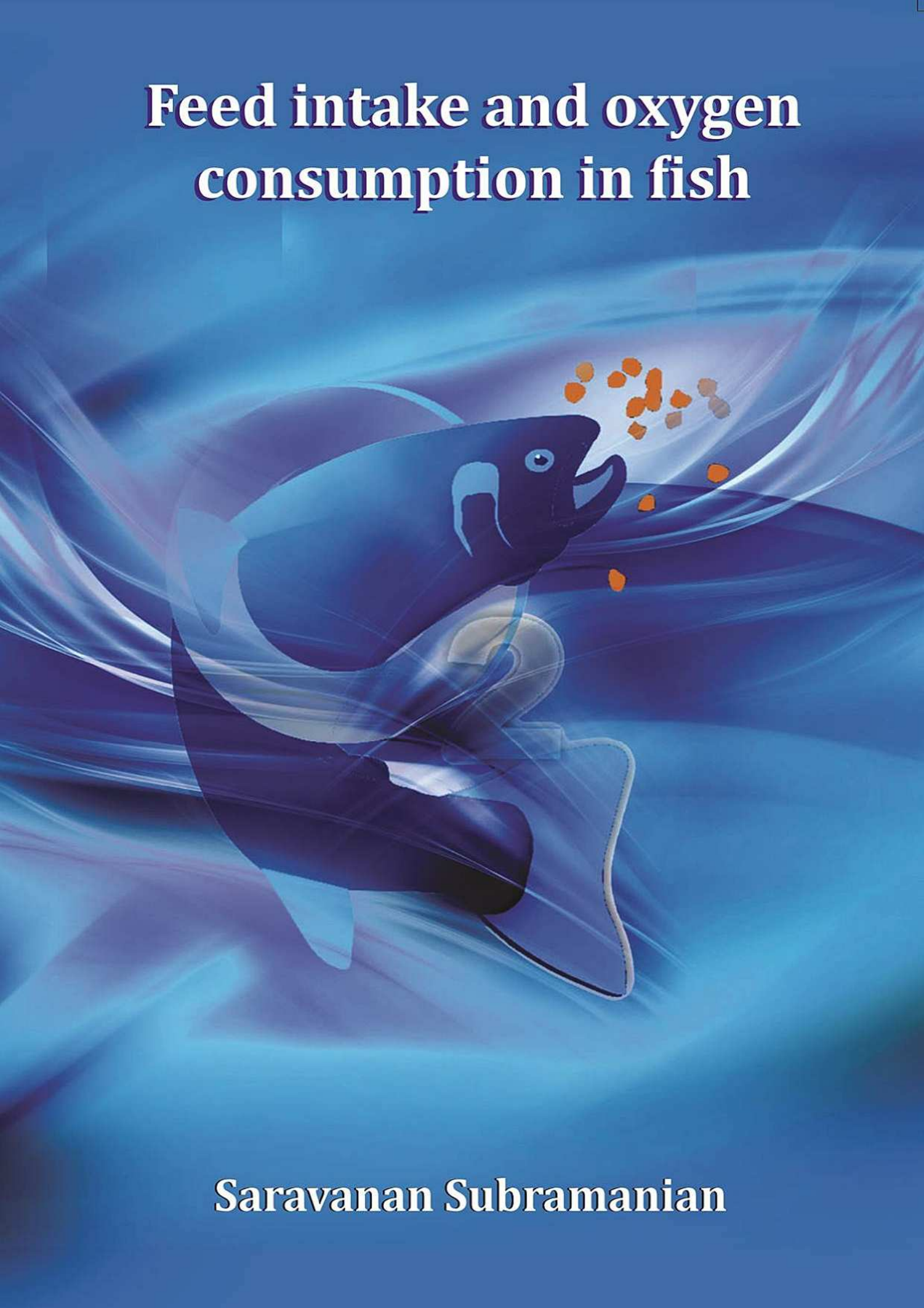
Feed intake and oxygen consumption in fish



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Thesis

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To my parents and pappu

Abstract

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In fish, the voluntary feed intake is influenced by dietary, environmental and/or physiological factors. It is well known that under hypoxia the concentration of oxygen in the water (DO) determines the feed intake of fish. However at non-limiting water DO levels (normoxia), several other mechanisms might play a role in feed intake regulation. Under hypoxia feed intake and oxygen consumption are interrelated. In this thesis we proposed the 'oxystatic' concept of feed intake regulation, which states that even at normoxia and in the absence of other constraints, the long term (weeks) voluntary feed intake of fish can be constrained by a set-point value of oxygen consumption. Dietary macronutrient composition affects the 'dietary oxygen demand' (i.e., amount of O_2 consumed per unit of feed). This oxystatic concept implies that fish fed to satiation with diets differing in 'dietary oxygen demand' (mg O_2/ g or kJ feed) will have a different digestible energy intake but a similar oxygen consumption. The validity of the oxystatic concept was assessed in two species, Nile tilapia and rainbow trout. These fish were fed diets which had large contrasts in nutrient composition (i.e., protein to energy ratio; type of the non-protein energy source (starch vs. fat); amino acid composition) in order to create contrasts in dietary oxygen demand. In all conducted studies with both species, the digestible energy intake was affected by the diet composition. However, in some studies oxygen consumption was similar and in others it differed between the diets, which respectively supports and contradicts the oxystatic concept. In all studies with both species, the digestible energy intake of tilapia and trout was negatively related to dietary oxygen demand and positively related to efficiency of oxygen utilization for energy retention. Furthermore it was observed in tilapia that the within-day variation in feed intake was affected by dietary macronutrient composition. The variation in within-day feed intake was related to pre-feeding oxygen levels. Based on the combined results, it is suggested that even at normoxia voluntary feed intake in fish is limited/determined by oxygen consumption and/or the oxidative metabolism. Overall, the oxystatic concept appears to be valid for certain conditions, but its generic application remains questionable. Yet, the oxystatic concept enables the combination of dietary, environmental and fish factors into one concept. Further it provides a conceptual insight for better understanding of feed intake regulation in fish.

Contents

CHAPTER 1

General Introduction

Aquaculture continues to be one of the fastest growing (6.3% per year) animal food producing sectors worldwide and produces 47% of food fish for human consumption (FAO, 2012). The increasing demand for food fish from aquaculture requires intensification. In intensive fish farming, feed is the main input and takes substantial share in the total production cost. Thus, the quality of feed and feeding management is important economic criterion for fish farmers. Feeding fish to their voluntary feed intake level is essential to maximize the growth rate. Compared to terrestrial animals, the *ad libitum* feeding of fish under farming conditions is relatively time consuming and a challenging task due to the difficulty in measuring the actual feed intake (Cho and Bureau, 1998; Jobling et al., 1995). In addition, determining the voluntary feed intake of fish is complicated, since feed intake varies with changes in the environmental, dietary and physiological (animal) factors. Consequently, as a common practise, fish are fed (often below their voluntary feed intake) with a pre-determined feed ration that may be insufficient to realise their full growth potential. Therefore, a thorough understanding of the factors affecting voluntary feed intake and their underlying mechanism is important for a good prediction of the feed ration and for optimizing the feeding management in fish farming.

Feed intake in fish

As in mammals, voluntary feed intake in fish is considered to be under the control of a central feeding system (located in the brain) with hunger and satiation signals being transmitted through complex networking of peripheral neural and humoral signals (Lin et al., 2000; Volkoff et al., 2005). Several neuropeptides, specific protein molecules, gastrointestinal peptides, hormones and blood metabolites operate the switch on-off mechanism of feed intake in fish (Volkoff and Peter, 2006; Volkoff et al., 2009). These feed intake signalling molecules in fish (like other animals) are controlled by a multitude of complex factors (Jobling et al., 2012). In addition, fish adjust the voluntary feed intake on its own time window (on daily, weekly, monthly basis) depending on their physiological state, which is also affected by the quality of feed (De la Higuera, 2001) and the prevailing environmental/water conditions (Kestemont and Baras, 2001) (fig. 1.1).

The factors affecting feed intake in fish can be conveniently divided into short term (within-day) and long term (weeks) controlling factors.

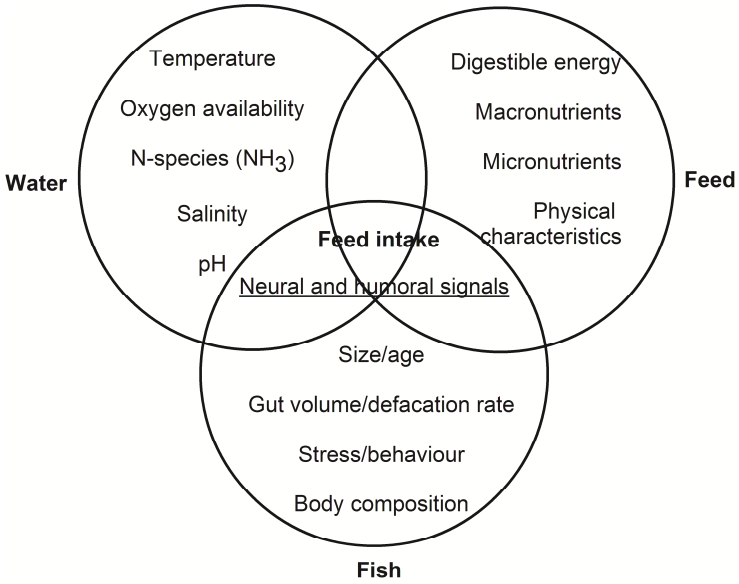

Figure 1.1 Factors influencing feed intake in fish

Short term control of feed intake

In short term, the maximum amount of feed a fish can ingest at a time is limited by its stomach (gut) volume and the gastrointestinal emptying rate (Grove et al., 1985; Jobling, 1981; Vahl, 1979). Therefore, dietary physical characteristics (like excess starch and fibre) can also determine feed intake due to increase in the dietary bulk. However, the stomach volume increases in fish fed bulky diet over experimental periods of few weeks (in rainbow trout (Hilton et al., 1983; Ruohonen and Grove, 1996); in plaice, (Jobling, 1982). In addition, the amount of feed intake often exceeds the normal level of intake after a period of feed deprivation (Rubio et al., 2010), which implies a flexibility in the gut volume. The gastrointestinal emptying rate is relatively fast in fish and varies widely between fish size, meal size and dietary characteristics (Güner and Davies, 2003; Hossain et al., 1998; Jobling, 1987). The gastrointestinal motility also depends on the environmental factors. For instance, the increase in water temperature increases the rate of evacuation (Brett and Higgs, 1970; Pérez-Casanova et al., 2009). Overall, the fish gut shows high morphological and functional plasticity towards the effect of dietary and environmental factors. Hence in long term, the physical limitation of gut will have minimal influence on the feed intake in fish.

Besides the physical limitations of the gut, a number of single nutrient/metabolite based mechanisms control feed intake. A well-known mechanism in mammals is based on signals generated by blood glucose. This mechanism is known as glucostatic theory (Mayer, 1953). However, the control of feed intake in fish by blood glucose is controversial. In rainbow trout hyperglycemia induced by the oral administration of glucose did not induce significant change in feed intake (Soengas and Aldegunde, 2004), whereas, increased blood glucose levels induced by the intraperitoneal administration

of glucose reduced feed intake in common carp and gold fish (Kuz'mina and Garina, 2001) and increased feed intake in rainbow trout (Polakof et al., 2008). It is usually considered that the circulatory glucose plays only a minor role in energy provision, and the low glucose turnover rates observed in fish seems to confirm this belief (West, 1994). Overall, the glucostatic regulation of feed intake in fish apply to short term feeding whereas its relevance in the long term feed intake is unclear. Next to blood glucose, the circulating level of amino acids in the blood control feed intake in mammals via specific receptors in the brain (aminostatic theory) (Gietzen et al., 2007; Mellinkoff et al., 1956). Compared to mammals, protein plays an important role in energy supply in fish (Kaushik and Medale, 1994; Weber and Haman, 1996). The changes in the amount of dietary protein (Gurure et al., 1995) as well the composition of amino acids in the diet affects feed intake in various fish species (see De la Higuera, 2001). However, the direct impact of blood amino acids level on feed intake and its controlling mechanism is studied to a lesser extent (Kuz'mina, 2005).

Long term control of feed intake

A long term controlling factor of feed intake is not mutually exclusive from the short term controlling factor but is considered to have an additive effect. Like other animals, fish consume feed to satisfy the energy required for maintenance, growth and reproduction (Cho and Kaushik, 1985; Kaushik and Medale, 1994). If the energy demand for maintenance and growth of fish is presumed to be constant, then fish will maintain a similar energy intake when fed diets varying in macronutrient composition. Indeed in fish, a number of studies show similar digestible energy intake when fish were fed with diets varying in amount and type of digestible energy (Bendiksen et al., 2002; Boujard and Médale, 1994; Bromley, 1980; Kaushik and Luquet, 1984; Yamamoto et al., 2000). In contrast, other studies in fish show different digestible energy intakes suggesting an involvement of other factors rather than energy requirement in the control of feed intake (Alanärä, 1996; Figueiredo-Silva et al., 2012; Helland and Grisdale-Helland, 1998). Besides energy requirement, an animal's demand for target lean growth (protein-stat) may control feed intake (Azevedo et al., 2004; Geurden et al., 2006; Millward, 1995; Peres and Oliva-Teles, 1999; Webster, 1993). In addition, Kennedy (1953) proposed the lipostatic control of feed intake in mammals which states that there is a set point for body fat reserve/adiposity which provides feedback signals for feed intake through leptin (Zhang et al., 1994). In fish, studies have shown that large fat stores reduce feed intake on a long term (Jobling, 1993; Jobling et al., 2002; Johansen et al., 2003; Shearer et al., 1997; Yamamoto et al., 2002), but the role of leptin in signalling adiposity in the feed intake regulation of fish is still unclear (Frøiland et al., 2012).

Overall, the majority of short- and long-term feed intake control mechanisms mentioned above could partly explain the feed intake response of fish to changes in the dietary factors (bulk, protein, starch, fat and energy), but fails to provide a systematic explanation towards the impact of environmental factors. A number of environmental factors (water dissolved oxygen, temperature, salinity, pH, ammonia etc.) affects feed intake in fish (see Kestemont and Baras, 2001). For instance, fish exposed to a high water ammonia levels show differences in feed intake (Schram et al., 2010), but the increase or decrease in feed intake lacks a comprehensive explanation. Similarly, the impact of other environmental factors on feed intake of fish is rather descriptive and lacks conceptual mechanism. As feed intake of fish is affected by both the dietary and environmental factors, knowledge on the combined effect of these factors and their control mechanisms on feed intake are essential. Since, oxygen consumption of fish is also influenced by several dietary (due to nutrient metabolism (Fu et al., 2007)) and environmental factors (e.g., temperature and nitrite effect on oxygen uptake capacity (Fry and Hart, 1948; Lewis and Morris, 1986)), it can be an unifying factor in explaining the control of feed intake in fish. Therefore, a thorough investigation on the link between oxygen consumption and feed intake in fish is needed for better understanding of feed intake control in fish.

Oxygen in control of feed intake

Availability of water oxygen

Oxygen is involved in all aspects of animal life. The amount of oxygen in water is about 20 times less than in the air and extracting oxygen from water is more difficult than from air (Kramer, 1983). Unlike in air, the amount of dissolved oxygen in water fluctuates greatly and is being the prime limiting factor for survival, feeding, growth and reproduction in water breathing fish (Kramer, 1987; Pauly, 2010). The impact of water dissolved oxygen on feed intake is widely documented in different fish species (Foss et al., 2002; Glencross, 2009; Pichavant et al., 2000; Thetmeyer et al., 1999; Tran-Duy et al., 2012). In general, feed intake decreases linearly with decreasing water oxygen levels. The minimum concentration of dissolved oxygen in water at which the physiological oxygen demand of fish for feed intake is limiting is termed '*incipient dissolved oxygen*' (iDO) (Tran-Duy et al., 2012). Below iDO, feed intake depends on the dissolved oxygen concentration of water but above iDO feed intake is independent of the water dissolved oxygen (fig. 1.2).

The mechanism controlling feed intake under hypoxic condition is that the availability of the oxygen in the water is insufficient to meet the oxygen demand of fish for feed intake. At normoxia the availability of dissolved oxygen from water is sufficient to meet oxygen demand of the fish and thus will not constrain feed intake.

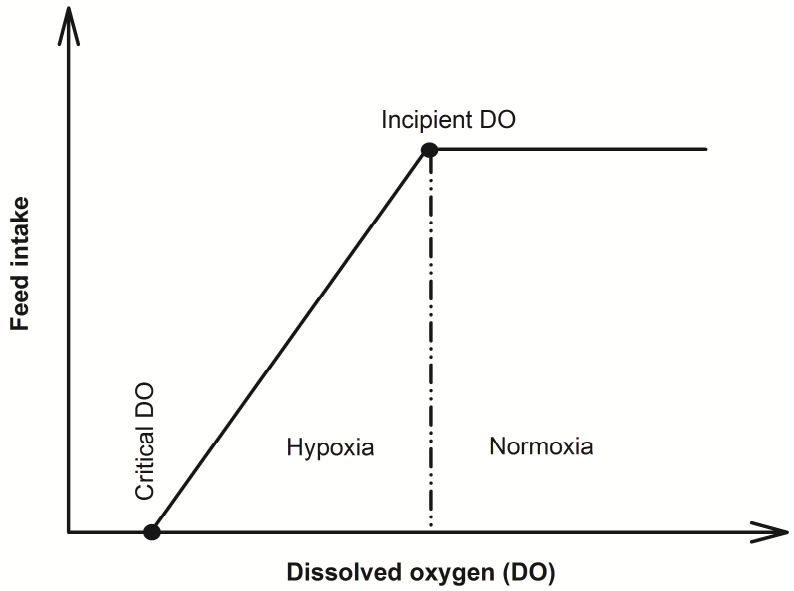

Figure 1.2 Relation between water dissolved oxygen level and feed intake in fish

Oxidative metabolism

Oxygen is essential for aerobic metabolism to derive energy (ATP) from protein, fat and carbohydrates for growth and maintenance. The consumption of feed gives rise to heat production which is proportionate to the post-feeding oxygen consumption of an animal (Kleiber, 1961). The oxidative metabolism of nutrients influences food/energy intake in mammals (Friedman and Tordoff, 1986; Scharrer and Langhans, 1986; Stubbs, 1996). The partitioning of ingested macronutrients (protein, carbohydrate and fat) between oxidation and storage/ deposition in the body provide feedback signals for energy intake (Stubbs, 1996). Compared to carbohydrate or protein, dietary fat is stored efficiently (less oxidised) in the body and the degree of oxidation is linked to their negative feedback on energy intake. Hence, the dietary fat is the least satiating than carbohydrate or protein (Stubbs, 1996). The protein and carbohydrate metabolism in the body is tightly regulated through obligatory oxidative disposal of nutrients resulting in a high satiating effect (Stubbs, 1998). Further, the oxidation of metabolic fuel is the common pathway providing signals for food intake regulation in mammals (Friedman et al., 1986).

Efficiency of oxygen utilization

Besides the direct involvement of the oxidative metabolism in the control of food intake, it is widely accepted that the enhanced oxidative metabolism due to food/energy intake has putative negative effects on the animal in the long term. This is due to reactive oxygen species, which may impair cell function and vitality, leading to ageing (Dowling and Simmons, 2009; Kirkwood and Shanley, 2005). The negative effect of enhanced oxidative metabolism on animal fitness is considered as an intrinsic cost of food intake

(Illius et al., 2002; Masoro, 2000). Based on such notion, Ketelaars and Tolkamp (1992) initially proposed the 'efficiency of oxygen utilization' as a cost-benefit model (oxygen consumption vs. net energy) in the control of feed intake in ruminants. According to this theory, feed intake has both benefits and costs. Thus the animal would strive to optimize the benefits of feed intake (net energy) against the intrinsic costs of feed intake (oxygen consumption). In other words, an animal will try to maximize its net energy intake per unit oxygen consumed as shown in ruminants fed with different feed stuffs (Tolkamp and Ketelaars, 1992; Ketelaars and Tolkamp, 1996). The validity of the concept of oxygen efficiency in the regulation of feed intake in a broader context has been argued (Emmans and Kyriazakis, 1995; Forbes, 2007). Since the oxidative metabolism of nutrients and its consequence are fundamental to all animals, the above mentioned concepts of oxidative metabolism and efficiency of oxygen utilization can also be applicable in the control of feed intake in fish.

Oxygen consumption

The maximum amount of oxygen that a fish can obtain/uptake from water for aerobic metabolism is referred as active metabolism. The minimum oxygen consumption needed to sustain the physiological activity of an unfed fish is referred as routine metabolism. The difference between the routine metabolism and active metabolism is considered as the metabolic scope or scope for activity within which the fish must perform all activities including feeding to grow and reproduce (Priede, 1985). The absolute metabolic scope (oxygen consumption) of fish can be constrained by anatomical (e.g., gill surface (Pauly, 1981)), physiological (e.g., cardiac output) and environmental (e.g., temperature (Fry and Hart, 1948)) factors. In certain fish species feeding and its associated cost alone can use the entire metabolic scope. For example, the rate of oxygen consumption of juvenile cod fed to satiation was close to the value of maximum oxygen consumption rate of active fish (Soofiani and Hawkins, 1982). Thus it can be assumed that the maximum feed intake of those fish can be constrained by the oxygen consumption. In line, Tran-Duy et al. (2008) studied the effect of changes in digestible energy (DE) source (fat vs. starch) in the diet on maximum feed intake in Nile tilapia and found a significant effect of DE source on the digestible energy intake of fish. A striking observation in that study was the similar heat production of fish, irrespective of differences in digestible energy intake and retained energy. Based on the observation of similar heat production, calculated as difference between metabolizable and retained energy, the authors postulated the involvement of limitation in oxygen uptake (e.g., gill surface area) for feed intake and nutrient processing. Similarly based on the oxygen uptake limitation in fish by diffusion at gill and gill surface area, van Dam and Pauly (1995), proposed the so called 'oxygen limitation theory'. This theory states that the

maximum rate of feed intake of fish is related to the capacity to deliver oxygen for feed/nutrient processing.

Taken together, even at non-limiting water oxygen levels (normoxia), many factors can set a limit on the oxygen consumption of fish such as a limited gill surface, a limited capacity of oxygen delivery to tissues and mitochondrial metabolism, and the negative consequences (reactive oxygen species) of oxidative metabolism. Moreover, in fish, the damage due to oxidative metabolism is assumed to be greater due to the presence of high level of polyunsaturated fatty acids (Bell et al., 1986) that are susceptible to oxidation (Gardner, 1989). Thus a fish should have a desired set-point value of oxygen consumption rate to perform all its functions, including feeding.

Hypothesis

In line with the above mentioned association between feed intake and oxygen consumption, we propose a hypothetical framework which could explain feed intake in fish under non-limiting water oxygen levels (above iDO or normoxia) as a function of oxygen consumption. Based on the observation of similar heat production together with differences in the digestible energy intake of Nile tilapia (Tran-Duy et al., 2008) we propose the concept of 'oxystatic' control of feed intake in fish, which states that the heat production (oxygen consumption) can control feed intake in fish. The relation between voluntary feed intake and oxygen consumption (fig. 1.3) illustrates two possible scenarios in the control of feed intake.

First, it is speculated that at non-limiting water oxygen levels (normoxia) and in the absence of other potential constraints on feed intake, the voluntary feed intake in fish can be constrained by a set-point value of heat production or oxygen consumption (assumption-1, fig. 1.3). This set-point value is below the maximum aerobic capacity of fish. The fish would strive not to exceed the set-point oxygen consumption by adjusting/ regulating its voluntary feed intake on a larger time scale (weeks/months). If this is true, then indeed one would expect to observe identical heat production/oxygen consumption when fish fed different diets varying in the nutrient composition.

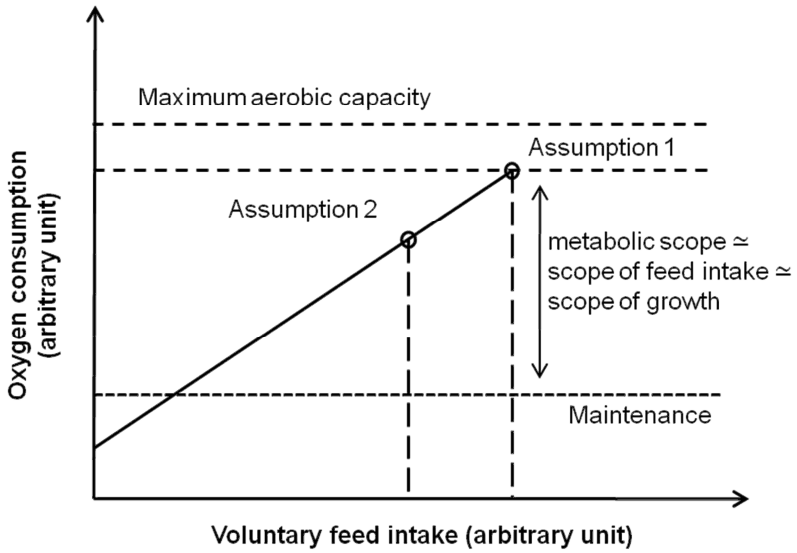

Figure 1.3 Assumptions on the relation between voluntary feed intake and oxygen consumption in fish

Second, it is assumed that in certain situations the set-point heat-production/oxygen consumption cannot be attained (assumption-2, fig. 1.3) when fish fed diets of very low quality so that other constrains (e.g., gut volume) might limit feed intake.

Also to test the 'oxystatic' concept, it is important that the normal oxygen uptake/aerobic capacity of fish should not be restrained by external factors (e.g., water oxygen level). In growing fish the amount of oxygen consumed per unit feed (mg O_2/ g or kJ feed) can be altered by the type of dietary macronutrient (protein, fat and carbohydrates). In addition, depending on the type of dietary energy substrate oxidation and growth composition, the oxygen consumption per unit feed (mg O_2/ g or kJ feed) and the utilization of consumed oxygen (net energy retained, kJ/mg O_2 consumed) can be altered. For instance, the deposition of body fat from dietary fat is considered to be relatively inexpensive (480 kJ/mol synthesized or 0.015 kJ expended per kJ synthesized), whereas the formation of fat from either carbohydrates (6,100 kJ/mol synthesized) or amino acids (12,800 kJ/mol synthesized) is more costly' (Reeds et al., 1982). This trend would also reflect in the oxygen consumption of an animal. Hence, fish fed diets having different macronutrients (e.g., different protein to energy ratio or changes in non-protein energy source (starch vs. fat)) will induce differences in oxygen consumption. This diet induced differences in oxygen consumption per unit feed intake is defined as dietary oxygen demand (i.e, mg O_2 consumed/kJ digestible energy intake), which is represented by the difference in the slope of the line between Diet A and Diet B in fig. 1.4.

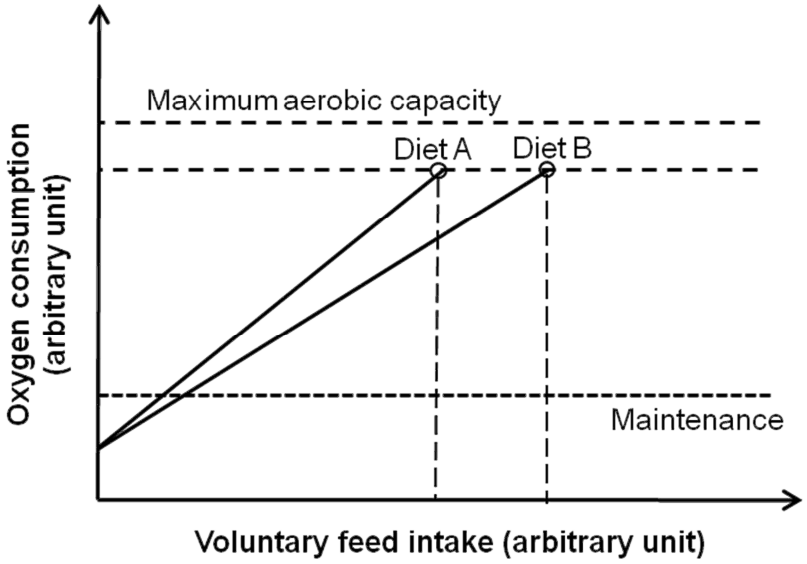

Figure 1.4 Hypothetical illustration on the effect of diet composition (diet A vs. diet B having high vs. low oxygen demand, respectively) on voluntary feed intake and oxygen consumption in fish

If the 'oxystatic' concept holds true, feeding fish with diets having a high oxygen demand per unit feed intake (Diet A; fig 4) compared to the diet having a low oxygen demand per unit feed intake (Diet B; fig 4) would result in a different voluntary feed intake, however at an identical heat production/oxygen consumption. In other words, feeding fish to satiation with different diets contrasting in dietary oxygen demand will result in a similar oxygen consumption, but will have different feed intake (fish fed with low oxygen demand diet will have higher feed intake).

