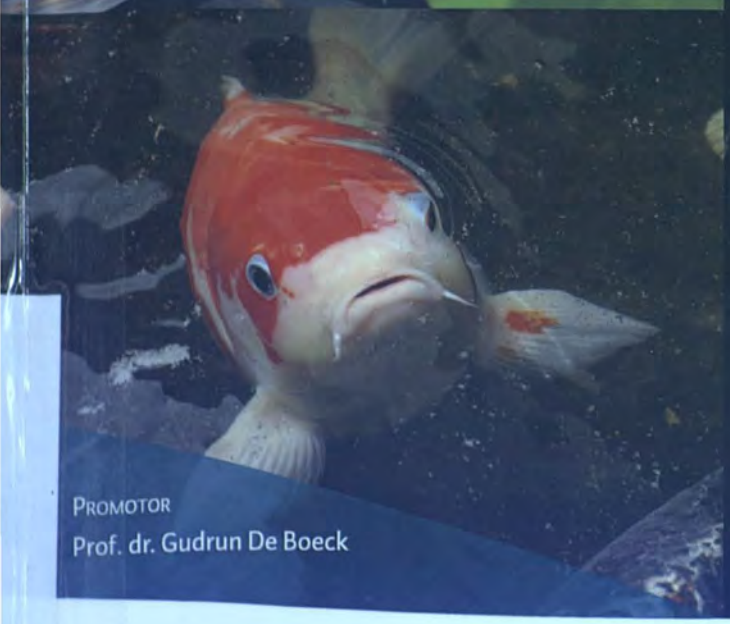
Metabolic strategies in freshwater teleost under stress

Consequences of feeding and swimming

Dissertation for the academic degree of Doctor in Science - Biology at the University of Antwerp to be defended by

Hon Jung Liew



PROMOTOR

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Faculty of Science
Department of Biology
Antwerpen 2013

Universiteit Antwerpen

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Metabole strategieën in zoetwater vissen onder stress – Invloed van voeding en zwemsnelheid

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Photograph description: Blue sky reflects off the surface waves created by a koi surfacing to feed in our backyard pond by Randy Heisch, Texas, United State of American.

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List of Abbreviations

AQ	-	Ammonia quotient
BACr	-	Branchial ammonia clearance rate
GR1	-	Glucocorticoid Receptor – 1
GR2	-	Glucocorticoid Receptor – 2
h	-	Hour
H^+ ATPase	-	Proton pump
h-PI	-	Hour-Post Implant
HSI	-	Hepatosomatic index
J_{amm}	-	Ammonia excretion
J_{urea}	-	Urea excretion
MO_2	-	Metabolic oxygen consumption
MR	-	Mineralocorticoid Receptor
MS222	-	Ethyl-3-aminobenzoate methanesulfonic acid
Na^+/K^+ ATPase (NKA)	-	Sodium pump
NKCC2	-	$Na^+/K^+/Cl^-$ co-transporter – 2
RIA	-	Radioimmunoassay
SDA	-	specific dynamic action
T_{amm}	-	Total ammonia (NH_3/NH_4^+)
TSH	-	Thyroid stimulating hormone
U_{crit}	-	Critical swimming speed

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

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 strategies in freshwater teleost under stress – 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 days food deprivation) and feeding (2.0% 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 elevation on these processes in common carp at different feeding regime (0.5% and 3.0% BW). 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 both feeding and swimming metabolisms 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 plasma ions levels. 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 adapt their osmorespiratory strategy sufficiently to minimize ion losses while maintaining gas exchange under exhaustive swimming.

Common carp were able to perform both feeding and swimming metabolisms independently while maintaining ion levels relatively stable without plasma

cortisol level increment. With that, common carp was selected to further investigate how carp respond during 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 hypercortisolemia 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, ammonia (J_{amm}) and urea excretion (J_{urea}) and stimulated protein metabolism as demonstrated by the increased ammonia quotient and endogenous nitrogenous waste levels (plasma ammonia and urea). Hypercortisolism stimulated ammonia self-detoxifying mechanisms by enhancing J_{amm} and J_{urea}, especially during exhaustive swimming. At a high swimming level, higher branchial ammonia clearance rates (BACr) in cortisol treated fish succeeded in eliminating the elevation of endogenous ammonia production and resulted in reduced plasma ammonia levels. Therefore, swimming performance was maintained.

