AGT-CC-3 RV: 5'-TCT-TTT-TCT-CGC-CGT-TCT-TG-3'	JX570879

Table 2 Sequences of corticoid receptors primers used in the qPCR assay.

Genes	Primers sequence	Genbank Acc. No.
β-actin	FW: 5'-GCT-ATG-TGG-CTC-TTG-ACT-TCG-A-3' RV: 5'-CCG-TCA-GGC-AGC-TCA-TAG-CT-3'	M24113
GR1	FW: 5'-AGA-CTG-AGA-GGC-GGA-GCT-ACT-G-3' RV: 5'-GGC-GGT-GTT-GGC-TCC-AT-3'	AJ879149
GR2	FW: 5'-GGA-GAA-CAA-CGG-TGG-GAC-TAA-AT-3' RV: 5'-GGC-TGG-TCC-CGA-TTA-GGA-A-3'	AM183668
MR	FW: 5'-TTC-CCT-GCA-GAA-CTC-AAA-GGA-3' RV: 5'-ACG-GAC-GGT-GAC-AGA-AAC-G-3'	AJ783704

Reference housekeeping gene (β-actin) was screened over 15 random samples and the threshold cycle (Ct) for β-actin exhibited only a small change across all time points and treatments. Therefore, β-actin was used for normalization as internal control to calculate relative mRNA expression by the standard curve method. Values were expressed as fold changes and Ct values were normalised to the housekeeping gene (β-actin) expression corrected for the efficiency of each primer set relative to control, and analysed according to the Pfaffl method Relative Expression Software tool (REST©) (Pfaffl, 2001; Pfaffl et al., 2002).

2.7 Statistics analysis

Normality was checked prior to the analysis by Shapiro-Wilk test. Homogeneity of variance was verified using Hartley test. Log-transformed data were applied if the requirements for ANOVA were not fulfilled. Effect of feeding regime was assessed by unpaired two-tail student *t*-test. One way ANOVA was performed to examine significant effects of implantation within and between treatments at different time points followed by multiple comparison of Tukey post-hoc test. Sample size for all biochemistry data was $n = 8$; while sample size for molecular assays were $n = 8$ (control and sham implanted groups) and $n = 4 - 5$ (cortisol implanted groups). The biochemical results showed that both control and sham implanted groups were not significant between time intervals. Therefore, samples from these two groups were randomly selected and pooled for the molecular assay. Level of significance was set at $P<0.05$. All data were expressed as mean values with standard error (Mean ± SEM).

3. Results

3.1 Plasma metabolites

Overall, both control and sham implanted groups exhibited no significant difference throughout entire experiment. Cortisol implanted fish exhibited a significant plasma cortisol elevation compared to control and sham fish. Cortisol levels peaked at 12h-PI and started to decrease at 24h-PI and returned to a level similar to control and sham groups at 168h-PI in both feeding regimes (Fig. 1a and 1b).

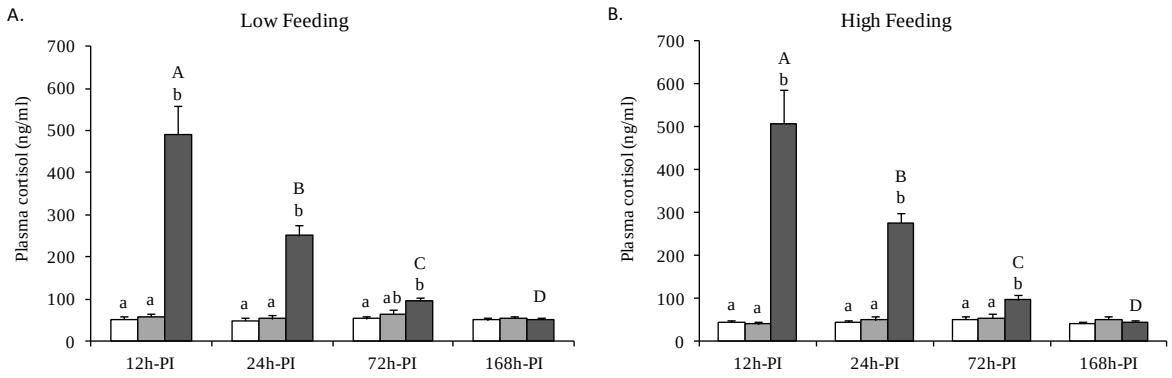

Fig. 1 Plasma cortisol levels of control carp without implants (open bars), sham implanted carp (light grey bars) and cortisol implanted carp (dark grey bars) at different feeding feeding regimes throughout 168h-PI. Small letters indicate significant differences between cortisol treatments. Capital letters indicate significant differences between time intervals.

A similar trend was observed for plasma glucose (Fig. 2a and 2b), although the glucose level still remained elevated as compared to control and sham implanted groups at 168h-PI in fish fed a low ration (Fig. 2a). Control fish that were fed a high ration exhibited relatively higher plasma glucose levels than the fish fed a low ration. An elevated plasma lactate level was only noticed during the first 12h-PI in cortisol implanted fish in both feeding regime and recovered to control levels afterwards (Fig. 2b and 2d).

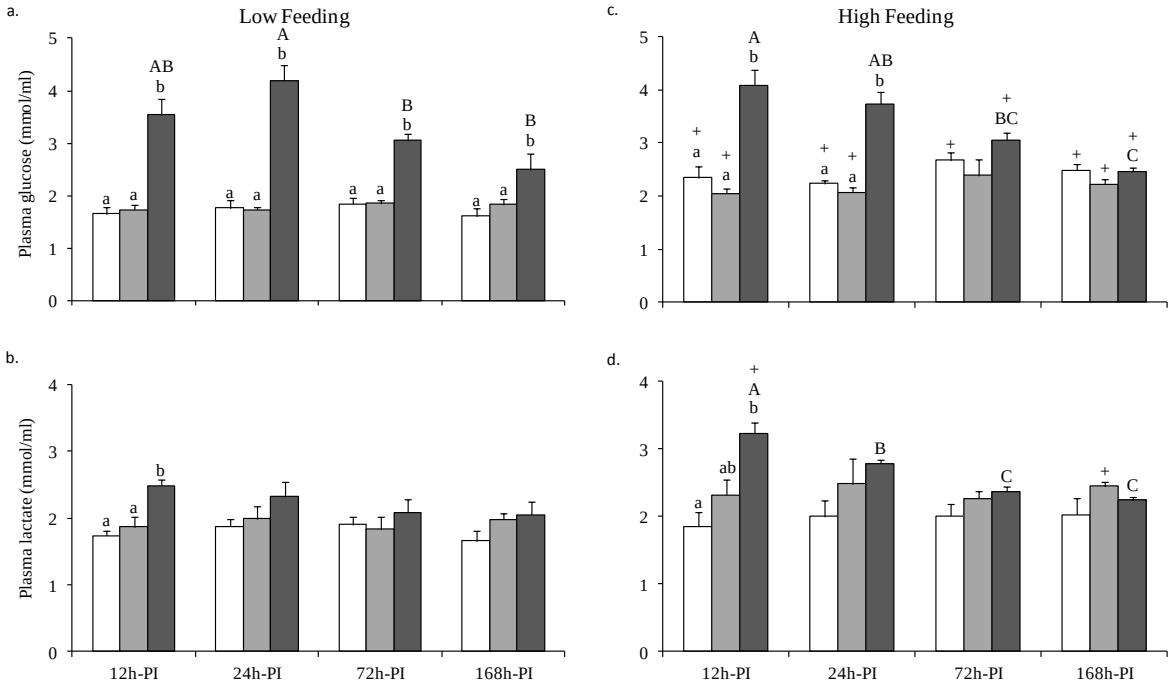

Fig. 2 Plasma glucose (a and c) and lactate (b and d) levels of control carp without implants (open bars), sham implanted carp (light grey bars) and cortisol implanted carp (dark grey bars) at different feeding feeding regimes throughout 168h-PI. Small letters indicate significant differences between cortisol treatments. Capital letters indicate significant differences between time intervals. A plus ($^{+}$) indicates significant differences between feeding regimes.

Overall, low feeding fish accumulated higher plasma ammonia levels than high feeding fish, especially in the fish implanted with cortisol (Fig. 3a and 3c). The level of ammonia was significantly elevated at 24h and 72h-PI in low feeding fish after being implanted with cortisol (Fig. 3a), while this was only seen at 24h-PI in high feeding fish (Fig. 3c). Cortisol implants only induced transient plasma urea elevation at first 12h-PI in low feeding fish (Fig. 3b). Contrary, control and sham implanted fish fed a high ration had higher plasma urea levels then cortisol implanted fish at 24h and 72h-PI (Fig. 3d), and in general higher urea levels than the fish fed a low ration (Fig. 3c).

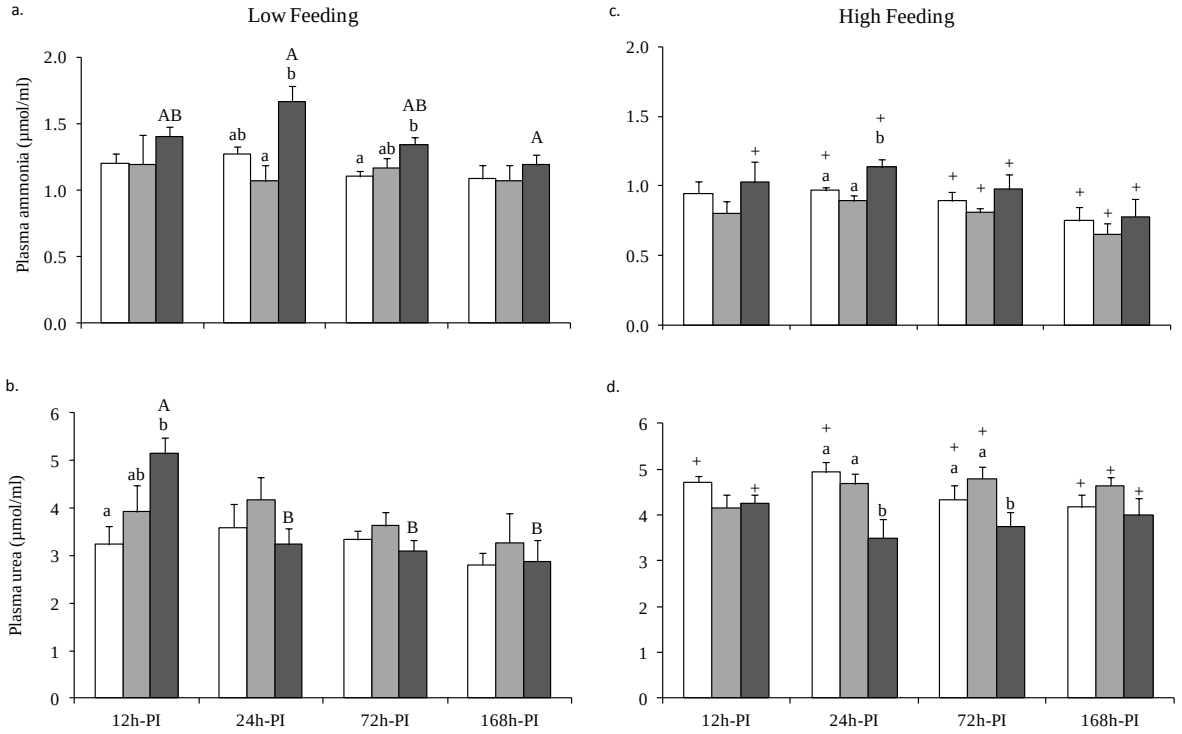

Fig. 3 Plasma ammonia (a and c) and urea (b and d) levels of control carp without implants (open bars), sham implanted carp (light grey bars) and cortisol implanted carp (dark grey bars) at different feeding feeding regimes throughout 168h-PI. Small letters indicate significant differences between cortisol treatments. Capital letters indicate significant differences between time intervals. A plus ($^+$) indicates significant differences between feeding regimes.

A decreased plasma osmolality level was recorded in low feeding fish implanted with cortisol at 24h-PI but osmolality recovered afterwards (Fig. 4a). Osmolality levels remained stable in high feeding fish (Fig. 4d). A feeding effect was observed at 168h-PI where high feeding fish implanted with cortisol exhibited higher osmolality levels than the low feeding fish. Overall, plasma ions (Na^+, Cl^- and K^+) remained at a relatively stable level (Fig. 4), although cortisol implanted fish seemed to display somewhat lower ions levels this was not significantly different. The only noticeable Na^+ loss was recorded in high feeding cortisol implanted fish at 24h-PI (Fig. 4e).

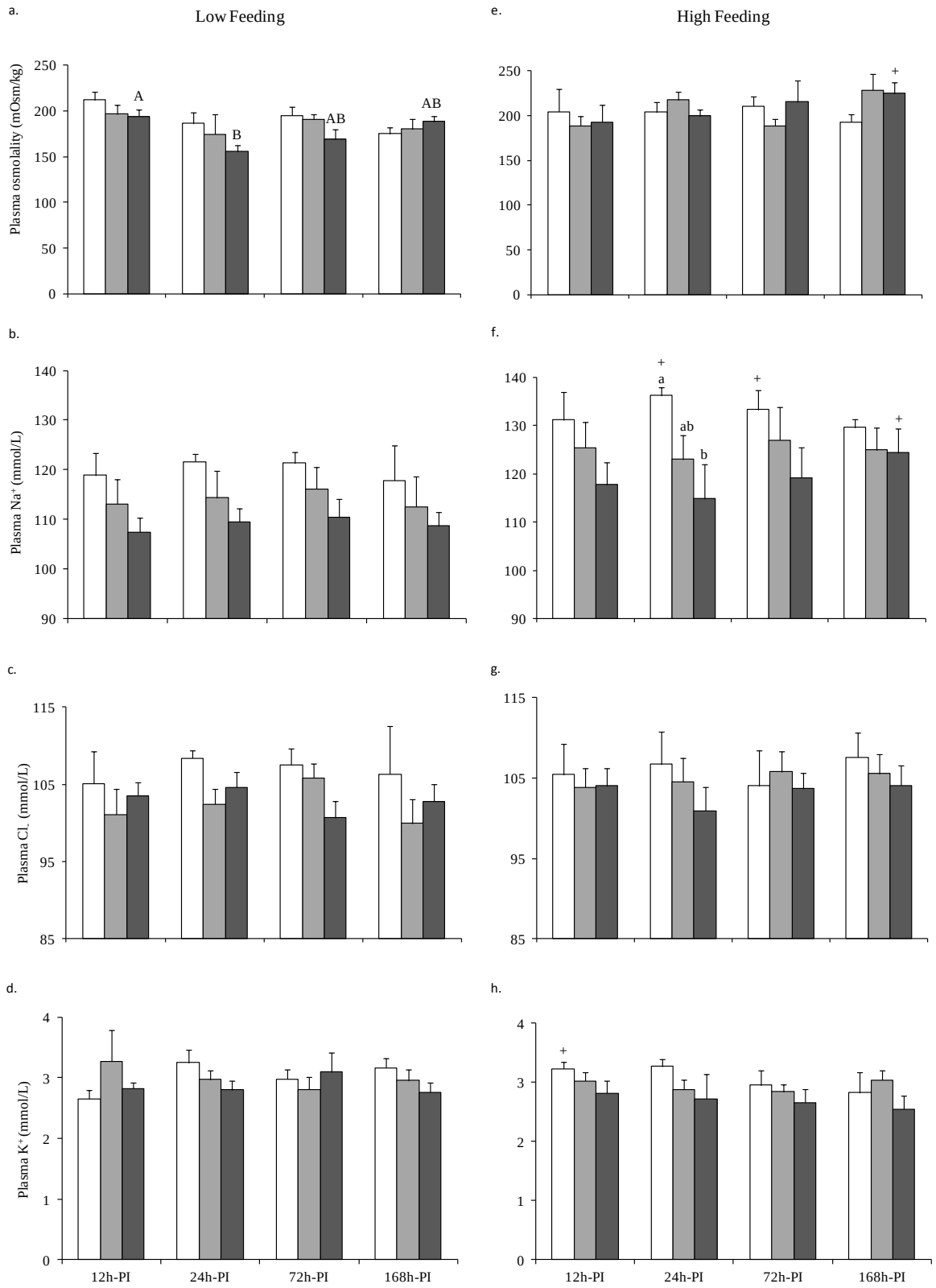

Fig. 4 Plasma osmolality (a and e), sodium, Na^+ (b and e), chloride, Cl^- (c and f) and potassium, K^+ (d and g) levels of control carp without implants (open bars), sham implanted carp (light grey bars) and cortisol implanted carp (dark grey bars) at different feeding feeding regimes throughout 168h-PI. Small letters indicate significant differences between cortisol treatments. Capital letters indicates significant differences between time intervals. A plus ($^+$) indicates significant differences between feeding regimes.

3.2 Gill and kidney ATPase activities

NKA activity increased significantly after cortisol implantation in the gill and kidney of both feeding regimes fish (Fig. 5). In the gill, cortisol induced a similar NKA activity trend with greater activity observed at 24h-PI in both feeding regimes fish (Fig. 5a and 5c). Although fish fed a high ration had greater gill NKA activities overall, a significant increase compared to low feeding fish was only recorded at 24h-PI and 72h-PI in sham and cortisol implanted fish; and at 72h control fish (Fig. 5c). In the kidney, the NKA activity was significantly induced at 12h-PI and 24h-PI in fish fed a low ration (Fig. 5b). This significant increase was only recorded at 24h-PI in fish fed a high ration (Fig. 5d).