Aim and outline of the thesis

In fish, it is widely known that feed intake causes an increase in oxygen consumption. However, the impact of enhanced oxygen consumption induced by the ingested nutrients on the feed intake regulation has never been explored. Therefore, the general aim of this thesis was to investigate the role of oxygen consumption in control of feed intake in fish. Elucidating this role is important to understand why and how fish adjust their voluntary feed intake (in long-term and short-term/within day variation) in response to changes in the diet composition.

The observation of similar heat production together with differences in digestible energy intake of Nile tilapia in the study of (Tran-Duy et al., 2008) was the trigger/basis for the concept of 'oxystatic' control of feed intake in fish proposed in this thesis. Therefore, the first objective was to verify the previous observation of similar heat production in other fish species (rainbow trout) under non-limiting water oxygen conditions (**chapter 2**).

The next step was to test the existence of 'oxystatic' control of feed intake by direct measurement of oxygen consumption in fish. The contrasts in dietary macronutrient composition (i.e., protein to energy ratio and non-protein energy source) were created to alter the dietary oxygen demand, which in turn will influence the oxygen consumption of the fish. Applying the changes in the macronutrient composition of diet, we tested the validity of the oxystatic concept in two species, Nile tilapia (**chapter 3**) and rainbow trout (**chapter 4**).

Next to alteration in the dietary macronutrient composition, the quality of dietary protein (amino acid composition) was expected to alter the dietary oxygen demand. A balanced amino acid diet will have a lower oxygen demand than an imbalanced amino acid diet. Therefore, this was tested in rainbow trout at two water oxygen levels (c**hapter 5**).

In chapters 2 to 5 the focus was on the involvement of heat production/oxygen consumption in the long term control of feed intake in fish. However, the feed intake response of fish observed over weeks might be a result of short term feed intake regulation (within-day effect). Therefore in **chapter 6**, we assessed the impact of dietary macronutrient composition on within-day variation in feed intake and oxygen consumption of Nile tilapia, to test whether the within-day (morning vs. afternoon meal) variation in feed intake induced by diet composition is related to the within-day variation in oxygen consumption of fish.

In the final **chapter 7**, the main results of all studies described in this thesis are discussed in line with the general objective and proposed the 'oxystatic' concept. Limitation and future perspectives of this concept and overall conclusions are presented.

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CHAPTER 2

Constraints on energy intake in fish:

the link between diet composition, energy metabolism, and energy intake in rainbow trout

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Abstract

The hypothesis was tested that fish fed to satiation with iso-energetic diets differing in composition will have different digestible energy intakes (DEI) but similar total heat production. Four iso-energetic diets (2 x 2 factorial design) were formulated having a contrast in i) the ratio of protein to energy (P/E): high ($H_{P/E}$) vs. low ($L_{P/E}$) and ii) the type of non-protein energy (NPE) source: fat vs. carbohydrate which were iso-energetically exchanged. Triplicate groups (35 fish/ tank) of rainbow trout were hand-fed each diet twice daily to satiation for 6 weeks under non-limiting water oxygen conditions. Feed intake (FI), DEI (kJ $kg^{-0.8}$ d^{-1}) and growth (g $kg^{-0.8}$ d^{-1}) of trout were affected by the interaction between P/E ratio and NPE source of the diet ($P<0.05$). Regardless of dietary P/E ratio, the inclusion of carbohydrate compared to fat as main NPE source reduced DEI and growth of trout by ~20%. The diet-induced differences in FI and DEI show that trout did not compensate for the dietary differences in digestible energy or digestible protein contents. Further, changes in body fat store and plasma glucose did not seem to exert a homeostatic feedback control on DEI. Independent of the diet composition, heat production of trout did not differ ($P>0.05$). Our data suggest that the control of DEI in trout might be a function of heat production, which in turn might reflect a physiological limit related with oxidative metabolism.

Introduction

Fish under farming conditions are mostly fed pre-set amounts of a single feed type so that the fish cannot compensate feed intake (FI) for the eventual lack of a particular nutrient or for energy content, which may lead to reduced growth. Thus, predicting the feed ration close to the voluntary FI level of fish as a function of diet composition and culture conditions is essential to maximize growth rate and feed use and also to minimize feed wastage in the aquatic environment. This requires a better understanding of the dietary, physiological, and environmental factors affecting FI and their underlying mechanisms.

Compared to mammals, mechanisms controlling FI are relatively less explored in fish. It was stated that "fish like other animals, eat to satisfy their energy requirements" (Cho and Kaushik, 1985). Indeed, among the dietary factors, the digestible energy (DE) content has been widely suggested to be a major determinant of FI control in several fish species such as rainbow trout, *Oncorhynchus mykiss* (Boujard and Médale, 1994; Kaushik and Luquet, 1984; Morales et al., 1994; Yamamoto et al., 2000), Atlantic salmon, *Salmo salar* (Bendiksen et al., 2002), Atlantic cod, *Gadus morhua* (Lekva et al., 2010), European seabass, *Dicentrarchus labrax* (Dias et al., 1998), turbot, *Scophthalmus maximus* (Bromley, 1980) and Channel catfish, *Ictalurus punctatus* (Page and Andrews, 1973).

In contrast, some studies have shown that fish do not regulate their FI based on dietary DE density as a whole, as seen in rainbow trout (Cláudia Figueiredo-Silva et al., 2011; Geurden et al., 2006), Atlantic salmon (Helland and Grisdale-Helland, 1998), Arctic charr, *Salvelinus alpinus* (Alanärä, 1996) and European seabass (Peres and Oliva-Teles, 1999), suggesting a possible role of energy or nutrient utilization and thus of DE source in FI regulation in fish. Recently, Tran Duy et al. (2008) studied the effect of changes in DE source (fat vs. starch) on FI in Nile tilapia, *Oreochromis niloticus* and found similar dry matter FI but different digestible energy intake (DEI) as affected by the DE source of the diet. One striking observation in that study was the similar total heat production of fish, irrespective of the diet-induced differences in ingested (DE) and retained (RE) energy. Based on the observation of similar heat production, calculated as the difference between metabolisable and retained energy, the authors postulated the involvement of heat production in the control of FI in Nile tilapia. Therefore, the present study further investigates the relation between heat production and the effect of macronutrient composition on FI and DEI in another teleost model, rainbow trout. We hypothesized that rainbow trout fed to satiation with iso-energetic diets, differing in protein to energy ratio (P/E) as well as in non-protein energy (NPE) source, would result in different DEI but with similar heat production.

Materials and Methods

The experiments were conducted following the Guidelines of the National Legislation on Animal Care of the French Ministry of Research (Decree 2001-464 of May 29, 2001) and were approved by the Ethics Committee of INRA (according to INRA 2002-36 of April 14, 2002).

Diets

Four diets were formulated in a 2 x 2 factorial design with protein to energy ratio (P/E) and non-protein energy (NPE) source as main factors, each consisting of two levels, being 'high' vs. 'low' and 'fat' vs. 'carbohydrate', respectively. The formulation and ingredient composition of diets are shown in Table 2.1. In order to have identical nutrient and energy density between diets, 15% of cellulose was included in the fat diets. We thus had four diets (Table 2.1) *viz.,* high P/E ratio with fat as energy source ($H_{P/E}F$), high P/E ratio with carbohydrate as energy source ($H_{P/E}C$), low P/E ratio with fat as energy source ($L_{P/E}F$) and low P/E ratio with carbohydrate as energy source ($L_{P/E}C$). As expected, all four diets resulted in similar digestible energy content (~18 kJ g^{-1}) and contrast in P/E ratio between $H_{P/E}$ diets (~26 mg kJ^{-1}) and $L_{P/E}$ diets (~14 mg kJ^{-1}). The ingredient mixtures of each diet were extruded through a 2 mm die, dried, sieved, and stored in plastic bags (feed extrusion plant, INRA Donzacq, France). The analyzed nutrient compositions of the four diets are detailed in Table 2.1.

Feeding trial and sampling

Rainbow trout (*O. mykiss)* were obtained from the same parental stock (INRA Lées-Athas fish farm, France) and were transferred to the experimental facilities of INRA (Donzacq, France) where they were acclimatized to the rearing conditions prior to the start of the feeding trial. The experimental setup consisted of 12 independent circular tanks (150 L) in a flow-through system (flow rate, 0.4 L sec^{-1}; water renewal in tank minimum 8 times per h) supplied with natural spring water having a temperature of 16 ± 1°C (mean ± SD), average pH (7.4), ammonia (<0.05 mg L^{-1}), nitrite (<0.02 mg L^{-1}), nitrate (<15 mg L^{-1}), dissolved oxygen (DO; > 8.5 and >7.0 mg L^{-1} respectively in inlet and outlet) under natural light regimen (February-April). At the start of experiment, fish (32.4 g initial body weight) were sorted for homogenous size and randomly allotted among the 12 tanks (35 fish/tank). Diets were assigned randomly to triplicate tanks and hand-fed twice daily to visual satiation (i.e., feed distributed until the fish stop displaying active feeding) in morning and afternoon. In total, the feeding trial lasted for 7 weeks, during the first 6 weeks (growth period) we assessed feed intake, growth and nutrient utilisation, and then fish were allowed to recover for 1 week (recovery period) before post-prandial sampling. During the growth period, mortality was monitored daily

and fish were group weighed every 2 weeks to calculate intermediate growth and feed intake. A random sample of 36 h feed deprived fish were euthanized (overdose of anaesthesia, 2-phenoxy-ethanol) and stored at -20ºC for subsequent analyses of whole body composition, at the beginning (35 fish) and end (8 fish/tank) of the growth period. At the end of the 6 weeks, all fish were counted and weighed to calculate the final body weight of fish. The fish were then continued to be fed their respective diets for a period of 1 week (recovery period) prior to post-prandial blood sampling. At 7 h post-feeding, nine fish per dietary treatment were sampled for blood. The blood was drawn from the caudal vein and transferred into a vial containing 20 µl anticoagulant (2g potassium oxalate + 1 g sodium fluoride in 100 ml distilled water). Blood samples were centrifuged (3000G, 10 min) and the plasma obtained were stored at -20° C until analyses of glucose and triglycerides.

Digestibility study

In parallel to the 6-week feeding trial, a separate 4-week digestibility trial was conducted at the INRA fish rearing unit (St Pée-sur-Nivelle, France) with rainbow trout from the same stock as in the feed intake study. Fifteen fish (mean body weight, 65 g) were stocked in 12 cylindro-conical tanks (60 L) connected to an automatic faeces collection unit (Choubert et al., 1982), the diets were assigned randomly among tanks in triplicates. The tanks received continuous supply of water (14 ± 1°C; mean ± SD) from the recirculation water system and were maintained at uniform conditions throughout the experiment. Prior to faeces collection, fish were acclimatized for a week to the experimental conditions and to their respective experimental diets. Diamol (acid insoluble ash, AIA) was added into the feed as inert marker for determining digestibility. Fish were fed twice daily (1.5% of body weight) and faeces collected twice daily over 3 weeks, pooled per tank and stored at -20°C.

Chemical analyses

Whole fish from each tank were ground, pooled and fresh moisture content was determined. Fish and faeces were subsequently freeze-dried before further analyses. The nutrient compositions of fish, diet and faeces were analyzed according to the following procedures. Feed, faeces and whole body samples were analyzed for dry matter (105ºC for 24 h), protein (Kjeldahl; N x 6.25) after acid digestion, fat content of feed and faeces (Folch et al., 1957) using dichloromethane instead of chloroform and the

Table 2.1 Formulation, ingredient composition and analyzed nutrient content of experimental diets

	Diets[1]			
	$H_{P/E}F$	$H_{P/E}C$	$L_{P/E}F$	$L_{P/E}C$
Ingredients (%)				
Protein mixture[2]	66.0	66.0	35.9	35.9
Oils[3]	11.0	1.0	19.1	9.1
Gelatinized maize starch[4]	5.0	30.0	24.3	49.3
Cellulose[5]	15.0	0.0	15.0	0.0
Other[6]	3.0	3.0	5.7	5.7
Analyzed nutrient content on DM basis (g kg^{-1})				
Dry matter (DM; g kg^{-1} diet)	938	924	949	947
Crude protein (N x 6.25)	519	511	276	261
Crude fat	152	34	207	143
Total carbohydrates[7]	254	380	444	528
Starch	49	303	246	456
Ash	75	75	73	68
Gross energy (GE; kJ g^{-1})	22.8	20.6	22.8	21.2
Digestible energy (DE; kJ g^{-1})	18.70	18.27	18.74	18.19
DP/DE (mg kJ^{-1})[8]	26.5	26.8	14.1	13.7

[1] $H_{P/E}F$ - High P/E ratio diet with fat as main non-protein energy source; $H_{P/E}C$ - High P/E ratio diet with carbohydrate as main non-protein energy source; $L_{P/E}F$ - Low P/E ratio diet with fat as main non-protein energy source; $L_{P/E}C$ - Low P/E ratio diet with carbohydrate as main non-protein energy source

[2] Protein mixture (% mixture): 50% fishmeal (Sopropêche 56100 Lorient, France), 16.5% soybean protein concentrate (Sopropêche 56100 Lorient, France) , 16.5% pea protein concentrate (Roquette 62080 Lestrem, France), 16.5% wheat gluten (Roquette 62080 Lestrem, France) and 0.5% DL methionine (Ajinomoto Eurolysine 75017 Paris, France).

[3] Oils: rapeseed oil (Daudruy 59640 Dunkerque, France) in $H_{P/E}$ diets; 5% (% diet) fish oil (Sopropêche 56100 Lorient, France) and the remaining part from rapeseed oil in $L_{P/E}$ diets.

[4] Gelatinized maize starch: Roquette 62080 Lestrem, France.

[5] Cellulose: Rettenmeier et Sohne 73494 Rosenberg, Germany

[6] Other (% diet): 2% Diamol (indigestible marker, Diamol GM, Franz Bertram Hamburg, Germany); 1% vitamin and mineral premix (INRA UPAE 78200 Jouy en Josas). For $L_{P/E}$-diets 0.4% $CaCO_3$, 1.8% $Ca(HPO_4)_2$, and 0.5% Na_2CO_3 were added.

[7] Calculated as, total carbohydrates (starch, free sugars, cellulose) = 1000 - (crude protein + crude fat + ash).

[8] DP/DE (Digestible protein to digestible energy ratio) = (Crude protein x % apparent digestibility coefficient of crude protein)/ (gross energy x % apparent digestibility coefficient of gross energy- see table 2.4).

fat content of fish by petroleum ether extraction (Soxhlet; 40-60ºC) and gross energy content by adiabatic bomb calorimeter (IKA-Werke C5000). Ash contents were determined by combustion in muffle furnace (550°C for 12 h). The same ash samples of feed and faeces were used to determine acid insoluble ash (ISO, 1981). Starch content was determined as glucose, using the amyloglucosidase/ hexokinase/glucose-6-phosphate dehydrogenase method after ethanol (40%) extraction and starch decomposition in dimethylsulfoxide/ HCl (ISO, 2005). Plasma glucose and triglycerides were determined following the procedures provided in the commercial kits, Glucose RTU (nº 61269) and Triglycérides (PAP 150 nº 61236) from Bio-Mérieux, Marcy-L'Etoile, France.

Calculations

The mean individual initial (W_i) and final (W_f) body weight of fish was obtained dividing the total initial and final fish biomass of the tank by the number of fish present in tank at start and end of study respectively. Absolute growth of fish (in g d^{-1}) was calculated as the difference between mean individual final (W_f) and initial (W_i) body weight of fish per tank divided by duration of experimental period (t). The geometric mean body weight (W_G; in g) is calculated as $\sqrt{(W_i \times W_f)}$, from which mean metabolic body weight (MBW_G; in $kg^{0.8}$) was calculated as $(W_G/1000)^{0.8}$. Growth rate on metabolic body weight (GR_{MBW}; in g $kg^{-0.8}$ d^{-1}) was calculated as $(W_f\text{-}W_i)/(MBW_G \times t)$. Daily growth coefficient (DGC, in % d^{-1}) was calculated as $100 \times (W_f^{1/3} - W_i^{1/3})/t$.

Absolute feed intake (FI_{ABS}; g DM $fish^{-1}$ d^{-1}) was calculated on dry matter (DM) basis as $FI_{tot}/(n \times t)$ where FI_{tot} is the total feed intake per tank (in g DM) over experimental period, n is the number of fish in tank and t is the experimental period. FI as fed (g $fish^{-1}$ d^{-1}) was calculated in similar way as FI_{ABS} but on as fed basis. Feed intake of fish expressed as a percentage of body weight (FI_{PCT}; % d^{-1}) was calculated as $(FI_{ABS}/ W_G) \times 100 /t$ and feed intake per metabolic body weight (FI_{MBW}; g DM $kg^{-0.8}$ d^{-1}) was calculated as FI_{ABS}/ MBW_G. Feed gain ratio (FGR; dry matter intake/wet weight gain) was calculated on DM basis as FI_{MBW}/ GR_{MBW}.

Apparent digestibility coefficients (ADC, in %) of dry matter, crude protein, crude fat, total carbohydrate, gross energy and ash were calculated for each tank using acid insoluble ash (AIA) as inert marker as described previously (Tran-Duy et al., 2008). Apparent digestibility coefficients were calculated as $ADC_X = (1- (AIA_{diet} /AIA_{faeces}) \times (X_{faeces} /X_{diet})) \times 100$, where X represents dry matter, crude protein, crude fat, total carbohydrate, gross energy and ash, AIA_{diet} and AIA_{faeces} are the AIA content in the diet and faeces, respectively and X_{diet} and X_{faeces} are the quantity of X in the diet and faeces, respectively.

The parameters of nitrogen balance (mg N $kg^{-0.8}$ d^{-1}) and energy balance (kJ $kg^{-0.8}$ d^{-1}) were calculated per tank, without changes as described earlier (Tran-Duy et al., 2008). The gross nitrogen intake (GNI) was calculated as product of total feed intake (g DM $kg^{-0.8}$ d^{-1}) and nitrogen content of feed (mg g^{-1}). The digestible nitrogen intake (DNI) was calculated as product of GNI and ADC of nitrogen (%). Faecal nitrogen loss (FN) was calculated as the difference between GNI and DNI. The retained nitrogen (RN) was calculated as the difference between nitrogen content of final and initial fish carcass. Branchial and urinary nitrogen loss (BUN) was calculated as difference between DNI and RN. Parameters of energy balance were calculated as follows: gross energy intake (GEI) as the product of feed intake (g DM $kg^{-0.8}$ d^{-1}) and energy content of the diet; digestible energy intake (DEI) as product of GEI and ADC of energy; metabolisable energy intake (MEI) was calculated as the difference between DEI and the branchial and urinary energy loss (BUE), which was estimated as BUE = (BUN×24.85)/1000, where 24.85 is the amount of energy (in kJ) equivalent to 1 g excreted nitrogen, assuming that all nitrogen is excreted as NH_3–N (Bureau, 2002); retained energy (RE) as the difference between energy content of final and initial fish carcass. The total heat production (H) was calculated as the difference between metabolisable energy intake (MEI) and retained (RE) energy from the energy balance. Similarly, the fat balance (mg $kg^{-0.8}$ d^{-1}) was calculated per tank. The gross fat intake (GFI) was calculated as product of total feed intake (g $kg^{-0.8}$ d^{-1}) and fat content of feed (mg g^{-1}). The digestible fat intake (DFI) was calculated as product of GFI and ADC of fat (%). Faecal fat loss (FF) was calculated as the difference between GFI and DFI. The retained fat (RF) was calculated as difference between fat content of final and initial fish carcass.

Statistical procedure

Statistical analyses were performed using SAS 9.2 (SAS Institute, Cary, NC, USA). Data were analyzed for the effect of P/E ratio, type of NPE source and their interaction by two-way ANOVA (PROC GLM). Normal distribution of the residuals was verified using Kolmogorov-Smirnov's test (PROC UNIVARIATE). The faecal fat loss (FF) overruled the assumption of normal distribution ($P < 0.05$) and logarithmic data transformation satisfied the assumptions. In the case of a significant interaction, post-hoc pair wise comparison of means was done using Tukey-Karmer test.

Results

Feed intake and growth

Feed intake (in g fish^{-1} d^{-1}, g DM fish^{-1} d^{-1}, % d^{-1}, and g DM kg$^{-0.8}$ d^{-1}), growth (in g d^{-1} and g kg$^{-0.8}$ d^{-1}), and feed gain ratio (FGR) were significantly affected by the P/E ratio and by the NPE source of the diet with a highly significant interaction between both factors (Table 2.2).

Within $H_{P/E}$ and $L_{P/E}$ groups, feed intakes were affected by the type of NPE, being lower in trout fed carbohydrate relative to fat as NPE source. The effect of NPE source on FI was greater with $L_{P/E}$ diets (~20% difference) than with $H_{P/E}$ diets (~11% difference); with lowest intakes registered in trout fed the $L_{P/E}C$ diet (11.6 g DM kg$^{-0.8}$ d^{-1}). Trout fed the diets containing fat as NPE source, i.e. $H_{P/E}F$ (16.6 g DM kg$^{-0.8}$ d^{-1}) and $L_{P/E}F$ (15.4 g DM kg$^{-0.8}$ d^{-1}) had similar dry matter intakes, irrespective of P/E ratio. Intakes of trout fed the diets with carbohydrate as NPE source were lower at low than at high P/E ratio. At high P/E ratio, growth (g kg$^{-0.8}$ d^{-1}) was not significantly different between groups fed diet $H_{P/E}F$ and $H_{P/E}C$, despite their different feed intakes. At low P/E intake, growth was lower in trout fed carbohydrate ($L_{P/E}C$) relative to fat ($L_{P/E}F$) as NPE source. The lowest growth was found in fish fed diet $L_{P/E}C$ (7.9 g kg$^{-0.8}$ d^{-1}), being 1.6 times lower than that of the $L_{P/E}F$ group. Remarkably, growth of trout fed the $L_{P/E}F$ diet (with only DP/DE of 14 mg kJ^{-1}) did not differ significantly from that of fish fed diet $H_{P/E}C$ (with DP/DE of 26 mg kJ^{-1}). The FGR was also affected by a significant interaction between both factors (NPE source and P/E ratio), being higher in trout fed carbohydrate compared to fat at the low P/E ratio, but not at the high P/E ratio at which FGR was not affected by the NPE source.

Body composition

The initial and final body compositions of the trout are shown in Table 2.3. Except for dry matter, other parameters (protein, fat, ash, and energy) of final body composition were affected ($P<0.01$) by P/E ratio of diet. Similarly, NPE source of diet affected ($P<0.01$) all parameters except protein and ash. There was no significant interaction between both effects on final body composition, except for ash content. Whole body protein content of fish fed $L_{P/E}$ diets was about 11% lower than in those fed with $H_{P/E}$ diets ($P<0.001$). Compared to initial body protein content, fish fed with $L_{P/E}$ diets had 7.5% lower protein content. Final body fat content increased in all groups compared to initial body fat content. Whole body fat content was 24% significantly higher in trout fed with $L_{P/E}$ diets (low P/E ratio) and 44% higher in groups fed diets containing fat as NPE source ($P<0.01$).

Table 2.2 Voluntary feed intake and growth performance of rainbow trout fed the experimental diets for 6 weeks[1]

	Diets[2]					*P*- value		
	$H_{P/E}F$	$H_{P/E}C$	$L_{P/E}F$	$L_{P/E}C$	Pooled SEM	P/E ratio	NPE source	P/E x NPE
Growth period (d)	42	42	42	42	-	-	-	-
No. of tanks	3	3	3	3	-	-	-	-
No. of fish / tank	35	35	35	35	-	-	-	-
Survival (%)	98.1	98.1	96.2	89.5	1.90	0.025	0.118	0.118
Initial body weight (g)	32.4	32.5	32.3	32.4	0.35	0.792	0.792	1.000
Final body weight (g)	103.7^{a}	96.6^{ab}	84.4^{b}	59.5^{c}	3.26	<0.001	0.001	0.025
Feed intake (FI)								
FI as fed (g fish^{-1} d^{-1})	1.82^{a}	1.59^{b}	1.53^{b}	1.00^{c}	0.044	<0.001	<0.001	0.010
FI_{PCT} (% d^{-1})	2.9^{a}	2.6^{b}	2.8^{ab}	2.2^{c}	0.05	<0.001	<0.001	0.018
FI_{ABS} (g DM fish^{-1} d^{-1})	1.70^{a}	1.46^{b}	1.45^{b}	0.95^{c}	0.042	<0.001	<0.001	0.013
FI_{MBW} (g DM kg $^{-0.8}$ d^{-1})	16.6^{a}	14.7^{b}	15.4^{ab}	11.6^{c}	0.29	<0.001	<0.001	0.012
Growth								
Absolute (g d^{-1})	1.70^{a}	1.53^{ab}	1.24^{b}	0.65^{c}	0.078	<0.001	0.001	0.026
GR_{MBW} (g kg$^{-0.8}$ d^{-1})	16.5^{a}	15.3^{ab}	13.2^{b}	7.9^{c}	0.60	<0.001	<0.001	0.009
DGC	3.6^{a}	3.3^{ab}	2.9^{b}	1.8^{c}	0.13	<0.001	<0.001	0.008
FGR (DM intake/ wt.gain)	1.01^{a}	0.96^{a}	1.17^{b}	1.48^{c}	0.037	<0.001	0.008	0.001

DM, dry matter; FI_{PCT}, Feed intake per percentage body weight; FI_{ABS}, Absolute feed intake; FI_{MBW}, Feed intake per metabolic body weight; DGC, Daily growth coefficient; FGR, Feed gain ratio. [1] Values represent least squares (LS) means (n=3), row means with different superscript letters were significantly different and assigned only if interaction effect was significant (P<0.05). [2] $H_{P/E}F$ - High P/E ratio diet with fat as main non-protein energy source; $H_{P/E}C$ - High P/E ratio diet with carbohydrate as main non-protein energy source; $L_{P/E}F$ - Low P/E ratio diet with fat as main non-protein energy source; $L_{P/E}C$ - Low P/E ratio diet with carbohydrate as main non-protein energy source.

Table 2.3 Effect of dietary treatments on final body composition (on fresh weight basis) of rainbow trout fed the experimental diets for 6 weeks1

Unit in g kg-1	Initial body composition	Final body composition							
		Diets2				Pooled SEM	*P*- value		
		$H_{P/E}F$	$H_{P/E}C$	$L_{P/E}F$	$L_{P/E}C$		P/E ratio	NPE source	P/E x NPE
Dry matter (DM)	220	278	251	285	257	4.1	0.125	<0.001	0.871
Protein	153	156	162	143	140	3.4	<0.001	0.632	0.263
Fat	34	94	61	111	81	3.9	0.001	<0.001	0.684
Ash	26	21^{a}	21^{a}	19^{b}	20^{ab}	0.5	0.008	0.223	0.042
Energy (kJ g^{-1})	5.0	7.5	6.3	8.1	6.8	0.16	0.015	<0.001	0.913

1 Values represent least squares (LS) means (n=3), row means with different superscript letters were significantly different and assigned only if interaction effect was significant (P<0·05).
2 $H_{P/E}F$ - High P/E ratio diet with fat as main non-protein energy source; $H_{P/E}C$ - High P/E ratio diet with carbohydrate as main non-protein energy source; $L_{P/E}F$ - Low P/E ratio diet with fat as main non-protein energy source; $L_{P/E}C$ - Low P/E ratio diet with carbohydrate as main non-protein energy source.