Chapter 4: Feeding granted readily energy and mineral available for ionoregulation leading to a stable ion balance. Hypercortisolism 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 hypercortisolemia 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 hypercortisolemia on non-genomic and genomic responses in carp. This study was only focused on resting fish fed at different rations.

Chapter 5: Cortisol implants immediately 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 higher in high feeding carp. In kidney, NKA was increased to a comparable level in both feeding groups, whereas H^+ ATPase activity was higher only in low feeding fish. Upregulation of Rhcg-1 enhanced branchial ammonia excretion efficiency.

Cortisol induced glucocorticoid (GR1 and GR2) and minerolocorticoid (MR) receptor expression in both kidney and liver. In the gill, GR2 and MR were significantly upregulated in high feeding carp, whereas only GR2 was significantly 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 corticosteroid receptors (24h-PI onward).

Overall, this thesis demonstrated that goldfish and common carp clearly show different physiological responses when swimming or when challenged with stress, even though both are close family members from the Cyprinidae. For goldfish, swimming was prioritized over feeding, whereas common carp exhibited a different strategy and increased oxygen consumption to perform both feeding and swimming metabolism independently. Both species displayed a mixture of opposing and identical compensation strategies such as (i) successfully dealing with the osmorespiratory compromise, likely by increasing gill functional surface area to improve gill permeability for gas exchange while reducing gill permeability to ion losses (Na^+ and Cl^-) during swimming; (ii) successfully make a trade-off between aerobic-anaerobic metabolism and accessing energy reserves at a different degree; (iii) common carp showed an incredible ammonia self-detoxifying capacity, they increased branchial ammonia clearance rate, and upregulated gill NKA and H^+ ATPase activities to improve ammonia excretion, especially in carp that ware most challeng with cortisol: high feeding carp treated with cortisol which were swum actively; (iv) low feeding carp minimized gill NKA and H^+ ATPase activities, but upregulated kidney NKA and H^+ ATPase activities for ion reabsorption which enabled them to maintain ion levels during food deprivation. It is clear that in highly fed fish, compensatory responses were more important in gills, while kidney played a more prominent role in fish on a low feeding ration. Cortisol induced immediate non-genomic responses followed by genomic action to compensate or re-strategize metabolic needs in common carp.

'Feeding is the main key in aquaculture and swimming is a key element to ensure fisheries 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 many 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 establishing a dominancy hierarchy 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 by 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., 2013c) 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 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 (Pagontta et al., 1994; Eros and 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 for fish health (Barton and Iwama, 1991; Pottinger, 1998; Ruane et al., 2002; Wojtaszek et al., 2002; Stolte et al., 2008a; Pankhurst, 2011).

However, most of the research mentioned above was 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., 2008a), although zebrafish are gaining importance as a model due to their fully annotated genome. Therefore, comparative research on swimming physiology and biochemistry on cyprinid species is needed. 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 from fasted fish rather than 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-off 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 that 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 hypercortisolemia on metabolic strategies and ionoregulation in common carp subject to different feeding and swimming regimes. We hypothesized that hypercortisolemia might exaggerate metabolic rate due to stress, thus inducing 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 ion losses. The

increase of gill NKA activity was most likely related to ammonia excretion rather than ion uptake per se in fed fish. Thereby, we hypothesized that swimming fish would improve gill ammonia excretion and clearance rates during hypercortisolemia, and thus subsequently would induce gill NKA and H^+ ATPase activities in comparison to low aerobic swimming 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 hypercortisolemia would upregulate kidney NKA and H^+ ATPase activities for ion reabsorption to maintain basal ion level (*Chapter 4*).

iii. Finally, we aimed to assess the effects of hypercortisolemia on non-genomic and genomic responses of common carp fed different feeding regimes. We hypothesized that (i) hypercortisolemia 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) hypercortisolemia would induce GR1, GR2 and MR expression and the effect would more prominent in liver and kidney of low feeding fish to signal glycogenolysis or gluconeogenic actions compared to high feeding fish. Immediate responses such as metabolic trade-offs were assessed as non-genomic responses to hypercortisolemia.