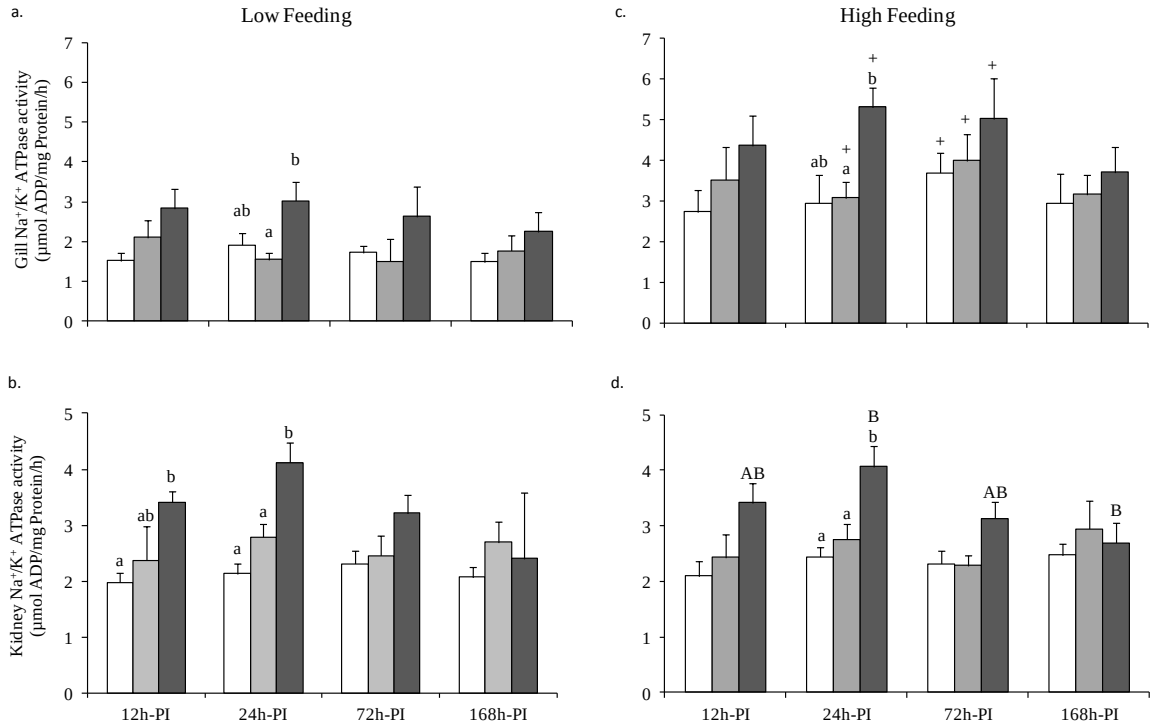

Fig. 5 Gill (a and c) and kidney (b and d) Na^+/K^+ ATPase activity levels of control carp without implants (open bars), sham implanted carp (light grey bars) and cortisol implanted carp (dark grey bars) at different feeding feeding regimes throughout 168h-PI. Small letters indicate significant differences between cortisol treatments. Capital letters indicate significant differences between time intervals. A plus ($^+$) indicates significant differences between feeding regimes.

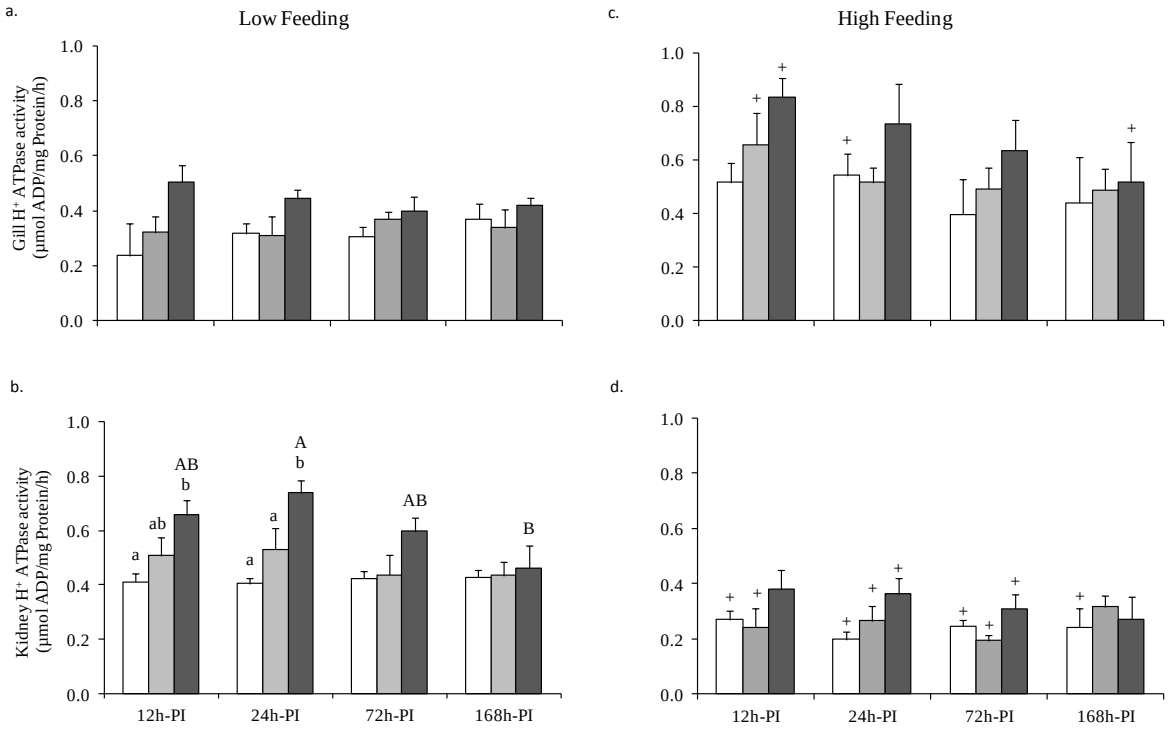

Fig. 6 Gill (a and c) and kidney (b and d) H^+ ATPase activity levels of control carp without implants (open bars), sham implanted carp (light grey bars) and cortisol implanted carp (dark grey bars) at different feeding feeding regimes throughout 168h-PI. Small letters indicate significant differences between cortisol treatments. Capital letters indicate significant differences between time intervals. A plus ($^+$) indicates significant differences between feeding regimes.

Gill H^+ ATPase activity remained unaltered after cortisol implantation, although there was a trend towards increased H^+ ATPase activity in cortisol implanted fish, but it was not significant (Fig. 6a and 6c). A feeding effect was observed at first 12h-PI with greater activity in sham and cortisol implanted fish fed a high ration (Fig. 6c). In the kidney, the opposite trend was observed with greater activity in fish fed a low ration and cortisol implantation significantly upregulated kidney H^+ ATPase activity at the first 24h-PI (Fig. 6b). In fish fed a high ration, the kidney H^+ ATPase activity remained stable (Fig. 6d).

3.3 Tissue metabolites

Cortisol induced liver protein mobilization in both feeding groups and mobilization was initiated at 12h-PI (Fig. 7b). In low feeding fish, cortisol significantly mobilized liver protein up to 72h-PI (Fig. 7a). In fish fed a high ration, this mobilization was observed for the first 24h-PI only (Fig. 7b). Surprisingly, muscle protein was maintained stable in low feeding fish (Fig. 8b), whilst a remarkable muscle protein mobilization was observed in high feeding fish implanted with cortisol at 24h-PI (Fig 8b). High feeding rates resulted in higher glycogen reserves in both liver and muscle (Fig. 7e and 8e). Cortisol induced liver glycogen deposition in both feeding groups (Fig. 7b and 7e). The effect of cortisol was more prominent in low feeding fish with a significant glycogen deposited observed from 24h-PI onward (Fig. 7b). Whereas in fish fed a high ration, the effect of cortisol induced glycogen deposition was only observed at 24h-PI (Fig. 7b). Muscle glycogen remained at a relatively stable level in low feeding fish (Fig. 8b), whereas muscle glycogen deposition was

remarkable in high feeding fish at 72h-PI (Fig. 8e). Overall, high feeding fish implanted with cortisol had higher muscle glycogen content than low feeding fish (Fig. 8e). Both liver and muscle lipid contents remained relatively stable in both feeding regimes (Fig. 7 and Fig. 8). A lipid mobilization was only observed in high feeding fish implanted with cortisol at 168h-PI as compared sham implanted fish (Fig. 7c).

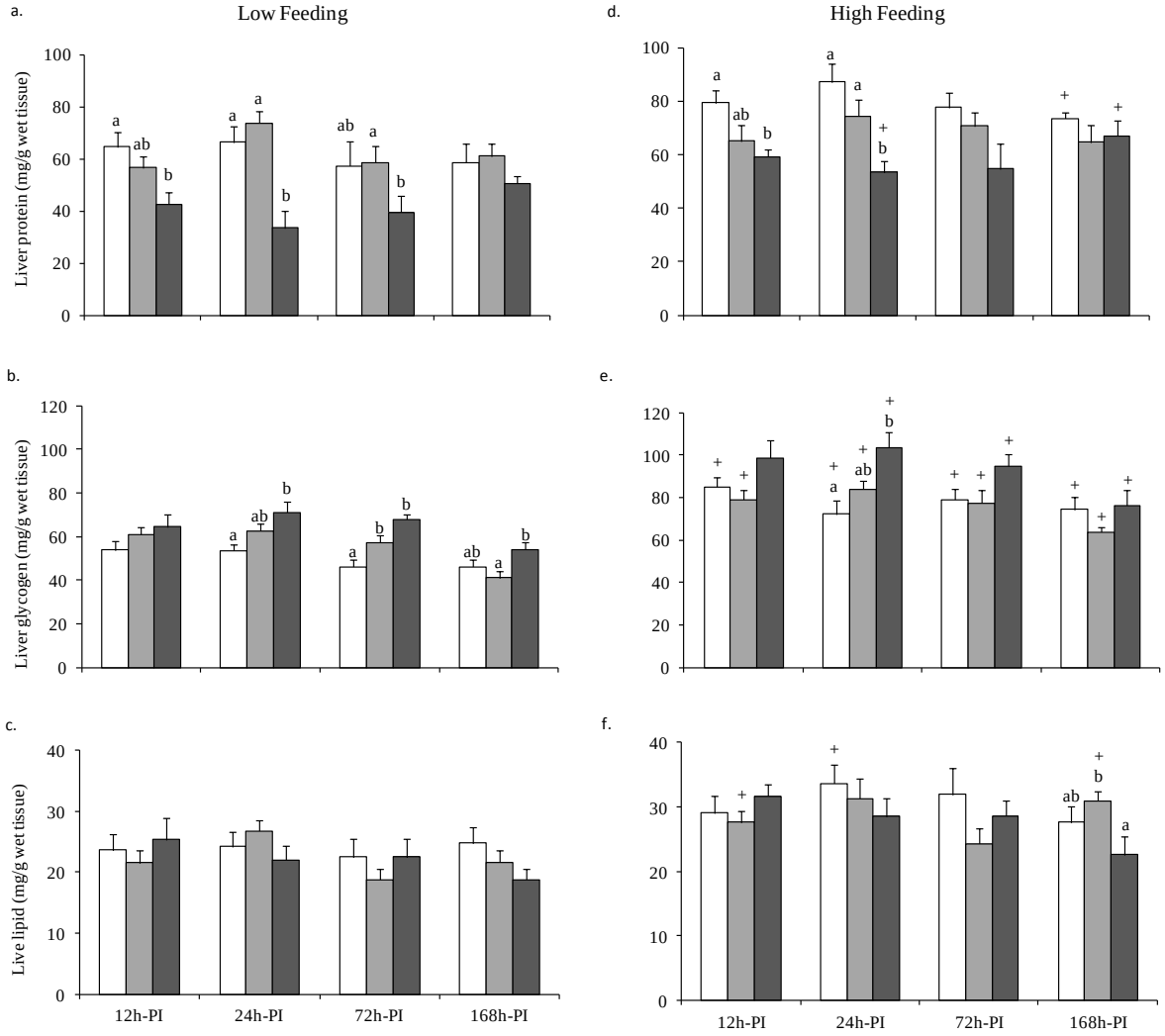

Fig. 7 Liver protein (a and d), glycogen (b and e) and lipid (c and f) of control carp without implants (open bars), sham implanted carp (light grey bars) and cortisol implanted carp (dark grey bars) at different feeding feeding regimes throughout 168h-PI. A plus ($^{+}$) indicates significant differences between feeding regimes. Small letters indicates significant differences between cortisol treatments.

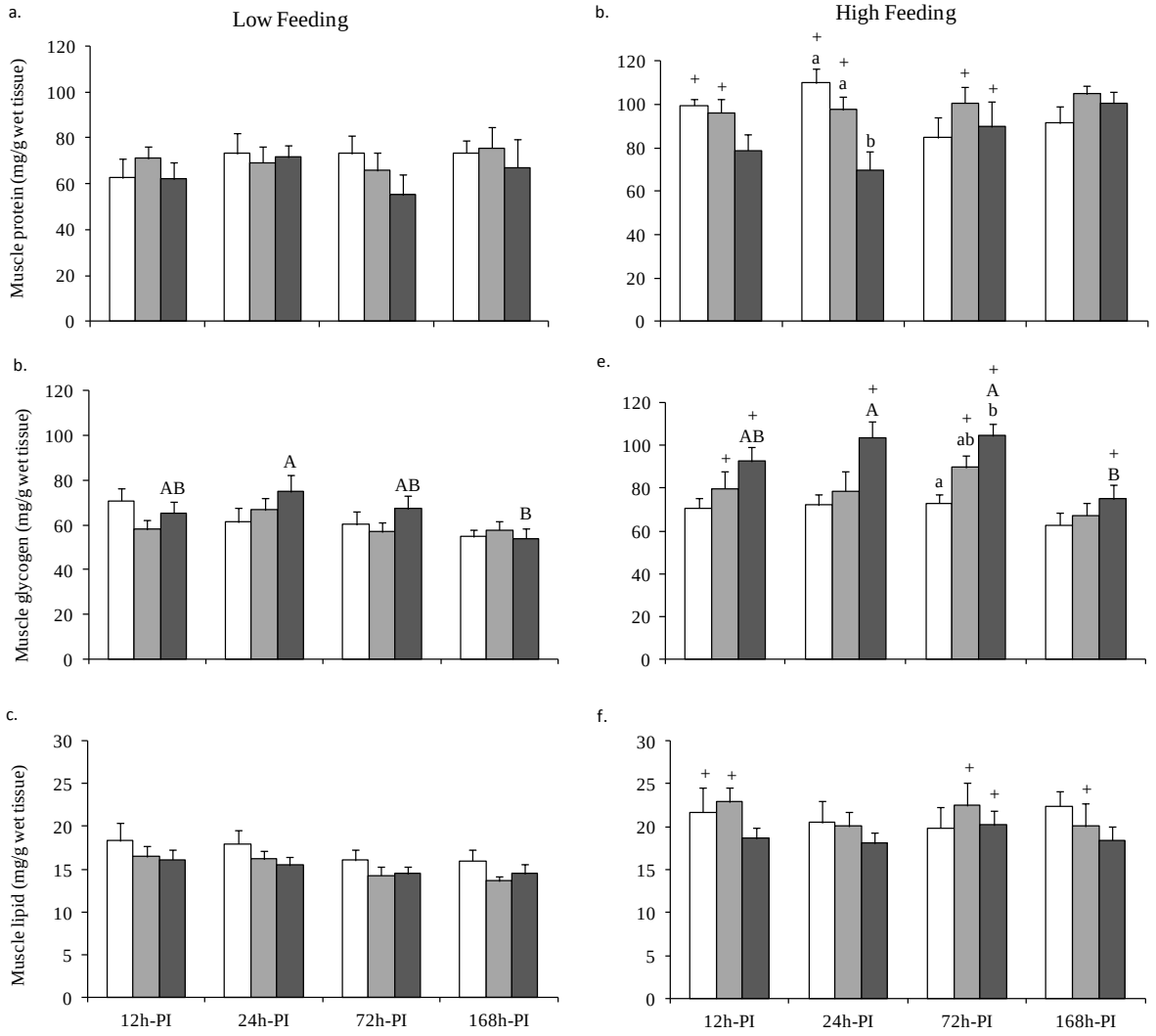

Fig. 8 Muscle protein (a and d), glycogen (b and e) and lipid (c and f) of control carp without implants (open bars), sham implanted carp (light grey bars) and cortisol implanted carp (dark grey bars) at different feeding feeding regimes throughout 168h-PI. Small letters indicates significant differences between cortisol treatments. Capital letters indicates significant differences between time intervals. A plus ($^{+}$) indicates significant differences between feeding regimes.

3.4 Gene expression

The expression of NKA and H^{+} ATPase mRNA in the gill and kidney is shown in Fig. 9. In the gill, neither feeding nor cortisol implantation have an effect on both NKA and H^{+} ATPase expression, although an increasing trend was observed during the first 24h-PI with cortisol implants (Fig. 9a and 9c). In the kidney, cortisol significantly upregulated NKA expression in fish fed a low ration at 72h-PI onward (Fig. 9b), while no effect was observed in fish fed a high ration (Fig. 9d). Similarly, no significant effect was observed in H^{+} ATPase expression over time, although an increasing trend was noticed in the high feeding regime (Fig. 9d). A fFeeding effect was seen at 72h-PI where the level of expression was significantly greater in high feeding fish compared to low feeding fish (Fig. 9d).

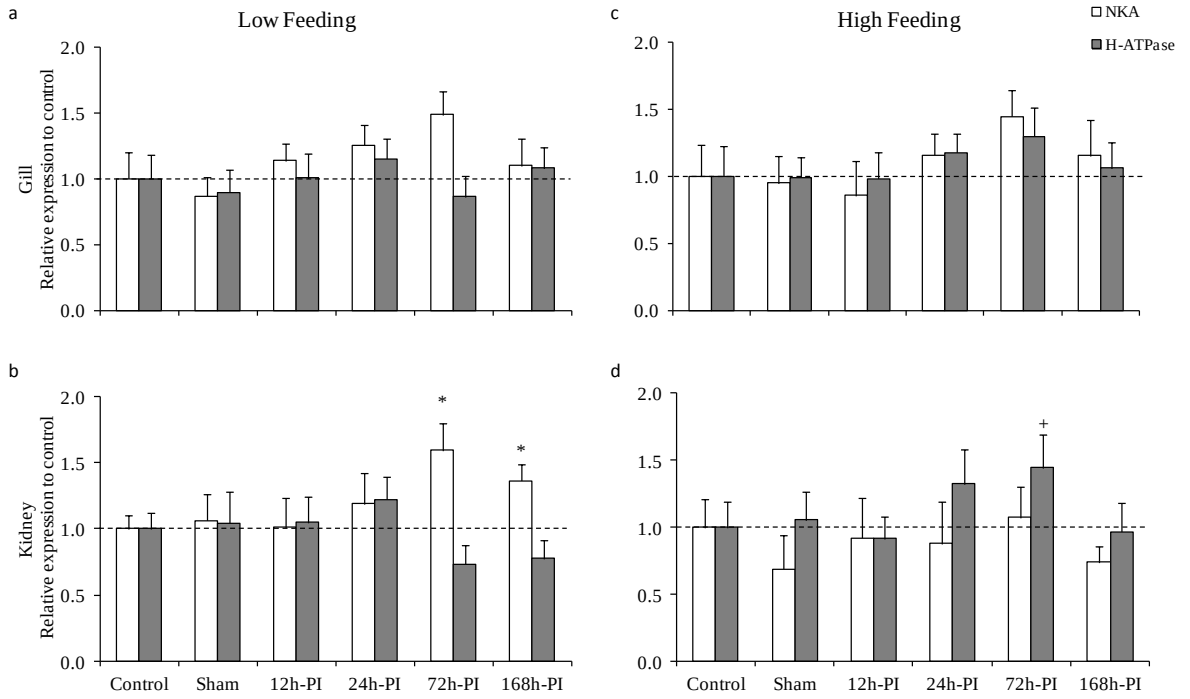

Fig. 9 mRNA Na^+/K^+ ATPase and H^+ ATPase expression levels in the gill (a and c) and kidney (b and d) of control carp without implants, sham implanted carp and cortisol implanted carp at different feeding feeding regimes throughout 168h-PI. An asterisk (*) indicates significant differences compared to the control group. A plus (+) indicates significant differences between feeding regimes.