Nitrogen, fat and energy balance

Table 2.4 presents the apparent nutrient and energy digestibility coefficients (ADC) used to calculate parameters of nitrogen, fat and energy balance presented in Table 2.5. Digestible nutrient intakes in terms of digestible nitrogen intake (DNI), digestible fat intake (DFI) and DEI were different between the dietary groups. DNI was affected (P<0.001) by P/E ratio and the source of NPE without interaction between both factors (P>0.3). The DNI was 54% higher with $H_{P/E}$ than $L_{P/E}$ diets and 18% lower in diets with carbohydrate compared to fat as NPE source. Despite the differences in DNI between both $H_{P/E}$ diets, RN was similar in trout fed the $H_{P/E}F$ and $H_{P/E}C$ diets. However, with $L_{P/E}$ diets, retained nitrogen (RN) differed significantly in line with their DNI. DFI was affected (P<0.05) by the interaction between P/E ratio and NPE source of diet, being the lowest and the highest respectively in $H_{P/E}C$ and $L_{P/E}F$ diets. In contrast to DFI, retained fat (RF) was only influenced by the dietary NPE source, with 46% higher RF in trout fed fat relative to carbohydrate diets.

The amount of voluntary DEI, as supplied from the different dietary macronutrients, is shown in fig 2.1. The DEI paralleled dry matter intake, showing a significant interaction between dietary P/E ratio and NPE source (Table 2.5). The lowest DEI were observed in

$L_{P/E}C$ fed groups, whereas DEI of trout fed diet $L_{P/E}F$ were not significantly different from those in $H_{P/E}$ groups. There was no significant difference in metabolisable energy intake (MEI) between $H_{P/E}F$ and $L_{P/E}F$ groups, both being higher than in groups fed carbohydrate as NPE source. However, retained energy (RE) was different and significantly affected by both P/E ratio and NPE source of diet, being lower in trout fed $L_{P/E}$- relative to $H_{P/E}$-diets and in trout fed carbohydrate relative to fat as NPE source. Although DEI and RE was different, the total heat production (H) was unaffected ($P>0.05$) by the P/E ratio, the NPE source and their interaction (Fig. 2.2).

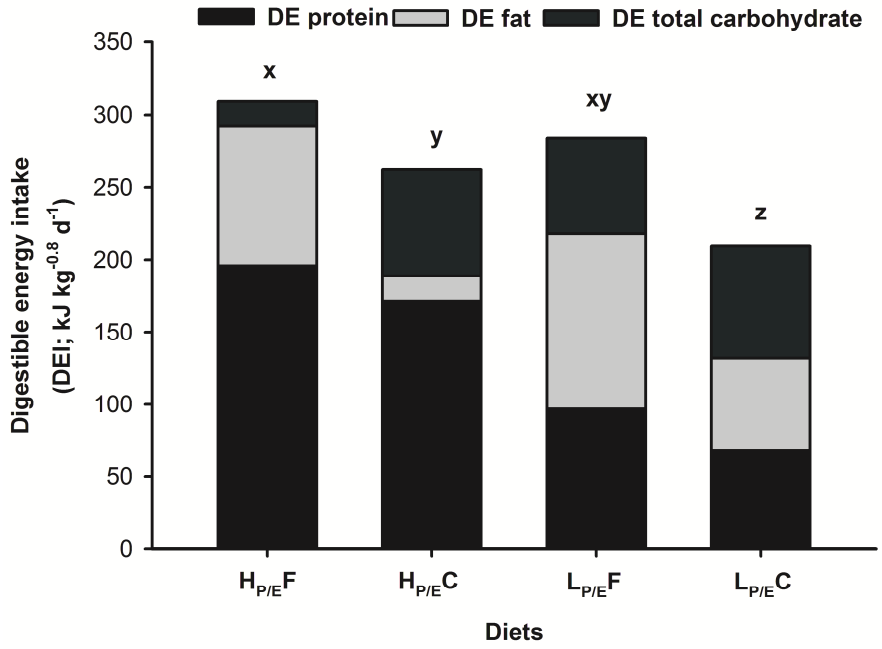

Figure 2.1 Effect of diet composition on digestible energy intake (DEI) in rainbow trout. Fish were fed to satiation with iso-energetic diets of different macronutrient composition having contrast in P/E ratio (high, $H_{P/E}$ vs. low, $L_{P/E}$) and NPE source (fat, F vs. carbohydrates, C) for 6 weeks. The bars show the amount of DEI derived from the digestible protein, fat and total carbohydrate (nitrogen-free extract) for each dietary group. Different superscripts indicate significant differences in total DEI.

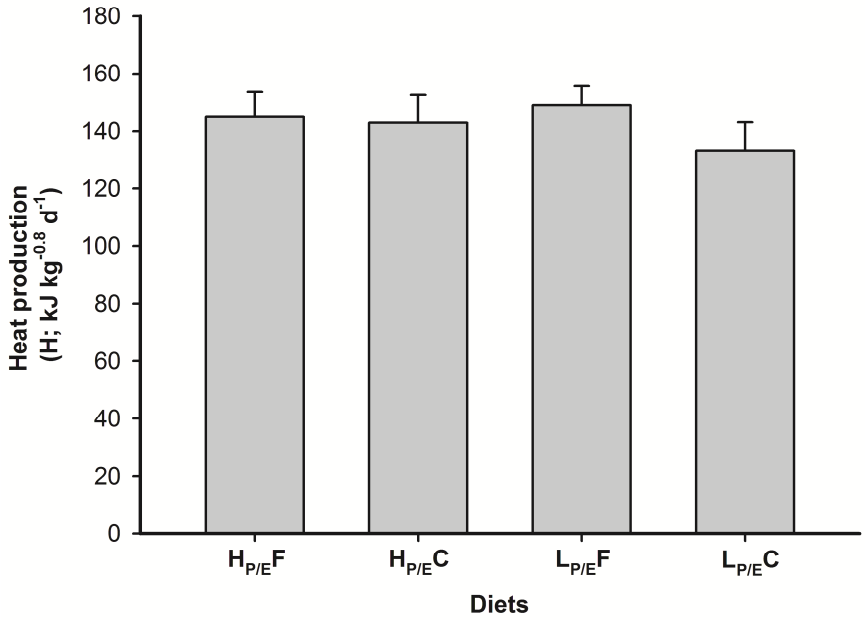

Figure 2.2 Effect of diet composition on heat production in rainbow trout. Heat production (H; least squares mean ± SD) in rainbow trout fed to satiation the iso-energetic diets of different macronutrient composition having contrast in P/E ratio (high, HP/E vs. low, LP/E) and NPE source (fat, F vs. carbohydrates, C). H was unaffected by P/E ratio, NPE source and their interaction effect (P>0.05).

Post-prandial glucose and triglyceride circulating levels

Figure 2.3 depicts the 7 h post-prandial plasma glucose and triglyceride (TAG) levels in rainbow trout fed the four experimental diets. The plasma glucose (g L^{-1}) was affected (P<0.001) by the dietary P/E ratio, NPE source and their interaction. Plasma glucose being higher in trout fed the $L_{P/E}$ compared to $H_{P/E}$ diets. The effect of NPE source on plasma glucose was significantly greater with the $L_{P/E}$ diets than $H_{P/E}$ diets. $H_{P/E}F$ and $H_{P/E}C$ diet showed similar plasma glucose levels and fish fed $L_{P/E}C$ diet attained the highest glucose levels. In contrast, TAG levels were affected by the NPE source (P=0.037), being higher in trout fed fat vs. carbohydrate, but not (P>0.05) by the P/E ratio. There was no interaction between P/E ratio and NPE source on plasma TAG.

Table 2.4 Apparent nutrient digestibility coefficient (%; ADC) in rainbow trout fed with four experimental diets[1]

	Diets[2]					*P*- value		
Unit in %	$H_{P/E}F$	$H_{P/E}C$	$L_{P/E}F$	$L_{P/E}C$	Pooled SEM	P/E ratio	NPE source	P/E x NPE
Dry matter (DM)	72.7[a]	83.8[b]	73.7[a]	80.1[b]	0.88	0.156	<0.001	0.027
Protein	95.5	95.9	96.1	95.2	0.24	0.750	0.338	0.028
Fat	96.7[a]	89.0[b]	95.8[a]	96.7[a]	0.38	<0.001	<0.001	<0.001
Total carbohydrates[3]	23.0[a]	76.4[b]	56.3[c]	74.0[b]	2.03	<0.001	<0.001	<0.001
Ash	34.0	36.5	32.7	33.8	1.99	0.346	0.396	0.709
Energy[3]	82.0[a]	88.7[b]	82.1[a]	85.7[c]	0.64	0.053	<0.001	0.040

[1] Values represent least squares (LS) means (n=3), row means with different superscript letters were significantly different and assigned only if interaction effect was significant (P<0·05).
[2] $H_{P/E}F$ - High P/E ratio diet with fat as main non-protein energy source; $H_{P/E}C$ - High P/E ratio diet with carbohydrate as main non-protein energy source; $L_{P/E}F$ - Low P/E ratio diet with fat as main non-protein energy source; $L_{P/E}C$ - Low P/E ratio diet with carbohydrate as main non-protein energy source.
[3] ADC of total carbohydrates and energy includes the effect of the added cellulose (indigestible) in diets $H_{P/E}F$ and $L_{P/E}F$.

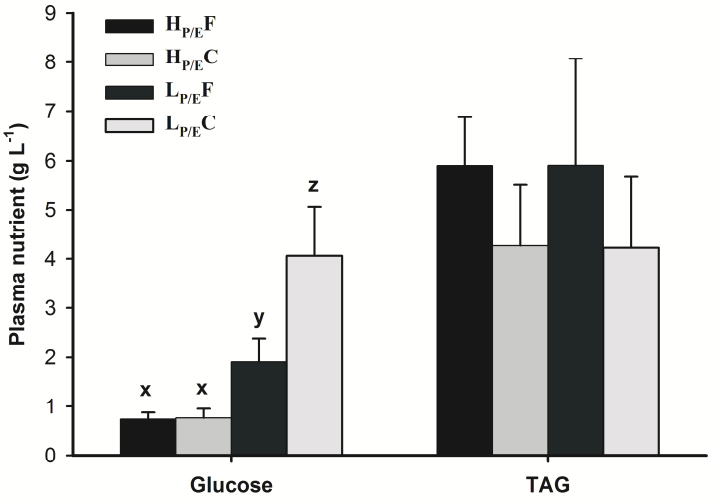

Figure 2.3 Effect of diet composition on post-prandial plasma glucose and triglycerides in rainbow trout. Seven hours post-prandial plasma levels (least squares mean ± SD) of glucose and triglycerides (TAG) of rainbow trout fed diets having contrast in P/E ratio and NPE source. Glucose was affected by dietary P/E ratio, NPE source and their interaction (P<0.001). In contrast, TAG levels were affected only by the NPE source (P=0.003) and not by P/E ratio and their interaction effect (P>0.05).

Table 2.5 Nitrogen, fat and energy balance in rainbow trout fed the experimental diets for 6 weeks[1]

	Diets[2]					*P*- value		
	$H_{P/E}F$	$H_{P/E}C$	$L_{P/E}F$	$L_{P/E}C$	Pooled SEM	P/E ratio	NPE source	P/E x NPE
Nitrogen balance (mg N $kg^{-0.8}$ d^{-1})								
GNI	1384	1204	680	484	15.1	<0.001	<0.001	0.593
FN	62.1	49.2	26.7	23.3	2.6	<0.001	0.011	0.103
DNI	1240	1068	620	437	13.7	<0.001	<0.001	0.393
BUN	905[a]	748[b]	367[c]	304[c]	16.3	<0.001	<0.001	0.021
RN	417[a]	408[a]	287[b]	157[c]	12.9	<0.001	<0.001	0.002
Fat balance (mg $kg^{-0.8}$ d^{-1})								
GFI	2532[a]	501[b]	3198[c]	1661[d]	55.8	<0.001	<0.001	0.002
FF	84[a]	55[a]	136[b]	55[a]	7.2	0.005	<0.001	0.004
DFI	2448[a]	446[b]	3062[c]	1606[d]	56.8	<0.001	<0.001	0.001
RF	2011	1133	2093	1072	101	0.919	<0.001	0.496
RF/DF	0.83	2.54	0.68	0.67	-	-	-	-
Energy balance (kJ $kg^{-0.8}$ d^{-1})								
GEI	380	303	352	246	6.6	<0.001	<0.001	0.055
FE	68	34	63	35	1.9	0.278	<0.001	0.133
DEI	311[a]	269[b]	289[ab]	211[c]	6.7	<0.001	<0.001	0.027
BUE	22[a]	19[b]	9[c]	7[c]	0.4	<0.001	<0.001	0.021
MEI	288[a]	250[b]	280[a]	203[c]	6.5	0.003	<0.001	0.018
RE	144	107	131	70	6.1	0.003	<0.001	0.083

SEM, Standard error mean; GNI, Gross nitrogen intake; FN, Faecal nitrogen loss; DNI, Digestible nitrogen intake; BUN, Branchial and urinary nitrogen loss; RN, Retained nitrogen; GFI, Gross fat intake; FF, Faecal fat loss; DFI, Digestible fat intake; RF, retained fat; RF/DF, fat efficiency; GEI, Gross energy intake; FE, faecal energy loss; DEI, digestible energy intake; BUE, branchial and urinary energy loss; MEI, metabolisable energy intake; RE, retained energy.

[1] Values represent least squares (LS) means (n=3), row means with different superscript letters were significantly different and assigned only if interaction effect was significant (P<0·05).

[2] $H_{P/E}F$ - High P/E ratio diet with fat as main non-protein energy source; $H_{P/E}C$ - High P/E ratio diet with carbohydrate as main non-protein energy source; $L_{P/E}F$ - Low P/E ratio diet with fat as main non-protein energy source; $L_{P/E}C$ - Low P/E ratio diet with carbohydrate as main non-protein energy source.

Discussion

In the present study, voluntary FI paralleled DEI due to the similar DE contents of the formulated diets. FI in rainbow trout as in several other fish species has been reported to be regulated by the total DE content of the diet (Boujard and Médale, 1994; Morales et al., 1994). The present data show that under satiation feeding conditions, rainbow trout consumed different amounts of DE, depending on the diet composition. These findings agree with previous reports in rainbow trout (Cláudia Figueiredo-Silva et al., 2011; Encarnacao et al., 2004; Geurden et al., 2006), highlighting the controversy on whether FI is adjusted to maintain a constant DEI in fish. In addition, these findings further suggest the involvement of dietary or physiological factors other than dietary DE content alone in the regulation of FI.

Independent of dietary DE level, FI has been shown to be directed by the animal's genetic growth potential in such a way that the animal will attempt to eat as much of a feed as needed to fulfill the nutrient requirements for achieving its (maximal) growth potential (Emmans and Kyriazakis, 1995). In this respect, intakes of specific nutrients such as protein have been shown to be separately regulated from energy intake, as shown in pig (Henry, 1985), poultry (Shariatmadari and Forbes, 1993) and rat (Sørensen et al., 2008). As a result, an excess of energy is ingested with low protein diets while an energy deficit may occur with high protein diets. Also fish have been reported to show hyperphagia and over-consume DE to compensate for reduced dietary protein as seen in Atlantic salmon (Helland and Grisdale-Helland, 1998). In contrast, protein levels above optimum do not seem to down-regulate DEI in rainbow trout (Geurden et al., 2006) in line with findings in mammalian carnivores used to deal with high protein intakes (Hewson-Hughes et al., 2011; Mayntz et al., 2009). The present low P/E ($L_{P/E}$) and high P/E ($H_{P/E}$) diets provided respectively 14 and 26 mg of digestible protein per kJ DE being, respectively, above and below the optimal DP/DE ratio of 17- 19 mg kJ^{-1} (Dias et al., 1999) or 21 mg kJ^{-1} (NRC, 2011) for rainbow trout. However, the similar or even decreased DEI in $L_{P/E}$- compared with the $H_{P/E}$-groups show that the trout fed the low P/E diets did not 'over-eat' energy to compensate for the reduced protein. In both cases, this resulted in lower digestible nitrogen intake (DNI) as well as lower weight and protein (RN) gain than with the high P/E diets.

According to the lipostatic theory of FI regulation [31], the failure of the trout fed $L_{P/E}$ diets to increase DEI and hence compensate DNI may be caused by the higher relative level of body fatness of fish fed the $L_{P/E}$ compared with $H_{P/E}$ diets. The negative effect of high body fat content on FI or DEI (Kennedy, 1953), mediated through the feedback mechanism of leptin is well documented in mammals (Woods et al., 1998). Adipostatic feedback control of FI has also been reported to occur in salmonid fish (Dias et al., 1999;

Johansen et al., 2003; Silverstein et al., 1999). However, diet-induced increases in the relative level of adiposity, which moreover varies depending on body size (Bureau et al., 2003), did not necessarily reduce appetite or energy intakes in rainbow trout (Gélineau et al., 2001; Geurden et al., 2006). Similarly, the observation of similar DEI in trout fed $H_{P/E}C$ and $L_{P/E}F$ diets, despite the difference in adiposity (61 and 111 g kg^{-1}, respectively), suggests a low feedback control of relative body fatness on DEI.

Interestingly, rainbow trout reduced intakes following the iso-energetic substitution of fat by carbohydrate, irrespective of the dietary P/E ratio. This might be due to physical constraints as the volume of feed a fish can eat depends on the stomach capacity and gut evacuation rate (Güner and Davies, 2003; Riche et al., 2004). The expansion of starch during feed extrusion reduces the bulk density of the pellets. As such, the lower density of diet $L_{P/E}C$ possibly limited the amount of FI during the first meals, but unlikely affected the long term (weeks) FI, as fish are known to increase stomach volume when fed high-bulk diets (Ruohonen and Grove, 1996). In addition, gut evacuation rate and hence the return of appetite are expected to be enhanced by the relatively high (16°C) water temperature (He and Wurtsbaugh, 1993). Another factor susceptible to reduce FI following the substitution of fat by carbohydrate is increased plasma glucose. The glucostatic theory implies that FI is controlled to maintain glucose homeostasis in blood through a feedback mechanism signaled by both hypothalamus and liver (Mayer, 1991). Thus, an increase or decrease in blood glucose level leads respectively to a down- or up-regulation of FI. Evidence in fish on glucostatic control of FI is highly ambiguous. For instance, high plasma glucose was found to either increase (Hemre et al., 1989) or decrease (Polakof et al., 2008; Volkoff and Peter, 2006) FI in fish. Our data on the relation between FI and plasma glucose also appear inconsistent as the substitution of fat by carbohydrate either increased ($L_{P/E}$-groups) or unmodified ($H_{P/E}$-groups) plasma glucose, whereas this led to reduced intakes in both groups. Moreover, voluntary FI between $H_{P/E}C$ and $L_{P/E}F$ groups were not significantly different, despite the differences in circulating plasma glucose.

Rather than a direct glucostatic or lipostatic feedback control of FI, some studies in mammals suggest that it is the overall metabolic utilization of the ingested nutrients which signals satiety and hence determines FI (Blundell and Tremblay, 1995; Nicolaidis, 2011; Woods and Ramsay, 2011). In other words, the degree of nutrient oxidation rather than the ingested amount of dietary energy *per se* would generate satiety (Stubbs and Tolkamp, 2006). In fish, the question whether and how dietary energy utilization (energy retention vs. expenditure/heat production) regulates the amount of DEI has received little attention. Interestingly, the energy balance of the present trout revealed no significant difference in heat production (133-149 kJ $kg^{-0.8}$ d^{-1}) between fish of the

different treatments, whereas the amount of energy retained (70 - 144 kJ $kg^{-0.8}$ d^{-1}) and DEI (211 - 311 kJ $kg^{-0.8}$ d^{-1}) were strongly affected by the dietary DE source. This confirms previous findings in Nile tilapia fed to satiation with diets varying in macronutrient supply and supports the hypothesis that heat production may set a limit to voluntary FI (Tran-Duy et al., 2008). This was also suggested in the very early works of Brobeck (Brobeck, 1957) in mammalian models, reporting that the important factor in FI regulation is not the food's energy value, but rather the amount of extra heat released during its assimilation. Further studies with homoeothermic vertebrates confirmed the relation between heat production and FI, yet mostly in relation with ambient temperature (Ferguson and Gous, 1997). Homoeothermic animals, when exposed to ambient temperature above the upper critical temperature, lower FI in order to avoid the excess heat production caused by the thermic effect of feeding [50]. As such, the extent to which the animal is able to dissipate heat to the environment will determine how much it will eat, as shown in pig (Ferguson and Gous, 2002) and broiler (Koh and Macleod, 1999). Since fish do not maintain constant body temperature, the amount of heat to be dissipated to the environment is not expected to control FI in fish in the same way as in homeotherms. Therefore, other more basic metabolic processes involved in heat production, shared by both homeo- and ectotherms, such as aspects related with oxygen use, may be implicated in the dietary control of FI in fish.

Theoretically, the amount of heat production by aerobic metabolism in animals parallels the amount of oxygen consumed (McLean, 1972). In mammals, several studies pointed at the difference between macronutrients in their contribution to oxidative metabolism and how these may relate to satiety (Nicolaidis, 2011; Stubbs and Tolkamp, 2006). In this respect, satiety and hence dietary FI control have been associated with the degree of hepatic oxidative metabolism (Friedman, 1998; Langhans, 2008) or the efficiency of oxygen use (Ketelaars and Tolkamp, 1996). The comparison of the heat production values observed in the present study (133-149 kJ/$kg^{0.8}$/d) with values calculated (i.e., H= MEI-RE) from literature for rainbow trout fed to satiation (e.g., 107 (Azevedo et al., 1998), 77-91 (Glencross et al., 2007), 93-112 (Glencross et al., 2008), 160 (Glencross, 2009) and 103-112 (Kim and Kaushik, 1992) kJ/$kg^{0.8}$/d), shows our values to be in the upper range, even after adjusting for the effect of temperature (positive curvilinear relationship between both variables, fig. 2.4). The present finding that heat production was similar irrespective of dietary composition in trout kept under normoxic condition, suggests that the DEI control in fish is a function of heat production. This might reflect a physiological limit related to oxidative metabolism. Various biological constrains might cause such a limit in fish even under normoxic water condition. For instance, the capacity of oxygen uptake by the fish (e.g. gill surface (Tran-Duy et al., 2008)), the capacity of oxygen transport (e.g. cardiac performance, hemoglobin affinity for O_2)

and/or constraints in oxidative metabolism at cellular level (e.g. mitochondrial respiration, production of reactive oxygen species). Measurements of oxygen consumption data are needed to further elucidate the role and possible limits set by heat production/oxidative metabolism on DEI. Therefore, ongoing studies in our laboratories further explore the relation between macronutrient-induced changes in feed/nutrient intake and oxygen consumption as well as the link with hepatic oxidative metabolism and hypothalamic satiety markers.

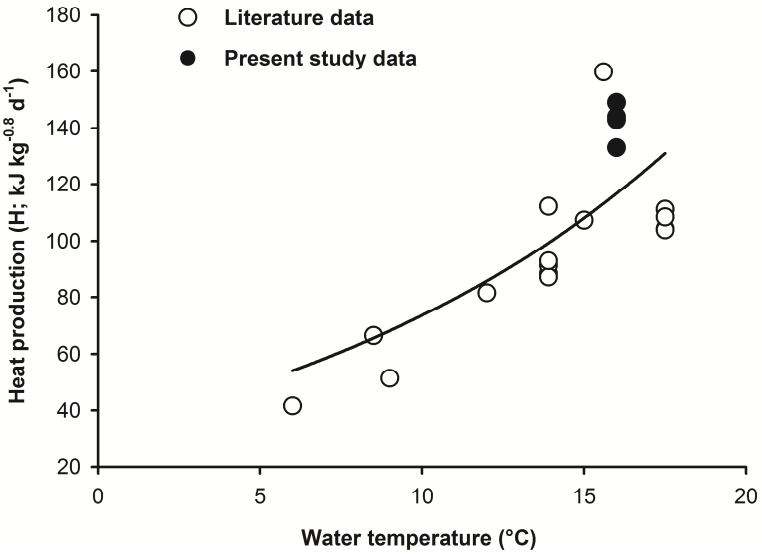

Figure 2.4 Relation between water temperature (T, °C) and heat production in rainbow trout fed to satiation The heat production values (H, kJ $kg^{-0.8}$ d^{-1}**)** are calculated for rainbow trout fed to satiation from literature data (e.g., Azevedo et al., 1998; Glencross et al., 2007; Glencross et al., 2008; Glencross, 2009; Kim and Kaushik, 1992) and from the present study. H was curvilinearly related to temperature, $H = 26.6 \times e^{0.0923 \times T}$, $R^2 = 0.73$.

In conclusion, the present study demonstrates that the macronutrient composition of the diet modifies voluntary DEI in rainbow trout. The observation that the rainbow trout had similar heat production, together with different DEI, is in line with the proposed hypothesis that DEI in fish might be controlled as a function of heat production, which might reflect a physiological limit related to oxidative metabolism.

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CHAPTER 3

Control of voluntary feed intake in fish: a role for dietary oxygen demand in Nile tilapia (*Oreochromis niloticus*) fed diets with different macronutrient profiles

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Abstract

It has been hypothesised that, at non-limiting water oxygen conditions, voluntary feed intake (FI) in fish is limited by the maximal physiological capacity of oxygen use (i.e., an *'oxystatic control of FI in fish'*). This implies, that fish will adjust FI when fed diets differing in oxygen demand, resulting in an identical oxygen consumption. Therefore, FI, digestible energy (DE) intake, energy balance and oxygen consumption were monitored at non-limiting water oxygen conditions in Nile tilapia fed diets with contrasting macronutrient composition. Diets were formulated in a 2 by 2 factorial design in order to create contrasts in oxygen demand: 2 ratios of digestible protein to digestible energy (DP/DE; 'high' *vs.* 'low'); and a contrast in type of non-protein energy source ('starch' *vs.* 'fat'). Triplicate groups of tilapia were fed twice daily each diet to satiation for 48 days. FI (g DM/kg$^{0.8}$/d) was significantly lower (9.5%) in tilapia fed the starch relative to fat diets. DP/DE ratio affected DE intakes ($P < 0.05$), being 11% lower with 'high' than with 'low' DP/DE ratio diets, which was in line with the 11.9% higher oxygen demand of these diets. Indeed, DE intakes of fish showed an inverse linear relation with the dietary oxygen demand (R^2, 0.81; $P < 0.001$). As hypothesized ('oxystatic' theory), oxygen consumption of fish was identical among three out of the four diets. All together, these results demonstrate the involvement of metabolic oxygen use and dietary oxygen demand in the control of FI in tilapia.

Introduction

Voluntary feed intake (FI) in fish, as in other animals, is controlled by a complex combination of nutritional, physiological and environmental factors (Fletcher, 1984). It has frequently been suggested that FI is controlled to maintain a relatively constant digestible energy (DE) intake i.e., to meet the DE requirements (Boujard, 1994; Cho, 1990; Kaushik, 1983; Lee and Putnam, 1973; Lekva et al., 2010). However few studies in fish have suggested the involvement of other nutritional factors in the control of FI (Geurden et al., 2006; Tran-Duy et al., 2008b). Studies that verify the importance of other well-known regulatory mechanisms of FI in mammals, such as glucostatic (Mayer, 1953) or lipostatic control (Kennedy, 1953) either lead to an ambiguous conclusion or show lesser impact in fish (Bellamy, 1968; Geurden et al., 2006) than in terrestrial animals. The effect of non-protein energy source (NPE; fat and starch) on FI in fish is unclear (Grisdale-Helland et al., 2008) and has not been systematically assessed at least at similar DP/DE ratio and DE content of diets.