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

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

Both goldfish (*Carassius auratus*) and common carp (*Cyprinus carpio*) 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, Taiwan and Thailand). 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 food searching, mate meeting 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 anaerobical 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 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; Liew et al., 2012). 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; McKenzie et al., 2003; Liew et al., 2012), effects on ion balance (Wang et al., 1994; Liew et al., 2013a), 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 specific dynamic action '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 the 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 the blood, and stored in tissue, especially liver (Soengas and Moon, 1998). The large proportion of protein dietary intake is hydrolyzed by cytoplasmic enzymes and absorbed across the apical and basolateral membranes of enterocytes into the 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 acid chains 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 tissues as energy reserves, while others are used for basal metabolism, somatic growth and reproduction. The use of energy reserves is influenced 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 responses and 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 endocrinological 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, kidney and gastrointestinal tract are organs that 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 ion losses by active reabsorption of ions 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 a 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 forming a large surface area that is highly permeable (Perry et al., 2003). Fish gills contain numerous 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 transports two K^+ into and three Na^+ out of animal cells into the intracellular fluid 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 (MO_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 gill also play a role in nitrogenous waste excretion. Unlike other vertebrates, teleosts produce large amounts of ammonia with approximately one molecule ammonia for every five molecules of O_2 consumed as a consequence of protein breakdown/catabolism

from either dietary intake or tissue reserve and they excrete it solely through the gill. 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 a Na^+ conductive channel that transports Na^+ and H^+ in opposite directions (Lin and Randall, 1991). The electrogenic H^+ ATPase on the apical membrane removes protons from the cell and generates a negative potential gradient on the inner side of the apical membrane which drives Na^+ influx through the Na^+ channel into the cell and releases H^+ from the cell (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 also 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

The kidney of freshwater fish acts as a final ion absorber prior to releasing hypotonic urine to the 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 larger 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 NKCC2 co-transporter located in the apical membrane and NKA in the basolateral membrane of tubule cells (Dantzler, 2003). The apical NKCC2 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), which is facilitated by the H^+ ATPase that creates a favourable electrochemical gradient for the Na^+ reabsorption (Perry et al., 2003).

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 energy mobilization are under endocrine control (Pickering, 1993; Schjolden et al., 2005). Plasma cortisol elevation is used as a stress indicator in fish (Czesny et al., 2003), which subsequently leads to increased levels of plasma glucose and plasma free fatty acids (Casillas and Smith, 1977; Mazeaud et al., 1977) as energy supply for increased metabolic demands during stress (Sheridan and Mommsen, 1991).

Cortisol is characterized as a multifunctional hormone involved in osmoregulation (McCormick, 2001), immunology response (Stolte et al., 2008b) and metabolic regulation (Vijayan et al., 1997; Barcellos et al., 2010). The cortisol 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 systemic and cellular level for homeostasis restoration (Bern and Madsen, 1992; Gilmour, 2005; Bury and Sturm, 2007) and is usually considered as a seawater-adapting hormone by increasing chloride cell proliferation and stimulating gill NKA expression and activity for osmoregulatory processes (Madsen, 1990; McCormick, 1995).