Overall, gill Rhesus glycoproteins (Rhbg, Rhcg-1 and Rhcg-2) expression was more prominent in high feeding fish, although no feeding effect was observed (Fig. 10a and 10b). In high feeding fish, an increasing trend of Rhbg, Rhcg-1 and Rhcg-2 expression was seen after cortisol implantation, but only Rhcg-2 expression was significantly increased from 72h-PI onward (Fig. 10b).

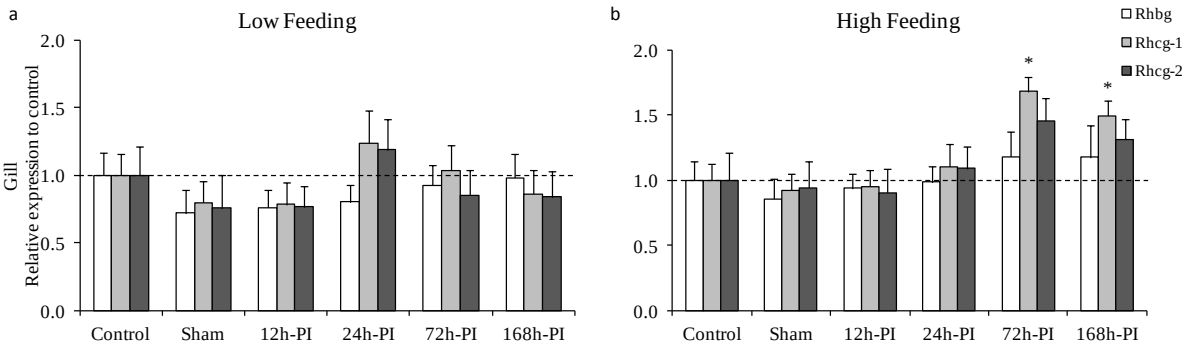

Fig. 10 mRNA Rhesus glycoprotein (Rhbg, Rhcg-1 and Rhcg-2) expression levels in the gill of control carp without implants, sham implanted carp and cortisol implanted carp at different feeding feeding regimes throughout 168h-PI. An asterisk (*) indicates a significant differences compared to the control group.

The corticoid receptors (GR1, GR2 and MR) expression pattern is summarized in Fig. 11. In the gill, GR1, GR2 and MR receptors were expressed in a similar trend in both feeding regimes (Fig. 11a and 11d), but were influenced by cortisol, especially in the high feeding fish (Fig. 11d). In the low feeding fish, GR2 was significantly upregulated by cortisol implantation at 72h-PI and returned to

control level at 168h-PI (Fig. 11a). In the high feeding fish, both GR2 and MR were upregulated at 72h-PI, while at 168h-PI only GR1 was upregulated (Fig. 11d).

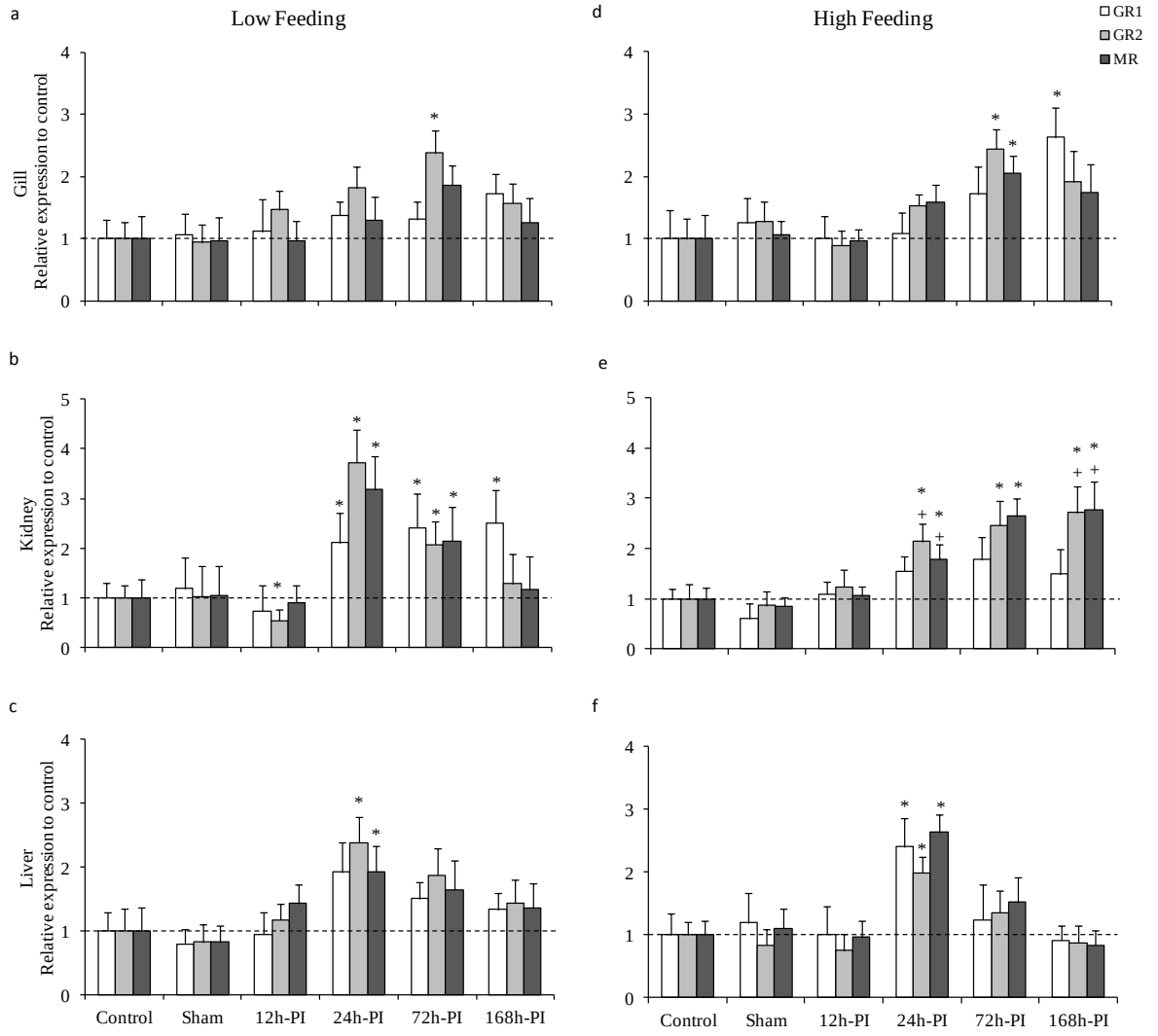

Fig. 11 mRNA glucocorticoid receptor-1 (GR1), glucocorticoid receptor-2 (GR2) and mineralocorticoid (MR) expression levels in the gill (a and d), kidney (b and e) and liver (c and f) of control carp without implants, sham implanted carp and cortisol implanted carp at different feeding feeding regimes throughout 168h-PI. An asterisk (*) indicates a significant differences compared to the control group. A plus ($^{+}$) indicates significant differences between feeding regimes.

In the kidney, GR1, GR2 and MR expression levels were more prominent in fish fed a low ration than high ratio (Fig. 11b and 11e). In the low feeding fish, kidney GR1 was significantly upregulated from 24h-PI onward. GR2 was significantly downregulated at 12h-PI and was significant upregulated at 24h-PI and 72h-PI (Fig. 11b). Similarly, MR expression levels were significantly upregulated at 24h-PI and 72h-PI (Fig. 11b). In the high feeding fish, GR2 and MR were remarkably upregulated from 24h-PI onward (Fig. 11e). A feeding effect was observed in GR2 and MR at 24h-PI with higher expression rates in low feeding fish, while the expression levels were greater in high feeding fish at 168h-PI (Fig. 11b and 11e). Liver corticoids showed a peak upregulation at 24h-PI (Fig. 11c and 11f). In the low feeding fish, GR2 and MR were significantly upregulated at 24h-PI (Fig. 11c),

whereas, a significant upregulation of GR1, GR2 and MR was detected at 24h-PI in high feeding fish (Fig. 11f).

4. Discussion

Our result showed that food limitation had no effect on the plasma cortisol changes that were induced by the cortisol implants. This result is similar with our previous study showing that plasma cortisol elevation was primarily caused by the cortisol implants without being affected by either food deprivation or exercise in common carp (Liew et al., 2013a; 2013b). Furthermore, our results are also in agreement with previous studies showing that food deprivation had no effect on plasma cortisol concentration in other species (Farbridge and Leatherland, 1992; Vijayan and Moon, 1992; 1994; Jørgensen et al., 1999). Cortisol level started to decrease and recovered to control level at 168h-PI likely associated with active cortisol catabolism and cortisol clearance, in combination with a negative feedback on endogenous cortisol production. Mommsen et al. (1999) and Teles et al. (2013) described that increased cortisol catabolism and negative feedback systems allowed teleosts to acclimatize to chronic cortisol elevation. A common response of carp responding to an overdose of cortisol hyperglycemia (Pottinger, 1998; Pottinger et al., 1999; 2000; 2010; Ruane et al., 2001; 2002; Liew et al., 2013b). Our result showed that fish fed a high ration have higher glucose level than fish fed a low ration during hypercortisol and the level of glucose recovered parallel to the cortisol recovery.

In conjunction with increased plasma ammonia and urea levels; liver protein was mobilized indicating that protein was used for gluconeogenesis. Cortisol induced protein use has been found before in salmonid species, Arctic char, sea raven and carp (Barton et al., 1995; Milligan, 1997; Vijayan et al., 1996; Liew et al., 2013b). Protein usage results increased endogenous ammonia levels. Carp that were fed a low ration retained more ammonia in plasma than the carp fed a high ration, even in carp that were not implanted with cortisol. Low plasma ammonia accumulation in high feeding fish indicated an enhanced detoxifying strategy by enhancing the excretion rate. This is not only stimulated by cortisol but also by feeding itself. In addition, a transient plasma lactate elevation was also seen in hypercortisol fish at 12h-PI which returned to control levels afterwards. The lactate was likely being used for glycogenesis as the observed liver and muscle glycogen levels were relatively higher in cortisol implanted fish from 24h-PI onward. This explained the fast recovery of the plasma lactate level in cortisol implanted fish. Similar cortisol induced liver glycogenesis has been reported previously in Japanese eel (Chan and Woo, 1978), rainbow trout (Hill and Fromm, 1968), killifish (Leach and Taylor, 1982) and common carp (Liew et al., 2013b).

Overall, plasma ions were maintained at a relatively stable level which was also reflected in the stable plasma osmolality. Although plasma Na^+ showed a downwards trend in cortisol treated fish, this Na^+ loss did not differ significantly. Feeding seems to contribute substantially to a sufficient ion uptake, and therefore the loss of Na^+ caused by hypercortisol was re-gained through dietary intake. Despite these stable ion levels, gill NKA activity was greater in the high feeding fish than low feeding fish. This increment in activity had been reported previously and was more associated with ammonia excretion during swimming rather than ion uptake per se (Liew et al. 2013a) as feeding granted sufficient ion uptake via the gastrointestinal system (Bucking and Wood, 2006; 2008; Bucking et al., 2010).

Cortisol stimulating gill NKA has been reported previously in cyprinids (Abo Hegab and Hanke, 1984), cichlids (Dang et al., 2000), salmonids (Shrimpton and McCormick, 1999; Wilson et al., 2002)

and catfish (Babitha and Peter, 2010). Similarly, the presents study confirmed that hypercortisol upregulated NKA activity in both gill and kidney in carp. In the gill, a greater NKA activity was detected in carp fed a high ration. These fish also retained less ammonia than the carp fed a low ration, while overall plasma ions and osmolality remained stable. This reflected the integration of gill NKA activity with transporters involved in apical ammonia excretion such as the NH_4^+/Na^+ and/or Na^+/H^+ exchanger. Enhanced gill ammonia excretion, subsequently created a basal electro potential gradient to promote Na^+ influx by upregulating NKA activity and/or Na^+ channels (Walsh, 1998; Salama et al., 1999; Wu et al., 2010; Zimmer et al., 2010; Liew et al., 2013a). The involvement of Na^+/H^+ exchanger, also explains the increase of gill H^+ ATPase activity which followed a similar trend as gill NKA activity with greater activity observed in the carp fed a high ration. Hypercortisol did not only modulate gill ionoregulation, but also induced increased kidney ionoregulation. An increase of kidney NKA activity was observed during the first 24h-PI in low feeding fish, and at 24h-PI in high feeding fish. Overall, kidney NKA activity detected in fish fed a low ration was comparable with the fish fed a high ration. A similar pattern was observed in for the H^+ ATPase activity, where hypercortisol increased H^+ ATPase activity at the first 24h-PI in low feeding fish. However, no effect was observed for the H^+ ATPase activity in the fish fed a high ration and the activities detected were much lower than in the low feeding fish. This again showed that feeding provided sufficient ion intake in the high feeding fish, therefore the activity in the kidney was maintained at control level. All ionoregulatory responses in the gill and the immediate response to hypercortisol in the kidney explained why plasma ions remained stable. Wood et al., (2009) and Bucking et al. (2010) noted that even though the rate of kidney ionoregulatory activity was lower than branchial activities, kidney ionoregulatory still played an important role in maintaining basal ions level. Nevertheless, a genomic response on the NKA mRNA expression in the kidney of low feeding fish was only observed at later stage of cortisol implantation.

The Rhesus glycoproteins examined in the present study also showed no obvious upregulation after cortisol implantation, and only Rhcg-1 was increased at 72h-PI in high feeding fish. The Rhcg-1 upregulation in the gill likely enhanced branchial ammonia excretion capacity, when endogenous ammonia levels were elevated. This is in parallel with previous studies reporting that cortisol regulates ionoregulatory and Rhesus glycoproteins gene expression in teleosts and may play a key role in regulating expression of branchial ammonia excretion (Kiilerich et al. 2007; Ivanis et al., 2008). This was proven by an *in vitro* gill culture of rainbow trout challenged to cortisol which activated the NH_4^+/Na^+ exchange metabolon and Rhesus glycoproteins to increase ammonia transport in the gill epithelial cells (Tsui et al., 2009). Also, increased ammonia excretion rates were noticed in trout and cyprinid species when challenged with ammonia exposure in parallel with increased plasma cortisol levels (Sinha et al., 2013), together with low plasma ammonia accumulation; stable ion levels and increased NKA and H^+ ATPase activities. This suggests that cortisol primarily stimulated non-genomic actions to cope with acute stress by activating the ionoregulatory metabolon in the gill. This confirms our first and second hypothesizes that cortisol does trigger gill NKA and H^+ ATPase activities, as well as Rhcg-1 expression in high feeding fish to facilitate ammonia excretion, while stimulating kidney NKA and H^+ ATPase activities in low feeding fish for ion reabsorption in common carp.

In the present study, the mRNA expression pattern of GR1, GR2 and MR in both gill and kidney were differently expressed in response to hypercortisol. According to Teles et al. (2013), gill is the primary action site of cortisol with the obvious important role in osmoregulation. They found that gill GR1, GR2 and MR levels were depressed in seabass 24h after being implanted with cortisol, but

the levels were upregulated thereafter up to 240h-PI. In our study, the gill GRs and MR were responding very sensitively even though plasma cortisol level was highly elevated during the first 12h-PI. In the low feeding fish, the only upregulation was detected for GR2 at 72h-PI. In the high feeding fish, GR2 and MR upregulation were observed at 72h-PI, followed by GR1 at 168h-PI. However, overall both feeding regimes exhibited an increasing trend of expression. Our finding is consistent with Yada et al., (2008) and Aruna et al., (2012a) who also found no significant difference in the GR1 and GR2 transcription at early stage, but later did find upregulation of GR2 and MR at 98h in trout and tilapia. They suggested the upregulation of GR2 and MR may play distinctive roles in hypo-and-hyper osmoregulatory mechanisms. Recently, the involvement of cortisol in ion-base regulation via the GR1, GR2 and MR receptors were proven in gills of tilapia where co-localization of GR1, GR2 and MR transcripts with the chloride cells was observed during seawater acclimation (Aruna et al., 2012b).

Cortisol is produced and released from the head kidney to signal and interacts neuroendocrine stress responses in teleosts (Tort, 2011). Our results showed that kidney was the most sensitive organ and responded first to hypercortisol, where GR2 downregulation was observed at 12h-PI in low feeding fish. This GR2 downregulation could reflect negative feedback due to the acute cortisol elevation which would decrease tissue responsiveness to acute cortisol stimulation (Hontela et al., 1992; Takahashi et al., 2006) or a shock response of the pituitary–inter-renal-axis due to extreme hypercortisol. However, the GR1, GR2 and MR started to be upregulated at 24h to 72h-PI and returned to control at 168h-PI, while GR1 still remained upregulated at 168h-PI. The similar decreasing trend concomitant with plasma cortisol levels showed that GR2 and MR were more sensitive to the decreasing trend in cortisol. In high feeding fish, upregulation of GR2 and MR responded at 24h-PI and remained upregulated up to 168h-PI. However, the levels of expression in low feeding fish were much higher at 24h-PI and lower at 168h-PI than in high feeding fish. Similarly, upregulation of GR1, GR2 and MR were also observed in seabass after 24h implanted with cortisol and GR1 continued to be upregulated untill 168h (Vazzana et al., 2010). This probably indicates the relationship between corticoids and increased NKA and H^+ ATPase activities for ionoregulation. Where, Takahashi and Sakamoto (2013) concluded that GRs may play a more important role in teleost osmoregulation than MR.

The liver is the tissue relevant for energetic and intermediary metabolism for all living organisms. It is not surprising that extra energy expenditure was needed during stress to compensate basal metabolic changes. Due to the cortisol elevation, our present results indicate that hypercortisol induced liver protein mobilization for gluconeogenesis. Additionally, this resulted in an increased plasma ammonia level. Extra energy mobilization during stress is always associated with growth depression due to limited energy expenditure for somatic growth. This phenomenon was referred to as 'high living cost' when observed in rainbow trout implanted with cortisol which not only depressed feeding performance and growth rate, but also depressed aggressive behaviour (Gregory and Wood, 1998; 1999; De Boeck et al., 2001).

Our results showed that the GR2 and MR were expressed in both feeding regimes at 24h-PI and returned to control level afterwards and GR1 expression was only noticed in high feeding fish. This expression pattern supports previous reports which noted that GR2 is the most sensitive corticoid receptor in transmitting the stress signal, followed by the MR and GR1 expression (Stolte et al., 2008a; 2008b). Moreover, liver GR2 and MR expression likely signaled energy mobilization for ionoregulation used in gill and kidney. Cortisol enhanced gluconeogenesis and mobilized glucose to fuel gill metabolism, including activation of gill NKA activity for hypo-osmoregulation, in tilapia

acclimated to seawater as well (Vijayan et al., 1997; Mommsen et al., 1999; McGuire et al., 2010). Also in our study increased plasma glucose paralleled with high cortisol levels and higher NKA and H^+ ATPase activity and mRNA expression in gill and kidney. Overall, the genomic expression of GR1, GR2 and MR mRNA were upregulated after the hypercortisol peak in gill, kidney and liver which supported our third hypothesis. However, in contrast to our earlier hypothesis, the receptors were not upregulated to a larger extend in low feeding fish, except in kidney.