Among the abiotic factors, dissolved oxygen (DO), pH, and ammonia are recognized to affect FI in fish (Kestemont, 2001). The effect of the availability of oxygen on FI has been relatively well documented. Several studies demonstrated that FI in fish decreases linearly with declining water DO content (Buentello, 2000; Glencross, 2009; Pichavant et al., 2000; Thetmeyer, 1999). The minimum DO level at which metabolic oxygen demand in fish limits FI is termed as incipient DO (iDO). Thus, below iDO, FI depends on DO concentration of water, whereas above iDO, FI is independent of water DO concentration. Recent studies in Nile tilapia (Tran-Duy et al., 2008b) and rainbow trout (our own unpublished data) under non-limiting DO showed differences in DE intake, when fish were fed to satiation with diets differing in NPE source (starch vs. fat). In addition, total heat production (considered theoretically as oxygen consumption) was found to be similar despite their difference in DE intake and retained energy. These data suggest that the difference in FI between the dietary groups might be caused by limitations in the maximum oxygen uptake or by the metabolic oxygen demand as induced by the nutrient processing. Since the amount of oxygen required to metabolize the dietary macronutrients depends on whether they are used for growth (protein, fat or glycogen) or production of ATP (McCue, 2006 ; Secor, 2009), feeding diets with different macronutrient composition results in different levels of metabolic or dietary oxygen demands (DOD), being defined here as the amount of oxygen (in mg) consumed per unit of digestible energy (kJ DE) intake. In terrestrial vertebrates, evidence on the role of oxygen as a regulatory factor in the control of FI has been studied at various levels such as oxygen efficiency of the whole animal (Ketelaars and Tolkamp, 1992; Ketelaars and Tolkamp, 1996) or oxidative metabolism in liver (Friedman, 1998) or in hypothalamus

(Coppola et al., 2007), whereas the link between oxygen use and voluntary FI has not been considered in fish nutrition.

It is postulated that, at non-limiting water DO, FI in fish may be limited by the maximal physiological capacity of oxygen use (for growth and maintenance). In order to verify this hypothesis, voluntary FI and oxygen consumption were monitored in Nile tilapia fed diets with contrasting macronutrient composition at non-limiting water DO conditions. The objective of this study is to verify the existence of an *'oxystatic control of FI in fish'*, i.e., intakes of diets with different oxygen demand are controlled by a physiological limit in metabolic oxygen use. If the hypothesis holds true, then fish will adjust intakes according to differences in dietary oxygen demand.

Materials and Methods

All procedures involving animals were carried out in accordance with the Dutch law on experimental animals and were approved by the Wageningen University Animal Experimental Committee.

Diets

Four iso-energetic diets were formulated according to a 2 x 2 factorial design to create contrasts in dietary oxygen demand (DOD) between diets (Table 3.1). The first factor was the digestible protein to digestible energy (DP/DE) ratio which was changed by modifying the dietary protein levels, 'low' (LP diets) *vs.* 'high' (HP diets). It is assumed that fish fed diets with low DP/DE ratio will have minimal use of protein as energy source, whereas at the high DP/DE ratio a substantial amount of protein will be used as energy source (LeGrow, 1986). Thus, the contrast in DP/DE ratio between diets will cause a difference in protein to fat deposition ratio in fish (Lee and Putnam, 1973) and thereby generate difference in DOD. The different DP/DE ratios (HP diets, 25 mg/kJ; LP diets, 14 mg/kJ) were created by exchanging an equal proportion (30%) of protein ingredient mixture (fish meal, wheat gluten, soya protein concentrate, pea protein concentrate and DL-methionine) by an equivalent amount of energy ingredient mixture (rapeseed oil, fish oil and gelatinized maize starch).

The second factor was the type of non-protein energy source (NPE): 'starch' *vs.* 'fat'. The oxygen demand of dietary starch and fat depends on whether it is used for ATP production through oxidation or deposited as an energy store (fat) in the body. The amount of oxygen required to deposit fat from dietary fat is lower than that required for lipogenesis from starch (Blaxter, 1989; Reeds, 1982). Therefore, diets were formulated to contain either starch (diets HPS and LPS) or fat (diets HPF and LPF) as major NPE source at both dietary DP/DE ratios. For the fat-diets, 10% of rapeseed oil was added as NPE source, whereas for the starch-diets it was exchanged by 25% of gelatinised maize

starch, assuming a similar digestible energy content of 10% rapeseed oil to that of 25% gelatinised maize starch. Furthermore, in order to have identical nutrient and energy density between these diets, 15% of cellulose was included in the fat-diets. The final ingredient compositions of diets are shown in Table 1. Diets were produced by Research Diet Services (Wijk bij Duurstede, The Netherlands). The ingredient mixture of each diet excluding major part of the oils, were mixed and hammer-milled (Condux LHM20/16, Hanau, Germany) through a 1 mm screen. Diets were processed by extrusion using a Clextral BC45 laboratory scale twin-screw extruder (Clextral, Firminy, France) with a 3 mm die, resulting in a pellet size of about 3 mm. In HPS and LPS diets, all oils were added to the mixture prior to extrusion. In HPF diet 6% of the oils and in LPF diet 9.1% of the oils were added to the mixture prior to extrusion. Following extrusion, pellets were dried in a tray-drier at 70°C for 3 h and cooled to ambient temperature. Finally, HPF and LPF diets were coated with the remaining part of the oils (5 and 10%, respectively) and stored at 4°C.

Fish stock and pre-experimental rearing conditions

A stock of 300 juvenile (mean body weight. 5 g) male Nile tilapia (NMT Manzala Silver strain) was obtained from a commercial fish breeder (Til Aqua International, Velden, The Netherlands) and reared at the experimental facilities ('De Haar Vissen') of the Wageningen University, The Netherlands. The fish were housed in six tanks (120 l) at a stocking density of 50 fish per tank. These tanks were connected to a common water recirculation unit comprised of trickling filter, settling tank and pump. Initially, fish were fed with a commercial starter feed (Skretting, F-10, MP Pro Aqua Brut; 1.0 mm, 57% crude protein, 15% crude fat) for about 6 weeks and thereafter with larger feed pellets (Skretting, F-1P Classic; 2.5 mm, 47% crude protein, 14% crude fat) until the fish reached a body weight of 40 g. During this pre-experimental period (10 weeks), fish were hand fed twice daily with a ration of about 10 g/kg$^{0.8}$/d. The fish were kept at optimal rearing conditions (water flow rate in tank, 6 l/min; temperature, 28°C; DO, >5mg/l; photoperiod, 12 light:12 dark hours).

Housing facility

The 48-day feeding trial was carried out in the Aquatic Metabolic unit of Aquaculture and Fisheries group, Wageningen University, The Netherlands. This metabolic unit consists of 12 metabolic tanks (90x60x45cm) in a series connected to a common water recirculation system consisting of trickling filter, oxygenation unit, a sump, a drum filter (Hydrotech 500®) and a cooling/heating system for maintaining uniform water quality throughout the study. Water was supplied to all tanks from a common inlet thus ensuring identical water quality and drained through individual tank outlets into the system. The oxygenation unit maintained the concentration of DO in water by injecting

Table 3.1 Formulation, ingredient composition and analyzed nutrient content of experimental diets

	Diets*			
	HPF	HPS	LPF	LPS
Ingredients (%)				
Rapeseed oil	11.0	1.0	14.1	4.1
Gelatinized maize starch†	5.0	30.0	24.29	49.29
Cellulose	15.0	-	15.0	-
Fish meal‡	33.0	33.0	18.0	18.0
Wheat gluten	10.89	10.89	5.94	5.94
Soya protein concentrate	10.89	10.89	5.94	5.94
Pea protein concentrate	10.89	10.89	5.94	5.94
Fish oil§	-	-	5.0	5.0
Calcium carbonate	-	-	0.36	0.36
Monocalcium phosphate	-	-	1.8	1.8
Sodium carbonate	-	-	0.45	0.45
DL-Methionine	0.33	0.33	0.18	0.18
Diamol‖	2.0	2.0	2.0	2.0
Vitamin-mineral premix¶	1.0	1.0	1.0	1.0
Analyzed nutrient content (g/kg DM)				
Dry matter (DM; g/kg)	963	931	946	925
Crude protein (N x 6.25)	534	541	295	299
Crude fat	170	70	232	132
Starch	38	294	234	476

Table 3.1 Formulation, ingredient composition and analyzed nutrient content of experimental diets (continued)

	Diets*			
	HPF	HPS	LPF	LPS
Total carbohydrates**	221	312	399	495
Ash	74	77	73	73
Gross energy (kJ/g) ††	23.1 (20.48)	20.8	23.1 (20.51)	20.8
Digestible nutrient content (g/kg DM)				
Protein (N x 6.25)	502	514	279	281
Fat	159	68	209	126
Total carbohydrates	27	270	219	460
Digestible energy (kJ/g)	18.6	19.5	18.6	19.5
DP/DE (mg/kJ)	27.0	26.4	15.0	14.4

DP/DE, digestible protein to digestible energy ratio

* HPF – High DP/DE ratio diet with fat as NPE source; HPS - High DP/DE ratio diet with starch as NPE source; LPF - Low DP/DE ratio diet with fat as NPE source; LPS - Low DP/DE ratio diet with starch as NPE source.

† Gelatinized maize starch (Merigel®100; Amylum Group, Belgium).

‡ Fish meal (999 LT fish meal - crude protein 72%, Triple Nine Fish protein, Esbjerg, Denmark).

§Fish oil (999 Fish Oil, Triple Nine Fish protein, Esbjerg, Denmark).

‖Diamol (Acid insoluble ash, as inert marker for digestibility measurement)- Diamol GM, Franz Bertram, Hamburg, Germany.

¶ Mineral premix composition (to supply, mg kg^{-1} feed): 50, iron (as $FeSO_4.7H_2O$); 30, zinc (as $ZnSO_4.7H_2O$); 0.1, cobalt (as $CoSO_4.7H_2O$); 10, copper (as $CuSO_4.5H_2O$); 0.5, selenium (as Na_2SeO_3); 20, manganese (as $MnSO_4.4H_2O$); 500, magnesium (as $MgSO_4.7H_2O$); 1, chromium (as $CrCl_3.6H_2O$); 2, iodine (as $CaIO_3.6H_2O$). Vitamin premix composition (to supply, mg or IU kg^{-1} feed): 10, thiamin; 10, riboflavin; 20, niacin; 40, pantothenic acid; 10, pyridoxine; 0.2, biotin; 2, folic acid; 0.015, cyanocobalamin; 1500, choline (as choline chloride); 100, ascorbyl phosphate; 3000 IU, retinyl palmitate; 2400 IU, cholecalciferol (Rovimix® D3-500, DSM Inc.); 100 IU, α-tocopheryl acetate; 10, menadione (as menadione sodium bisulfite, 51%); 400, Inositol; 100, anti-oxidant BHT (E 321); 1000, calcium propionate.

** Calculated as, total carbohydrates (starch, free sugars and non-starch polysaccharides) = 1000 - (crude protein + crude fat + ash).

†† Gross energy value measured including energy from added cellulose, values within parenthesis represents energy value calculated excluding energy from added cellulose (15%).

pure oxygen into the common inlet, which was regulated by a mass flow controller (Brooks® Model 5850S, Brooks instruments, The Netherlands) and microprocessor (Brooks® Read out and control electronics model 0154, Brooks instruments, The Netherlands). Each metabolic tank was equipped with a water flow meter (MAGFLOW® MAG 5000, Danfoss A/S, Nordborg, Denmark) to regulate and monitor water flow. The volume of water within tanks was kept identical (200 l) by adjusting the standpipe. The water surface of each tank was covered with a water resistant floating panel to prevent gas exchange between water and air. Within the floating panel, a circular feeding hatch (18.5cm diameter) with removable floating lid was used to feed the fish. The inlet and outlet of each metabolic tank were linked to two separate sampling pipe lines. One sampling pipe led to an auto-analyzer (SANplusSYSTEM, Skalar, The Netherlands) to continuously measure nitrite, nitrate, total-ammonia nitrogen (TAN), urea and CO_2. The other sampling pipe led to a common measuring hub to continuously measure dissolved oxygen (WTW-Trioximatic® 700 IQ, WTW GmbH, Weilheim, Germany), pH (WTW-SensoLyt DW® (SEA) 700 IQ, WTW GmbH) and conductivity (WTW TetraCon325® 700 IQ, WTW GmbH) of water. The oxygen measurements from each metabolic tank were regulated by an electromagnetic valve (ASCO model 24/50 6 WFT, ASCO/Joucomatic, Scherpenzeel, The Netherlands) which controlled the water flow from inlet and outlet of each tank to the common measuring hub. These electromagnetic valves were controlled by algorithmic program via. a user interface (HTBasic, Version 9.5, TransEra Corp.) and the measured values of dissolved oxygen, water flow, pH and conductivity were automatically recorded in personal computer.

In addition, the outlet of each tank was connected to a swirl separator (44 cm height, 24.5 cm diameter; AquaOptima AS, Trondheim, Norway) to collect faeces for determination of nutrient digestibility. The faeces were collected in a detachable 250 ml bottle at the bottom of the swirl separator. To minimize the bacterial decomposition of faeces, the bottle was kept under ice. During feeding, another set of bottles were used in the swirl separator to collect the uneaten feed pellets flushed out from the tanks.

Experimental procedure

At the start of the experiment, 240 fish (mean body weight 40 g) from the stocking tanks (unfed for about 36 hours) were taken out, anaesthetized (0.2 g/l tricaine methane sulfonate (MS-222, Finquel®, Argent chemical lab., Washington) with 0.4 g/l sodium bicarbonate as buffer), weighed individually and randomly distributed among the 12 metabolic tanks (20 fish/tank). The respective diets were assigned randomly to triplicate tanks. Twenty fish were sacrificed with an excess dose of anesthesia (0.8 g/l tricaine methane sulfonate with 1.6 g/l sodium bicarbonate as buffer) for initial body composition, kept in plastic bags, sealed and stored at -20°C until further analysis.

During the experimental period (48 days), fish were hand fed with their respective diets twice daily to apparent satiation for an hour (09.00 to 10.00 and 16.00 to17.00 hrs). At the end of each feeding session the uneaten pellets were collected and counted to determine feed intake accurately. Feed fed and uneaten feed were recorded for each feeding. From the second week of the trial, 30 min prior to each feeding, faeces were collected from swirl separator and transferred to aluminum trays and stored at -20°C until further analysis. A representative sample (50 g) of each diet was collected twice weekly and stored at 4°C.

The fish were kept under optimal water quality parameters (mean ± SD) during the entire study period with photoperiod (12 light: 12 dark hours), temperature (27.7 ± 0.29ºC), pH (6.8 ± 0.11), dissolved oxygen at tank inlet (8.8 ± 0.75 mg/l) and outlet (5.6 ± 0.58 mg/l), conductivity (2821 ± 99 µS/cm), nitrite (0.02 ± 0.01 mg N/l), nitrate (85 ± 0.5 mg N/l) and TAN (0.12 ± 0.06 mg N/l). After 20 days from the start of the experiment, as the DO level in tank outlets dropped below 5 mg/l, especially during postprandial hours, pure oxygen was injected into the common inlet until the end of the experiment, in order to ensure sufficient DO availability to the fish.

The volume of water (Vt) and water flow (Wf) were kept constant at 200 l and 7 l/min respectively in all tanks. Thus, the rate of replenishment (Vt/ Wf) of entire tank water is achieved in about 30 min. The water was sampled for duration of 5 min from common inlet and outlet of each tank and flushed over the oxygen electrode for measuring oxygen concentration. Thus within an hour, oxygen was measured twice in common inlet and outlet of 4 tanks. The oxygen measurements were performed in a continuous cycle of 2 days (48 hours; from 08.00 to 08.00 hrs) in a set of 4 tanks consisting of all dietary treatments. Consequently, in six days, oxygen measurement was undertaken in all 12 tanks. This procedure was repeated till the end of the experiment resulting in 5 cycles of 48-hour oxygen measurements for each tank. The oxygen electrode was calibrated once every week.

At the end of the experiment, fish were starved for about 36 hours prior to handling. Fish from each tank were anaesthetized and weighed individually for final body weight. Eight fish from each tank were randomly sampled for analysis of final body composition and handled in similar way as initial body composition samples.

Analytical procedure

Frozen fish samples were homogenized twice through a 4.5 mm die in a meat mincer (Gastromaschinen, GmbH model TW-R 70, Feuma) and sub-samples were taken immediately for dry matter and protein analysis. The rest of the homogenized fish samples and faeces (pooled per tank) were then freeze-dried and finely ground using a

blender. Prior to fat analysis, feed and faecal samples were hydrolysed by boiling for 1 hour with 3N HCl. Proximate composition of feed, fish carcass and faeces were analyzed in triplicate for dry matter, protein (Kjeldahl method), fat (Soxhlett method), ash, acid-insoluble ash, energy (Bomb-calorimeter) as described elsewhere (Santos et al., 2010). Starch content was determined as glucose, using the amyloglucosidase/ hexokinase/glucose-6-phosphate dehydrogenase method after ethanol (40%) extraction and starch decomposition in dimethylsulfoxide/ HCl (ISO., 2005).

Calculations

Weight gain rate of fish (g/d) was calculated as the difference between average individual final (W_f) and initial (W_i) body weight of fish per tank divided by the duration of the experimental period (t). The geometric mean body weight (W_G; in g) was calculated as $\sqrt{(W_i \times W_f)}$. Growth rate on metabolic body weight (GR_{MBW}; in g/kg$^{0.8}$/d) was calculated as $(W_f - W_i)/(MBW_G \times t)$, where MBW_G is the mean metabolic body weight of fish (in kg$^{0.8}$), which was calculated as $(W_G /1000)^{0.8}$ and t, the duration (days) of the growth study. Lean body growth of fish (in g/d) was calculated as the difference between $(W_f - W_{f\text{-}fat})$ and $(W_i - W_{i\text{-}fat})$ divided by (t), where $W_{f\text{-}fat}$ and $W_{i\text{-}fat}$ are the crude fat content of final and initial fish carcass respectively, expressed on fresh weight basis. Daily growth coefficient (DGC, in %/d) was calculated as $100 \times (W_f^{1/3} - W_i^{1/3})/t$.

Daily absolute feed intake (FI_{ABS}; g DM/fish/d) was calculated on dry matter basis as $FI_{tot}/ (n \times t)$, where FI_{tot} is the total feed intake per tank (in g DM) over the experimental period corrected for dead fish and uneaten pellets, n is the number of fish per tank, and t is the experimental period. FI as fed (g/fish/d) was calculated in similar way as FI_{ABS} but on as fed basis. Feed intake of fish expressed as a percentage of body weight (FI_{PCT}; g DM/100 g fish/d) was calculated as $(FI_{ABS}/W_G) \times 100$ and feed intake per metabolic body weight (FI_{MBW}; g DM/kg$^{0.8}$/d) was calculated as FI_{ABS}/MBW_G. Feed gain ratio (FGR; dry matter intake/wet weight gain) was calculated as FI_{MBW}/MBW_G.

Apparent digestibility coefficient (ADC; in %) of dry matter, protein, fat, total carbohydrate, gross energy and ash were calculated for each tank according to Tran Duy et al. (2008b), using acid insoluble ash (AIA) as inert marker. Digestible nutrient intake (g or kJ/kg$^{0.8}$/d) was calculated as $FI_{MBW} \times Feed_Z \times (ADC_Z/100)$, where $Feed_Z$ is the nutrient content in feed on dry matter basis (in g), ADC_Z is the apparent digestibility of nutrients (in %) and Z represents dry matter, protein, fat, total carbohydrate, energy and ash.

The parameters of nitrogen balance, fat balance and energy balance were calculated per tank and expressed in mg N/kg$^{0.7}$/d, mg/kg$^{0.9}$/d and kJ/kg$^{0.8}$/d respectively. The gross nitrogen intake (GN) was calculated as product of total feed intake (g DM/kg$^{0.7}$/d) and

nitrogen content of feed (mg/g). The digestible nitrogen intake (DN) was calculated as product of GN and ADC of nitrogen (%). Faecal nitrogen loss (FN) was calculated as the difference between GN and DN. The retained nitrogen (RN) was calculated as the difference between nitrogen content of final and initial fish carcass. Branchial and urinary nitrogen loss (BUN) was calculated as difference between DN and RN. Parameters of fat balance were calculated as follows: gross fat intake (GF) was calculated as product of total feed intake (g DM/$kg^{0.9}$/d) and fat content of feed (mg/g). The digestible fat intake (DF) was calculated as product of GF and ADC of fat (%). Faecal fat loss (FF) was calculated as the difference between GF and DF. The retained fat (RF) was calculated as the difference between fat content of final and initial fish carcass. Parameters of energy balance were calculated as follows: gross energy intake (GE) as the product of feed intake (g DM/$kg^{0.8}$/d) and energy content of the diet; digestible energy intake (DE) as product of GE and ADC of energy; branchial and urinary energy loss (BUE) as product of ammonia-N and urea-N with their corresponding energy value of 24.9 and 22.5 kJ/ g N (Bureau, 2002). Ammonia-N and urea-N were calculated from BUN based on the measured averaged ratio ammonia-N to urea-N excretion of 9 to 1 over diets (our own unpublished data). Metabolizable energy intake (ME) as the difference between DE and BUE; retained energy (RE) as the difference between energy content of final and initial fish carcass; and heat production (H) as the difference between ME and RE; retained energy as protein (RE_p) as product of retained protein (RN x 6.25) and 23.7, where 23.7 is the energy content of 1g of protein (Brafield, 1985); retained energy as fat (RE_f) as difference between RE and RE_p, assuming total retained energy only in the form of fat and protein.

The oxygen consumption of the fish was calculated per tank and expressed as mg O_2/$kg^{0.8}$/min adopting the formula used for calculating ammonia excretion in fish (Kaushik, 1980): $OXt = ((V_L \times \Delta C) + (C_t \times \Delta W))/(t \times W_{mean})$, where OXt is the O_2 consumption of fish per unit time (mg O_2/$kg^{0.8}$/min), V_L is the volume of water in metabolic tank (in l), ΔC is the variation in O_2 concentration in outlet between two consecutive measurements ($C_i - C_{i\text{-}t}$), C_t is the mean O_2 concentration of inlet minus outlet between two consecutive intervals ($C_i - C_{i\text{-}t}/2$), ΔW is the water flow per unit time (l/min), t is the unit of increment in time (min) between two consecutive oxygen measurements, and W_{mean}, is the average predicted metabolic body weight of fish ($kg^{0.8}$) during the measurement days. W_{mean} was calculated as $(W_p/1000)^{0.8}$ where, W_p is the predicted daily body weight of individual fish, estimated as $W_{i(1\text{-}48)} + DFI_{i(1\text{-}48)}/ FGR_{tank}$, where $DFI_{i(1\text{-}48)}$ is the daily feed intake per fish per tank (in g/fish), W_i is the average initial body weight of fish, i indicates the i^{th} day of experiment, and FGR_{tank} is the feed gain ratio of each tank calculated for the entire experimental period.

Dietary oxygen demand (mg O_2/kJ or mg O_2/g) for each diet was calculated by dividing mean daily oxygen consumption (mg O_2/$kg^{0.8}$/d) of fish in each tank by their respective DE (kJ/$kg^{0.8}$/d) or daily dry feed intakes (g DM/$kg^{0.8}$/d). Similarly, efficiency of oxygen utilization for energy retention (i.e., oxygen efficiency; kJ RE/mg O_2 consumed) was calculated by dividing RE (kJ/$kg^{0.8}$/d) of fish within each tank by their respective mean daily oxygen consumption (mg O_2/$kg^{0.8}$/d).

Statistical procedures

Statistical analyses were performed using SAS 9.1 (SAS Institute, Cary, NC, USA). The homogeneity of variances among groups was checked by Levene's *F* test (PROC ANOVA). All variables met the assumption of equal variances ($P > 0.05$). The parameters related with feed intake, oxygen consumption, growth and nutrient utilization were subjected to a two-way analysis of variance (ANOVA) in order to test the effect of DP/DE ratio, type of NPE and their interaction (PROC GLM). Normal distribution of the residuals was verified using Kolmogorov-Smirnov's test (PROC UNIVARIATE). The total digestible carbohydrate intake and retained fat (RF) overruled the assumption of normal distribution ($P < 0.05$) and logarithmic data transformation satisfied the assumptions. When the interaction between DP/DE and NPE was significant ($P < 0.05$), comparison of means was performed using Tukey-Kramer test. A linear regression (PROC REG) analyzed the relation between dietary oxygen demand or oxygen efficiency and DE intake of each treatment unit.

Results

Growth

The survival of the fish during the experimental period was above 98% and did not differ among the dietary treatments ($P > 0.05$). Data on growth and feed utilization of the fish over the entire study period are reported in Table 3.2. Mean initial body weight was not different among dietary groups ($P > 0.05$). Mean final body weights were higher for fish fed with fat as NPE ($P < 0.02$), as was the growth rate expressed per unit metabolic body weight, being 9.3% higher in fish fed the fat relative to starch diets. A similar trend was observed for overall growth rate (DGC) or lean body growth. While growth parameters were not affected by the DP/DE ratio of diet ($P > 0.05$), FGR was significantly improved in fish fed high DP/DE ratio diets ($P < 0.01$). Similarly, protein efficiency ratio (PER) was affected by dietary DP/DE ratio ($P < 0.001$) with higher efficiency in LP than HP diets. There were no interaction effects between DP/DE ratio and NPE on any of the growth parameters.

Table 3.2 Growth performance of Nile tilapia fed the experimental diets for 48 days (*n* 3)

	Diets*					*P*- value		
	HPF	HPS	LPF	LPS	SEM	DP/DE ratio	NPE	DP/DE x NPE
Growth period (d)	48	48	48	48	-	-	-	-
No. of tanks	3	3	3	3	-	-	-	-
No. of fish / tank	20	20	20	20	-	-	-	-
Initial body weight (g)	40.6	40.1	40.6	41.0	0.50	0.386	0.945	0.397
Final body weight (g)	240.8	213.7	249.8	221.0	8.41	0.360	0.010	0.927
Growth								
Weight gain rate (g/d)	4.2	3.6	4.4	3.8	0.18	0.392	0.011	0.887
GR_{MBW} ($g/kg^{0.8}/d$)	26.6	24.3	27.3	24.6	0.80	0.503	0.014	0.801
Lean growth (g/d)	3.6	3.2	3.5	3.1	0.14	0.635	0.016	0.909
DGC (%/d)	5.8	5.3	6.0	5.4	0.16	0.471	0.013	0.848
FGR	0.88	0.89	0.92	0.93	0.010	0.003	0.432	0.995
PER† (%)	2.12	2.08	3.69	3.60	0.032	<0.001	0.088	0.554

SEM, Standard error mean; DP/DE, digestible protein to digestible energy ratio; NPE, non-protein energy source; $GR_{MBW,}$ Growth expressed in metabolic body weight; DGC, Daily growth coefficient; FGR, Feed gain ratio; PER, Protein efficiency ratio.

* HPF – High DP/DE ratio diet with fat as NPE source; HPS - High DP/DE ratio diet with starch as NPE source; LPF - Low DP/DE ratio diet with fat as NPE source; LPS - Low DP/DE ratio diet with starch as NPE source.

† PER = wet weight gain/protein intake.

Feed intake and digestible nutrient intake

Feed intake and digestible nutrient intake of Nile tilapia are shown in Table 3.3. FI (expressed as fed, ABS, PCT and MBW of fish) was affected by the type of NPE source (P <0.03) and to a lesser extent by the DP/DE ratio (P =0.07), which disappeared when FI was expressed on DM basis. Although not significantly different, fish fed LP diets had approximately 6% higher FI than HP diets. On the other hand, tilapia fed diets containing starch as the main NPE had significantly reduced FI (9.5%) compared to those fed the fat diets.

Table 3.3 Feed intake, digestible nutrient intake (on dry matter basis, except dry matter) of Nile tilapia and dietary oxygen demand of experimental diets (*n* 3).