Doyle and Epstein (1972) were the first scientists to suggest that cortisol is associated with seawater adaptation and this was later confirmed and associated with an increased efflux of ions and gill epithelium permeability (Laurent and Dunel, 1980; Perry and Laurent, 1989). For example, tilapia larvae treated with cortisol had improved survival and osmoregulatory capacity after a transfer to seawater (Hwang and Wu, 1993). On the other hand, cortisol is known to induce chloride cell proliferation in the gill epithelia of freshwater teleosts as well (Doyle and Epstein, 1972; Perry and Wood, 1985; Perry and Walsh, 1989; McCormick, 1990; Sloman et al., 2001). Cortisol significantly increased the overall density and size of the apical chloride cells in freshwater rainbow trout (Laurent and Perry, 1990; Perry et al., 1992; Bindon et al., 1994) and concomitantly stimulated the whole-body ion uptake (Laurent and Perry, 1990; Goss et al., 1992a; 1992b). It is becoming increasingly clear that cortisol does play a role in promoting ion uptake in freshwater acclimated fish by maintaining transport proteins that are important for ion uptake, including gill H^+ ATP (Lin and Randall, 1993) and Na^+/K^+ ATPase (McCormick, 2001; McCormick et al., 2008; Kumai and Perry, 2012). Taken together, these studies strongly suggest that cortisol is indeed playing an important role in osmoregulation in both seawater and freshwater-acclimated fish. Indeed, cortisol increases unidirectional Na^+ influx in freshwater and Na^+ efflux in seawater fish gills. These unidirectional Na^+ fluxes in cortisol treated fish allowed them to maintain ion homeostasis (Perry et al., 1992; Goss et al., 1992b).

McCormick (2001) suggested that the action of cortisol occurs 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. In brown trout, prolactin injection does inhibit the hypoosmoregulatory action, but it did not affect the capacity of cortisol to increase gill NKA activity. Cortisol and prolactin 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). However, cortisol has also been found to rapidly decrease the release of prolactin from the tilapia pituitary (Borski et al., 1991).

Overall, the control of the osmoregulatory system of teleosts involves hypophysial hormones such as prolactin and growth hormone, 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 acclimated teleost. The basic actions of prolactin in freshwater teleost are to decrease osmotic permeability to water and to increase Na^+ and Cl^- uptake across the transport epithelia by increasing the number of chloride cells in the gill (Manzon, 2002). Growth hormone promotes the development of hypoosmoregulation during smoltification by stimulating chloride cell development and NKA activity in the gill of salmonids, which occurs in synergism with cortisol (Madsen, 1990; McCormick, 2001). Cortisol is the major corticosteroid produced by the interrenal tissue of teleost fish (Mancera and McCormick, 2007). Cortisol is known for its role in regulating hydromineral balance in euryhaline teleosts adapted to seawater, by modifying the number and the morphology of chloride cells and by increasing gill NKA activity (McCormick, 1995; Eckert et al., 2001; Chasiotis and Kelly, 2012; McGuire et al., 2012). However, the role of cortisol is not just limited to seawater fish and is also important in freshwater fish. In recent years, evidence suggests the role for cortisol in ion uptake in freshwater fish or fish adapted to low salinity (Marshall and Grosell, 2005; Babitha and Peter, 2010; Chasiotis and Kelly, 2012; Liew et al., 2012). This was recently proven by *in vivo* cortisol treatment which resulted in an increased gill NKA activity and improved salinity tolerance (McCormick et al., 2008). At mRNA level, cortisol significantly upregulated gill NKA α1a and α1b which increased in response to freshwater and seawater acclimation in Atlantic salmon (McCormick et al., 2008). An induction of chloride cell proliferation associated with an increase in number and size, as well as an increase in NKA activity was reported in freshwater tilapia (Dang et al., 2000), north african catfish (Babitha and Peter, 2012) and goldfish (Babitha and Peter, 2010) treated with cortisol. These new evidences suggest that cortisol plays the role of a 'dual-osmoregulatory' action. Cortisol in its classic role improves salinity tolerance and Na^+ secretion in cooperation with growth hormone and insulin-like growth factor-I in seawater adapted teleost. When cooperating with prolactin, cortisol acts to increase ion uptake in hypo-osmotic environments by increasing gill NKA activity (Mancera and McCormick, 2007).

Chapter 1

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

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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 (J_{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 J_{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 lead 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.,