5. Conclusion

As a conclusion, we can summarize that cortisol plays an important role in regulating non-genomic ionoregulatory and metabolic responses in carp during early stages of high cortisol as an immediate response. A rapid upregulation of gill NKA and H^+ ATPase activities in carp fed a high ration seem associated with ammonia excretion due to protein mobilization. In the kidney, the upregulation of NKA and H^+ ATPase activities were more pronounced in fish fed a low ration clearly to enhance ion reabsorption due to the limited dietary ion intake. This enabled carp to maintain plasma osmolality and ions at a relative stable level. Additionally, cortisol caused a repartitioning of energy for gluconeogenesis and glycogenesis. Secondly, genomic responses to the high cortisol happened in a later stage after the peak of cortisol had passed, suggesting that these genomic responses require a more prolonged cortisol elevation. In low feeding fish, kidney NKA mRNA expression was upregulated for ionoregulation, while in high feeding fish gill Rhcg-1 was upregulated for ammonia excretion. The GR1, GR2 and MR mRNA expression pattern showed tissue specific responses with kidney as most sensitive organ, especially in low feeding fish. In combination with the increased activity of ion transporters, this suggests a contribution of GR1, GR2 and MR in renal ion reabsorption. Our results also revealed gill GRs and MR expressed during the plasma cortisol recovery period. This occurred simultaneously with the increase in Rhesus glycoprotein mRNA expression but well after NKA and H^+ ATPase activities increased. Further immunohistochemistry and *in situ* hybridization studies would be valuable in order to address the relationship between corticoid receptors and ionoregulation in common carp.

General Discussion

Goldfish and common carp are two closely related species from the Cyprinid family. As expected short-term food deprivation did not impair swimming capacity in both species. However, both species exhibited fundamentally different metabolic strategies to cope with the increasing metabolic demand of simultaneously sustaining feeding and swimming metabolism. To do that, goldfish sacrificed digestion metabolism by channeling oxygen intake towards swimming metabolism, which was clearly prioritized. Besides sufficient oxygen delivery, goldfish mobilized protein from both liver and muscle to fuel swimming expenditure which coincided with an increased AQ and plasma ammonia level. Goldfish, a member of *Carassius* seem to conserve liver glycogen while mobilizing muscle glycogen during exhaustive swimming. Conserving glycogen is a key survival trait for *Carassius* encountering anoxia. They are an extremely anoxia tolerant species, and rely completely on their glycogen stores during anoxia fuelling anaerobic metabolism and converting lactate to ethanol (Nilsson, 1990). Therefore, it is advantageous for *Carassius* to rely on protein fuel as much as possible while delaying the activation of anaerobic metabolism under normoxic conditions (Sollid et al., 2005) (**Chapter 1**).

In contrast in common carp, both digestion and swimming metabolism were performed independently, and extra oxygen was consumed and delivered to the digestion metabolism and the swimming metabolism simultaneously. In terms of fuel preference, the primary fuel mobilized during fasting was glycogen with a remarkable depletion in both liver and muscle followed by liver protein. Interestingly, fed common carp not only mobilized liver glycogen but also muscle glycogen, muscle protein as well as small contribution from muscle lipid during exhaustive swimming. The small contribution of muscle lipid detected in common carp swum to exhaustion likely supported earlier hypotheses that intra-and-extra muscular lipid was oxidized to fuel ATP, phoshocreatine and glycogen production in white muscle tissue (Wang et al., 1994; Richards et al., 2002) (**Chapter 1**). The use of protein as primary metabolic fuel had been reported previously in salmonid and tilapia (Kutty, 1972; Van den Thillart, 1986; Davison, 1989; Smith and Houlihan, 1995; Jobling, 1994; Weber and Haman, 1996). In contrast, a rather different conclusion was reached by Lauff and Wood (1996a, 1996b, 1997) who showed that in rainbow trout lipid was the main fuel, followed by carbohydrate mobilization, whilst protein contribution declined as swimming speed increased. Others found that glycogen was the most important fuel due to recruitment of white muscle during active swimming which resulted in plasma lactate elevation due to anaerobic metabolism (Wood, 1991; Moyes and West, 1995; Milligan, 1996; Weber and Haman, 1996; Milligan et al., 2000).

Moreover, the use of protein certainly induced endogenous ammonia production and increased ammonia excretion rate in actively swimming common carp. An efficient ammonia excretion and gill ammonia clearance rate served as ammonia self-detoxifying strategies to eliminate plasma ammonia accumulation to sustain swimming capacity, especially in carp implanted with cortisol (**Chapter 3**). Plasma ammonia elevation impairs swimming capacity in rainbow trout, thus it should be avoided (Beaumont et al., 1995; McKenzie et al., 2003; De Boeck et al., 2006). As feeding granted dietary ion and energy intake, the upregulation of gill NKA seem likely associated with ammonia excretion facilitated by Na^+/NH_4^+ exchanger and $Na^+/NH_4^+/2Cl^-$ co-transporter which created a Na^+ potential gradient to promote ammonia excretion (Alam and Frankel, 2006; Yang et al., 2010). With this respect, feeding seems not only to contribute to basic ion and energy homeostasis but also stimulates ammonia excretion (**Chapter 2 & 4**). Cortisol elevation did not

impair swimming capacity in cyprinids but cortisol elevation did induce metabolic re-organisation to sustain metabolism demands (**Chapter 1 & 3**).

As expected, exhaustive swimming induced a plasma cortisol elevation in goldfish. This elevated cortisol level following exhaustive swimming is in line with previous studies in rainbow trout (Pagnotta et al., 1994; Eros and Milligan, 1996; Milligan et al., 2000; Kieffer, 2010). Surprisingly, this elevation was not seen in common carp where plasma cortisol levels remained unchanged at approximately 50 ng/ml, either when resting or when forced to swim (**Chapter 2 & 3**).

As expected in order to conserve energy expenditure during food deprivation, both fasting species exhibited similar ionoregulation strategies by minimized gill NKA activity, even though these fish were swum to exhaustion. For the fed fish, gill NKA activity was upregulated in response to feeding and swimming in both species. Overall plasma ion levels in both fasting and feeding fish were maintained at a stable level which illustrates a successful 'osmorespiratory compromise' during swimming. The trend of gill NKA activity in goldfish followed the trend for plasma cortisol levels in goldfish (**Chapter 2**).

In regard to the osmorespiratory compromise, it will be interesting to examine the gill functional surface area (gill remodeling) which is responsible for both gas exchange versus ion loss, as well as to compare blood volume (plasma) after exhaustive swimming with either low aerobic swimming or resting fish. This could enable us to understand more in detail how common carp succeed in compromising between ionoregulation-and-respiration when swimming during cortisol elevation. Although there is increased evidence demonstrating that cortisol is involved in ionoregulation in freshwater fish by increasing gill, kidney and gut NKA (McCormick, 2011) these kind of studies were so far only performed on rainbow trout by Randall et al. (1972), Wood and Randall (1973a, 1973 b), Booth (1978, 1979), Farrell et al. (1980), Gonzalaz and McDonald (1992, 1994). They found that rainbow trout only utilized a fraction of their total gill surface area when oxygen demand is low, thereby limiting the surface area available to ion loss at rest, and limiting blood flow in the perfused lamellae. During active swimming, rainbow trout recruited more gill functional surface area for gas exchange, which subsequently increased Na^+ loss. However, this did not seem to occur in common carp (**Chapter 2**), except in fish implanted with cortisol on a low feeding ration, especially when swum to exhaustion (**Chapter 4**). Therefore, it would be interesting to examine the gill osmorespiratory compromise on a physiological-and-biochemical level in combination with the latest histochemistry-and-electromicroscopy.

A meal allows the fish not only to generate energy for growth and reproduction, but feeding also provides a large ion load (Bucking and Wood, 2006a; Bucking and Wood, 2007; Taylor et al., 2007) and challenges water balance (Bucking and Wood, 2006b). Internal high Na^+ load alone is able to induce various elements of the seawater ionoregulatory phenotypes in freshwater rainbow trout fed with a high Na^+ diet (11% NaCl) (Perry et al., 2006). However, on a limited dietary ion intake, cortisol induced kidney NKA and H^+ ATPase activities for ion reabsorption in common carp (**Chapter 4 & 5**). Cortisol induced rapid upregulation of gill NKA and H^+ ATPase activities in carp fed a high ration which we believe is associated with ammonia excretion, while in kidney the NKA and H^+ ATPase activities in carp fed at low ration enhanced ion reabsorption. Cortisol clearly induced hyperglycemia and the level recovered to control levels together with cortisol recovery. The cortisol effect on energy metabolism still remains difficult to characterize and appears to be species specific and dependent on the multitude of experimental procedures. Fundamentally, cortisol induced hyperglycemia via gluconeogenesis (Mommsen et al., 1999). Lipid mobilization is suggested as a possible energy sources for cortisol induced actions in rainbow trout to conserve protein and

glycogen (De Boeck et al., 2001). On the other hand, cortisol also promoted proteolytic processes by activating protein catabolism to generate free amino acids (Mommsen et al., 1999), thus providing energy for osmoregulation (Takei and Loretz, 2006).

After a first series of fast non-genomic responses, cortisol induced genomic responses such as the upregulation of kidney NKA mRNA expression for Na^+ reabsorption in low feeding fish and gill Rhcg-1 in the high feeding fish facilitating ammonia excretion. The gill GRs and MR expressed an increasing trend at a later stage of cortisol elevation. They might signal to other ionoregulatory processes including NKA, H^+ ATPase and Rhesus glycoprotines mRNA expression for a further adaptive phase (**Chapter 5**). Sea bass acclimated to different environmental salinities showed an adaptive phase with recruitment of new NKA, chloride cells and an energy redistribution in parallel with cortisol elevation (Sangio-Alvarellos et al., 2005). Also, Senegalese sole challenged with hypersalinity required three days to adapt to osmotic stress and regulated glucocorticoids to increase ATP availability for the NKA (Arjona et al., 2008). The different expression pattern of GR1, GR2 and MR observed in tilapia challenged to different salinities illustrated that cortisol signaling serves different physiological functions (Aruna et al., 2012). In rainbow trout, cortisol induced tissue specific GRs and MR mRNA expression level related to tissue function during plasma cortisol elevation and the recovery period (Teles et al., 2012). The GR1, GR2 and MR mRNA expression pattern exhibited in this study in common carp also showed that corticoid receptors expression was tissue specific. Results showed that kidney was the most sensitive organ to cortisol and GR and MR receptor genes expressed to a greater extend in low feeding fish than high feeding fish. Together with the greater activity of ionoregulatory enzymes this suggests a contribution of GR1, GR2 and MR in renal ion reabsorption. Whether or not corticoid receptors are involved directly or indirectly in ionoregulation in common carp should be addressed in future research by knockdown of these receptors and by performing histochemistry studies on ionoregulatory organs, while monitor plasma ion levels and NKA and H^+ ATPase activities.

General conclusion

Overall, this thesis demonstrated that goldfish and common carp clearly showed different physiological responses, even though both are close family members from the Cyprinidae. Goldfish prioritize swimming metabolism over digestion, while common carp increase aerobic metabolism to sustain both digestion and swimming. Feeding ration (1 week fasting, 0.5%, 2.0% and 3.0% of BW) or cortisol elevation did not influence swimming capacity. Goldfish protected liver stores glycogen stores, even during fasting or exhaustive swimming, while common carp did not. Protein catabolism was important in both species, and fish fed at a high feeding ration had better ammonia excreting capacities than fasting fish or fish feeding at a low ration resulting in lower plasma ammonia values. High feeding fish protected ion homeostasis mainly by upregulating branchial transport mechanisms, while at lower feeding rations the kidney played an increasingly important role. Since high feeding fish get sufficient ions from their food, upregulation of branchial NKA and H^+ ATPase is probably more related to ammonia excretion than ion uptake per se. Both fish species succeeded very well in balancing gas uptake versus ion losses. Only in low feeding cortisol treated fish swimming at high speeds the osmorespiratory compromise could not be sustained. When cortisol was elevated, common carp showed quick non-genomic changes in metabolism and ionoregulation. Genomic responses developed much slower, and upregulation of genes was only observed after a prolonged cortisol elevation. Our results indicate that the glucocorticoid receptors might not only play an important role in energy metabolism and branchial ionoregulation, but are also important in the regulation of renal ion transport, especially in fish on a limited food supply.

Bibliography

Abo Hegab, S., Hanke, W., 1984. The significance of cortisol for osmoregulation in carp (*Cyprinus carpio*) and tilapia (*Saratherodon mossambicus*). Gen. Comp. Endocrinol. 54, 409-417.

Alam, M., Frankel, T.L., 2006. Gill ATPase activities of silver perch, *Bidyanus bidyanus* (Mitchell), and golden perch, *Macquaria ambigua* (Richardson): effects of environmental salt and ammonia. Aquaculture 251, 118-133.

Alsop, D., Vijayan, M.M., 2008. Development of the corticosteroid stress axis and receptor expression in zebrafish. Am. J. Physiol. Regul. Integr. Comp. Physiol. 294, 711-719.

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

Altimiras, J., Claireaux, G., Sandblom, E., Farrell, A.P., McKenzie, D.J., Axelsson, M., 2008. Gastrointestinal blood flow and postprandial metabolism in swimming sea bass *Dicentrarchus labrax*. Physiol. Biochem. Zool. 81, 663-672.

Alvarenga, C.M.D., Volpato, G.L., 1995. Agonistic profile and metabolism in alevins of the Nile tilapia. Physiol. Behav. 57, 75-80.

Anderson, D.E., Reid, S.D., Moon, T.W., Perry, S.F., 1991. Metabolic effects associated with chronically elevated cortisol in rainbow trout (*Oncorhynchus mykiss*). Can. J. Fish. Aquat. Sci. 48, 1811-1817.

Arjona, F.J., Vargas-Chacoff, L., Martín del Río, M.P., Flik, G., Mancera, J.M., Klaren, P.H.M., 2008. The involvement of thyroid hormones and cortisol in the osmotic acclimation of *Solea senegalensis*. Gen. Comp. Endocrinol. 155, 796-803.

Aruna, A., Nagarajan, G., Chang, C.F., 2012a. Involvement of corticotrophin-releasing hormone and cortosteroid receptors in the brain-pituitary-gill of tilapia during the course of seawater acclimation. J. Endocrinol. 24, 818-830.

Aruna, A., Nagarajan, G., Chang, C.F., 2012b. Differential expression patterns and localization of glucocorticoid and minerolocorticoid receptor transcripts in the osmoregulatory organ of tilapia during salinity stress. Gen. Comp. Endocrinol. 179, 465-476.

Ashley, P.J., 2006. Fish welfare: current issues in aquaculture. Appl. Anim. Behav. Sci. 104, 199-235.

Babitha, G.S., Peter, M.C.S., 2010. Cortisol promotes and integrates the osmotic competence of the organs in North African catfish (*Clarias gariepinus* Burchell): Evidence from *in vivo* and *in situ* approaches. Gen. Comp. Endocrinol. 168, 14-21.

Bandeen, J., Leatherland, J.F., 1997. Changes in the proximate composition of juvenile white suckers following re-feeding after a prolonged fast. Aquac. Internat. 5, 327-337.

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., Iwama, G.K., 1991. Physiological changes in fish from stress in aquaculture with emphasis on the response and effects of corticosteroids. Ann. Rev. Fish Dis. 1, 3-26.

Barton, K.N., Gerrits, M.F., Ballantyne, J.S., 1995. Effects of exercise on plasma nonesterified fatty acids and free amino acids in Arctic char (*Salvelinus alpines*). J. Exp. Zool. 271, 183-189.

Beamish, F.W.H., 1966. Swimming endurance of some northwest Atlantic fishes. J. Fisher. Res. Bd. Can. 23, 341-347.

Beamish, F.W.H., 1978. Swimming capacity. *In*: Hoar, W.S., Randall, J.D. (Eds.), Fish Physiology, vol. 7. Academic Press Inc, New York, pp 101-187.

Beamish, F.W.H., Trippel, E.A., 1990. Heat increment: a static or dynamic dimension in bioenergetic models? Trans. Am. Fish. Soc. 119, 649-661.

Beaumont, M.W., Butler, P.J., Taylor, E.W., 1995. Plasma ammonia concentration in brown trout (*Salmon trutta*) exposed to sublethal copper in soft, acidic water and its relationship to decreased swimming performance. J. Exp. Biol. 189, 2213-3220.

Beaumont, M.W., Butler, P.J., Taylor, E.W., 2000a. Exposure of brown trout, *Salmo trutta*, to a sublethal concentration of copper in soft acidic water: effects upon muscle metabolism and membrane potential. Aquat. Toxicol. 51, 259-272.

Beaumont, M.W., Taylor, E.W., Butler, P.J., 2000b. The resting membrane potential of whiter muscle from brown trout (*Salmo trutta*) exposed to copper in soft, acidic water. J. Exp. Biol. 203, 2229-2236.

Bern, H.A., Madsen, S.S., 1992. A selective survey of the endocrine system of rainbow trout (*Oncorhynchus mykiss*) with emphasis on the hormonal regulation of ion balance. Aquaculture 100, 237-262.

Black, E.C., 1955. Blood levels of hemoglobin and lactate in some freshwater fishes following exercise. J. Fish. Res. Bd. Can. 12, 917-929.

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

Blom, S., Andersson, T.B., Forlin, L., 2000. Effects of food deprivation and handling stress on head kidney 17alphahydroxyprogesterone 21-hydroxylase activity, plasma cortisol and the activities of liver detoxification enzymes in rainbow trout. Aquat. Toxicol. 48, 265-274.

Booth, J.L., 1978. The distribution of blood flow in gills offish: application of a new technique to rainbow trout (*Salmo gairdneri*). J. Exp. Biol. 73, 119-129.

Booth, J.L., 1979. The effects of oxygen supply, epinephrine and acetylcholine on the distribution of blood flow in trout gills. J. Exp. Biol. 83, 31-39.

Borski, R.J., Helms, L.M.H., Richam, N.H., Grau, E.G., 1991. Cortisol rapidly reduces prolactin release and cAMP and $^{45}Ca^{2+}$ accumulation in the cichlid fish pituitary in vitro. Proc. Natl. Acad. Sci. 88, 2758-2762.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities utilizing the principle of protein dye binding. Anal. Biochem. 72, 248-254.

Brett, J.R., 1964. The respiratory metabolism and swimming performance of young sockeye salmon. J. Fish Res. Board Can. 21, 1183-1226.