	Diets*					***P*- value**		
	HPF	HPS	LPF	LPS	SEM	DP/DE ratio	NPE	DP/DE x NPE
Feed intake (FI)								
FI as fed (g/fish/d)	3.8	3.4	4.2	3.8	0.17	0.064	0.038	0.745
FI_{ABS} (g DM/fish/d)	3.7	3.2	4.0	3.5	0.16	0.100	0.015	0.839
FI_{PCT} (g DM/100g fish/d)	3.7	3.5	4.0	3.7	0.11	0.069	0.028	0.727
FI_{MBW} (g DM/kg $^{0.8}$/d)	23.4	21.5	25.1	22.8	0.74	0.074	0.023	0.759
Digestible nutrient intake (g or kJ/kg								
Dry matter	16.8	19.0	18.5	20.5	0.48	0.010	0.002	0.787
Protein	11.7	11.1	7.0	6.4	0.21	<0.001	0.018	0.912
Fat	3.7	1.5	5.2	2.9	0.13	<0.001	<0.001	0.684
Total carbohydrate	0.6	5.8	5.5	10.5	0.16	<0.001	<0.001	0.551
Ash	0.67	0.69	0.75	0.76	0.015	0.001	0.319	0.754
Energy	435	420	468	446	11.8	0.035	0.156	0.775
Dietary oxygen demand								
mg O_2/ g DM intake	366	399	311	365	8.3	<0.001	<0.001	0.265
mg O_2/ kJ DE intake	19.7	20.5	16.7	18.7	0.37	<0.001	0.005	0.163

SEM, Standard error mean; DP/DE, digestible protein to digestible energy ratio; NPE, non-protein energy source; FIABS, absolute feed intake; FIPCT, feed intake expressed in percentage body weight of fish; FIMBW, feed intake expressed in metabolic body weight.
* HPF – High DP/DE ratio diet with fat as NPE source; HPS - High DP/DE ratio diet with starch as NPE source; LPF - Low DP/DE ratio diet with fat as NPE source; LPS - Low DP/DE ratio diet with starch as NPE source.

Digestible dry matter, protein, fat and total carbohydrate (without cellulose) intakes were affected by both DP/DE ratio and NPE of diets (P <0.02). As expected per the protocol, digestible protein intake was 41% lower with LP than with HP diets. Digestible fat and carbohydrate intakes were also affected by both DP/DE ratio and NPE of diets (P <0.001). In contrast to digestible macronutrient intakes, DE intake was not affected by the source of NPE (P >0.05), but was affected by the dietary DP/DE ratio, being higher at low than at high DP/DE ratio (P <0.05). There was no interaction between the effect of DP/DE ratio and of NPE on any of the observed FI and digestible nutrient intake variables (P >0.05).

Oxygen consumption

The oxygen consumption (mg O_2/$kg^{0.8}$/min) of Nile tilapia (fig. 3.1), affected by both DP/DE ratio and NPE of diet (P <0.01), showed a significant interaction between both effects (P =0.01). The multiple means comparison (Tukey's test) showed that except for the LPF (mean ± SD; 5.4 ± 0.14) diet group, oxygen consumption was similar in the other three diet groups (HPF, 5.9 ± 0.05; HPS, 6.0 ± 0.04; LPS, 5.8 ± 0.04).

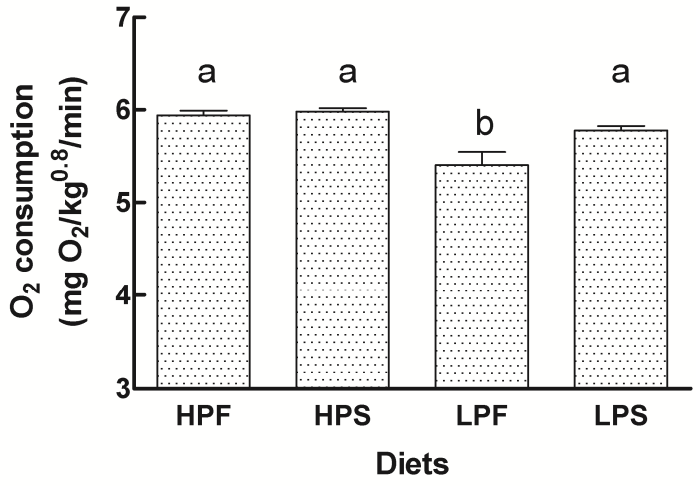

Figure 3.1 Effect of diets on mean oxygen consumption (*mg O_2/$kg^{0.8}$/min*) of Nile tilapia. Each bar shows overall mean with standard deviation represented by error bars. Bars labeled with different lower case letters are significantly different (*n* 3; P <0·05).

Dietary oxygen demand

The dietary oxygen demand of diets expressed both on DM and DE intake (Table 3.3) were influenced by DP/DE ratio and NPE of diet (P <0.01). The HP diets induced a 11% higher oxygen demand than LP diets. Similarly, starch diets led to a 11% and 7% higher oxygen demand per unit DM and DE intake respectively, compared to fat diets. No interaction effect was observed for dietary oxygen demand. Intriguingly, DE intake showed a significant inverse linear relation (R^2 =0.81) with dietary oxygen demand of

the diets (fig. 3.2). Fish fed the LPF diet with the lowest dietary oxygen demand, had highest DE intakes, followed by LPS, HPF and HPS groups.

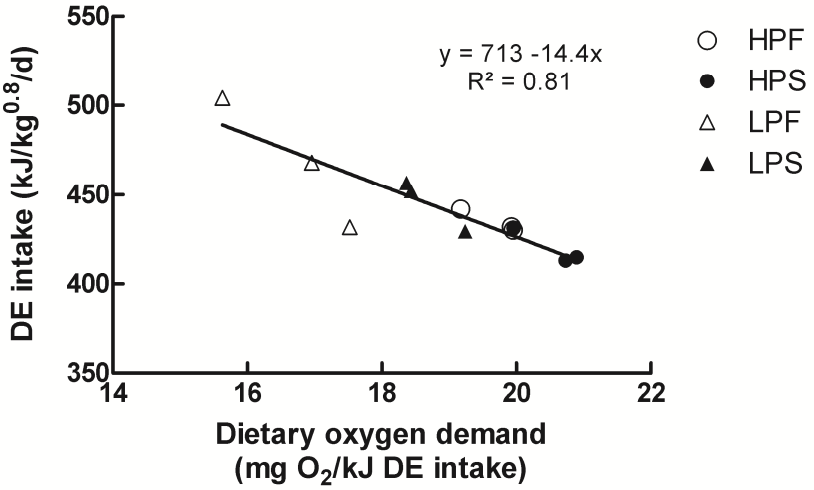

Figure 3.2 Relation between dietary oxygen demand and DE intake of Nile tilapia fed diets differing in DP/DE ratio and NPE source. For every unit increase in dietary oxygen demand, DE intake of Nile tilapia would decrease with 14. 4 kJ (*n* 12; P <0.001).

Body composition

The initial and final whole body compositions of tilapia are presented in Table 3.4. Dietary DP/DE ratio had a significant effect on final body composition of tilapia. Similar effect was observed for the dietary NPE source, except for dry matter and gross energy content where no effect was found (P >0.05). The fat content of fish fed the LP diets were 37% higher than fish fed the HP diets. Consequently, LP-fed fish had about 19% higher energy deposit per unit body weight compared to HP-fed fish. There was no interaction effect between DP/DE ratio and NPE on final body composition, except for protein content (P <0.05).

Nitrogen, fat and energy balance

Nitrogen, fat and energy balances are shown in Table 3.5. All parameters of the nitrogen balance (GN, FN, DN, BUN and RN) were affected by the DP/DE ratio of the diets (P <0.02) and, except for RN (P =0.063), also by NPE (P <0.02). GN and DN intake was about 41% lower with LP compared to HP diets. HP diets resulted in higher FN (33.5%) and BUN loss (59%) than LP diets. RN represented 37% and 58% in terms of DN intake in HP and LP diets, respectively. GN, DN and RN were 7.1%, 6.7% and 6.2% higher, respectively, in fish fed fat compared to starch diets. No interaction effect between NPE and DP/DE ratio was observed except for FN (P <0.05).

Table 3.4 Effect of DP/DE ratio of diet and NPE source (fat vs. starch) on final body composition (on fresh weight basis) of Nile tilapia (*n* 3).

Unit in (g/ kg)	Initial body composition	Final body composition							
		Diets*					*P*- value		
		HPF	HPS	LPF	LPS	SEM	DP/DE ratio	NPE	DP/DE x NPE
Dry matter	243	307	299	347	333	5.2	<0.001	0.067	0.563
Protein	154	153[a]	161[b]	143[c]	143[c]	1.4	<0.001	0.019	0.038
Fat	56	124	109	166	153	4.9	<0.001	0.019	0.777
Ash	34	32	29	33	32	0.7	0.016	0.024	0.469
Energy(kJ/g)	5.6	8.5	8.1	10.1	9.6	0.2	<0.001	0.100	0.919

SEM, Standard error mean; DP/DE, digestible protein to digestible energy ratio; NPE, non-protein energy source

* HPF – High DP/DE ratio diet with fat as NPE source; HPS - High DP/DE ratio diet with starch as NPE source; LPF - Low DP/DE ratio diet with fat as NPE source; LPS - Low DP/DE ratio diet with starch as NPE source.

[a,b,c] Values represent LS means, row means with different superscript letters were significantly different and assigned only if interaction effect was significant (P <0·05).

Variables of fat balance (GF, FF, DF and RF) were affected by DP/DE ratio and NPE of diet (P <0.01) without interaction, except for FF. RF was significantly (P <0.05) different between HP and LP groups, being about 30% higher in LP compared to HP diet groups. The RF/DF (i.e., fat efficiency) was found to be above 1 for starch diet groups (2.0, HPS; 1.5, LPS) and close to 1 for fat diet groups.

The DP/DE ratio of the diet had no effect on GE, FE and H (P >0.05), but affected DE and ME intakes being about 11% higher in fish fed LP relative to HP diets. On the other hand, the source of NPE did not affect DE and ME intakes, but showed a lesser effect on RE_p (P =0.063). Although ME intakes of fish fed starch and fat diets were found to be similar, the higher RE with fat diets resulted in a 10% lower heat production (H) than with starch diets. There was no interaction between DP/DE ratio and NPE of diet on any of the energy balance parameters (P >0.05).

Table 3.5 Nitrogen, fat and energy balance in Nile tilapia fed the four experimental diets for 48 days (*n* 3)

	Diets*					*P*- value		
	HPF	HPS	LPF	LPS	SEM	DP/DE ratio	NPE	DP/DE x NPE
Nitrogen balance (mg N/kg$^{0.7}$/d)								
GN	1587	1470	944	864	32.1	<0.001	0.015	0.575
FN	97^{a}	72^{b}	52^{c}	55^{c}	3.9	<0.001	0.019	0.009
DN	1490	1398	891	809	28.6	<0.001	0.016	0.858
BUN	975	901	403	371	17.1	<0.001	0.014	0.256
RN	515	497	488	439	15.0	0.022	0.054	0.329
RN/DN	0.35	0.36	0.55	0.54	-	-	-	-
Fat balance (mg/kg $^{0.9}$/d)								
GF	5007	1924	7347	3821	192.8	<0.001	<0.001	0.284
FF	250^{a}	59^{b}	598^{c}	151^{ab}	33.6	<0.001	<0.001	0.005
DF	4692	1849	6595	3630	152.9	<0.001	<0.001	0.701
RF	4634	3719	6453	5462	279.8	<0.001	0.005	0.625
RF/DF	0.99	2.01	0.98	1.50	-	-	-	-
Energy balance (kJ/kg$^{0.8}$/d)								
GE	541	449	582	475	16.8	0.081	<0.001	0.666
FE	106	29	114	29	5.5	0.515	<0.001	0.479
DE	435	420	468	446	11.8	0.035	0.156	0.775
BUE	30	28	12	12	0.5	<0.001	0.017	0.276
ME	404	392	456	435	11.4	0.003	0.175	0.729
H	163	180	157	175	6.0	0.335	0.020	0.982
RE	241	211	298	260	11.0	0.001	0.015	0.713
RE_p	96	93	91	82	2.7	0.016	0.063	0.297
RE_f	145	118	207	178	8.8	<0.001	0.012	0.893

SEM, Standard error mean; DP/DE, digestible protein to digestible energy ratio; NPE, non-protein energy source; GN, Gross nitrogen intake; FN, Faecal nitrogen loss; DN, Digestible nitrogen intake; BUN, Branchial and urinary nitrogen loss; RN, Retained nitrogen; GF, Gross fat intake; FF, Faecal fat loss; DF, Digestible fat intake; RF, retained fat; RF/DF, fat efficiency; GE, Gross energy intake; FE, faecal energy loss; DE, digestible energy intake; BUE, branchial and urinary energy loss; ME, metabolizable energy intake; H, heat production; RE, retained energy; RE_p, retained energy as protein; RE_f, retained energy as fat

* HPF – High DP/DE ratio diet with fat as NPE source; HPS - High DP/DE ratio diet with starch as NPE source; LPF - Low DP/DE ratio diet with fat as NPE source; LPS - Low DP/DE ratio diet with starch as NPE source.

[a,b,c] Values represent LS means, row means with different superscript letters were significantly different and assigned only if interaction effect was significant (P <0·05).

Discussion

Indispensable criteria

Two main criteria need to be fulfilled to investigate the possible role of dietary oxygen demand (DOD) on FI regulation: (i) the availability of dissolved oxygen from water (DO) should not be limiting for the fish and (ii) the experimental diets should generate differences in oxygen demand in the species concerned.

It is well documented in many fish species that a reduction in concentration of water oxygen lowers FI (Buentello, 2000; Glencross, 2009; Pichavant et al., 2000; Thetmeyer, 1999). The mean incipient DO concentration inside the tank for Nile tilapia has recently been reported to be 2.6 and 5.0 mg/l for small (60-100 g) and big (200-270 g) fish, respectively (Tran-Duy et al., 2008a), below which FI decreases. Cho (1992) however underlined the importance of considering the rate of replenishment of oxygen per unit time (mg/l/s) rather than the mean oxygen concentration inside the tank. In the present study, oxygen was kept at an average of 8.8 mg/l in inlet and 5.6 mg/l in outlet water, indicating a DO concentration higher than 5.6 mg/l inside the tank. This concentration, together with the 30 min total replenishments, ensured sufficient oxygen availability for the fish (40-250g) throughout the experiment.

As intended, the diets generated differences in DOD (kJ per g DM or DE intake), related to both the DP/DE ratio (HP>LP) and NPE source (starch>fat). The metabolic fate of a specific dietary nutrient for energy production depends on the relative proportions of energy-yielding nutrients and on the nutritional status of the fish. The high oxygen demand for the HP diets agrees with post-feeding oxygen consumption data reported in fish fed high protein (Jobling, 1980; Peres, 1999) and also with the use of protein for ATP production in fish (Alsop and Wood, 1997). The low oxygen demand in tilapia fed the high fat diets, as observed in other studies (Cho, 1992; Seth, 2009), suggests that the majority of the dietary fat was used for fat deposition rather than for ATP production, as reflected by their higher level of body adiposity. In terms of ATP (oxygen) demand, the formation of fat from dietary lipids is considered to be less expensive than from either starch or protein (Blaxter, 1989; Reeds, 1982), which likely explains the increase in dietary oxygen demand observed following the replacement of fat by starch as non-protein energy source.

Control of feed intake

FI in several fish species, including Nile tilapia, has been found to be regulated by the dietary DE level in order to maintain a constant DE intake irrespective of diet composition, provided all essential nutrients are present in adequate amounts and in the right proportions (Boujard, 1994; Cho, 1990; Kaushik, 1983; Kubaryk, 1980; Lee and

Putnam, 1973; Lekva et al., 2010). In case of a very low dietary DE density, FI can be limited by the excessive bulk relative to stomach volume induced by the physical characteristics of the feed (Lovell, 1979). Some studies however reported an increase in stomach volume to allow increased dry matter intakes as shown in rainbow trout 35% over a 10-week period (Ruohonen, 1996), and in plaice (Jobling, 1982). In the present study, tilapia fed the LP compared to HP diets with similar DE concentration displayed a 11% higher DE intake. The current finding that the tilapia did not adjust FI for constant DE intake is in line with observations in other studies (Alanärä, 1996; Geurden et al., 2006; Helland, 1998; Peres, 1999), showing the absence of dietary DE intake compensations and hence suggests a role of factors other than DE in the control of FI. A possible adjustment to make up for the low protein supply in the LP diets is one explanation for this.

Some studies in mammals suggest that an animal seeks to eat until it reaches the maximum protein deposition as determined by its genetic growth potential (Birkett, 2001; Webster, 1993). In fish also, some studies propose that FI is controlled in order to achieve the maximal protein growth rather than to fulfill the daily energy needs (Azevedo, 2001; Gélineau, 2001; Geurden et al., 2006). If it is indeed the maximal growth potential which determines FI, one would expect to have similar lean body growth, irrespective of diet composition. In contrast, results of the current study showed differences in lean growth and RN between fish groups, which does not comply with above claims.

Like in mammals, reduced FI in fish fed high-fat diets has been attributed to increased adiposity or high body fat contents (Boujard et al., 2004; Johansen et al., 2003; Metcalfe, 1992; Shearer et al., 1997; Silverstein, 1999). In the present study, high growth (GR_{MBW}) in fat groups resulted from the high deposition of body fat (lipid gain), as seen in other fish species fed high levels of dietary fat (Azevedo, 2004a; Grisdale-Helland et al., 2008; Vergara, 1999). However, tilapia fed either the HPF or LPF diet did not reduce FI. On the contrary, these groups in fact had a higher FI, despite their high body fat content. Similar observations have been made in other fish such as turbot (Saether, 2001) and rainbow trout (Geurden et al., 2006). Our results suggest that FI in Nile tilapia is not related to adiposity, which suggest the need for further studies on the lipostatic control of FI in poikilotherms.

Our previous observations showing different DE intakes concurrent with similar heat productions in Nile tilapia (Tran-Duy et al., 2008b) and rainbow trout (our own unpublished data) suggest that DE intake might be limited and thus controlled by either constraints in the physiological capacity of oxygen uptake or metabolic oxygen use by the fish. This forms the basis of the proposed oxystatic theory, which to our knowledge

has never been considered before in fish. The concept of the 'oxystatic control of FI in fish' tested here assumes that (maximal) FI is limited by the (maximal) capacity of oxygen use in the fish. In this view, it is expected to find similar oxygen consumption if fish are fed to satiation with diets differing in macronutrient composition. Thus, changes in the dietary oxygen demand (by changing the macronutrient composition) are expected to induce differences in FI. Indeed in the current study, the amount of oxygen consumed per unit metabolic body weight was similar for three out of the four diets. As such, the reduced DE intakes of fish fed the HP diets, which had a higher dietary oxygen demand than LP diets but which resulted in equal oxygen consumption by the fish, possibly stem from physiological constraints in oxygen use, in line with the 'oxystatic control of FI in fish'. Also in mammals, high levels of dietary protein have been reported to produce a higher satiating effect than fat (Anderson and Moore, 2004; Friedman, 1998; Stubbs, 1996), which has been attributed to their limited storage capacity and hence their (obligatory) partitioning towards oxidation. Interestingly, tilapia fed the LPF diet however consumed a lower amount of oxygen than fish from the other three dietary treatments. Moreover, tilapia fed this low oxygen-demanding diet displayed the highest FI. According to the oxystatic theory, LPF-fed fish could have eaten more since oxygen consumption did not reach its upper limit. As such, it is believed that FI in tilapia fed the

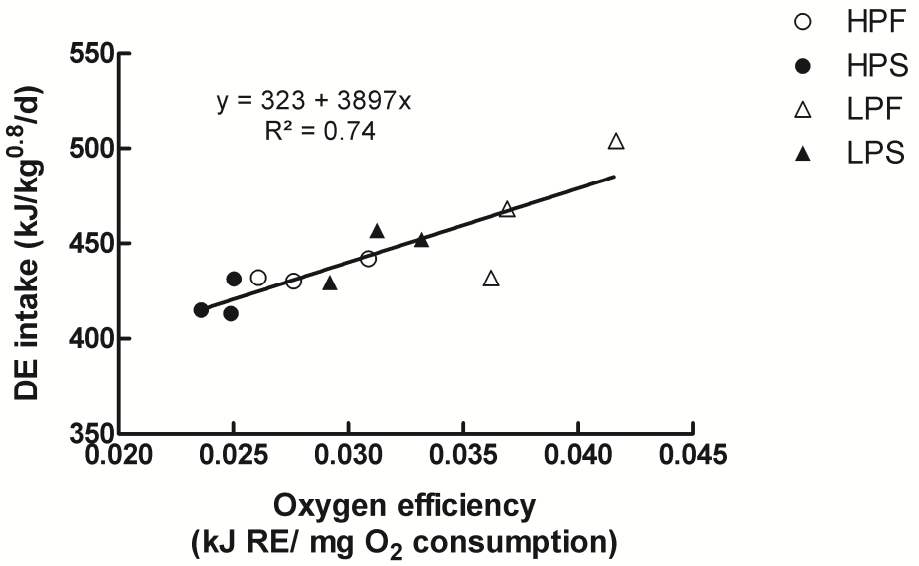

Figure 3.3 Relation between oxygen efficiency and DE intake of Nile tilapia fed diets differing in DP/DE ratio and NPE source. The DE intake of tilapia increases linearly with increasing efficiency of oxygen utilization for energy retention (*n* 12; P <0.001).

LPF diet was limited by other constraints of physical (stomach capacity) or metabolic (lipid/protein gain) origin rather than by the maximum capacity of oxygen use.

Our data demonstrate that DE intake in tilapia is not only significantly related to the dietary oxygen demand, but also to the amount of energy retained per unit of oxygen consumed (figs. 3.2 & 3.3). DE intakes decreased with increasing dietary oxygen demand but also increased linearly with increasing oxygen efficiency (i.e., amount of energy retained per unit of oxygen consumed). This parallels the finding in ruminants, that metabolizable energy intake increases with increasing oxygen efficiency (Ketelaars and Tolkamp, 1992). Based on these observation in ruminants, Ketelaars and Tolkamp postulated the 'oxygen efficiency theory' in the control of FI (Ketelaars and Tolkamp, 1992; Ketelaars and Tolkamp, 1996). According to this theory, FI entails both benefits (energy gain) and costs (measured as oxygen consumption) to the animal, which strives to optimize its FI close to the value of maximum efficiency of oxygen utilization for energy gain. The current results however do not allow to conclude whether FI in Nile tilapia is regulated as a function of (maximizing) oxygen efficiency or by a limit set by the (maximum) capacity of oxygen use by the fish.

In summary, the FI of Nile tilapia was related to dietary macronutrient-induced changes in oxygen demand. As such, even under normoxic conditions, oxygen consumption of fish appears to play a role in the dietary control of FI in tilapia. Further studies are warranted to explore other environmental and nutritional factors affecting oxygen use in fish and their metabolic implications in regulating FI in fish.

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CHAPTER 4

Voluntary feed intake in rainbow trout is regulated by diet-induced differences in oxygen use

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Abstract

This study investigated the hypothesis that the voluntary feed intake in fish is regulated by diet-induced differences in oxygen use. Four diets were prepared with a similar digestible protein to digestible energy ratio (18 mg/kJ), but which differed in the composition of non-protein energy source. This replacement of fat (F) by starch (S) was aimed to create a diet-induced difference in oxygen use (per unit of feed): *viz.* diets F30-S70, F50-S50, F65-S35 and F80-S20 with digestible fat providing respectively 28, 49, 65 and 81% of the non-protein digestible energy (NPDE). Each diet was fed to satiation to triplicate groups of twenty rainbow trout for 6 wk. As expected, diet-induced oxygen use decreased linearly (R^2=0.89; P<0.001) with increasing NPDE as fat. The digestible and metabolizable energy intake of trout slightly increased with increasing NPDE as fat (i.e., decreasing starch content) (R^2=0.30; P=0.08 and R^2=0.34; P=0.05, respectively). Oxygen consumption of trout fed to satiation declined with increasing dietary NPDE as fat (R^2=0.48; P=0.01). The inverse relation between digestible energy intake of trout and the diet-induced oxygen use (R^2=0.33; P=0.05), suggests a possible role of diet-induced oxygen use in feed intake regulation as shown by the replacement of dietary fat by starch.

Introduction

In fish, factors influencing feed intake is extensively documented but the underlying mechanism that regulates feed intake has been less intensively studied compared to mammals (Houlihan et al., 2001). It has been often suggested that fish, like other animals, adjust their voluntary feed intake according to the digestible energy content of the diet in order to meet a predefined energy requirement (Kaushik et al., 1981; Rasmussen et al., 2000; Yamamoto et al., 2000). However, recent findings in rainbow trout (Alvarez et al., 1998; Encarnação et al., 2004; Figueiredo-Silva et al., 2012a; Geurden et al., 2006; Morales et al., 1994; Saravanan et al., 2012a) and in other teleosts (Alvarez et al., 1998; Saravanan et al., 2012b; Tran-Duy et al., 2008) contradict the notion that feed intake is adjusted to have a constant digestible energy intake. Similarly, the demand for a target lean growth or protein deposition rather than for a predefined energy requirement has been proposed to regulate feed intake in fish (Azevedo et al., 2004; Encarnação et al., 2004; Geurden et al., 2006; Peres and Oliva-Teles, 1999). However, several other fish studies did not show an equal protein deposition under satiation feeding (Saravanan et al., 2012b; Saravanan et al., 2012a). The diet-induced difference in the voluntary intake levels observed in various fish species (Borges et al., 2009; Da Vies, 1989; Figueiredo-Silva et al., 2012a; Gélineau et al., 2001; Saravanan et al., 2012a) might be related to the type and level of dietary non-protein digestible energy (NPDE) source (starch *vs.* fat) as suggested in mammals (see review Blundell et al., 1996). The mechanism by which the NPDE source affects voluntary feed intake in fish has been, so far, little explored. In terrestrial animals, dietary starch and fat exert their effects on feed intake via feedback mechanisms mediated by circulating glucose (Mayer, 1955) (glucostatic regulation) and body fat store (Kennedy, 1953) (lipostatic regulation), respectively. These chemostatic control mechanisms of feed intake show inconsistent outcomes in fish (Saravanan et al., 2012b; Saravanan et al., 2012a). Alternatively, the thermostatic regulation of feed intake in homeotherms proposes the intake to be controlled by the animal's need for body heat and its ability to dissipate the extra heat to the environment generated as a by-product of food processing (Brobeck, 1948; Strominger and Brobeck, 1953). As such, the concept of thermostatic control and its more recent revision, "heat dissipation limit theory (Speakman and Król, 2005)" in the feed intake regulation of fish is debatable because of its ectothermic nature. Besides, in homeotherms under thermoneutral condition, differences in diet-induced thermogenesis are suggested to be involved in the regulation of food intake (Westerterp-plantenga et al., 1990; Westerterp, 2004). As such, the heat produced by food processing in animals varies with the dietary macronutrient (protein, fat, and starch) composition (Karst et al., 1984), which implies a difference in the diet-induced oxygen use (per unit of feed). Further, the basic biochemical processes of oxidative

nutrient metabolism are analogous between homeotherms and poikilotherms (endotherms and ectotherms). An enhanced oxidative metabolism over a long term has been suggested to impart putative negative effects (e.g., the buildup of reactive oxygen species) on the animal (Fridovich, 1998; Sohal and Weindruch, 1996). In view of the above findings, we have previously proposed a role of oxygen consumption in the control of voluntary feed intake in Nile tilapia fed with diets highly varying in the macronutrient composition (Saravanan et al., 2012b). In that study, Nile tilapia linearly adjusted the feed intake (digestible energy intake) depending on the diet-induced differences in oxygen use and moreover showed a similar oxygen consumption in some of the diet groups, which suggests that physiological factors related to oxygen consumption perhaps, constrained the feed intake.

The present study was designed to test the role of diet-induced differences in oxygen use (O_2 per unit of feed) on the voluntary feed intake in rainbow trout. To this end, the feed intake (digestible energy intake) and oxygen consumption were monitored in rainbow trout fed to apparent satiation with diets contrasting in the percentage of NPDE source (starch *vs.* fat) and with a similar digestible protein to digestible energy ratio. The contrast in fat and starch is expected to create a difference in diet-induced oxygen use. In addition, growth, body composition, nutrient partitioning (nitrogen, fat, and energy balance) and postprandial nutrient (plasma glucose and triglycerides) concentrations were observed to evaluate their involvement on the regulation of feed intake in rainbow trout.

Materials and Methods

The study was conducted at De Haar vissen, Wageningen University in accordance with the Dutch law on experimental animals and as approved by the Ethical Committee of Wageningen University for animal experiments.