Brett, J.R., 1967. Swimming performance of sockeye salmon (*Oncorhynchus nerka*) in relation to fatigue time and temperature. J. Fish. Res. Bd. Can. 24, 1731-1741.

Brown, C., Gardner, C., Braithwaite, V.A., 2005. Differential stress responses in fish from areas of high and low-predation pressure. J. Comp. Physiol. 175, 305-312.

Brown, J.R., Cameron, J.N., 1991. The relation between specific dynamic action and protein synthesis rates in channel catfish. Physiol. Zool. 64, 298-309.

Bucking, C., Fitzpatrick, J.L., Nadella, S.R., McGaw, I.J., Wood, C.M., 2011. Assimilation of water and dietary ions by the gastrointestinal tract during digestion in seawater-acclimated rainbow trout. J. Comp. Physiol. B 181, 615-630.

Bucking, C., Landman, M.J., Wood, C.M., 2010. The role of the kidney in compensating the alkaline tide, electrolyte load, and fluid balance disturbance associated with feeding in the freshwater rainbow trout. Comp. Biochem. Physiol. A 156, 74-83.

Bucking, C., Wood, C.M., 2006a. Gastrointestinal processing of Na^+, Cl^-, and K^+ during digestion: implications for homeostatic balance in freshwater rainbow trout. Am. J. Physiol. Integr. Comp. Physiol. 291, 1764-1772.

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

Bucking, C., Wood, C.M., 2007. Gastrointestinal transport of Ca^{2+} and Mg^{2+} during the digestion of a single meal in the freshwater rainbow trout. J. Comp. Physiol. B 177, 349-360.

Bucking, C., Wood, C.M., 2008. The alkaline tide and ammonia excretion after voluntary feeding in freshwater rainbow trout. J. Exp. Biol. 211, 2533-2541.

Bucking, C., Wood, C.M., 2012. Digestion of a single meal affects gene expression of ion and ammonia transporters and glutamine synthesise activity in the gastrointestinal tract of freshwater rainbow trout. J. Comp. Physiol. B 181, 341-350.

Buddington, R.K., Krogdahl, A., Bakke-McKellep, A.M., 1997. The intestines of carnivorous fish: structure and functions and relations with diet. Acta Physiol. Scand. Suppl. 638, 67-80.

Buddington, R.R., Chen, J.W., Diamond, J.M., 1987. Genetic and phenotypic adaptation of intestinal nutrient transport to diet in fish. J. Physiol. 393, 261-281.

Bui, P., Bagherie-Lachidan, M., Kelly, S.P., 2010. Cortisol differentially alters claudin isoforms in cultured puffer fish gill epithelia. Mol. Cell. Endocrionol. 317, 120-126.

Bury, N.R., Sturm, A., 2007. Evolution of the corticosteroid receptor signaling pathway in fish. Gen. Comp. Endocrinol. 153, 47-56.

Bury, N.R., Sturm, A., Le Rouzic, P., Lethimonier, C., Ducouret, B., Guiguen, Y., Robinson-Rechavi, M., Laudet, V., Rafestin-Oblin, M.E., Prunet, P., 2003. Evidence for two distinct functional glucocorticoid receptors in teleost fish. J. Mol. Endocrinol. 31, 141-156.

Caruso, G., Maricchiolo, G., Micale, V., Genovese, L., Caruso, R., Denaro, M.G., 2010. Physiological responses to starvation in the European eel (*Anguilla Anguilla*): effects on haematological, biochemical, non-specific immune parameters and skin structures. Fish Physiol. Biochem. 36, 71-83.

Casillas, E., Smith, L.S., 1977. Effect of stress on blood coagulation and haematology in rainbow trout (*Salmo gairdneri*). J. Fish Biol. 10, 481-491.

Chan, D.K., Woo, N.Y., 1978. Effect of cortisol on the metabolism of the eel, *Anguilla japonica*. Gen. Comp. Endocrinol. 35, 205-215.

Charmandari, E., Tsigos, C., Chrousos, G., 2005. Endocrinology of the stress response. Ann. Rev. Physiol. 65, 259-284.

Chasiotis, H., Kelly, S.P., 2012. Effects of elevated circulating cortisol levels on hydromineral status and gill tight junction protein abundance in the stenohaline goldfish. Gen. Comp. Endocrinol. 175, 277-283.

Clements, K.D., Raubenheimer, D., 2006. Feeding and Nutrition. *In*: Evans, D.H., Claiborne, J.B. (Eds.), The physiology of fishes, 3rd edition, CRC, Taylor and Francis, Boca Raton, FL, pp 47-74.

Collins, A.L., Anderson, T.A., 1997. The influence of changes in food availability on the activity of key degradative and metabolic enzymes in the liver and epaxial muscle of the golden perch. J. Fish Biol. 50, 1158-1165.

Correa, S.A., Fernandes, M.O., Iseki, K.K., Negrao, J.A., 2003. Effect of the establishment of dominance relationships on cortisol and other metabolic parameters in Nile tilapia (*Oreochromis niloticus*). Braz. J. Med. Biol. Res. 36, 1725-1731.

Cui, Z., Wang, Y., 2007. Temporal changes in body mass, body composition and metabolism of gibel carp *Carassius auratus gibelio* during food deprivation. J. Appl. Ichthyol. 23, 215-220.

Culter, C.P., Brezillion, S., Bekir, S., Sterling, R., Cramb, G., 2009. The molecular basis of osmoregulation, and ion and water transport in teleostean fish. In: Handy, R.D., Bury, N.R., Flik, G., (Eds.), Osmoregulation and ion transport: Intergrating physiological, molecular and environmental aspects. Essential reviews in experimental biology, Vol. 1. Pp. 63-107.

Czesny, S., Rinchard, J., Garcia Abiado, M.A., Dabrowski, K., 2003. The effect of fasting, prolonged swimming, and predator presence on energy utilization and stress in juvenile walleye (*Stizostedion vitreum*). Physiol. Behav. 79, 597-603.

Dabrowska, H., Dabrowski, K., Meyer-Burgdorff, K., Hanke, W., Gunther, K,D., 1991. The effect of large doses of vitamin C and magnesium on stress responses in common carp, *Cyprinus carpio*. Comp. Biochem. Physiol. A 99, 681-685.

Dang, Z.C., Lock, R.A.C., Wendelaar Bonga, S.E., 2000. Na^+, K^+-ATPase immunoreactivity in branchial chloride cells of *Oreochromis mossambicus* exposed to copper. J. Exp. Biol. 203, 379-387.

Dantzler, W.H., 2003. Regulation of renal proximal tubule transport: sodium chloride and organic anions. Comp. Biochem. Physiol. A 163, 453-478.

Davison, W., 1989. Training and its effects on teleost fish. Comp. Biochem. Physiol. A 94, 1-10.

Davison, W., Goldspink, G., 1978. The effect of training on the swimming muscle of the goldfish (*Carassius auratus*). J. Exp. Biol. 74, 115-122.

De Boeck, G., Alsop, D., Wood, C.M. 2001. Cortisol effects on aerobic and anaerobic metabolism, nitrogen excretion, and whole-body composition in juvenile rainbow trout. Physiol. Biochem. Zool. 76, 858-868.

De Boeck, G., De Smet, H., Blust, R., 1995. The effect of sublethal levels of copper on oxygen consumption and ammonia excretion in the common carp, *Cyprinus carpio*. Aquat. Toxicol. 32, 127-141.

De Boeck, G., Smolders, R., Blust, R., 2010. Copper toxicity in gibel carp *Carassius auratus gibelio*: Importance of sodium and glycogen. Comp. Biochem. Physiol. C 152, 332-337.

De Boeck, G., van der Ven, K., Hattink, J., Blust, R., 2006. Swimming performance and energy metabolism of rainbow trout, common carp and gibel carp respond differently to sublethal copper exposure. Aquat. Toxicol. 80, 92-100.

De Boeck, G., Vlaeminck, A., van der Linden, A., Blust, R., 2000a. Salt stress and resistance to hypoxic challenges in the common carp (*Cyprinus carpio* L.). J. Exp. Biol. 57, 761-776.

De Boeck, G., Vlaeminck, A., van der Linden, A., Blust, R., 2000b. The energy metabolism of common carp (*Cyprinus carpio*) when exposed to salt stress: an increase in energy expenditure or effects of starvation? Physiol. Biochem. Zool. 73, 102-111.

De Boeck, G., Vleaminck, A., Balm, P.H.M., Lock, R.A.C., Wachter, B.D., Blust, R., 2001b. Morphological and metabolic changes in common carp, *Cyprinus carpio*, during short-term

copper exposure: interactions between Cu^{2+} and plasma cortisol elevation. Environ. Toxicol. Chem. 20, 378-381.

de la Torre, F.R., Ferrari, L., Salibian, A., 1999. Enzyme activities as biomarkers of freshwater pollution: response of fish branchial Na^{+} K-ATPase and liver transaminases. Environ. Toxicol. 14, 313-319.

De Smet, H., Blust, R., 2001. Stress responses and changes in protein metabolism in carp *Cyprinus carpio* during cadmium exposure. Ecotoxicol. Environ. Saf. 48, 255-262.

Doucett, R.R., Booth, R.K., Power, G., McKinley, R.S., 1999. Effects of the spawning migration on the nutritional status of anadromous Atlantic salmon (*Salmo sa*lar): insights from stable-isotope analysis. Can. J. Fish. Aquat. Sci. 56, 2172-2180.

Dupont-Prinet, A., Claireaux, G., McKenzie, D.J., 2009. Effects of feeding and hypoxia on cardiac performance and gastro-intestinal blood flow during critical speed swimming in the sea bass *Dicentrarchus labrax*. Comp. Biochem. Physiol. A 154, 233-240.

Eckert, S.M., Yada, T., Shepherd, B.S., Stetson, M.H., Hirano, T., Grau, E.G., 2001. Hormonal control of osmoregulation in the channel catfish *Ictalurus punctatus*. Gen. Comp. Endocrinol. 122, 270-286.

Eros, S. Milligan, C.L., 1996. The effect of cortisol on recovery from exhaustive exercise in rainbow trout: potential mechanisms of action. Physiol. Zool. 69, 1196-1214.

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.

Farbridge, K.J., Leatherland, J.F., 1992. Plasma growth hormone levels in fed and fasted rainbow trout (*Oncorhynchus mykiss*) are decreased following handling stress. Fish Physiol. Biochem. 10, 67-73.

Farrell, A.P., 2007. Cardiovascular performance during prolonged swimming tests with salmonids: a perspective on temperature effects and potential analytical pitfalls. Philos. Trans. R. Soc. B 362, 2017-2030.

Farrell, A.P., 2008. Comparisons of swimming performance in rainbow trout using constant acceleration and critical swimming speed tests. J. Fish Biol. 72, 693-710.

Farrell, A.P., Gallaugher, P.E., Fraser, J., Pike, D., Bowering, P., Hadwin, A.K.M., Parkhouse,W., Routledge, R., 2001. Successful recovery of the physiological status of coho salmon onboard a commercial gillnet vessel by means of a newly designed revival box. Can. J. Fish. Aquat. Sci. 58, 1932-1946.

Ferrari, L., Eissa, B.L., Salibian, A., 2011. Energy balance of juvenile *Cyprinus carpio* after a short-term exposure to sublathal water-borne cadmium. Fish Physiol. Biochem. 37, 853-862.

Flick, G., Klaren, P.H.M., Van den Burg, E.H., Metz, J.R., Huising, M.O., 2006. CRF and stress in fish. Gen. Comp. Endocrinol. 164, 36-44.

Foster, G.D., Moon, T.W., 1986. Cortisol and liver metabolism of immature American eels, *Anguilla rostrata* (LeSueur). Fish Physiol. Biochem. 1, 113-124.

Fu, S.J., Xie, X.J., Cao, Z.D., 2005. Effect of fasting on resting metabolic rate and postprandial metabolic in *Silurus meridionalis*. J. Fish Biol. 67, 279-285.

Fu, S.J., Zeng, L.Q., Li X.M., Pang, X., Cao, Z.D., Peng, J.L., Wang, Y.X., 2009. Effect of meal size on excess post-exercise oxygen consumption in fishes with different locomotive and digestion performance. J. Comp. Physiol. B 197, 509-517.

Fu, S.J., Zeng, L.Q., Li, X.M., Pang, X., Cao, Z.D., Peng, J.L., Wang, Y.X., 2009. The behavioural, digestive and metabolic characteristics of fishes with different foraging strategies. J. Exp. Biol. 212, 2296-2302.

Gallaugher, P.E., Thorarensen, H., Kiessling, A., Farrell, A.P., 2001. Effects of high intensity exercise training on cardiovascular function, oxygen uptake, internal oxygen transport and osmotic balance in Chinook salmon (*Oncorhynchus tshawytscha*) during critical speed swimming. J. Exp. Biol. 204, 2861-2872.

Gamperl, A.K., Vijayan, M.M., Boutilier, R.G., 1994. Experimental control of stress hormone levels in fishes: techniques and applications. Rev. Fish Biol. Fish. 4, 215-225.

Gilmour, K.M., 2005. Mineralocorticoid receptors and hormones: fishing for answers. Endocrinology 146, 44-46.

Gilmour, K.M., Wilson, R.W., Sloman, K.A., 2005. The integration of behaviour into comparative physiology. Physiol. Biochem. Zool. 78, 669-678.

Gingerich, A.J., Philipp, D.P., Suski, C.D., 2009. 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.

Gonzalez, R.J., McDonald, D.G., 1994. The relationship between oxygen uptake and ion loss in fish from diverse habitats. J. Exp. Biol. 190, 95-108.

Goss, G.G., Laurent, P., Perry, S.F., 1992. Evidence for a morphological component in acid-base regulation during environmental hypercapnia in the brown bullhead (*Ictalurus nebulosus*). Cell Tissue Res. 268, 539-552.

Greenwood, A.K., Butler, P.C., White, R.B., DeMarco, U., Pearce, D., Fernald, R.D., 2003. Multiple corticosteroid ceceptors in a teleost fish: distinct sequences, expression patterns, and transcriptional activities. Endocrinol. 32, 627-648.

Gregory, T.R., Wood, C.M., 1998. Individual variation and interrelationships between swimming performance, growth rate, and feeding in juvenile rainbow trout (*Oncorhynchus mykiss*). Can. J. Fish. Aquat. Sci. 55, 1583-1590.

Gregory, T.R., Wood, C.M., 1999. The effects of chronic plasma cortisol elevation on the feeding behavior, growth, competition ability, and swimming performance of juvenile rainbow trout. Physiol. Biochem. Zool. 72, 286-295.

Guderley, H., Lapointe, D., Dedard, M., Dutil, J., 2003. Metabolic priorities during starvation: enzyme sparing in liver and white muscle of Atlantic cod, *Gadus morhua* L. Comp. Biochem. Physiol. A 135, 347-356.

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.

Gutzska, M.A., Drazen, J.C., Robison, B.H., 2004. Digestive chitinolytic activity in marine fishes of Monterey Bay, California. Comp. Biochem. Physiol. A 139, 351-358.

Hammer, C. 1995. Fatigue and exercise tests with fish. Comp. Biochem. Physiol. A 112, 1-20.

Handy, R.D., 2003. Chronic effects of copper exposure versus endocrine toxicity: Two sides of the same toxicological process? Comp. Biochem. Physiol. A 132, 25-38.

Heath, A.G., 1995. Water pollution and fish physiology. Lewis, CRC, Boca Raton, Fla.

Hickman, C.P., Trump, B.F., 1969. The kidney. *In*: Hoar, W.S., Randall, D.J. (Eds.), Fish Physiology, Academic Press, New York, 91, 91-239.

Hicks, J.W.H., Bennett, A.F., 2004. Eat and run: prioritization of oxygen delivery during elevated metabolic states. Res. Physiol. Neaurol. 144, 215-244.

Hill, C.W., Fromm, P.O., 1968. Response of the interregnal gland of rainbow trout (*Salmo gairdneri*) to stress. Gen. Comp. Endocrinol. 11, 69-77.

Holloway, A.C., Reddy, P.K., Sheridan, M.A., Leatherland, J.F., 1994. Diurnal rhythms of plasma growth hormone, somatostatin, thyroid hormones, cortisol and glucose concentrations in rainbow trout, *Oncorhynchus mykiss*, during progressive food deprivation. Biol. Rhythm Res. 25, 415-432.

Hontela, A., Rasmussen, J.B., Audet, C., Chevalier, G., 1992. Impaired cortisol stress response in fish from environments polluted by PAHs, PCBs and mercury. Arch. Environ. Contam. Toxicol. 22, 278-283.

Houlihan, D.F., Hall, S.J., Gray, C.G., Noble, B.S., 1988. Growth rates and protein turnover in Atlantic cod, *Gadus morhua*. Can. J. Fish. Aquat. Sci. 45, 951-964.

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.

Hunt von Herbing, I., White, F., 2002. The effects of body mass and feeding on metabolic rate in small juvenile Atlantic cod. J. Fish Biol. 61, 945-958.

Hwang, P.P., Lee, T.H., 2007. New insights into fish ion regulation and mitochondrion-rich cells. Comp. Biochem.and Physiol. A 148, 479-497.

Hwang, P.P., Lee, T.H., Lin, L.Y., 2011. Ion regulation in fish gills: recent progress in the cellular and molecular mechanisms. Am. J. Physiol. Reg. Integr. Comp. Physiol. 301, R28-R47.

Ivanis, G., Esbaugh, A.J., Perry, S.F., 2008. Branchial expression and localization of SLC9A2 and SLC9A3 sodium/hydrogen exchangers and their possible role in acid-base regulation in freshwater rainbow trout (*Oncorhynchus mykiss*). J. Exp. Biol. 211, 2467-2477.

Iwama, G.K., McGeer, J.C., Pawluk, M.P., 1989. The effects of five fish anesthetics on acid-base balance, hematocrit, blood gases, cortisol, and adrenaline in rainbow trout. Can. J. Fish. Aquat. Sci. 67, 2065-2073.

Jobling, M., 1980. Effects of starvation on proximate chemical composition and energy utilization of plaice, *Pleuronectes platessa* L. J. Fish Biol. 17, 325-334.

Jobling, M., 1981. The influences of feeding on the metabolic rate of fishes: a short review. J. Fish Biol. 18, 385-400.

Jobling, M., 1994. Fish Bioenergetics. Chapman & Hall, London, pp 309.

Jones, D.R., Randall, D.J., 1978. The respiratory and circulatory and system during exercise. In: Hoar, W.S., Randall, D.J. (Eds.), Fish Physiology, vol. 7. Academic Press, New York. pp. 425-501.

Jørgensen, E.H., Bye, B.E., Jobling, M., 1999. Influence of nutritional status on biomarker responses to PCB in the Arctic charr (*Salvelinus alpinus*). Aquat. Toxicol. 44, 233-244.

Jørgensen, E.H., Vijayan, M.M., Aluru, N., Maule, A.G., 2002. Fasting modifies Aroclor 1254 impact on plasma cortisol, glucose and lactate responses to a handling disturbance in Arctic charr. Comp. Biochem. Physiol. C 132, 235-245.

Jourdan-Pineau, H., Dupont-Prinet, A., Claireaux, G., McKenzie, D.J., 2010. An investigation of metabolic prioritization in the European sea bass, *Dicentrarchus labrax*. Physiol. Biochem. Zool. 83, 68-77.

Kajimura, M., Croke, S.J., Glover, C.N., Wood, C.M., 2004. Dogmas and controversies in the handling of nitrogenous wastes: the effect of feeding and fasting on the excretion of ammonia, urea and other nitrogenous waste products in rainbow trout. J. Exp. Biol. 207, 1993-2002.

Kaushik, S.J., Teles, A.D., 1985. Effect of digestible energy on nitrogen and energy balance in rainbow trout. Aquaculture 50, 89-101.

Kelley, K.M., Haigwood, J.T., Perez, M., Galima, M.M., 2001. Serum insulin-like growth factor binding proteins (IGFBPs) as markers for anabolicycatabolic condition in fishes. Comp. Biochem. Physiol. B 129, 229-236.

Kelly, S.P., Chow, I.N.K., Woo, N.Y.S., 1999. Haloplasticity of black seabream (*Mylio macrocephalus*): hypersaline to freshwater acclimation. J. Exp. Zool. 283, 226-241.

Kieffer, J.D., 2000. Limits to exhaustive exercise in fish. Comp. Biochem. Physiol. A 126, 161-179.

Kieffer, J.D., 2010. Perspective – exercise in fish: 50+ years and going strong. Comp. Biochem. Physiol. A 156, 163-168.

Kieffer, J.D., Alsop, D., Wood, C.M., 1998. A respirometric analysis of fuel use during aerobic swimming at different temperatures in rainbow trout (*Oncorhynchus mykiss*). J. Exp. Biol. 201, 3123-3133.

Kieffer, J.D., Cooke, S.J., 2009. Physiology and organismal performance of centrarchids. *In*: Cooke, S.J., Philipp, D.P. (Eds.), Centrarchid Fishes: diversity, biology and conservation. Wiley-Blackwell, pp. 207-263.

Kiilerich, P., Kristiansen, K., Madsen, S., 2007. Hormone receptors in gills of smolting Atlantic salmon, *Salmon salar*: expression of growth hormone, prolactin, mineralocorticoid and glucocorticoid receptors and 11β-hydroxysteroid dehydrogenase type 2. Gen. Comp. Endocrinol. 152, 295-303.

Kiilerich, P., Milla, S., Sturm, A., Valotaire, C., Chevolleau, S., Giton, F., Terrien, X., Fiet, J., Fostier, A., Debrauwer, L., Prunet, P., 2011. Implication of the mineraloorticoid axis in rainbow trout osmoregulation during salinity acclimation. J. Endocrinol. 209, 221-349.

Kolok, A.S., 1999. Interindividual variation in the prolonged locomotor performance of ectothermic vertebrates: a comparison of fish and herpetofaunal methodologies and a brief review of the recent fish literature. Can. J. Fish. Aquat. Sci. 56, 700-710.

Kristiansen, H.R., Rankin, J.C., 2001. Discrimination between endogenous and exogenous water sources in juvenile rainbow trout fed extruded dry feed. Aquat. Living Resour. 14, 359-366.

Kubokawa, K., Watanabe, T., Yoshioka, M., Iwata, M., 1999. Effects of acute stress on plasma cortisol, sex steroid hormone and glucose levels in male and female sockeye salmon during the breeding season. Aquaculture, 172, 335-349.

Kumai, Y., Nesan, D., Vijayan, M.M., Perry, S.F., 2012. Cortisol regulates Na^+ uptake in zebrafish, *Dario rerio*, larvae *via* the glucocorticoid receptor. Mol. Cell. Endocrinol. 364, 113-125.

Kunwar, P.S., Tudorache, C., Eyckmans, M., Blust, R., De Boeck, G., 2009. Influence of food ratio, copper exposure and exercise on the energy metabolism of common carp (*Cyprinus carpio*). Comp. Biochem. Physiol. C 149, 113-119.

Kutty, M.N., 1972. Respiratory quotient and ammonia excretion in *Tilapia mossambica*. Mar. Biol. 16, 126-133.

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

Laiz-Carrion, R., Martin Del Rio, M.P., Miguez, J.M., Mancer, J.M., Soengas, J.L., 2003. Influence of cortiol on osmoregulation and energy metabolism in gilthead seabream *Sparus aurata*. J. Exp. Zool. A 298, 105-118.

Larsson, A., Lewander, K., 1973. Metabolic effects of starvation in the eel, *Anguilla anguilla*. Comp. Biochem. Physiol. A 44, 367-374.

Lauff, R.F., Wood, C.M., 1996a. Respiratory gas exchange, nitrogenous waste excretion and fuel usage during starvation in juvenile rainbow trout. J. Comp. Physiol. B 165, 542-551.

Lauff, R.F., Wood, C.M., 1996b. Respiratory gas exchange, nitrogenous waste excretion and fuel usage during aerobic swimming in juvenile rainbow trout. J. Comp. Physiol. B 166, 501-509.

Lauff, R.F., Wood, C.M., 1997. Effects of training on respiratory gas exchange, nitrogenous waste excretion, and fuel usage during aerobic swimming in juvenile rainbow trout (*Oncorhynchus mykiss*). Can. J. Fish. Aquat. Sci. 54, 566-571.

Leach, G.J., Taylor, M.H., 1982. The effects of cortisol treatment on carbohydrate and protein metabolism in *Fundulus heteroclitus*. Gen. Comp. Endocrinol. 48, 76-83.

Lee, C.G., Devlin, R.H., Farrell, A.P., 2003. Swimming performance, oxygen consumption and excess post-exercise oxygen consumption in adult transgenic and ocean-ranched coho salmon. J. Fish Biol. 62, 753-766.

Leung, K.M.Y., Chu, J.C.W., Wu, R.S.S., 1999. Interacting effects of water temperature and dietary protein levels on post-prandial ammonia excretion by the areolated grouper *Epinephelus areolatus* (Forskal). Aquaculture Res. 30, 793-798.

Li, X.M., Cao, Z.D., Fu, S.J., 2010. The effect of exercise training on the metabolic interaction between feeding and locomotion in the juvenile southern catfish (*Silurus meridionalis* Chen). J. Exp. Zool. A 313, 557-563.

Li, X.M., Cao, Z.D., Fu, S.J., 2010. The effect of exercise training on the metabolic interaction between feeding and locomotion in the juvenile southern catfish (*Silurus meridionalis* Chen). J. Exp. Zool. A 313, 557-563.

Liew, H.J., Chiarella, D., Pelle, A., Faggio, C., Blust, R., De Boeck, G., 2013b. Cortisol emphasizes the metabolic strategies employed by common carp, *Cyprinus carpio* at different feeding and swimming regimes. Comp. Biochem. Physiol. A (*Under Revision*).

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. A 163, 327-335.

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

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

Lingwood, D., Harauz, G., Ballantyne, J.S., 2006. Decoupling the Na^+-K^+-ATPase in vivo: A possible new role in the gills of freshwater fishes. Comp. Biochem. Physiol. A 144, 451-457.

Lowery, M.S., Somero, G.N., 1990. Starvation effects on protein synthesis in red and white muscle of the barred sand bass *Palabrax nebulifer*. Physiol. Zool. 63, 630-648.

Lurman, G.J., Bock, C.H., Portner,H.O., 2007. An examination of the metabolic processes underpinning critical swimming in Atlantic cod (*Gadus morhua* L.) using *in vivo* ^{31}P-NMR spectroscopy. J. Exp. Biol. 210, 3749-3756.

Lyndon, A.R., Houlihan, D.F., Hall, S.J., 1992. The effect of short-term fasting and a single meal on protein synthesis and oxygen consumption in cod, *Gadus morhua*. J. Comp. Physiol. B 162, 209-215.

Madsen, S.S., 1990. Cortisol treatment improves the development of hypoosmoregulatory mechanisms in immature rainbow trout (*Salmo gairdneri*). Fish Physiol. Biochem. 8, 271-281.

Mancera, J.M., Laiz-Carrión, R., Martín del Río, M.P., 2002. Osmoregulatory action of PRL, GH and cortisol in the gilthead sea bream (*Sparus aurata* L.). Gen. Comp. Endocrinol. 129, 95-103.

Marshall, D.M., 2010. Starvation physiology: Reviewing the different strategies animals use to survive a common challenge. Comp. Biochem. Physiol. A 156, 1-18.

Marshall, W.S., Grosell, M., 2006. Ion transport, osmoregulation and acid-base balance. *In*: Evan, D.H., Claiborne, J.B. (Eds.), The physiology of fishes, 3rd edition. CRC Press, Boca Raton, Florida, pp 177-230.

Martin, S.A.M., Cash, P., Blaney, S., Houlihan, D.F., 2001. Proteome analysis of rainbow trout (*Oncorhynchus mykiss*) liver proteins during short term starvation. Fish Physiol. Biochem. 24, 259-270.

Martinez, M., Guderley, H., Dutil, J., Winger, P.D., He, P., Walsh, S.J., 2003. Condition, prolonged swimming performance and muscle metabolic capacities of cod *Gadus morhua*. J. Exp. Biol. 203, 503-511.

Mazeaud, M.M., Mazeaud, F., Donalden, B.M., 1977. Primary and secondary effects of stress in fish. Some new data with a general review. Trans. Am. Fish. Soc. 106, 201-212.

McCarthy, I.D., Houlihan, D.F., Carter, C.G., Moutou, K., 1993. Variation in individual food consumption rates of fish and its implications for the study of fish nutrition and physiology. Proc. Nutr. Soc. 52, 427-436.

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

McCormick, S.D., 1995. Hormonal control of gill Na^+, K^+-ATPase and chloride cell function. *In*: Wood, C.M., Shuttleworth, T.J., (Eds.), Cellular and molecular approaches to fish ionic regulation. Academic Press, San Diego, C.A., pp 285-315.

McCormick, S.D., 2001. Endocrine control of osmoregulation in teleost fish. Am. Zool. 41, 781-794.

McCormick, S.D., 2011. The hormonal control of osmoregulation in teleost fish. *In*: Farrell, A.P. (Ed.), Encyclopedia of fish physiology: from genome to environment, vol. 2, Academic Press, San Diego, pp. 1466-1473.

McCormick, S.D., Regish, A., O'Dea, M.F., Shrimpton, J.M., 2008. Are we missing a mineralocorticoid in teleost fish? Effects of cortisol, deoxycorticosterone and aldosterone on osmoregulation, gill Na^+, K^+-ATPase activity and isoform mRNA levels in Atlantic salmon. Gen. Comp. Endocrinol. 157, 35-40.

McCue, M.D., 2010. Starvation physiology: reviewing the different strategies animals use to survive a common challenge. Comp. Biochem. Physiol. A 156, 1-18.

McDonald, M.D., Walsh, P.J., Wood, C.M., 2002. Branchial and renal excretion of urea and urea analogues in the plainfin midshipman, *Porichthys notatus*. J. Comp. Physiol. B 172, 699-712.

McDonald, M.D., Wood, C.M., 2004. The effect of chronic cortisol elevation on urea metabolism and excretion in the rainbow trout (*Oncorhynchus mykiss*). J. Comp. Physiol. B 174, 71-81.

McGuire, A., Aluru, N., Akihiro, T., Weil, R., Wilson, J.M., Vijayan, M.M., 2010. Hyperosmotic shock adaptation by cortisol involves upregulation of branchial osmotic tress transcription factor 1 gene expression in Mozambique Tilapia. Gen. Comp. Endocrinol. 165, 321-329.

McKenzie, D.J., Hale, M., Domenici, P., 2007. Locomotion in primitive fishes. *In*: McKenzie, D.J., Farrell, A.P., Brauner, C.J. (Eds.), Primitive fishes. Elsevier Publishers, Amsterdam, pp 319-380.

McKenzie, D.J., Shingles, A., Taylor, E.W., 2003. Sub-lethal plasma ammonia accumulation and the exercise performance of salmonids. Comp. Biochem. Physiol. A 135, 515-526.

McKenzie, D.L., Martinez, R., Morales, A., Acosta, J., Morales, R., Taylor, E.W., Steffensen, J.F., Estrada, M.P., 2003. Effects of growth hormone transgenesis on metabolic rate, exercise performance and hypoxia tolerance in tilapia hybrids. J. Fish Biol. 63, 398-409.

McMillan, D.N., Houlihan, D.F., 1988. The effect of refeeding on tissue protein-synthesis in rainbow trout. Physiol. Zool. 61, 429-441.

Meher, T., Wieser, W., 1994. Energetics and metabolic correlates of starvation in juvenile perch (*Perca fluviailis*). J. Fish Biol. 45, 325-333.

Metz, J.R., van den Burg, E.H., Wendelaar Bonga, S.E., Flik, G., 2003. Regulation of branchial Na^+/K^+-ATPase in common carp *Cyprinus carpio* L. acclimated to different temperatures. J. Exp. Biol. 206, 2273-2280.

Milligan, C.L., 1996. Metabolic recovery from exhaustive exercise in rainbow trout. Comp. Biochem. Physiol. A 113, 51-60.

Milligan, C.L., 1997. The role of cortisol in amino acid mobilization and metabolism following exhaustive exercise in rainbow trout (*Oncorhynchus mykiss Walbaum*). Fish Physiol. Biochem. 16, 119-128.

Milligan, C.L., Hooke, G.B., Johnson, C., 2000. Sustained swimming at low velocity following a bout of exhaustive exercise enhances metabolic recovery in rainbow trout. J. Exp. Biol. 203, 921-926.

Mommsen, T.P., Danulat, E., Walsh, P.J., 1992. Metabolic actions of glucogan and dexamethasone in liver of the ureogenic teleost *Opsanus beta*. Gen. Comp. Endocrinol. 85, 316-326.

Mommsen, T.P., French, C.J., Hochachka, P.W., 1980. Sites and patterns of protein and amino acid utilization during the spawning migration of salmon. Can. J. Zool. 58, 1785-1799.

Mommsen, T.P., Vijayan, M.M., Moon, T.W., 1999. Cortisol in teleosts: dynamics, mechanisms of action, and metabolic regulation. Rev. Fish. Biol. Fish. 9, 211-268.

Mommsen, T.P., Vijayan, M.M., Moon, T.W., 1999. Cortisol in teleosts: dynamics, mechanisms of action and metabolic regulation. Rev. Fish Biol. Fish. 9, 211-268.

Moon, T.W., Johnstan, I.A., 1980. Starvation and the activities of glycolytic and gluconeogenic enzymes in skeletal muscles and liver of the plaice, *Pleuronectes platessa*. J. Comp. Physiol. 136, 31-38.

Morgan, J.D., Iwana, G.K., 1996. Cortisol induced changes in oxygen consumption and ionic regulation in coastal cutthroat trout (*Oncorhynchus clarki clarki*) parr. Fish Physiol. Biochem. 15, 385-394.

Moyes, C.D., West, T.G., 1995. Exercise metabolism in fish. *In*: Hochachka, P.W., Mommsen, T.P. (Eds.), Biochemistry and Molecular Biology of Fishes, vol. 4, Elsevier Press, Amsterdam, pp. 367-392.

Navarro, I., Blasco, J., Banos, N., Gutierrez, J., 1997. Effects of fasting and feeding on plasma amino acid levels in brown trout. Fish Physiol. Biochem. 16, 303-309.

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., Wood, C.M., 2007. Ammonia excretion in rainbow trout (*Oncorhynchus mykiss*): evidence for Rh glycoprotein and H^+-ATPase involvement. Physiol. Genomics. 31, 463-474.

Nawata, C.M., Wood, C.M., 2009. mRNA analysis of the physiological responses to ammonia infusion in rainbow trout. J. Comp. Physiol. B 179, 799-810.

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, 1049-1050.

Naylor, R.L., Goldburg, R.J., Primavera, J.H., Kautsky, N., Beveridge, M.C.M., Clay, J., Folke, C., Lubchenco, J., Mooney, H., Troell, M., 2000. Effect of aquaculture on world fish supplies. Nature 405, 1017-1024.

Nelson, J.A., Gotwalt, P.S., Reidy, S.P., Webber, D.M., 2002. Beyond U_{crit}: matching swimming performance tests to the physiological ecology of the animal including a new fish drag strip. Comp. Biochem. Physiol. A 133, 289-302.

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.

Nilsson, G.E., 1992. Evidence for a role of GABA in metabolic depression during anoxia in crucian carp (*Carassius carassius*). J. Exp. Biol. 164, 243-259.

Nilsson, G.E., 2007. Gill remodeling in fish: a new fashion or an ancient secret? J. Exp. Biol. 201, 2403-2409.

Nilsson, G.E., Renshaw, G.M.C., 2004. Hypoxic survival strategies in two fishes: extreme anoxia tolerance in the North European crucian carp and natural hypoxic preconditioning in a coral-reef shark. J. Exp. Biol. 207, 3131-3339.

Overli, O., Kotzian, S., Winberg, S., 2002. Effects of cortisol on aggression and locomotor activity in rainbow trout. Horm. Behav. 42, 53-61.

Pagnotta, A., Brooks, L., Milligan, L., 1994. The potential regulatory role of cortisol in the recovery from exhaustive exercise in rainbow trout. Can. J. Zool. 72, 2136-2146.

Pang, X., Cao, Z.D., Fu, S.J., 2011. The effects of temperature on metabolic interaction between digestion and locomotion in juveniles of three cyprinid fish (*Carassius auratus, Cyprinus carpio* and *Spinibarbus sinensis*). Comp. Biochem. Physiol. A 159, 253-260.

Pang, X., Cao, Z.D., Peng, J.L., Fu, S.J., 2010. The effects of feeding on the swimming performance and metabolic response of juvenile southern catfish, *Silurus meridionalis*, acclimated at different temperatures. Comp. Biochem. Physiol. A 155, 253-258.

Pankhurst, N.W., 2011. The endocrinology of stress in fish: An environmental perspective. Gen. Comp. Endocrinol. 170, 265-275.

Pankurst, N.W., 2011. The endocrinology of stress in fish: An environmental perspective. Gen. Comp. Endocrinol. 170, 265-275.

Parwez, I., Goswami, S.V., 1985. Effects of prolactin, adrenocorticotrophin, neurohypophysial peptides, cortisol, and androgens on some osmoregulatory parameters of the hypophysectomized catfish, *Heteropneustes fossilis* (Block). Gen. Comp.Endocrinol. 58, 51-68.

Peake, S., Barth, C., McKinley, R.S., 1997. Effect of recovery parameters on critical swimming speed of juvenile rainbow trout (*Oncorhynchus mykiss*). Can. J. Zool. 72, 1724-1727.