Diets

Four diets, with the aim to have a similar digestible protein to digestible energy ratio (~18 mg/kJ) and contrasting in the percentage of non-protein energy source (starch *vs.* fat), were formulated by replacing an iso-energetic (iso-digestible energy) amount of two test ingredients: gelatinized maize starch *vs.* rapeseed oil. The test ingredients were exchanged with the assumption that the digestible energy value of 12.5 g maize starch (87% apparent digestibility coefficient) is equal to that of 5.0 g rapeseed oil (95% apparent digestibility coefficient). To the basal ingredient composition (62.5%), different ratios of the test ingredients (maize starch *vs.* rapeseed oil) were added. These test ingredients were exchanged iso-energetically to create contrasts in percentage of non-protein energy (F, fat *vs.* S, starch) (Supplemental Table): *viz.* Diet F30-S70, 0%

rapeseed oil + 37.5% maize starch; Diet F50-S50, 5% rapeseed oil + 25% maize starch; Diet F65-S35, 10% rapeseed oil + 12.5% maize starch; and Diet F80-S20, 15% rapeseed oil + 0% maize starch. Due to the difference in energy content (kJ/g) between rapeseed oil and maize starch, an incomplete mass balance of 92.5, 85.0, and 77.5%, respectively occurred to diet F50-S50, F65-S35, and F80-S20. In order to complete the mass balance to 100%, the amount of both the basal and the test ingredients were adjusted in those diets in an equal proportion without affecting the aimed digestible protein to digestible energy ratio and contrast in non-protein energy type. Diamol (acid insoluble ash) was added as inert marker to measure the digestibility. The ingredient mixture were extruded (Clextral BC45, twin screw extruder) through a 3 mm die, dried (70°C for 3 h), vacuum coated with oils and stored at 4°C (Research Diet Service B.V., Wijk bij Duurstede, The Netherlands). The analyzed gross and digestible nutrient content of the diets are shown in Table 4.1. The observed digestible protein to digestible energy ratio of diets varied from 18 to 19.8 mg/kJ. The percent fat in the NPDE was 28% in diet F30-S70, 49% in diet F50-S50, 65% in diet F65-S35, and 81% in diet F80-S20.

Fish, housing conditions, and feeding

At the start of the experiment, 240 unfed (feed-deprived for about 36 h) juvenile rainbow trout (*Oncorhynchus mykiss;* supplied by Forrel BV, The Netherlands) were individually weighed (under sedation; 2-phenoxy ethanol, 0.25 mL/L water) and randomly distributed among the 12 metabolic tanks (20 fish/tank). Each tank was assigned randomly to one of the four experimental diets forming triplicates per diet. The details of the aquatic metabolism unit are described elsewhere (Saravanan et al., 2012b), in brief, the entire unit was connected to a recirculating aquaculture system with the facilities to collect fish feces and to measure the oxygen consumption. Throughout the experiment, the environmental/water quality parameters (mean ± SD) were maintained in an optimal conditions for rainbow trout; photoperiod 12 light: 12 dark h, water volume (150 L/tank), water flow (7 L/min), water temperature (13.7 ± 0.1ºC), pH (7.42 ± 0.2), dissolved oxygen of water at the tank inlet (10.3 ± 0.3 mg/L), conductivity (2.9 ± 0.2 µS/cm), nitrite (<0.15 mgN/L), nitrate (<250 mgN/L) and total ammonia nitrogen (<0.5 mgN/L). The fish were hand fed with their respective diets twice daily to apparent satiation for an hour (09.00 to 10.00 and 16.00 to 17.00 hrs). Feed given and uneaten pellets were counted and registered at each feeding.

Supplemental Table Ingredient composition of the experimental diets[1]

	Diets			
	F30-S70	F50-S50	F65-S35	F80-S20
Test ingredients		%		
Rapeseed oil	0.00	5.41	11.77	19.36
Gelatinized maize starch	37.50	27.03	14.71	0.00
Basal ingredients		%		
Fish meal	16.50	17.84	19.41	21.29
Soy protein concentrate	7.50	8.11	8.82	9.68
Pea protein	7.50	8.11	8.82	9.68
Wheat gluten	7.50	8.11	8.82	9.68
Wheat flour	16.00	17.30	18.82	20.65
Fish oil	3.00	3.24	3.53	3.87
DL-methionine	0.25	0.27	0.29	0.32
L-Lysine HCl	0.05	0.05	0.06	0.07
L-Threonine	0.05	0.05	0.06	0.07
Calcium carbonate	0.20	0.22	0.24	0.26
Mono-calcium phosphate	1.45	1.57	1.71	1.87
Diamol[2]	1.50	1.62	1.77	1.94
Vitamin mineral premix[3]	1.00	1.08	1.18	1.29

[1] F30-S70, F50-S50, F65-S35, and F80-S20, respectively: diet with fat providing 28, 49, 65, and 81% of the non-protein digestible energy. F, fat; S, starch.

[2] Diamol (Acid insoluble ash, as inert marker for digestibility measurement)- Diamol GM, Franz Bertram, Hamburg, Germany.

[3] Mineral premix composition (to supply, mg per kg feed): 50, iron (as $FeSO_4.7H_2O$); 30, zinc (as $ZnSO_4.7H_2O$); 0.1, cobalt (as $CoSO_4.7H_2O$); 10, copper (as $CuSO_4.5H_2O$); 0.5, selenium (as Na_2SeO_3); 20, manganese (as $MnSO_4.4H_2O$); 500, magnesium (as $MgSO_4.7H_2O$); 1, chromium (as $CrCl_3.6H_2O$); 2, iodine (as $CaIO_3.6H_2O$). Vitamin premix composition (to supply, mg or IU per kg feed): 10, thiamin; 10, riboflavin; 20, niacin; 40, pantothenic acid; 10, pyridoxine; 0.2, biotin; 2, folic acid; 0.015, cyanocobalamin; 1500, choline (as choline chloride); 100, ascorbyl phosphate; 3000 IU, retinyl palmitate; 2400 IU, cholecalciferol (Rovimix® D3-500, DSM Inc.); 100 IU, α-tocopheryl acetate; 10, menadione (as menadione sodium bisulfite, 51%); 400, Inositol; 100, anti-oxidant BHT (E 321); 1000, calcium propionate.

Table 4.1 Analyzed nutrient content of the experimental diets[1]

	Diets			
	F30-S70	F50-S50	F65-S35	F80-S20
Dry matter, *g/kg*	947	948	959	954
Crude protein (N x 6.25), *g/kg DM*	336	363	397	431
Crude fat, *g/kg DM*	71	133	198	283
Starch, *g/kg DM*	493	402	285	164
Ash, *g/kg DM*	60	64	70	71
Gross energy, *kJ/g DM*	19.9	21.4	23.1	25.1
Digestible protein[2], *g/kg DM*	317	344	377	411
Digestible fat[2], *g/kg DM*	61	123	186	267
Digestible total carbohydrate[2], *g/kg DM*	354	298	229	146
Digestible energy[2], *kJ/g DM*	16.0	18.1	20.3	22.9
Non-protein digestible energy, *kJ/g DM*	8.5	10.0	11.3	13.0
Digestible protein to digestible energy ratio, *mg/kJ*	19.8	19.0	18.6	18.0
Fat as non-protein digestible energy, *%*	28	49	65	81

[1] F30-S70, F50-S50, F65-S35, and F80-S20, respectively: diet with fat providing 28, 49, 65, and 81% of the non-protein digestible energy. F, fat; S, starch.
[2] Calculated as product of respective nutrient/energy content in feed and their measured percentage apparent digestibility.

Sampling and measurements

At the start of experiment 20 fish were euthanized (2-phenoxy ethanol, 1.5 mL/L water) and stored at -20ºC for the analysis of the initial whole body composition. The entire experiment lasted for 7 wk, over the first 6 wk period (nutrient balance period), feed intake, growth, digestibility, oxygen consumption, and final body composition were determined. After 1 wk of recovery period (end of 7 wk), blood samples were collected. Fish feces were collected using swirl separator as previously described (Saravanan et al., 2012b) and stored at -20ºC until further analysis. From the second week onwards, the water was sampled automatically for a duration of 5 min from the common inlet and the outlet of each tank and flushed over the oxygen electrode (WTW-Trioximatic® 700 IQ, WTW GmbH, Weilheim, Germany) to measure dissolved oxygen concentration. The oxygen measurements were performed in a continuous cycle of 2 d (48 h; from 08.00 to 08.00 h) in a set of 4 tanks consisting of all the dietary treatments. Thus, in 6 d dissolved oxygen concentrations were measured in all 12 tanks. The oxygen electrode was

calibrated once a week. This procedure was repeated until the end of the experiment, which resulted in 5 cycles of 48 h oxygen measurements per tank. At the end of 6 wk, the fish (feed-deprived for about 36 h) were individually weighed and in addition, 6 fish per tank were euthanized and stored at -20ºC for final body composition analysis. The remaining fish in each tank were then continued to be fed with their respective diets for 1 wk (recovery period) prior to post-prandial blood sampling. After 6 h post-feeding, 5-6 fish from each tank were sampled for blood under sedation. The blood (1 mL) was collected from the caudal part, mixed to 20 µL anti-coagulant (potassium oxalate + sodium fluoride), and centrifuged (3000x *g*, 10 min). The plasma obtained was then stored at -20ºC until analysis.

Chemical analyses

Chemical analysis of the feeds, feces and fish carcasses were done in triplicates for dry matter (ISO 6469/NEN 3332), crude protein (Nx6.25; Kjeldahl, ISO 5983/NEN 3145), crude fat (Soxhlett, ISO-DIS 6492), ash (ISO 5984/NEN 3329), acid-insoluble ash (ISO 5985) and gross energy (adiabatic bomb calorimetry, IKA-calorimeter C 7000). The starch content of the feed was determined enzymatically as glucose (ISO 15914). Plasma glucose and triglycerides (TGs) were determined following the protocol provided in the commercial kits, Glucose (RTU n° 61269) and TGs (PAP 150 n° 61236) from Bio-Merieux, Marcy-L'Etoile, France.

Calculations

Apparent digestibility coefficient (%)= $[(1 - D_{AIA}/ F_{AIA} \times F_N/ D_N) \times 100]$, where D_{AIA} and F_{AIA} are the acid insoluble ash content (% dry matter) in the diet and feces, respectively, and F_N and D_N are the amount of nutrient in 1 gram dry matter of the feces and diet, respectively. The comparative slaughter method was used to determine the nitrogen (N) (mg N/($kg^{0.7}$ · d)), fat (g/($kg^{0.9}$ · d)) and energy balance (kJ/($kg^{0.8}$ · d)) as previously described (Saravanan et al., 2012b). The only exception was the branchial and urinary energy loss, which was calculated as BUN × 24.9/1000, where, BUN is the branchial and urinary nitrogen loss and 24.9 is the amount of energy in kJ/g NH_3-N, assuming all nitrogen was lost as ammonia (Bureau, 2002). Oxygen consumption of the fish was calculated per tank with the difference in measured concentration of oxygen between inlet and outlet, and the rate of water flow in the tank using the formula shown elsewhere (Saravanan et al., 2012b) without modification.

Statistical analysis

For all dependent variables, the tank was considered as the experimental unit. Data were tested for the relation between the dependent variable (Y; e.g., growth, feed intake etc.) and the measured percentage of fat in NPDE of the diets as an independent variable

(X, %; 28, 49, 65, 81) according to the linear regression model: $Y_i = \alpha + \beta X + \varepsilon_i$, where α, β, and ε represents intercept, slope and error term respectively (i = 1, 2, 3,…12). Data were analyzed using the general linear model procedure in SAS 9.2 (SAS institute, Cary, NC, USA). Linear regression results were reported when $P<0.1$.

Results

Feed intake

The mean percentage survival of fish, about 93% over all the treatments, was not affected by replacing non-protein energy source of the diet ($P=0.33$). Feed intake was significantly affected by replacing dietary non-protein energy source (Table 4.2). The absolute feed intake (g DM/(fish · d)) and feed intake per unit metabolic body weight (g DM/($kg^{0.8}$ · d)) of the trout decreased linearly with increasing percent of fat in NPDE of the diet ($P<0.001$). The feed intake of trout in the low fat diet (F30-S70) was 34% greater than in those fed the high fat diet (F80-S20).

Growth and body composition

Growth performance of the trout and all the measured body composition parameters were significantly affected by replacing non-protein energy source of the diet (Table 4.2). The final body weight and the growth of trout increased with increasing percent of fat in NPDE ($P<0.001$). The growth of trout fed F80-S20 was 21% higher than in the group fed the F30-S70 diet. Feed conversion ratio reduced linearly from 1.16 to 0.70 with increasing percent fat from 28 to 81% in NPDE of the diet ($P<0.001$).

The final body dry matter, crude fat and energy content increased ($P<0.001$), whereas, the crude protein ($P<0.01$) and ash ($P=0.002$) decreased linearly with increasing percent of fat in NPDE of the diet. Compared to the initial body fat content (72 g/kg) of trout, the final body fat content almost doubled (141 g/kg) in the group fed the F80-S20 diet with 81% NPDE as fat. The lowest final body fat content (81 g/kg) was observed in the group which received the F30-S70 diet with 28% NPDE as fat.

Table 4.2 Feed intake, growth, and body composition of rainbow trout fed diets with varying levels of fat and starch for 6 wk[1]

	Diets					Regression analysis		
	F30-S70	F50-S50	F65-S35	F80-S20	Pooled SEM	β (SE)	R^2	*P*-value
Initial body weight, *g*	51.3	51.0	50.8	51.3	0.36	-	-	0.92
Final body weight, *g*	134.9	143.4	153.4	160.4	3.28	0.49 (0.08)	0.81	<0.001
Feed intake (FI)								
Absolute FI, *g DM/(fish · d)*	2.3	2.1	2.0	1.8	0.07	- 0.01 (0.002)	0.75	<0.001
FI_{MBW}[2], *g DM/(kg$^{0.8}$ · d)*	16.8	15.4	14.0	12.5	0.38	- 0.08 (0.009)	0.89	<0.001
Growth								
Absolute, *g/d*	2.0	2.2	2.4	2.6	0.08	0.01 (0.002)	0.81	<0.001
$Growth_{MBW}$[2], *g/(kg$^{0.8}$ · d)*	14.6	15.7	17.0	17.7	0.41	0.06 (0.01)	0.81	<0.001
Feed conversion, *g DM intake/ g wet weight gain*	1.16	0.98	0.82	0.70	0.02	- 0.01 (0.001)	0.98	<0.001
Final body composition[3], *g/kg wet weight*								
Dry matter	275	299	307	320	2.8	0.82 (0.08)	0.92	<0.001
Crude protein	166	164	158	157	2.5	- 0.18 (0.06)	0.50	0.010
Crude fat	81	111	123	141	3.1	1.10 (0.08)	0.95	<0.001
Ash	22	21	21	20	0.3	- 0.04 (0.009)	0.63	0.002
Energy, *kJ/g wet weight*	7.0	8.1	8.6	9.1	0.15	0.04 (0.004)	0.91	<0.001

[1] Values are least-squares mean; (*n*=3). Linear regression results were reported when P<0.1. F30-S70, F50-S50, F65-S35, and F80-S20, respectively: diet with fat providing 28, 49, 65, and 81% of the non-protein digestible energy. F, fat; S, starch.
[2] Expressed in metabolic body weight (MBW), calculated using geometric mean body weight i.e., $(\sqrt{(\text{Final body weight x Initial body weight})}/1000)^{0.8}$.
[3] Initial body composition of rainbow trout at start of the experiment (g/kg wet weight): dry matter, 254; crude protein, 156; crude fat, 72; ash, 23; energy 6.4 kJ/g wet weight.

Nitrogen, fat, and energy balance

Gross and digestible nitrogen intake were not affected by the replacement of the dietary non-protein energy source (P>0.2) with intakes of 695 and 659 mgN/($kg^{0.7}$ · d), respectively (Table 4.3). Despite the similar digestible nitrogen intake, the retained nitrogen and nitrogen retention efficiency increased (P<0.01) with increasing percent of fat in NPDE of the diet. In line with the increasing amount of fat in the diet, gross and digestible fat intake and fat retention of the trout increased (P<0.001), but the fat retention efficiency decreased linearly (P<0.001). The mean fat retention efficiency increased linearly with increasing fat in NPDE of the diets.

The gross energy intake of the trout tended to decrease linearly (P=0.05), whereas, digestible (P=0.08) and metabolizable energy intake (P=0.05) increased with increasing percent of fat in NPDE of the diet; with regression coefficient of digestible and metabolizable energy intake of 0.30 and 0.34 kJ/($kg^{0.8}$ · d per %), respectively. The differences in feed intake or gross energy intake between the dietary treatments did not relate to the growth of trout, but the difference in the digestible energy intake of trout was related to the growth (P=0.004; fig 4.1A). The heat production decreased with increasing fat in NPDE (P<0.001). The higher metabolizable energy intake together with the lower heat production resulted in an increased energy retention and growth (Table 4.2 and 4.3). Similarly, the energy retained as fat and protein increased with increasing fat in NPDE (P<0.001).

Plasma glucose and triglycerides

At 6 h post-feeding, plasma glucose tended to decrease linearly (P=0.09) and plasma TGs tended to increase linearly (P=0.06; fig 4.1B) with increasing NPDE as fat.

Oxygen consumption and diet-induced oxygen use

The oxygen consumption (mg O_2/(kg · min) and mg O_2/($kg^{0.8}$ · min)) of rainbow trout was linearly affected (P<0.01) by non-protein energy source of the diet (Table 4.4). Mean oxygen consumption of rainbow trout decreased with increasing percent inclusion of digestible energy as fat in the diet.

The diet-induced oxygen use (mg O_2/kJ digestible energy intake) decreased (P<0.001) with increasing percentage of fat in NPDE (Table 4.4). The digestible energy intake of trout reduced with increasing diet-induced oxygen use (P=0.05; Fig 4.1C).

Table 4.3 Nitrogen, fat, and energy balance of rainbow trout fed diets with varying levels of fat and starch for 6 wk1

	Diets					Regression analysis		
	F30-S70	F50-S50	F65-S35	F80-S20	Pooled SEM	β (SE)	R^2	*P*-value
Nitrogen balance *mg N/(kg$^{0.7}$ · d)*								
Gross nitrogen intake	706	700	695	677	19.0	-	-	0.27
Digestible nitrogen intake (DNI)	667	663	661	645	17.5	-	-	0.38
Retained nitrogen (RN)	314	331	341	352	7.7	0.72 (0.18)	0.62	0.002
Nitrogen efficiency (RN/DNI), *%*	47	50	52	55	1.1	0.14 (0.03)	0.75	<0.001
Fat balance *g/(kg$^{0.9}$ · d)*								
Gross fat intake	1.5	2.6	3.5	4.5	0.09	0.06 (0.002)	0.99	<0.001
Digestible fat intake (DFI)	1.3	2.4	3.3	4.2	0.07	0.06 (0.002)	0.99	<0.001
Retained fat (RF)	1.6	2.7	3.2	3.9	0.12	0.04 (0.003)	0.95	<0.001
Fat efficiency (RF/DFI), *%*	124	110	97	92	2.5	- 0.61 (0.06)	0.90	<0.001
Energy balance *kJ/(kg$^{0.8}$ · d)*								
Gross energy intake	338	330	322	313	8.3	- 0.43 (0.20)	0.33	0.05
Digestible energy intake (DEI)	271	279	284	285	6.6	0.30 (0.16)	0.27	0.08
Metabolizable energy intake	260	268	274	276	6.2	0.34 (0.15)	0.34	0.05
Heat production	152	126	108	92	4.5	- 1.11 (0.10)	0.92	<0.001
Retained energy	108	143	166	184	4.9	1.45 (0.12)	0.94	<0.001
Retained energy as protein	60	63	64	66	1.4	0.13 (0.03)	0.60	0.003
Retained energy as fat	48	80	102	118	4.5	1.32 (0.11)	0.94	<0.001

[1] Values are least-squares mean; (*n*=3). Linear regression results were reported when P<0.1. F30-S70, F50-S50, F65-S35, and F80-S20, respectively: diet with fat providing 28, 49, 65, and 81% of the non-protein digestible energy. F, fat; S, starch.

Table 4.4 Oxygen consumption and diet-induced oxygen use in rainbow trout fed diets with varying levels of fat and starch for 6 wk1

	Diets					Regression analysis		
	F30-S70	F50-S50	F65-S35	F80-S20	Pooled SEM	β (SE)	R^2	*P*-value
O_2 consumption, *mg O_2/(kg · min)*	5.26	5.10	4.95	4.67	0.11	- 0.01 (0.003)	0.61	0.003
O_2 consumption, *mg O_2/(kg$^{0.8}$ · min)*	3.28	3.21	3.13	2.99	0.07	- 0.005 (0.002)	0.48	0.013
Diet-induced oxygen use[2], *mg O_2/kJ DEI*	17.5	16.6	15.9	15.1	0.21	- 0.04 (0.005)	0.89	<0.001
Retained energy/oxygen consumed[3], *J/ mg O_2*	23	31	37	43	0.86	0.37 (0.02)	0.97	<0.001

[1] Values are least-squares mean, (*n*=3). F30-S70, F50-S50, F65-S35, and F80-S20, respectively: diet with fat providing 28, 49, 65, and 81% of the non-protein digestible energy. F, fat; S, starch.
[2] Diet-induced oxygen use = O_2 consumption (mg O_2/(kg$^{0.8}$ · d))/ digestible energy intake (DEI; kJ/(kg$^{0.8}$ · d)).
[3] Retained energy/oxygen consumed = Retained energy (RE; J/(kg$^{0.8}$ · d))/ O_2 consumption (mg O_2/(kg$^{0.8}$ · d)).

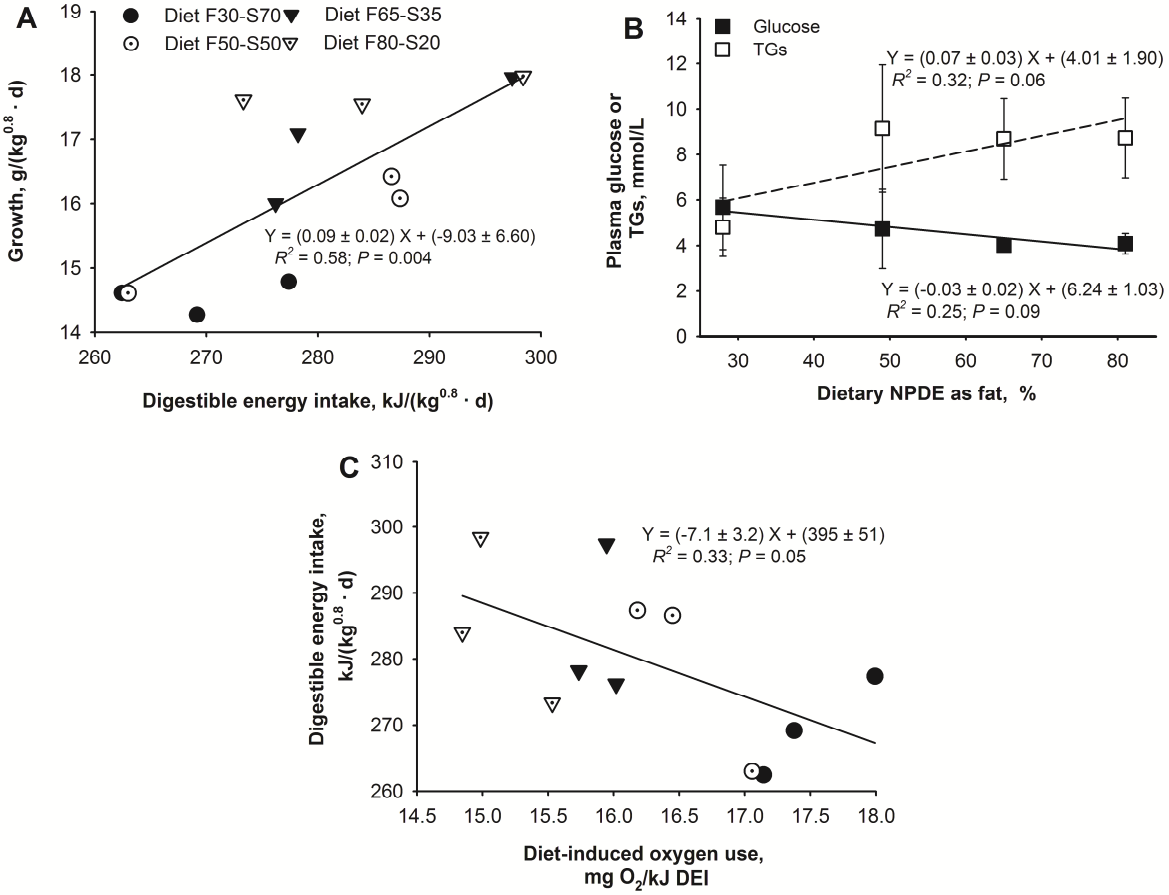

Figure 4.1 Relations between digestible energy intake and growth (**A**), percent of dietary NPDE as fat and 6 h postprandial plasma glucose and TGs concentration (**B**), and diet-induced oxygen use and digestible energy intake in rainbow trout fed diets with varying levels of fat and starch as NPDE sources for 6 wk. Values are mean ± SD, *n*=3 (**B**) or in equations, mean ± SE, *n*=12 (**A,C**). DEI, digestible energy intake; F30-S70, F50-S50, F65-S35, and F80-S20 respectively: diet with fat providing 28, 49, 65, and 81% of the NPDE; NPDE, non-protein digestible energy; TGs, triglycerides.

All measured parameters were tested for curvilinearity but for none was the quadratic function significant (*P*>0.10).

Discussion

The digestible energy intake (DEI) range in the present study is within that seen in our previous study with rainbow trout (211-311 kJ/($kg^{0.8} \cdot d$)), where DEI was found to be significantly increased following the replacement of starch by fat (Saravanan et al., 2012a). The present data confirm the effect of non-protein energy source on DEI, be it to a lesser degree since trout in the present study consumed a larger amount (dry matter) of the less energy-dense (starch) diets than of the high energy-dense (fat) diets. In general, literature in fish suggests that feed intake is regulated to meet the digestible energy requirement, or in other words, fish maintain a relatively constant DEI (Boujard, 1994; Cho, 1990; Lee and Putnam, 1973). However, the effect of diet on the digestible energy intake of fish in the present and also in other studies (Borges et al., 2009; Da Vies, 1989; Figueiredo-Silva et al., 2012a; Gélineau et al., 2001; Peres and Oliva-Teles, 1999; Saravanan et al., 2012a) contradicts with the above suggestion.

In mammals, the influence of dietary non-protein energy source on feed (energy) intake has been related to the direct effects of blood glucose (Mayer, 1955) and body fat level (Kennedy, 1953). The non-significant relation between DEI and postprandial plasma nutrient concentrations suggests that there were no effects of either postprandial (6 h) plasma glucose (P>0.1) or TGs concentration (P>0.1; data not shown) on DEI of the trout. The lack of a visible effect of glucostatic feedback on DEI has been previously suggested in rainbow trout (Saravanan et al., 2012a) and in Atlantic cod (Hemre et al., 1989). Also, an increased body fat level in the trout fed diets with a high fat level did not negatively affect DEI, as would be expected to occur via lipostatic feedback mechanisms. On the contrary, our data show slightly an increased DEI with increasing body fat content, in line with other reports in fish (Figueiredo-Silva et al., 2012c; Gélineau et al., 2001; Saravanan et al., 2012b; Saravanan et al., 2012a). This suggests a lesser impact of lipostatic feedback on DEI in the juvenile trout. Similarly, studies in mammals have shown that the intake of dietary fat energy exerts a weaker satiety effect than carbohydrates (Rolls and Hammer, 1995), resulting in a high DEI (Blundell et al., 1996) as also seen in fish (Encarnação et al., 2004; Saravanan et al., 2012a). This difference in the satiety effect between fat and starch has been associated with their post-absorptive metabolic fate, in particular the partitioning between storage and oxidation (Stubbs, 1996). Irrespective of the fat intake, trout in the present study predominantly deposited dietary fat into body fat with a high fat retention efficiency (>92%). This confirms the overall low utilization of ingested fat for energy production through oxidation and the large capacity of trout to store body fat without compromising DEI as previously reported in this species (Figueiredo-Silva et al., 2012c; Figueiredo-Silva et al., 2012b; Geurden et al., 2006; Saravanan et al., 2012a) and in other fish (Schrama et al., 2012).