Peake, S.J., 2008. Gait transition speed as an alternate measure of maximum aerobic capacity in fishes. J. Fish Biol. 72, 645-655.

Peake, S.J., Farrell, A.P., 2006. Fatigue is a behavioural response in respirometer-confined smallmouth bass. J. Fish Biol. 68, 1742-1755.

Perry, S.F., Beyers, M.L., Johnson, D.A., 2000. Cloning and molecular characterisation of the trout (*Oncorhynchus mykiss*) vacuolar H^+-ATPase B subunit. J. Exp. Biol. 203, 459-470.

Perry, S.F., Fryer, J.N., 1997. Proton pumps in the fish gill and kidney. Fish Physiol. Biochem. 17, 363-369.

Perry, S.F., Goss, G.G., 1994. The effects of experimentally altered gill chloride cell surface on acid-base regulation in rainbow trout during metabolic alkalosis. J. Comp. Physiol. A 164, 327-336.

Perry, S.F., Shahsavarani, A., Georgalis, T., Bayaa, M., Furimsky, M., Thomas, S.L.Y., 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.

Perry, S.T., Rivero-Lopez, L., McNeill, B., Wilson, J., 2006. Fooling a freshwater fish: how dietary salt transforms the rainbow trout gill into a seawater gill phenotype. J. Exp. Biol. 209, 4591-4596.

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

Pfaffl, M.W., 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 29, 220-2007.

Pfaffl, M.W., Horgan, G.W., Dempfle, L., 2002. Relative expression software tool (REST©) for group-wise comparison and statistical analysis of the relative expression results in real-time PCR. Nucleic Acids Res. 30, 1–10.

Pickering, A.D., 1993. Endocrine-induced pathology in stressed salmonid fish. Fish. Res. 17, 35-50.

Plaut, I., 2001. Critical swimming speed: its ecological relevance. Comp. Biochem. Physiol. A 131, 41-50.

Polakof, S., Arjona, F.J., Sangiao-Alvarellos, S., Miguez, J.M., Martin del Rio, M.P., Mancera, J.M., Soengas, J.L., 2006. Food deprivation alters osmoregulatory and metabolic responses to salinity acclimation in gilthead sea bream *Sparus aurata*. J. Comp. Physiol. B 176, 441-452.

Postlethwaite, E.K., McDonald, D.G., 1995. Mechanisms of Na^+ and Cl^- regulation in freshwater-adapted rainbow trout (*Oncorhynchus mykiss*) during exercise and stress. J. Exp. Biol. 198, 295-304.

Pottinger, T.G., 1998. Changes in blood cortisol, glucose and lactate in carp retained in angler's keepnets. J. Fish Biol. 53, 728-742.

Pottinger, T.G., 2010. A multivariate comparison of the stress response in three salmonid and three cyprinid species: evidence for inter-family differences. J. Fish Biol. 76, 601-621.

Pottinger, T.G., Carrick, T.R., Appleby, A., Yeomans, W.E., 2000. High blood cortisol levels and low cortisol receptor affinity: is the chub, *Leuciscus cephalus*, a cortisol-resistant teleost? Gen. Comp. Endocrinol. 120, 108-117.

Pottinger, T.G., Rand-Weaver, M., Sumpter, J.P., 2003. Overwinter fasting and re-feeding in rainbow trout: plasma growth hormone and cortisol levels in relation to energy mobilization. Comp. Biochem. Physiol. B 136, 403-417.

Pottinger, T.G., Yeomans, W.E., Carrick, T.R., 1999. Plasma cortisol and 17β-oestradiol levels in roach exposed to acute and chronic stress. J. Fish Biol. 54, 525-532.

Rahmatullah, M., Boyde, T.R.C., 1980. Improvements in the determination of urea using diacetyl monoxime: methods with and without deproteinisation. Clin. Chem. Acta 107, 3-9.

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

Reddy, P.K., Vijayan, M.M., Leatherland, J.F., moon, T.W., 1995. Does RU486 modify hormonal responses to handling stressor and cortisol treatment in fed and fasted rainbow trout? J. Fish Biol. 46, 341-359.

Reidy, S.P., Kerr, S.R., Nelson, J.A., 2000. Aerobic and anaerobic swimming performance of individual Atlantic cod. J. Exp. Biol. 203, 347-357.

Richards, J.G., Heigenhauser, G.J.F., Wood, C.M., 2002. Lipid oxidation fuels recovery from exhaustive exercise in white muscle of rainbow trout. Am. J. Physiol. 282, R89-R99.

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

Roy, B., Rai, U., 2009. Genomic qnd non-genomic effect of cortisol on phagocytosis in freshwater teleost *Channa punctatus*: An in vitro study. Steroids 74, 449-455.

Ruane, N.M., Huisman, E.A., Komen, J., 2001. Plasma cortisol and metabolite level profiles in two isogenic strains of common carp, during confinement. J. Fish Biol. 59, 1-12.

Ruane, N.M., Huisman, E.A., Komen, J., 2002. The influence of feeding history on the acute stress response of common carp (*Cyprinus carpio*). Aquaculture 201, 245-257.

Sakamoto, T., McCormick, S.D., 2006. Prolactin and growth hormone in fish osmoregulation. Gen. Comp. Endocrinol. 147, 24-30.

Salama, A., Morgan, I.J., Wood, C.M., 1999. The linkage between Na^+ uptake and ammonia excretion in rainbow trout: kinetic analysis, the effects of $(NH_4)_2SO_4$ and NH_4HCO_3 infusion and the influence of gill boundary layer pH. J. Exp. Biol. 202, 697-709.

Sangiao-Alvarellos, S., Arjona, F.J., Martín del Río, M.PMíguez, J.M., Mancera, J.M., Soengas, J.L ., 2005. Time course of osmoregulatory and metabolic changes during osmotic acclimation in *Sparus auratus*. J. Exp. Biol. 208, 4291-4304.

Schjolden, J., Stoskhus, S., Winberg, S., 2005. Dose individual variation in stress responses and agonistic behaviour reflect divergent stress coping strategies in juvenile rainbow trout? Physiol. Biochem. Zool. 78, 715-723.

Secor, S.M., Wopten, J.A., Cox, C.L., 2007. Effects of meal size, meal type, and body temperature on the specific dynamic action of anurans. J. Comp. Physiol. B 177, 165-182.

Seidelin, M., Madsen, S.S., 1997. Prolactin antagonizes the seawater-adaptive effect of cortisol and growth hormone in anadromous brown trout (*Salmo trutta*). Zool. Sci. 14, 249-256.

Seidelin, M., Madsen, S.S., Byrialsen, A., Kristiansen, K., 1999. Effects of insulin-like growth factor-I and cortisol on Na1, K1-ATPase expression in osmoregulatory tissues of brown trout (*Salmo trutta*). Gen. Comp. Endocrinol. 113, 331-342.

Sheridan, M.A., Mommsen, T.P., 1991. Effects of nutritional state on in vivo lipid and carbohydrate metabolism of coho salmon, *Oncorhynchus kisutch*. Gen. Comp. Endocrinol. 81, 473-483.

Sherwani, F.A., Parwez, I., 2008. Plasma thyroxine and cortisol profiles and gill and kidney Na^+/K^+-ATPase and SDH activities during acclimation of the catfish *Heteropneustes fossilis* (bloch) to higher salinity, with special reference to the effects of exogenous cortisol on hypo-os;oregulatory ability of the catfish. Zool. Sci. 25, 164-171.

Shrimpton, J.M., McCormick, S.D., 1999. Responsiveness of gill Na^+/K^+-ATPase to control is related to gill corticosteroid receptor concentration in juvenile rainbow trout. J. Exp. Biol. 202, 987-995.

Sigurgisladottir, S., Lall, S.O.P., Parrish, C.C., Ackmqn, R.G., 1992. Cholestane as a digestibility marker in the absorption of polyunsaturated fatty-acid ethyl-esters in Atlantic salmon. Lipids 27, 418-424.

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 gold fish (*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 glycoprotein, ammonia excretion and Na^+ fluxes in three freshwater teleosts when exposed chronically to high environmental ammonia. J. Exp. Biol. Doi:10.1242/jeb.084574.

Sire, M.F., Vernier, J.M., 1992. Intestinal absorption of protein in teleost fish. Comp. Biochem. Physiol. A 103, 771-781.

Sloman, K.A., Desforges, P.R., Gilmour, K.M., 2001. Evidence for a mineralocorticoid-like receptor linked to branchial chloride cell proliferation in freshwater rainbow trout. J. Exp. Biol. 204, 3953-3961.

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 142, 217-223.

Smith, R.W., Houlihan, D.F., 1995. Protein synthesis and oxygen consumption in fish cells. J. Comp. Physiol. B 165, 93-101.

Soengas, J.L., Moon, T.W., 1998. Transport and metabolism of glucose in isolated enterocytes of the black bullhead *Ictalurus melas*: effects of diet and hormones. J. Exp. Biol. 201, 3263-3273.

Sollid, J., Weber, R.E., Nilsson, G.E., 2005. Temperature alters the respiratory surface area of crucian carp *Carassius carassius* and goldfish *Carassius auratus*. J. Exp. Biol. 208, 1109-1116.

Stolte, E.H., Chadzinska, M., Przybylska, D., Flik, G., Savelkoul, H.F.J., Verburg-van Kemenade, B.M.L., 2009. The immune response differentially regulates Hsp70 and glucocorticoid receptor expression *in vitro* and *in vivo* in common carp (*Cyprinus carpio* L.). Fish Shellfish Immunol. 27, 9-15.

Stolte, E.H., de Mazon, A.F., Leon-Koosterziel, K.M., Jesiak, M., Bury, N.R., Sturm, A., Savelkoul, H.F.J., van Kemenade, B.M.L.V., Flik, G. 2008a. Cortisosteroid receptors involved in stress regulation in common carp *Cyprinus carpio*. J. Endocrinol. 198:403-417.

Stolte, E.H., Nabuurs, S.B., Bury, N.R., Sturm, A., Flik, G., Savelkoul, H.F.J., Verburg-van Kemenade, B.M.L., 2008b. Stress and innate immunity in carp: Corticosteroid receptors and pro-inflammatory cytokines. Mol. Immunol. 46, 70-79.

Stone, D.A.J., Allan, G.L. Anderson, A.J., 2003. Carbohydrate utilization by juvenile silver perch, *Bidyanus bidyanus* (Mitchell). III. The protein-sparing effect of wheat starch-based carbohydrates. Aquaculture Res. 34, 123-134.

Storer, J.H., 1967. Starvation and the effects of cortisol in the goldfish (*Carassius auratus* L.). Comp. Biochem. Physiol. 20, 939-948.

Sturm, A., Bury, N.R., Dengreville, L., Fagart, J., Flouriot, G., Rafestin-Oblin, M.E., Prunet, P., 2004. 11-Deoxycorticosterone is a potent agonist of the rainbow trout (*Oncorhynchus mykiss*) mineralocorticoid receptor. Endocrinology 146, 47-55.

Suarez, R.K., Mommsen, T.P., 1987. Gluconeogenesis in teleosts fishes. Can. J. Zool. 65:1869-1882.

Sugita, T., Shmeno, S., Nakano, N., Hosokawa, H., Masumoto, T., 2000. Response of enzyme activities and metabolic intermediate concentrations to a short-time exercise and following resting in muscle and hepatopancreas of carp. Fish. Sci. 66, 594-598.

Sumpter, J.P., 1992. Control of growth of rainbow trout (*Oncorhynchusmikiss*). Aquaculture, 92: 299-320.

Sumpter, J.P., Le Bail, P.Y., Pickering, A.D., Pottinger, T.G., Carragher, J.F., 1991. The effect of starvation on growth and plasma growth hormone concentrations of rainbow trout, *Oncorhynchus mykiss*. Gen. Comp. Endocrinol. 83, 94-102.

Suski, C.D., Cooke, S.J., Tufts, B.L., 2007. Failure of low-velocity swimming to enhance recovery from exhaustive exercise in largemouth bass (*Micropterus salmoides*). Physiol. Biochem. Zool. 80, 78-87.

Takahashi, H., Sakamota, T., Hyodo, S., Shepherd, B.S., Kaneko, T., Grau, E.G., 2006. Expression of glucocorticoid receptor in the intestine of a euryhaline teleost, the Mozambique tilapia (*Oreochromis mossambicus*): effect of seawater exposure and cortisol treatment. Life Sci. 78, 2329-2335.

Takahashi, H., Sakamoto, T., 2013. The role of 'minerolocorticoids' in teleost fish: Relative importance of glucocorticoid signaling in the osmoregulation and 'central' actions of mineralocorticoid receptor. Gen. Comp. Endocrinol. 181, 223-228.

Takei, Y., Loretz, C.A., 2006. Endocrinology. *In*: Evan, D.H., Claiborne, J.B. (Eds.), The physiology of fishes, 3rd edition. CRC Press, Boca Raton, Florida, pp 272-307.

Teles, M., Tridico, R., Callol, A., Fierro-Castro, C., Tort, L., 2013. Differential expression of the corticosteroid receptors GR1, GR2 and MR in rainbow trout organs with slow release cortisol implants. Comp. Biochem. Physiol. A 164, 506-514.

Tipsmark, C.K., Madsen, S.S., 2009. Distinct hormonal regulation of *Na^+, K^+-atpase* genes in the gill of Atlantic salmon (*Salmo salar* L.). J. Endocrinol. 203, 301-310.

Tocher, D.R., 2003. Metabolism and functions of lipids and fatty acids in teleosts fish. Rev. Fish. Sci. 11, 107-184.

Tort, L., 2011. Stress and immune modulation in fish. Dev. Comp. Immunol. 35, 1366-1375.

Tsui, T.K.N., Hung, C.Y.C., Nawata, C.M., Wilson, J.M., Wright, P.A., Wood, C.M., 2009. Ammonia transport in cultured gill epithelium of freshwater rainbow trout: the importance of Rhesus glycoproteins and the presence of an apical Na^+/NH_4^+ exchange complex. J. Exp. Biol. 212, 878-892.

Tudorache, C., Blust, R., De Boeck, G., 2007. Swimming capacity and energetics of migrating and non-migrating morphs of three-spined stickleback *Gasterosteus aculeatus* L. and their ecological implications. J. Fish Biol. 78, 1448-1456.

Tudorache, C., Blust, R., De Boeck, G., 2008. Social interactions, predation behaviour and fast start performance are affected by ammonia exposure in brown trout (*Salmo trutta* L.). Aquat. Toxicol. 90, 145-153.

Tudorache, C., Jordan, A.D., Svendsen, J.C., Domenici, P., De Boeck, G., Steffernsen, J.F., 2009. Pectoral fin beat frequency predicts oxygen consumption during spontaneous activity in a labriform swimming fish (*Embiotoca lateralis*). Environ. Biol. Fish 84, 121-127.

Tudorache, C., O'Keefe, R.A., Benfey, T.J., 2010a. The effect of temperature and ammonia exposure on swimming performance of brook charr (*Salvelinus fontinalis*). Comp. Biochem. Physiol. A 156, 523-528.

Tudorache, C., O'Keefe, R.A., Benfey, T.J., 2010b. Flume length and post-exercise impingement affect anaerobic metabolism in brook charr *Salvelinus fontinalis*. J. Fish Biol. 76, 729-733.

Tudorache, C., Viaenen, P., Blust, R., De Boeck, G., 2007. Longer flumes increase critical swimming speeds by increasing burst-glide swimming duration in carp *Cyprinus carpio,* L. J. Fish Biol. 71, 1630-1638.

Uchida, K., Kaneko, T., Tagawa, M., Hirano, T., 1998. Localisation of cortisol receptor in branchial chloride cells in chum salmon fry. Gen. Comp. Endocrinol. 109, 175-185.

Ultsch, G.R., 1989. Ecology and physiology of hibernation and overwintering among freshwater fishes, turtles, and snakes. Biol. Rev. 64, 435-516.

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. A 67, 329-335.

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. A 67, 329-335.

Umminger, B.L., Gist, D.H., 1973. Effects of termal acclimation on physiological responses to handling stress, cortisol and aldosterone injections in the goldfish, *Carassius auratus*. Comp. Biochem. Physiol. 44, 967-977.

Van der Boon, J., van den Thillart, G.E.E.J.M., Addink, A.D.F., 1991. The effects of cortisol administration on intermediary metabolism in teleost fish. Comp. Biochem. Physiol. A 100, 47-53.

Van Dijk, P.L.M., an den Thillart, G.E.E.J.M., Wendelaar Bonga, S.E., 1993. Is there a synergistic effect between steady-state exercise and water acidification in carp? J. Fish Biol. 42, 673-681.

van Raaij, M.T.M., van den Thillart, G.E.E.J., Vianen, G.J., Pit, D.S.S., Balm, P.H.M., Steffens, A.B., 1996. Substrate mobilization and hormone changes in rainbow trout (*Oncorhynchus mykiss*, L.) and common carp (*Cyprinus carpio*, L.) during deep hypoxia and subsequent recovery. J. Comp. Physiol. B 166, 443-452.

Vazzana, M., Vizzini, A., sanfratello, M.A., Celi, M., Salerno, G., Parrinello, N., 2010. Differential expression of two glucocorticoid receptors in seabass (teleost fish) head kidney after exogenous cortisol inoculation. Comp. Biochem. Physiol. A 157, 49-54.

Veillette, P.A., Young, G., 2005. Tissue culture of sockeye salmon intestine: functional response of Na^+-K^+-ATPase to cortisol. Am. J. Physiol. Regul. Inter. Comp. Physiol. 288, 1598-1605.

ven den Thillart, G.E.E.J.M., 1986. Energy metabolism of swimming trout (*Salmo gairdneri*). J. Comp. Physiol. 156, 511-520.

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

Videler, J., 1993. Fish Swimming, 1st edition. Chapman & Hall, London, UK.

Vijayan, M.M., Ballantyne, J.S., Leather, J.F., 1991. Cortisol-induced changes in some aspects of the intermediary metabolism of *Salvelinus fontinalis*. Gen. Comp. Endocrinol. 82, 476-486.

Vijayan, M.M., Foster, G.F., Moon, T.W., 1993. Cortisol implantation alters hepatic metabolism and hormonal responsiveness in the sea raven. Fish Physiol. Biochem. 12, 327-335.

Vijayan, M.M., Leatherland, J.F., 1989. Cortisol-induced changes in plasma glucose, protein, and thyroid hormone levels, and liver glycogen content of coho salmon (*Oncorhynchus kisutch* Walbaum). Can. J. Zool. 67, 2746-2750.

Vijayan, M.M., Moon, T.W., 1992. Acute handling stress alters hepatic glycogen metabolism in food-deprived rainbow trout (*Oncorhynchus mykiss*). Can. J. Fish. Aquat. Sci. 49, 2260-2266.

Vijayan, M.M., Moon, T.W., 1994. The stress response and the plasma disappearance of corticorsteroid and glucose in a marine teleost, the sea raven. Can. J. Zool. 72, 379-386.

Vijayan, M.M., Morgan, J.D., Sakamoto, T., Grau, E.G., Iwana, G.K., 1996. Food-deprivation affects seawater acclimation in tilapia: hormonal and metabolic changes. J. Exp. Biol. 199, 2467-2475.

Vijayan, M.M., Pereira, C., Grau, E.G., Iwana, G.K., 1997. Metabolic responses associated with confinement stress in tilapia: the role of cortisol. Comp. Biochem. Physiol. C 116, 89-95.

Walsh, P.J., 1998. Nitrogen excretion and metabolism. In: Evan, D.H. (Eds), The Physiology of Fishes, 2nd Eds, Boca Raton, FL. CRC Press. pp 199-214.

Wang, Y., Heigenhauser, G.J.F., Wood, C.M., 1994. Integrated responses to exhaustive exercise and recovery in rainbow trout white muscle: acid-base, phosphogen, carbohydrate, lipid, ammonia, fluid volume, and electrolyte metabolism. J. Exp. Biol. 195, 227-258.

Weber, J.M., Haman, F., 1996. Pathways for metabolic fuels and oxygen in high performance fish. Comp. Biochem. Physiol. A 113, 33-38.

Wendelaar-Bonga, S.E.W., 1997. The stress response in fish. Physiol. Rev. 77, 591-625.

Wieser, W., Krumschnabel, G., Ojwand-Okwor, J.P., 1992. The energetics of starvation and growth after refeeding in juveniles of three cyprinid species. Environ. Biol. Fish. 33, 63-71.

Wilson, J.M., Whiteley, N.M., Randall, D.J., 2002. Ionoregulatory changes in the gill epithelia of coho salmon during seawater acclimation. Physiol. Biochem. Zool. 75, 237-249.

Wojtaszek, J., Dziewulska-Szwajkowska, D., Lozinska-Gabska, M., Adamowicz, A., Dzugaj, A., 2002. Hematological effects of high dose of cortisol on the carp (*Cyprinus carpio* L.): cortisol effect on the carp blood. Gen. Comp. Endocrinol. 125, 176-183.

Wood, C.M., 1991. Acid-base and ion balance, metabolism and their interactions, after exhaustive exercise in fish. J. Exp. Biol. 160, 285-308.

Wood, C.M., 2001. Influence of feeding, exercise and temperature on nitrogen metabolism and excretion. *In*: Wright, P.A., Anderson, P.M. (Eds.), Nitrogen Excretion. Fish Physiology Series. Academic Press, New York, pp. 201-237.

Wood, C.M., Bucking, C., Grosell, M., 2010. Acid-base responses to feeding and intestinal Cl^- uptake in freshwater- and seawater-acclimated killifish, *Fundulus heteroclitus*, an agastric euryhaline teleost. J. Exp. Biol. 213, 2681-2692.

Wood, C.M., Iftikar, F.I., Scott, G.R., De Boeck, G., Sloman, K.A., Matey, V., Domingos, F.X.V., Duarte, R.M., Almeida-Val, V.M., Val, A.L., 2009. Regulation of gill transcellular permeability and renal function during acute hypoxia in the Amazonian Oscar (*Astronotus ocellatus*): new angles to osmorespiratory compromise. J. Exp. Biol. 212, 1949-1964.

Wood, C.M., Kajimura, M., Mommsen, T.P., Walsh, P.J., 2005. Alkaline tide and nitrogen conservation after feeding in the elasmobranch *Squalus acanthias*. J. Exp. Biol. 208, 2693-2705.

Wood, C.M., Perry, S.F., 1985. Respiratory, circulatory, and metabolic adjustments to exercise in fish. *In*: Gilles, R. (Ed.), Circulation, Respiration and Metabolism , Proceedings in Life Sciences, pp 2-22.

Wood, C.M., Randall, D.J., 1973a. The influence of swimming activity on sodium balance in the rainbow trout (*Salmo gairdneri*). J. Comp. Physiol. 82, 207-233.

Wood, C.M., Randall, D.J., 1973b. The influence of swimming activity on water balance in the rainbow trout (*Salmo gairdneri*). J. Comp. Physiol. 82, 257-276.

Wright, P., Felskie, A., Anderson, P., 1995. Induction of ornithine-urea cycle enzyme and nitrogen metabolism and excretion in rainbow trout (*Oncorhynchus mykiss*) during early life stages. J. Exp. Biol. 198, 127-135.

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.

Wu, S.C., Horng, J.L., Liu, S.A., Hwang, P.P., Wen, Z.H., Lin, S.C., Lin, L.Y., 2010. Ammonium-dependent sodium uptake in mitochondrion rich cells of medaka (*Oryzias latipes*) larvae. Am. J. Physiol. Cell Physiol. 298, 237-250.

Yada, T., Hyodo, S., Schreck, C.B., 2008. Effects of seawater acclimation on mRNA levels of corticosteroid receptor genes in os;oregulatory and immune systems in trout. Gen. Comp. Endocrinol. 156, 622-627.

Yang, W., Xiang, F., Sun, H., Chen, Y., Minter, E., Yang, Z., 2010. Changes in the selected hematological parameters and gill Na^+/K^+ ATPase activity of juvenile crucian carp *Carassius auratus* during elevated ammonia exposure and the post-exposure recovery. Biochem. Syst. Ecol. 38, 557-562.

Yin, Z., Lam, T.J. Sin, Y.M., 1995. The effect of crowding stress on the non-specific immune response in fancy carp (*Cyprinus carpio* L.). Fish Shellfish Immun. 5, 519-528.

Zare, S., Greenaway, P., 1998. The effect of moulting and sodium depletion on sodium transport and the activities of Na^+K^+-ATPase, and V-ATPase in freshwater crayfish *Cherax destructor* (Crustacea: Parastacidae). Comp. Biochem. Physiol. A 119, 739-745.

Zeng, L.Q., Li, F.J., Li, X.M., Cao, Z.D., Fu, S.J., Zhang, Y.G., 2012. The effects of starvation on digestive tract function and structure in juvenile southern catfish (*Silurus meridionalis* Chen). Comp. Biochem. Physiol. A, 162, 200-211.

Zhang, W., Cao, Z.D., Fu, S.J., 2012. The effects of dissolved oxygen levels on the metabolic interaction between digestion and locomotion in Cyprinid fishes with different locomotive and digestion performances. J. Comp. Physiol. B 182, 641-650.

Zhang, W., Cao, Z.D., Peng, J.L., Chen, B.J., Fu, S.J., 2010. The effects of dissolved oxygen level on the metabolic interaction between digestion and locomotion in juvenile southern catfish (*Silurus meridionalis* Chen). Comp. Biochem. Physiol. A 157, 212–219.

Zhou, B., Kelly, S.P., Ianowski, J.P., Wood, C.M. 2003. Effects of cortisol and prolactin on Na^+ and Cl^- transport in cultured branchial epithelia from FW rainbow trout. Am. J. Physiol. Integr. Comp. Physiol. 285:1305-1316.

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.

CIRRICULUM VITAE

Academic and Professional Background

1999 – 2000	Diploma Fisheries Science Universiti Putra Malaysia (Terengganu)
2000 – 2003	Bachelor of Agrotechnology – Aquaculture College University of Science and Technology Malaysia
2004 – 2006	Master of Science – Aquaculture College University of Science and Technology Malaysia
2007 – 2008	Science Officer Institute of Tropical Aquaculture Universiti Malaysia Terengganu
2008 – Present	Assistant Researcher/Lecturer Institute of Tropical Aquaculture Universiti Malaysia Terengganu
2009 - Present	Phd in Science – Fish physiology University of Antwerp, Belgium

Peer Review Paper

1. **Hon Jung Liew**, Daniela Chiarella, Antonella Pelle, Caterina Faggio, Ronny Blust, Gudrun De Boeck. **2013**. Cortisol emphasizes the metabolic strategies employed by common carp, *Cyprinus carpio* at different feeding and swimming regimes. Comp. Biochem. Physiol. (*Under Revision*).
2. **Hon Jung Liew**, Amit Kumar Sinha, Nathalie Mauro, Marjan Diricx, Veerle M. Darras, Ronny Blust, Gudrun De Boeck. **2013**. Feeding and swimming modulate iono-and-hormonal regulation differently in goldfish *Carassius auratus* and common carp, *Cyprinus carpio*. Comp. Biochem. Physiol. A 165, 13-21.
3. **Hon Jung Liew**, Gudrun De Boeck, Chris M. Wood. **2013**. An in vitro study of urea, water, ion and CO_2/HCO_3^- transport in the gastrointestinal tract of the dogfish shark (*Squalus acanthias*): the influence of feeding. J. Exp. Biol. 216, 2063-2072.
4. **Hon Jung Liew**, Amit Kumar Sinha, Nawata C. Michele, Ronny Blust, Chris M. Wood, Gudrun De Boeck. **2013**. Differential responses in ammonia excretion, sodium fluxes and gill permeability explain different sensitivities to acute high environmental ammonia in three freshwater teleosts. Aquat. Toxicol. 126, 63-76.
5. Chris M. Wood, **Hon Jung Liew**, Gudrun De Boeck, Patrick J. Walsh. **2013**. A perfusion study of the handling of urea and urea analogues by the gills of the dogfish shark (*Squalus acanthias*). PeerJ 1:e33; DOI:10.7717/peerj.33.
6. Amit Kumar Sinha, **Hon Jung Liew**, Nawata C. Michele, Ronny Blust, Chris M. Wood, Gudrun De Boeck. **2013**. Modulation of Rh glycoproteins, ammonia excretion and Na^+ fluxes in three freshwater fish teleosts when exposed chronically to high environmental ammonia. J. Exp. Biol. 216, 2917-2930.

7. Yeong Yik Sung, **Hon Jung Liew**, Abol Munafi Ambok Bolong, Mohd. Effendy Abdul Wahid, Thomas H MacRae. **2013**. The induction of Hsp70 synthesis by non-lethal heat shock confers thermotolerance and resistance to lethal ammonia stress in common carp, *Cyprinus carpio* (Linn). Aquacult. Res. 1-7; DOI:101111/are.12116.
8. **Hon Jung Liew**, Amit Kumar Sinha, Nathalie Mauro, Marjan Diricx, Ronny Blust, Gudrun De Boeck. **2012**. Fasting goldfish, *Carassius auratus* and common carp, *Cyprinus carpio*, use different metabolic strategies when swimming. Comp. Biochem. Physiol. A 163, 327-335.
9. Amit Kumar Sinha, Marjan Diricx, Lai Pong Chan, **Hon Jung Liew**, Vikas Kumar, Ronny Blust, Gudrun De Boeck. **2012**. Expression pattern of potential biomarker genes related to growth, ion regulation and stress in response to ammonia exposure, food deprivation and exercise in common carp (*Cyprinus carpio*). Aquat. Toxicol. 122-123, 93-105.
10. Amit Kumar Sinha, **Hon Jung Liew**, Marjan Diricx, Vikas Kumar, Veerle M. Darras, Ronny Blust, Gudrun De Boeck. **2012**. Combined effects of high environmental ammonia, starvation and exercise on hormonal and ion-regulatory response in goldfish (*Carassius auratus* L.). Aquat. Toxicol. 114-115, 153-164.
11. Amit Kumar Sinha, **Hon Jung Liew**, Marjan Diricx, Ronny Blust, Gudrun De Boeck. **2012**. The interactive effects of ammonia exposure, nutritional status and exercise on metabolic and physiological responses in gold fish (*Carassius auratus* L.). Aquat. Toxicol. 109, 33-46.
12. **Liew H.J.**, Varuthan M., Nasution Z., Ambak M.A. **2012**. A comparison of lipid content and fatty acids profile of hybrid tilapia, *Oreochromis niloticus* cultured in freshwater and marine conditions. J. Sustain. Sci. Manage. 7, 145-152.
13. Hua Thai Nhan, **Liew Hon Jung**, Mohd. Azmi Ambak, Gordon J. Watson, Hii Yii Siang. **2010**. Evidence for sexual attraction pheromones released by male tropical donkey's ear abalone (*Haliotis asinine*), (L.). Invertebr. Reprod. Dev. 54, 169-176.
14. **Liew, H.J.,** A. Ambak, A.B. Abol-Munafi, T.S. Chuah. **2006**. Embryonic development of clownfish *Amphiprion ocellaris* under laboratory-reared conditions. J. Sustain. Sci. Manage. 1:64-73.

Monographs

1. **Liew, H.J,** Ambak, M.A., Abol-Munafi, A.B. **2010**. False Clownfish - Breeding, Behavioral, Embryonic and Larval Development, Rearing and Management of clownfish Under Captivity. LAP Lambert Academic Publishing GmbH & Co KG, Saarbrücken, Germany. 87p.

Scientific Conferences Abstract

1. **Hon Jung Liew**, Angela Fazio, Sofie Moyson, Nathalie Van Dooren, Aline Delcroix, Caterina Faggio, Gudrun De Boeck. **2013**. Kidney compensates ion losses in copper exposed exercised shubunkin (*Carassius auratus auratus*). Society for Experimental Biology (SEB) Annual Main Meeting, 3rd – 6th July 2013, Valencia, Spain.
2. **Hon Jung Liew**, Gudrun De Boeck, Chris M. Wood. **2013**. *In vitro* study of urea and ion transport in gastrointestinal tract of Dogfish shark (*Squalus acanthias*): the influence of feeding. Society for Experimental Biology (SEB) Annual Main Meeting, 3rd – 6th July 2013, Valencia, Spain.
3. **Hon Jung Liew**, Antonella Pelle, Daniella Chiarella, Gudrun De Boeck. **2012**. Cortisol elevation in common carp *Cyprinus carpio* improves ammonia excretion capacity during swimming at different feeding regimes, but diverts energy use towards protein and anaerobic metabolism.

Society for Experimental Biology (SEB) Annual Main Meeting, 29th June – 2nd July 2012, Salzburg, Austria.

4. Chris M. Wood, C. Michele Nawata, Amit Kumar Sinha, **Hon Jung Liew**, Gudrun De Boeck. **2012**. Ammonia excretion, Na^+ uptake, cortisol, and intracellular signalling during high environmental ammonia exposure in fish. Society for Experimental Biology (SEB) Annual Main Meeting, 29th June – 2nd July 2012, Salzburg, Austria.
5. **Hon Jung Liew**, Amit K. Sinha, Veerle M. Darras, Ronny Blust, Gudrun De Boeck. **2011**. Combined impact of feeding and exhaustive swimming on iono- and hormonal regulation of goldfish *Carassius auratus*. International Fisheries Symposium, 3rd – 5th October 2011. Kuala Terengganu, Malaysia.
6. Gudrun De Boeck, **Hon Jung Liew**, Amit K. Sinha, C. Michele Nawata, Chris M. Wood. **2011**. Consequences of elevated environmental ammonia for Na^+ transport and ammonia/urea excretion in freshwater fish. International Fisheries Symposium, 3rd – 5th October 2011. Kuala Terengganu, Malaysia.
7. **Hon Jung Liew**, Amit Kumar Sinha, Gudrun De Boeck. **2011**. Effects of food deprivation and exhaustive swimming impacts on swimming performance, metabolic responses and the energy budget of goldfish, *Carassius auratus*. Society for Experimental Biology (SEB) Annual Main Meeting, 1st – 4th July 2011. Glasgow, United Kingdom.
8. Amit K. Sinha, Gudrun De Boeck, Ronny Blust, Marjan Diricx, **Hon Jung Liew**. **2011**. The interactive effects of ammonia exposure, nutritional status and exercise on the ecological fitness of gold fish (*Carassius auratus* L.). Society for Experimental Biology (SEB) Annual Main Meeting, 1st – 4th July 2011. Glasgow, United Kingdom.

Awards

1. Society for Experimental Biology (COST) Travel Grant **2013**, Marine Conservation and Physiology, The Company of Biology, United Kingdom.
2. Society for Experimental Biology (SEB) Travel Grant **2013**, The Company of Biology, United Kingdom.
3. Society for Experimental Biology (SEB) Travel Grant **2012**, The Company of Biology, United Kingdom.
4. 1st Prize Alltech Young Scientist Award **2010**, United State of America – Country Categories. Timing of first exogenous feeding of false clownfish *Amphiprion ocellaris* larvae.
5. Doctorate scholarship (Skim Latihan Akademik IPTA) **2009 – 2013**, Ministry of High Education Malaysia (MoHE).
6. Research & Development Award **2008**, Universiti Malaysia Terengganu, Malaysia.
7. Excellent Research Group Award **2008**, Universiti Malaysia Terengganu, Malaysia.
8. Vice Chancellor Special Award **2008**, Category Research Award, Universiti Malaysia Terengganu, Malaysia.
9. Malaysia Technology Exhibition (MTE) Bronze Medal Award **2007** – Culture of Seawater Tilapia as Potential Marine Food Fish, Kuala Lumpur, Malaysia.
10. National Science Fellowship (NSF) **2004 – 2006,** Ministry of Science, Technology and Innovation (MOSTI).

International Research Collaboration

1. Research topic: Effect of hypercortisolism and feeding on metabolism response, ionoregulation and genes expression in common carp, *Cyprinus carpio*.
   Research member: Gudrun De Boeck (University of Antwerp, Belgium); Angela Fazio, Caterina Faggio (University of Messina, Italy).
   Research venue: **February – June 2012**
   Systemic Physiological and Ecotoxicological Research, University of Antwerp, Belgium.

2. Research topic:
   1. An *in vitro* study of urea, water, ion and CO_2/HCO_3^- transport in the gastrointestinal tract of the dogfish shark (*Squalus acanthias*): the influence of feeding.
   2. A Perfusion Study of the Handling of Urea and Urea Analogues by the Gills of the Dogfish Shark (*Squalus acanthias*) - Urea Handling in Shark Gills.

   Research member: Gudrun De Boeck (University of Antwerp, Belgium); Chris M. Wood, (University of McMaster, Canada), Patrick J. Walsh (University of Ottawa, Canada).
   Research venue: **29th July – 30th August 2011**
   Bamfield Marine Science Centre, Vancouver, Canada.

3. Research topic: Combined effects of feeding, swimming and hypercortisolism on swimming, ionoregulation and metabolism response in common carp, *Cyprinus carpio*.
   Research member: Gudrun De Boeck (University of Antwerp, Belgium); Antonella Pelle, Daniela Chiarela, Caterina Faggio (University of Messina, Italy).
   Research Venue: **April – June 2011**
   Systemic Physiological and Ecotoxicological Research, University of Antwerp, Belgium.

4. Research topic: Comparative studies of nitrogen metabolism and sodium transport in three species of freshwater fish: common carp, goldfish and rainbow trout.
   Research member: Amit Kumar Sinha, Gudrun De Boeck (University of Antwerp, Belgium); Chris M. Wood, Michele C. Nawata (University of McMaster, Canada).
   Research venue: **March – April 2011**;
   Systemic Physiological and Ecotoxicological Research, University of Antwerp, Belgium.