The changes in non-protein energy source altered the diet-induced oxygen use which increased with increasing dietary starch and decreasing dietary fat, in accordance with our earlier observations in Nile tilapia (Saravanan et al., 2012b). This may be attributed to the differences in the metabolic use of absorbed glucose and fatty acids, for instance the synthesis of fat from dietary starch demanding more oxygen than from dietary fat (Reeds, 1982). The fat retention efficiency greater than 100% (110-124%) substantiates the occurrence of *de novo* lipogenesis in trout fed the high starch diets. This probably contributes to the high oxygen use of starch-rich diets, as seen in tilapia, which displayed even a higher fat retentions (>200%). Of interest, the present range in diet-induced oxygen use in trout (15.1-17.5 mg O_2/kJ DEI) is only slightly below the range found in tilapia (16.7-20.5 mg O_2/kJ DEI), despite the overall higher DEI and the differences in metabolic handling of starch in tilapia compared to trout (Figueiredo-Silva et al., 2012b).

Our previous studies suggested that the feed intake or DEI in fish can be constrained by a set-point value of oxygen consumption (on a time scale larger than weeks). This is based on the observations in trout and Nile tilapia that the feeding of diets differing in the macronutrient composition resulted in different DEI but with an equal heat production (Saravanan et al., 2012a; Tran-Duy et al., 2008) or with an equal oxygen consumption (Saravanan et al., 2012b). The trout in the present study however did not consume an equal amount of oxygen. This clearly suggests that oxygen consumption did not impose a physiological constraint on the feed intake or DEI in this study. Yet, DEI was negatively related to the diet-induced oxygen use. This is consistent with our previous data in Nile tilapia, providing further support to a possible role of diet-induced oxygen use in the regulation of feed intake in fish (Saravanan et al., 2012b). Still, the rate of decrease in DEI per unit increase in diet-induced oxygen use (i.e. slope of line) was 100% larger in tilapia (~14.4 kJ/(kg$^{0.8}$ · d per diet-induced oxygen use) than in the rainbow trout (~7.1 kJ/(kg$^{0.8}$ · d per diet-induced oxygen use). This difference in the slope warrants further confirmation, but may be related to inter-species differences with tilapia handling carbohydrates differently than rainbow trout at a moderately low protein intake (Figueiredo-Silva et al., 2012b).

In mammals, under thermoneutral condition, the consumption of a high protein diet result in a greater satiety together with the high diet-induced thermogenesis/energy expenditure (Veldhorst et al., 2008). This increase in satiety effect of a high-thermogenic protein diet at rest is conceived due to an increase in oxygen consumption and to a lesser extent by the body heat (thermogenesis) (Westerterp-Plantenga et al., 1999; Westerterp-plantenga et al., 1990). In the same way, DEI of the trout in the present study reduced with increasing diet-induced oxygen use (per unit of feed). This suggests a possible role for diet-induced oxygen use in the regulation of feed intake/DEI in

poikilotherms. In the view of the present results and bearing in mind the key-role of oxygen use as the basis of diet-induced thermogenesis, we hypothesize that also in homeotherms, in the absence of other potential intake constraints; the DEI is regulated by oxidative metabolism. Similarly, the satiating power of a nutrient has been proposed to be determined by their degree of hepatic oxidative metabolism as outlined in the hepatic oxidation theory in mammals (Allen and Bradford, 2012).

In order to create a strong contrast in the type of non-protein energy, the diet with the lowest level of fat had 49% of starch, which is uncommon in the feed for rainbow trout. The general consensus in fish nutrition that a carnivorous fish like rainbow trout are glucose intolerant is debatable (Moon, 2001). In the current study, none of the observed parameters showed a curvilinear response, which suggest no negative effect of high starch. Also, the postprandial plasma glucose concentrations (4.1 to 5.8 mmol/L) were well within the range of values (3.8 to 11 mmol/L) reported in the literature (Bergot, 1979; Kaushik and de Oliva Teles, 1985). Iso-energetic replacement of starch by fat in the diets always coincides with alterations in the dietary energy density. In this study, we chose not to include a dietary filler (e.g., cellulose), because of its possible effect on the feed intake at a high inclusion level (Bromley and Adkins, 1984). Consequently, the dietary concentrations differed also for other nutrients than starch and fat, but their ratio to digestible energy (e.g., digestible protein to digestible energy ratio) was kept comparable between the diets. As for all studies applying changes in the diet composition, the suggested impacts of type of non-protein energy source in the current study might also be due to other confounding changes in the diets (e.g., nutrient density, protein content etc.).

In summary, the present study shows that the DEI of trout increased with increasing replacement of dietary starch by fat as non-protein energy source, but to a lesser extent than previously reported for rainbow trout (Figueiredo-Silva et al., 2012c; Geurden et al., 2006; Saravanan et al., 2012a). In agreement with the observations in Nile tilapia (Saravanan et al., 2012b), the DEI was inversely related to diet-induced oxygen use, which suggests a possible role of diet-induced oxygen use in feed intake regulation as shown by the replacement of dietary fat by starch.

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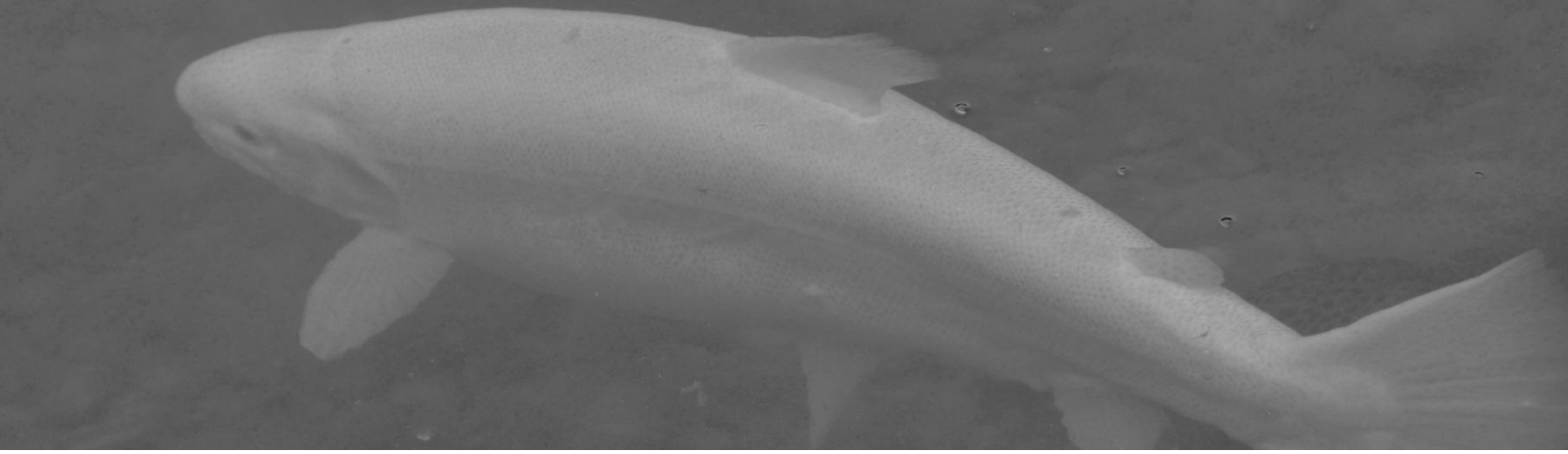

CHAPTER 5

Oxygen consumption constrains food intake in fish fed diets varying in essential amino acid composition

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Abstract

Compromisation of food intake when confronted with diets deficient in essential amino acids is a common response of fish and other animals, but the underlying physiological factors are poorly understood. We hypothesize that oxygen consumption of fish is a possible physiological factor constraining food intake. To verify, we assessed the food intake and oxygen consumption of rainbow trout fed to satiation with diets which differed in essential amino acid (methionine and lysine) compositions: a *balanced vs.* an *imbalanced amino acid diet.* Both diets were tested at two water oxygen levels: *hypoxia vs. normoxia*. Trout consumed 29% less food under hypoxia compared to normoxia ($p < 0.001$). Under both hypoxia and normoxia trout significantly reduced food intake by 11% and 16% respectively when fed the imbalanced compared to the balanced amino acid diet. Oxygen consumption of the trout per unit body mass remained identical for both diet groups not only under hypoxia but also under normoxia ($p > 0.05$). This difference in food intake between diets under normoxia together with the identical oxygen consumption supports the hypothesis that food intake in fish can be constrained by a set-point value of oxygen consumption, as seen here on a six-week time scale.

Introduction

The majority of animals, including fish, show a reduction in food intake when the food has an imbalanced essential amino acid composition (D'Mello, 2003; De la Higuera, 2001; Fortes-Silva et al., 2012). It is not clear which physiological factor (constraint) forces an animal to compromise its food intake when confronted with a dietary amino acid deficiency (Potier et al., 2009). In rodents, physiological factors such as changes in postprandial blood and brain concentration of free amino acids and ammonia (liberated by deamination) down regulate food intake when fed with amino acid deficient diets (Anderson, 1979; Gietzen et al., 2007; Mellinkoff et al., 1956; Noda and Chikamori, 1976; Peters and Harper, 1985). Intake of an amino acid imbalanced diet results in a less efficient use of amino acids for protein synthesis. The most limiting amino acid is best utilised while others get wasted, which leads to a greater ammoniagenesis and ureagenesis. The carbon remnants of these amino acids are either oxidized or used in *de novo lipogenesis*, which increases the oxygen consumption (Elango et al., 2008; Kaczanowski and Beamish, 1996). Compared to terrestrial animals, oxygen is a relatively scarce resource for fish and moreover, gill breathing in water requires more energy than air-breathing (Kramer, 1987).

Therefore, we hypothesize that oxygen consumption is one of the possible physiological factors (constraints) which can limit food intake in fish. We propose that even at normoxic conditions and in the absence of other potential constraints on food intake, food intake in fish can be constrained by a set-point value of oxygen consumption (on a time scale of larger than weeks). In the present study, we assessed the food intake and oxygen consumption of rainbow trout fed diets, which were contrasting in their composition of two essential amino acids (methionine and lysine; *a balanced vs. an imbalanced diet*). Both diets were tested at two different water dissolved oxygen (DO) concentrations (*hypoxia vs. normoxia*). The conceptual illustration of the hypothesis tested is shown in figure 1. The dietary deficiency of both lysine and methionine in the current study was used with the purpose to create a contrast in oxygen consumption per unit food intake (i.e. a difference in slope as depicted in fig. 5.1). In addition to normoxia, we measured food intake and oxygen consumption under hypoxia as a positive control in order to verify the effect of dietary amino acid induced changes in oxygen consumption on food intake, at limiting water DO levels.

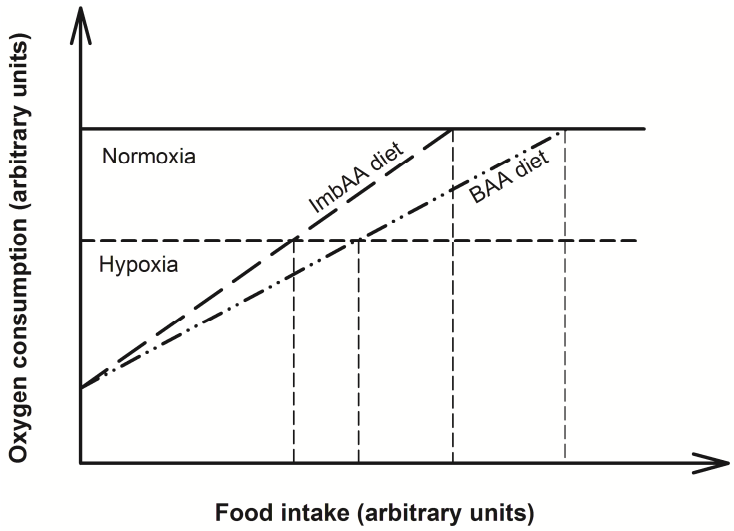

Figure 5.1 Conceptual illustration of the hypothesis tested in the present study

It is hypothesized that under non-limiting water oxygen level (normoxia) food intake of fish fed a diet deficient in essential amino acids is compromised by a physiological constraint in oxygen consumption. To test the hypothesis, fish were fed under normoxia with diets contrasting in essential amino acids (lysine and methionine) composition: an imbalanced (ImbAA) *vs.* a balanced amino acid (BAA) diet. The difference in amino acid composition of the diet is expected to create differences in metabolism, which will alter the amount of oxygen consumption per unit of food intake. This amount of oxygen consumption per unit of food is higher at the ImbAA diet than at the BAA diet (as indicated by the differences in the slope of lines). As such: 1) If oxygen consumption is constraining the food intake, then the food intake between ImbAA and BAA fed fish will be different but the oxygen consumption will be similar; 2) If oxygen consumption is not constraining the food intake, then food intake between ImbAA and BAA fed fish will be different but also the oxygen consumption. Further, to verify the effect of dietary amino acid induced changes in oxygen consumption on food intake, we measured food intake under limiting water oxygen level (hypoxia) as a positive control.

Materials and Methods

Ethics statement

This study complied with the Dutch law on experimental animals and was approved by the ethical committee for animal experiments of Wageningen University (DEC: 2011016.d).

Fish and housing conditions

Three hundred and sixty juvenile rainbow trout (*Oncorhynchus mykiss;* Mohnen Aquaculture, Germany) with an average initial body weight of 52.5 g ± 1.2 g (mean ± SD) were randomly allocated among 12 tanks (30 fish tank^{-1}) of the aquatic metabolism unit (De Haar Vissen, Wageningen University). The tanks were connected to a closed

recirculation system, equipped to adjust and monitor water flow and oxygen concentration in the inflow water of each tank of the fish housing facility, as for details reported elsewhere (Saravanan et al., 2012). Throughout the experiment, fish were reared under the following conditions: photoperiod (12 L: 12 D), water temperature (13.8 ± 0.4°C), pH (7.5 ± 0.3), conductivity (2.9 ± 0.2 mS cm^{-1}), nitrite (<0.08 mg N l^{-1}), nitrate (<32 mg N l^{-1}), total-NH_3-N (<0.15 mg N l^{-1}).

Treatments

Treatments were designed in a 2 by 2 factorial setup with dietary amino acid composition and water dissolved oxygen (DO) levels as main factors, each consisting of two levels; an amino acid 'balanced' *vs.* 'imbalanced' diet and 'normoxia' *vs.* 'hypoxia', respectively. The contrast in dietary amino acid composition was made sufficiently large in order to alter the oxygen consumption required per unit of food, being 'high' with an imbalanced (deficient) amino acid diet and 'low' with a balanced (adequate) amino acid diet. Thus, two diets differing in the limiting amino acid content (i.e. lysine and methionine) were prepared: a balanced amino acid diet which meet the lysine (5.1 g per 16 g N) and methionine (2.4 g per 16 g N) requirement for rainbow trout (NRC, 2011), and an imbalanced amino acid diet which was 47% deficient in lysine (2.7 g per 16 g N) and 31% deficient in methionine (1.6 g per 16 g N) requirement. The ingredient composition of the experimental diets is given in table 5.1. The diets were prepared (Research Diet Service B.V., Wijk bij Duurstede) by extrusion process (Clextral BC45, twin screw extruder) with a 3 mm die, dried (70°C for 3 h), vacuum coated with oil and stored at 4°C. The dietary nutrient compositions are shown in table 5.2.

The difference in dissolved oxygen (DO) level in the water was created by adjusting the rate of water flow into the tanks. The water volume was kept constant at 200 l in all tanks. For the normoxia groups, the rate of water inflow into tank (mean ± SD) was kept at 7.9 ± 0.03 l min^{-1} with mean water DO level of 10.2 ± 0.2 mg l^{-1}, and the mean DO content in the outflowing water remained above 8.0 mg l^{-1}. When necessary, pure oxygen was injected into the inflow water in order to maintain outflow DO content above 8.0 mg l^{-1}. The hypoxia condition was created by reducing the rate of water inflow (1.94 ± 0.02 l min^{-1}) with a mean DO content of 9.8 ± 0.2 mg l^{-1}, and the mean DO content in the outflowing water remained below 6.0 mg l^{-1} and above 4 mg l^{-1}. The level of DO content for the hypoxia treatment was decided based on the reported incipient DO level of about 6.0 mg l^{-1} for rainbow trout (Pedersen, 1987). The applied DO level in hypoxia treatment is expected to reduce food intake but with very minimal level of discomfort for rainbow trout. The welfare of fish was assessed daily by observing the food intake (at tank level) and general behaviour of fish.

Table 5.1 Ingredient composition of the experimental diets

Ingredients (%)	Balanced diet	Imbalanced diet
Wheat gluten	26	26
Soy protein concentrate	14	14
Lysine HCl	1.3	-
DL-methionine	0.4	-
L-glutamic acid	-	1.7
Gelatinized maize starch*	11	11
Wheat	28.5	28.5
Fish oil†	11.8	11.8
Mono-calcium phosphate	3	3
Calcium phosphate	1	1
Diamol‡	2	2
Vitamin-mineral premix§	1	1

* Gelatinised maize starch (Merigel® 100; Amylum Group).
† Fish oil (999 Fish Oil; Triple Nine Fish protein).
‡ Diamol (acid-insoluble ash, as inert marker for digestibility measurement) – Diamol GM; Franz Bertram.
§ Vitamin-mineral premix composition is reported elsewhere (Saravanan et al., 2012)

Experimental procedure

The treatments were randomly assigned among 12 tanks to have triplicates for each treatment group. During the experimental period of 6 weeks, fish were hand-fed with their respective diets twice daily to apparent satiation for an hour (09.00-10.00 and 16.00-17.00 hrs). At each feeding session, feed given and uneaten feed were recorded; in addition, uneaten pellets were collected and counted to determine the food intake accurately. Faeces were collected to determine nutrient digestibility in a similar way as described earlier (Saravanan et al., 2012). The oxygen consumption of fish was monitored for the entire experimental period. The concentration of oxygen in the inlet and outlet of each tank was automatically measured at 5 min intervals using an electrode (WTW-Trioximatic® 700 IQ, WTW GmbH, Weilheim, Germany) and data were recorded in a personal computer using interface (HTBasic, Version 9.5, TransEra Corp.). The oxygen measurement was performed in a continuous cycle of 2 consecutive days (48 h; from 08.00 to 08.00 hrs) in a set of four tanks comprising all treatments. Consequently, in 6 days, oxygen concentrations were measured in all the 12 tanks. The oxygen electrode was calibrated once every week. In addition, one continuous 48 h

Table 5.2 Analysed nutrient and amino acid composition of the amino acid imbalanced and balanced diets

	Balanced diet	Imbalanced diet
Dry matter (DM; g kg^{-1})	976	965
Crude protein (g $kgDM^{-1}$)	388	377
Crude fat (g $kgDM^{-1}$)	144	137
Starch (g $kgDM^{-1}$)	296	301
Ash (g $kgDM^{-1}$)	73	72
Digestible energy* (kJ gDM^{-1})	17.95	17.52
Amino acid composition (% crude protein)		
Arginine	3.94	4.11
Histidine	1.80	1.88
Isoleucine	3.32	3.53
Leucine	6.03	6.37
Lysine	***5.13***†	***2.73***
Methionine	***2.35***†	***1.62***
Phenylalanine	4.23	4.46
Threonine	2.53	2.68
Tryptophan	0.77	0.85
Valine	3.58	3.82
Cysteine	1.52	1.64
Tyrosine	2.58	2.73
Glutamic acid	25.03	30.58

* Digestible energy determined under normoxia treatment of the present study (Saravanan et al., 2012)
† For rainbow trout the estimated requirement for lysine, vary from 4.5 to 6.3% crude protein (NRC, 2011) and for methionine, it varies from 1.8 to 2.14% crude protein depending on level of cysteine (Kim et al., 1992; NRC, 2011).

(from 08.00 to 08.00 hrs) measurement of total ammonia nitrogen was performed in all the 12 tanks.

The water was continuously sampled at 3 min intervals from a common inlet and outlet of each tank using an auto-sampler (SANplusSYSTEM, Skalar, The Netherlands) and the concentration of total ammonia nitrogen was determined with colorimetric method (Krom, 1980) following the manufacturer's (Skalar) protocol. The oxygen consumption (mg $kg^{-0.8}$ h^{-1}) (Saravanan et al., 2012) and the total ammonia nitrogen excretion (mg N $kg^{-0.8}$ d^{-1}) (Kaushik, 1980) of fish were calculated using the formula as described

previously. Fish were weighed under sedation (0.25 ml l^{-1}, 2-phenoxy ethanol) at the start and end of the experiment 36 h after the last meal. Fish used to determine initial (15 fish) and final body composition (8 fish $tank^{-1}$), and the remaining fish at the end of experiment were euthanized by an overdose of anaesthesia (1 ml l^{-1}, 2-phenoxy ethanol). Fish samples for body composition were stored at -20°C until further analysis.

Chemical analysis

Whole fish samples were pooled per tank, ground, and subsequently freeze-dried before analysis. Analyses of fish were done in triplicates for dry matter (105°C for 24 h), crude protein (Kjeldahl; N x 6.25), crude fat (Soxhlet; 40-60ºC) and energy (Bomb calorimetry) as described previously (Saravanan et al., 2012). Nutrient compositions of the diets were determined using the same methods. The amino acid composition of the diets were analysed (AGROBIO, Rennes, France) in an amino acid analyser (Biochrom 30; Pharmacia Biochrom Ltd) according to standard methods (Moore and Stein, 1951).

Calculation and statistical analysis

All parameters of fish growth performance, food intake and body composition were calculated as per formulae mentioned earlier (Saravanan et al., 2012). Values were expressed as mean ± SD. Two-way ANOVA was used to assess the effect of dietary amino acid composition (diet), water DO level and their interaction (PROC GLM; SAS 9.2, SAS Institute) and was followed by post-hoc Tukey test, if interaction was significant ($p < 0.05$). Normal distributions of the residuals were verified using Kolmogorov-Smirnov test (PROC UNIVARIATE).

Results

Food intake and growth

Food intake of trout was clearly affected by the diet ($p < 0.001$; fig. 5.2A). Under hypoxia, the food intake of trout was 11% lower when fed the imbalanced compared to the balanced amino acid diet. Similarly, under normoxia, the food intake was 16% lower for fish fed the imbalanced compared to the balanced diet. Regardless of the dietary amino acid composition, trout kept under hypoxia showed a 29% lower food intake than under normoxia (fig. 5.2A). The difference in food intake of trout between both diets was greater under normoxia than hypoxia, as indicated by the significant interaction between water DO level and diet. The digestible protein intake and digestible energy intake of trout paralleled the food intake. The intakes of specific amino acids (glutamic acid, lysine, and methionine) of trout were in line with the created contrast in amino acids between the diets (table 5.3).

At end of the 6 weeks, the survival of fish was above 98% and was not different between treatments (p = 0.73). Trout fed the amino acid balanced diet had better growth than trout fed the amino acid imbalanced diet. Likewise, trout kept under normoxia had a larger final body weight and higher growth compared to fish kept under hypoxia (table 5.3).

Body composition and nutrient utilisation

As expected, the difference in amino acid composition of the diet altered the nitrogen utilisation by the rainbow trout as well as their body composition (table 5.3). The total ammonia nitrogen excretion per unit digestible protein intake was affected by the diet (p = 0.006), being 45% higher in trout fed the imbalanced than in those fed the balanced amino acid diet. The protein retention efficiency was thus significantly lower (24%) in trout fed the imbalanced than in those fed the balanced amino acid diet. Both nitrogen excretion and retention efficiency were unaffected by the water DO level and its interaction with diet (p > 0.05; table 5.3). Both under hypoxia and normoxia, trout fed the amino acid imbalanced diet showed 14% more body fat than fish fed the balanced diet, despite their lower food intake.

Oxygen consumption

The oxygen consumption of trout was lower under hypoxia than normoxia, which was due to the applied contrast in water DO levels (fig. 5.2B). Under hypoxia, the oxygen consumption of trout was identical at the balanced (142.5 mg $kg^{-0.8}$ h^{-1}) and imbalance amino acid (142.6 mg $kg^{-0.8}$ h^{-1}) diet (p = 0.36). Despite the differences in food intake under normoxic conditions, the oxygen consumption of trout fed the balanced (171.9 mg $kg^{-0.8}$ h^{-1}) or the imbalanced amino acid (169.2 mg $kg^{-0.8}$ h^{-1}) diet were equal (p = 0.36). There was no significant interaction effect between water DO level and diet on the oxygen consumption of trout (p = 0.31).

Discussion

We found that irrespective of the water DO levels, the rainbow trout fed the amino acid imbalanced diet did not increase food intake to compensate for the inadequate amount of lysine and methionine supplied by this diet. Growth was reduced in trout fed the amino acid imbalanced diet, in agreement with data from most of the amino acid requirement studies in fish (Kim et al., 1992; Rodehutscord et al., 1997; Rumsey et al., 1983; Walton et al., 1984). In mammals, the capacity to sense specific nutrients in the diet has been well documented (Gietzen et al., 2007). Similarly, the changes in diet selection pattern when fed diets differing in amino acid composition have been reported in some fish species (Fortes-Silva et al., 2012; Yamamoto et al., 2001). The difference in food intake between the amino acid balanced and imbalanced diet groups validates that

trout are able to detect the presence of specific dietary essential amino acid. Nevertheless, our aim was to understand the mechanisms involved in the reduced food intake of trout fed the imbalance compared to the balanced amino acid diet, and this under conditions of contrasting water DO levels (hypoxia *vs.* normoxia).

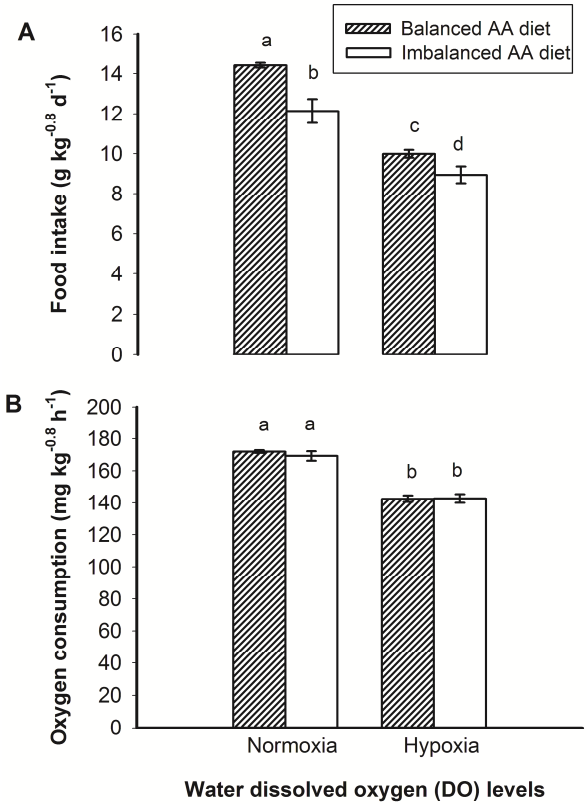

Figure 5.2. Effect of diet and dissolved oxygen on food intake and oxygen consumption in rainbow trout. Rainbow trout fed to satiation with a balanced amino acid diet and an imbalanced amino acid diet at two levels of water dissolved oxygen (DO): hypoxia *vs.* normoxia. (**A**) Food intake was affected by dietary amino acid composition ($p < 0.001$), water DO level ($p < 0.001$), and the interaction between both factors ($p = 0.02$). (**B**) Oxygen consumption was affected by water DO level ($p < 0.001$) but unaffected by dietary amino acid composition ($p = 0.36$) and the interaction between both factors ($p = 0.31$). Values are mean ± SD ($n = 3$, group of 30 fish tank^{-1}).

Regulation of food intake in fish is influenced by various physiological conditions, which interact with dietary and environmental factors (Fletcher, 1984). Among environmental factors, the water DO level is known to influence food intake and growth of fish (Buentello et al., 2000; Davis, 1975; Evans, 2007; Glencross, 2009; Mallekh and Lagardère, 2002; Pichavant et al., 2001; Thetmeyer et al., 1999; van Dam and Pauly, 1995), as confirmed in the current study by the lower food intake in trout under hypoxia

compared to normoxia. Reduced food intake under limited DO conditions (hypoxia) has been explained by a reduced metabolic scope of oxygen for aerobic activities and metabolism, including those related to food processing (Glencross, 2009; Tran-Duy et al., 2008). Our data further show an effect of dietary amino acid composition on food intake under hypoxia. The lower food intake in trout fed the imbalanced amino acid diet relative to the balanced diet, while accompanied by similar oxygen consumption, is possibly caused by the imposed limitation in oxygen availability combined with increased oxidative metabolism for nutrient processing when fed the amino acid imbalanced diet. Indeed, averaged over both hypoxia and normoxia, the oxygen consumption per gram digestible protein intake (DPI) of trout fed the amino acid imbalanced diet was 992 mg O_2 per g DPI, which was about 18% higher than when fed the amino acid balanced diet (841 mg O_2 per g DPI).

The higher oxygen consumption in the amino acid imbalanced diet groups is likely to be attributed to an obligatory increase in amino acid deamination and the further processing of amino acid carbon skeleton towards *de novo lipogenesis* (Kaczanowski and Beamish, 1996). This is confirmed in the current study by the higher ammonia excretion, lower nitrogen retention and increased body fat content of trout fed the imbalanced compared to the balanced amino acid diet. Thus, the higher oxygen demand for metabolism along with the imposed limitation of oxygen availability under hypoxia is likely to constrain the food intake of trout, explaining the lower intake of imbalanced amino acid diet.

Of interest, exactly similar observations were made under normoxia as under hypoxia. Even at non-limiting water DO level, rainbow trout reduced their food intake when fed the imbalanced amino acid diet compared to the balanced diet. Moreover, even under normoxia, trout of both dietary groups displayed identical oxygen consumption, although the oxygen consumption was higher than those seen under hypoxia. Unlike under hypoxia, this diet-specific reduction in food intake points toward a constraint imposed by factors other than by the availability of water DO (i.e., supply of oxygen to the fish).

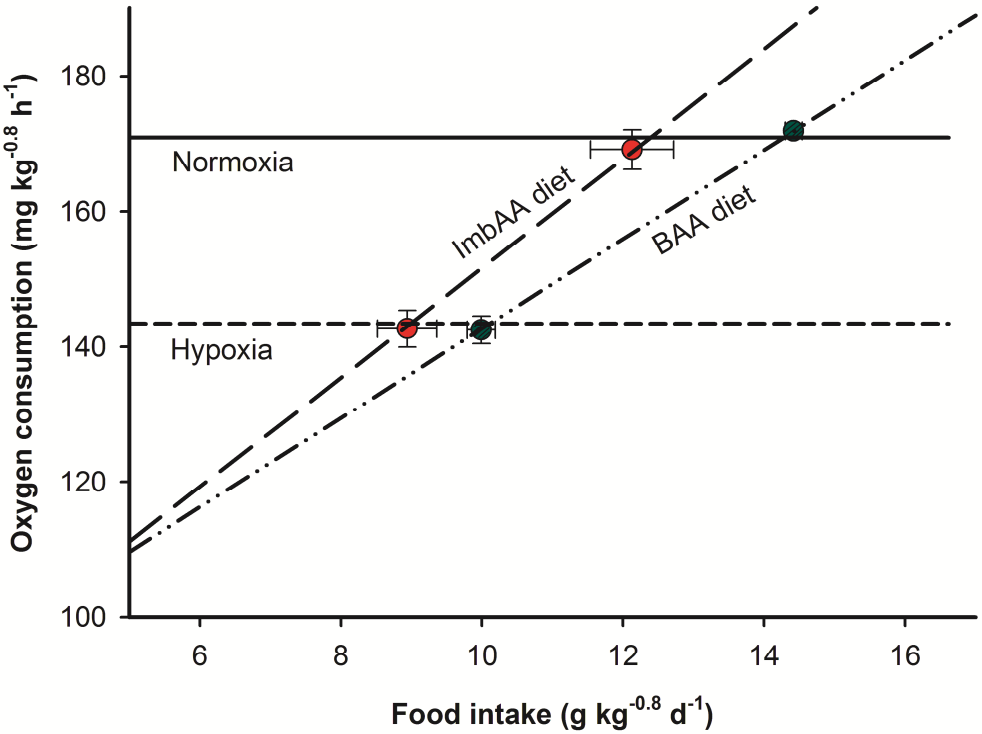

Figure 5.3 Food intake and oxygen consumption of trout in relation to the proposed hypothesis (figure 5.1). The measured food intake and oxygen consumption of rainbow trout fed to satiation with a balanced amino acid diet and an imbalanced amino acid diet at two levels of water dissolved oxygen: hypoxia *vs.* normoxia. Values are mean ± SD (n = 3).

As mentioned above, the metabolic handling of the amino acid imbalanced diet under normoxia also requires higher oxygen consumption per unit of digested protein intake compared to the balanced diet. As such, the equal oxygen consumption of trout in both the dietary groups together with the differences in voluntary food intake (fig. 5.3) suggests that the oxygen consumption may act as a physiological factor constraining food intake. This is in conformity with our hypothesis as shown in figure 1, that there is set-point oxygen consumption over a period of weeks. The question remaining is what dictates the set-point oxygen consumption. An increased oxidative metabolism has been suggested to exert a negative effect on the organism with cellular damage, for instances due to the production of reactive oxygen species (Dowling and Simmons, 2009). This implies the oxygen consumption as an intrinsic cost of food intake (Illius et al., 2002; Ketelaars and Tolkamp, 1996). Therefore, reducing food intake of a high oxygen demanding diet, such as the amino acid imbalanced diet might be a strategy of fish to avoid the negative effects related to increased oxygen use. In support of the present results, a previous study (Saravanan et al., 2012) in Nile tilapia similarly showed an equal oxygen consumption (in three out of four dietary treatments) together with altered intake levels of diets which differed in the amount of oxygen consumed per unit

of digestible energy intake, created by changes in dietary macronutrients. This and the present findings (fig. 5.3) of diet-induced differences in food intake in concert with similar oxygen consumptions, obtained at normoxia in both species, suggest that physiological constraints related to oxygen consumption might play a role in the control of food intake in fish, even under non-limiting DO conditions.

Table 5.3 Fish performance, metabolic parameters and body composition of rainbow trout fed to satiation with balanced amino acid diet and imbalanced amino acid diet at two levels of water dissolved oxygen: hypoxia vs. normoxia for 42 days.

	Normoxia		Hypoxia			*P* value		
	Balanced diet	Imbalanced diet	Balanced diet	Imbalanced diet	Pooled SEM	Water DO level	Diet	Water DO x Diet
Fish performance								
Initial body weight (g)	53.0	52.0	53.3	51.9	0.72	0.844	0.143	0.828
Final body weight (g)	144.4^{a}	109.5^{b}	110.8^{c}	92.5^{b}	2.52	<0.001	<0.001	0.011
Growth (g $kg^{-0.8}$ d^{-1})	15.3^{a}	10.8^{b}	10.7^{b}	8.2^{c}	0.29	<0.001	<0.001	0.010
Food intake (g DM $fish^{-1}$ d^{-1})	2.1^{a}	1.5^{b}	1.3^{c}	1.1^{d}	0.04	<0.001	<0.001	0.006
Digestible energy intake (kJ $kg^{-0.8}$ d^{-1})	259^{a}	213^{b}	185^{c}	162^{d}	4.1	<0.001	<0.001	0.021
Digestible protein intake (g $kg^{-0.8}$ d^{-1})	5.4^{a}	4.4^{b}	3.7^{c}	3.2^{d}	0.08	<0.001	<0.001	0.014
Glutamic acid intake (mg $kg^{-0.8}$ d^{-1})	1400^{a}	1399^{a}	970^{b}	1031^{b}	24.9	<0.001	0.263	0.249
Lysine intake (mg $kg^{-0.8}$ d^{-1})	287^{a}	125^{b}	199^{c}	92^{d}	2.5	<0.001	<0.001	<0.001
Methionine intake (mg $kg^{-0.8}$ d^{-1})	131^{a}	74^{b}	91^{c}	55^{d}	1.4	<0.001	<0.001	<0.001

Table 5.3 Fish performance, metabolic parameters and body composition of rainbow trout fed to satiation with balanced amino acid diet and imbalanced amino acid diet at two levels of water dissolved oxygen: hypoxia vs. normoxia for 42 days (continued)

	Normoxia		Hypoxia			*P* value		
	Balanced diet	Imbalanced diet	Balanced diet	Imbalanced diet	Pooled SEM	Water DO level	Diet	Water DO x Diet
Metabolic parameters								
Total ammonia nitrogen loss (mg N per g DPI)	50	73	49	71	6.2	0.779	0.006	0.965
Protein retention efficiency (%)*	44.9	34.3	45.2	33.9	0.48	0.998	<0.001	0.482
Final body composition*								
Protein (g kg^{-1})	164.3^{a}	145.1^{b}	164.2^{a}	140.1^{b}	0.94	0.028	<0.001	0.033
Fat (g kg^{-1})	108.0	123.0	99.2	113.1	3.36	0.024	0.003	0.882

SEM, Standard error mean; DO, dissolved oxygen; DM, dry matter; DPI, digestible protein intake.
Mean values in a row with unlike superscript were significantly different and assigned only if the interaction effect was significant ($p < 0.05$).
* Protein retention efficiency (%) = (Wet protein gain/ protein intake in dry weight) x 100
** Initial body composition (g kg wet $weight^{-1}$): 157, protein and 80.5, fat.

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CHAPTER 6

Effect of dietary macronutrients on within-day variation in feed intake of Nile tilapia

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Abstract

In the present study we investigated the effect of dietary protein level and non-protein energy source (fat vs. starch) on the within-day variation (morning vs. afternoon) in feed intake and digestible energy (DE) intake of Nile tilapia. Triplicate groups (20 fish/tank) of fish were fed with one of the four iso-energetic diets contrasting in protein level (high; ~27 mg digestible protein/kJ DE vs. low; ~14 mg digestible protein/kJ DE) and in the type of non-protein energy source ('starch' vs. 'fat') for 48 days. Fish were hand-fed twice daily in morning (09.00 hrs) and afternoon (16.00 hrs) to apparent satiation for an hour. Apparent nutrient digestibility, oxygen consumption, and non-fecal nitrogen (ammonia and urea) excretion were measured. The feed intake of tilapia was affected by the dietary protein level and NPE source, and their impact differed between feeding sessions (morning vs. afternoon) ($P<0.01$). Feed intake in the morning and afternoon feeding were respectively affected by the type of dietary NPE source and protein level ($P<0.05$). At morning, feed and digestible energy intake was higher in group fed the diets with fat as NPE source than starch, regardless of protein levels in the diet. These differences in intake at the morning feeding were not related to the pre-feeding oxygen consumption of tilapia ($P>0.05$). Compared to the low protein diet, tilapia fed the high protein in the morning showed a significant reduction (10%) in digestible energy intake during afternoon feeding. The digestible energy intake of tilapia at afternoon feeding was inversely related to the two and one hour pre-feeding oxygen consumption ($P<0.05$). Together, these results suggest that the pre-feeding metabolic status (oxygen consumption) of the fish influences the within-day variation in feed intake.

Introduction

To attain the full growth potential, fish needs to be fed to their level of daily voluntary feed intake. Under farming conditions, fish are often fed twice or thrice daily in feeding sessions with a given amount of feed ration. Fish fed with different diets to satiation or given access to self-feeding show differences in feed intake between feeding sessions of a day (Gélineau et al., 2002; Tran-Duy et al., 2008). However, factors that determine these differences in feed intake remain little understood. Therefore, understanding the factors affecting feed intake of fish between feeding sessions of a day will help in better prediction of the feed ration, which is important for feed management under practical conditions of fish farming. Feeding in fish usually follows a diel (24 h) pattern, with distinct active feeding moments directed by the endogenous biological rhythm (Aranda et al., 1999; Bolliet et al., 2001; López-Olmeda and Sánchez-Vázquez, 2010; Sánchez–Vázquez et al., 1999). A number of physiological, dietary and environmental factors affect the feeding frequency and daily feed intake (Houlihan et al., 2001). Several studies in fish have focused on factors such as water temperature, dietary energy density, dietary bulk and their impact on physical limitation of stomach and the rate of gut evacuation/ emptying in relation to feed intake in a meal (Elliott, 1975; Fletcher, 1984; Jobling, 1980; 1981; Pérez-Casanova et al., 2009; Ruohonen et al., 1997; Sánchez-Vázquez et al., 2001; Shiau et al., 1988). However, the impact of dietary macronutrients on within-day differences in feed intake (i.e., between feeding sessions of a day) is relatively less studied in fish. In mammals, the impact of dietary macronutrients on food intake between meals is well documented (Anderson and Moore, 2004; Saris and Tarnopolsky, 2003). For instance, the intake of protein, fat, or carbohydrate rich diet in a meal is shown to differently affect food intake in subsequent meal, which is linked to different metabolic effects (e.g., satiety) induced by dietary macronutrients (Halton and Hu, 2004).

We have recently reported that macronutrient composition of the diet affects total daily feed and digestible energy (DE) intake in Nile tilapia (Saravanan et al., 2012). The focus of the aforementioned study was primarily on the long-term (weeks) regulation of feed intake in fish without addressing the effects on the short-term (within-day) regulation of feed intake. The objectives of the present study are: (i) to assess the effect of dietary macronutrient composition (protein level and non-protein energy source) on within-day variation (morning *vs.* afternoon) in feed intake of Nile tilapia and (ii) to find whether these relate to the pre-feeding metabolic status of the fish in terms of oxygen consumption and non-fecal nitrogen (ammonia and urea) excretion.

Materials and Methods

Here we mention only the important information on materials and methods relevant to this present study and for specific details of the experiment the reader is re-directed to refer our previous study (Saravanan et al., 2012). The present study was approved by Wageningen University Animal Experimental Committee and conducted in accordance with the Dutch law on experimental animals.

Diets

Briefly, four iso-energetic diets (~19 kJ DE/g) were formulated (2x2 factorial setup) to have contrast in (i) amount of protein: high (HP; ~27 mg digestible protein/kJ DE) *vs.* low (LP; ~14 mg digestible protein/kJ DE) and (ii) type of non-protein energy (NPE) source: fat (F) *vs.* starch (S) which were iso-energetically exchanged. Thus, four diets having either a high protein with fat (HPF) or starch (HPS) as NPE source or a low protein with fat (LPF) or starch (LPS) as NPE source. Details on ingredient composition, diet preparation, and nutrient analysis have been reported earlier (Saravanan et al., 2012). To ensure an identical nutrient and energy density between the diets, cellulose was used as ingestible filler in the fat diets. The analyzed nutrient compositions of the four diets are presented in Table 6.1.

Experimental procedure

A group of 240 juvenile Nile tilapia (*Oreochromis niloticus*) with mean body weight of 40.6 ± 0.8 g per fish (mean±SD) was randomly allocated among 12 metabolic tanks (20 fish/tank). Each experimental diet was fed to triplicate groups of fish for 48 days. Fish were hand fed with their respective diets twice daily to apparent satiation for an hour in morning (09.00 to 10.00 hrs) and afternoon (16.00 to 17.00 hrs). At the end of each feeding session, the uneaten pellets were collected and counted to determine the feed intake accurately. Thus, the feed intake of fish was recorded separately during morning and afternoon feeding. Fish were weighed at the beginning and at the end of the study to determine growth and weight gain. Apparent digestibility of diets were determined to calculate digestible energy intake of the fish (Saravanan et al., 2012). Throughout the experiment, the fish were reared under optimal water quality parameters: photoperiod (12L:12D), mean water temperature (28 ºC), water flow in tank (7.0 l/min), dissolved oxygen (8.8 mg/1), pH (6.8) and conductivity (2.8 mS/cm). During the experiment, within-day oxygen consumption and non-fecal nitrogen excretion (ammonia and urea) of fish were determined. The concentration of oxygen in inlet and outlet of each tank was automatically measured at 5 min intervals using an electrode (WTW-Trioximatic® 700 IQ, WTW GmbH, Weilheim, Germany) and the data

Table 6.1 Analyzed nutrient composition (g/kg dry matter) of experimental diets fed to Nile tilapia

	Diets [b]			
	HPF	HPS	LPF	LPS
Dry matter (g, DM)	963	931	946	925
Crude protein	534	541	295	299
Crude fat	170	70	232	132
Starch	38	294	234	476
Ash	74	77	73	73
Digestible protein	502	514	279	281
Digestible energy (kJ/g)	18.6	19.5	18.6	19.5
DP/DE (mg/kJ)	27.0	26.4	15.0	14.4

[a] Diet formulation, ingredient, and digestible nutrient composition of diet are reported previously (Saravanan et al., 2012)
[b] HPF – high protein diet with fat as NPE source; HPS - high protein diet with starch as NPE source; LPF - low protein diet with fat as NPE source; LPS - low protein diet with starch as NPE source.
DP/DE, digestible protein to digestible energy ratio

were recorded in a personal computer using interface (HTBasic, Version 9.5, TransEra Corp.). Similarly, water was continuously sampled at intervals of 3 min from a common inlet and from the outlet of each tank using an auto-sampler (SANplusSYSTEM, Skalar, The Netherlands) and the concentration of total ammonia nitrogen (TAN) and urea-nitrogen determined with colorimetric methods (Krom, 1980; Wybenga et al., 1971) following the manufacturer's (Skalar) protocol. The oxygen consumption was measured in a continuous cycle of 2 days (48 h; from 08.00 to 08.00 hrs) in a set of four tanks consisting of all the diet groups. Thus, over 6 days, oxygen consumption was measured in all 12 tanks and during the entire growth study, 5 cycles of 48 h oxygen consumption measurements were determined for each tank. Similarly, a continuous 48 h (from 08.00 to 08.00 hrs) measurement of TAN and urea were performed in all 12 tanks to determine the non-fecal nitrogen (branchial and urinary nitrogen, BUN) loss.

Calculation and statistics

Feed and digestible energy intake, and oxygen consumption and nitrogen excretion (Kaushik, 1980) of fish were calculated using the formula as described previously (Saravanan et al., 2012). Statistical analyses were performed using SAS 9.2 (SAS Institute, Cary, NC, USA). The effect of feeding session, protein level, and NPE source on feed and digestible energy intake was tested by F-tests using 3-way ANOVA, with mean feed and digestible energy intake within a tank taken as repeated measurements. At

each feeding session, the effect of protein level, NPE source, and their interaction on all parameters were tested using 2-way ANOVA for non-repeated measurements. The relation between pre-feeding hourly oxygen consumption of the fish and digestible energy intake at each feeding session was tested (PROC CORR). For all tests, the level of significance was set at $P<0.05$.

Results

Feed and digestible energy intake

The feed and digestible energy intake of tilapia was affected by the 3-way interaction effect of dietary protein level, NPE source, and feeding sessions ($P<0.01$; fig. 6.1A, B). This implies that depending on the feeding session, the effect of dietary protein level and NPE source on feed and digestible energy intake was different. Tilapia showed a significantly higher feed intake at afternoon compared with morning feeding in all the diet groups except in those fed HPF diets (Table 6.2).

At the morning feeding, the amount of feed consumed by the tilapia was unaffected by dietary protein level ($P>0.05$), but affected by the NPE source ($P<0.05$), being 15% higher in tilapia fed the fat diets (HPF & LPF) compared to starch (HPS & LPS) diets (Table 6.2). A similar pattern was seen for digestible energy intake (fig. 6.1A), being 10% higher in group fed fat than starch diets ($P=0.07$). Both feed and digestible energy intake were unaffected by the interaction effect of dietary protein level and NPE source ($P>0.05$).

At the afternoon feeding, feed and digestible energy intake of tilapia was affected by the dietary protein level with a strong interaction effect with NPE source ($P<0.05$) (Table 6.2; fig. 6.1B). The fish fed high protein diets (HPF & HPS) had a 10% lower digestible energy intake than the fish fed low protein diets (LPF & LPS). In other words, fish fed high protein diet in the morning significantly reduced their digestible energy intake in the afternoon feeding or subsequent meal.

Further, the digestible protein intake of tilapia at each feeding session paralleled with the contrasts in the amount of dietary protein levels, being lower in fish fed low protein diets (LPF & LPS) compared with high protein diets (HPF & HPS). The digestible protein intake of tilapia was only affected ($P<0.01$) by the NPE source in group fed diets with high protein at morning feeding (Table 6.2).

Table 6.2 Within-day feed intake, digestible energy intake, and daily mean total ammonia, and urea excretion of Nile tilapia fed the experimental diets for 48 days (*n* 3)

	Diets [a]					P- value		
	HPF	HPS	LPF	LPS	SEM	Protein	NPE	Protein x NPE
Feed intake (FI; g DM/kg$^{0.8}$/meal)								
Morning FI	12.3^{X}	10.2^{X}	11.7^{X}	10.7^{X}	0.49	0.984	0.014	0.321
Afternoon FI	11.1^{aY}	11.4^{aY}	13.4^{bX}	12.1^{abY}	0.28	<0.001	0.094	0.026
Digestible protein intake (DPI; g/kg$^{0.8}$/meal)								
Morning DPI	6.2^{aX}	5.2^{bX}	3.3^{cX}	3.0^{cX}	0.15	<0.001	0.004	0.060
Afternoon DPI	5.6^{aX}	5.8^{aX}	3.7^{bX}	3.4^{bX}	0.10	<0.001	0.690	0.018
Metabolic parameters								
TAN excretion (mg N/kg$^{0.8}$/d)	698^{a}	547^{b}	183^{c}	185^{c}	27.2	<0.001	0.025	0.023
Urea excretion (mg N/kg$^{0.8}$/d)	75	58	20	18	7.5	<0.001	0.232	0.371

[a] HPF – high protein diet with fat as NPE source; HPS - high protein diet with starch as NPE source; LPF - low protein diet with fat as NPE source; LPS - low protein diet with starch as NPE source.

Mean values within a row having different superscript (^{a,b,c}) are significantly different and assigned only if the interaction effect was significant (P<0·05).

Mean values of feed intake and digestible protein intake within a column having different superscript (^{X,Y}) shows significant difference between morning and afternoon.

SEM, pooled standard error mean; NPE, non-protein energy source; DM, dry matter

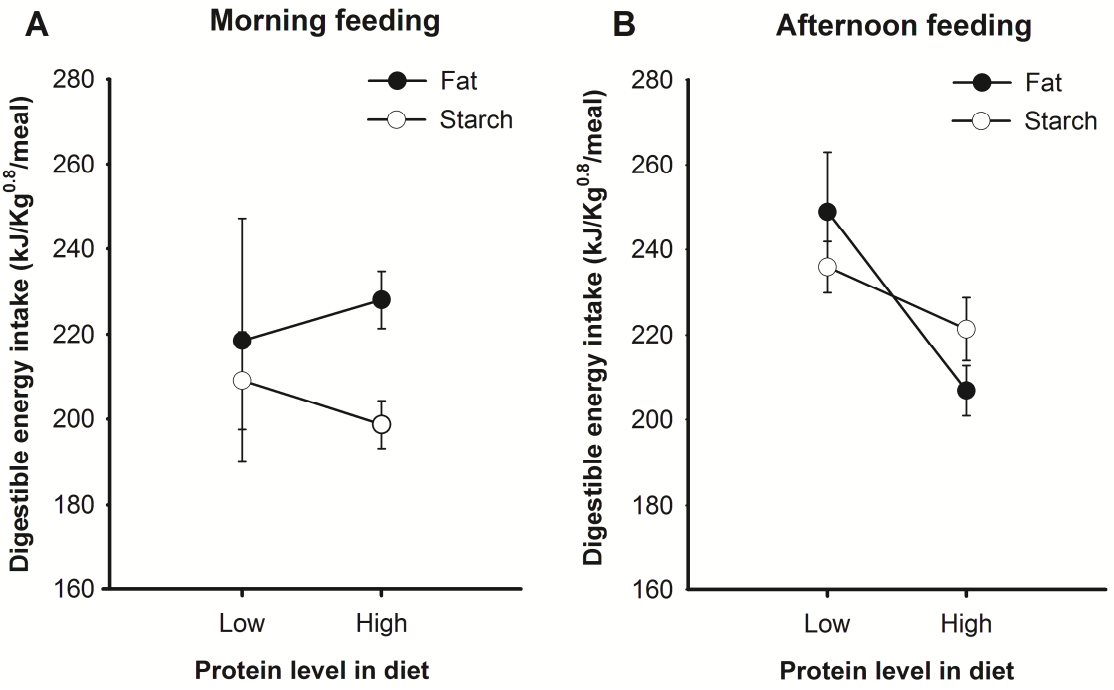

Figure 6.1. Mean (± SD) digestible energy intake of Nile tilapia at morning (A) and afternoon (B) fed with diets varying in the amounts of protein and non-protein energy source (fat *vs.* starch). At morning feeding, the digestible energy intake was slightly affected (P=0.07) by the dietary non-protein energy source (NPE; fat vs. starch) and unaffected by the protein level and its interaction effect with NPE source (P>0.05). At afternoon feeding, the digestible energy intake was affected by the dietary protein level (P<0.001) and unaffected by the NPE source and the interaction effect (P>0.05).

Oxygen consumption and non-fecal nitrogen excretion

The oxygen consumption pattern of Nile tilapia over a 24-hr cycle is shown in Fig. 6.2A. The value of oxygen consumption measured over an hour is represented herein as the mean value in that respective hour; for e.g. mean value of oxygen consumption between 08.00 and 09.00 hrs is represented at 08.30 hrs. After the morning feeding at 09.00 hrs, the oxygen consumption of tilapia increased in all the diet groups. A clear deviation in the pattern of oxygen consumption emerged after 2 and 3 hour feeding in fish fed diet with starch (HPS & LPS) and fat (HPF & LPF) as main NPE source, respectively. Following the morning feeding, the oxygen consumption of tilapia leveled-off at about 4 hours after meal (13.00 hrs) in the groups fed the low protein diets, but continued to increase in the groups fed the high protein diets, particularly in the HPF diet group. After the afternoon feeding (16.00 hrs), the oxygen consumption of tilapia once again increased, attained a peak point, and thereafter gradually decreased in all the diet

groups. The peak oxygen consumption of tilapia was quite similar among the diet groups, except LPF diet group which showed the lowest peak for oxygen consumption. The mean daily oxygen consumption of tilapia was significantly affected by the dietary protein, NPE source and their interaction (data already reported in Saravanan et al., 2012).

The total ammonia nitrogen (TAN) and urea-nitrogen excretion patterns of tilapia over a 24-hr showed a strong distinction between high and low protein diets (fig. 6.2B, C). Five hours after morning feeding, the TAN excretion of tilapia fed the high protein diet diverged differently; being high in fat (HPF) compared with starch (HPS) diet groups. This difference in TAN excretion was not noticeable between low protein diet groups (LPF & LPS). Similar observations were made for the urea-nitrogen excretion. The mean daily TAN excretion of tilapia was affected by the dietary protein, NPE source and their interaction effect ($P<0.05$; Table 6.2).

The TAN excretion was similar ($P>0.05$) in tilapia fed low protein diet (LPF & LPS), however, at high protein diet, TAN excretion was significantly affected by the type of dietary NPE source. The urea excretion of tilapia was affected by the dietary protein level ($P<0.001$) and unaffected by the type of NPE source ($P>0.05$; Table 6.2). In all the diet groups, ammonia and urea contributed approximately 90% and 10% respectively, in the total non-fecal nitrogen excretion of the Nile tilapia.

Oxygen consumption vs. digestible energy intake

The digestible energy intake of tilapia at the morning feeding was not significantly related to each of the seventeen pre-feeding hourly (from 16:30 to 08:30 hrs) oxygen consumption. For instance, there was no relation between one hour pre-feeding oxygen consumption of fish at 08:30 hrs and digestible energy intake at the morning feeding (fig. 6.3A). Similarly, the digestible energy intake of tilapia at the afternoon feeding was not related ($P>0.05$) to each of the five, pre-feeding hourly (from 09:30 to 13:30 hrs) oxygen consumption. Of interest, the digestible energy intake of tilapia at the afternoon feeding was inversely related to two (14:30 hrs) and one (15:30 hrs; fig. 6.3B) hour pre-feeding oxygen consumption ($P<0.05$). In other words, the high pre-feeding oxygen consumption or metabolic status of tilapia resulted in reduced digestible energy intake.

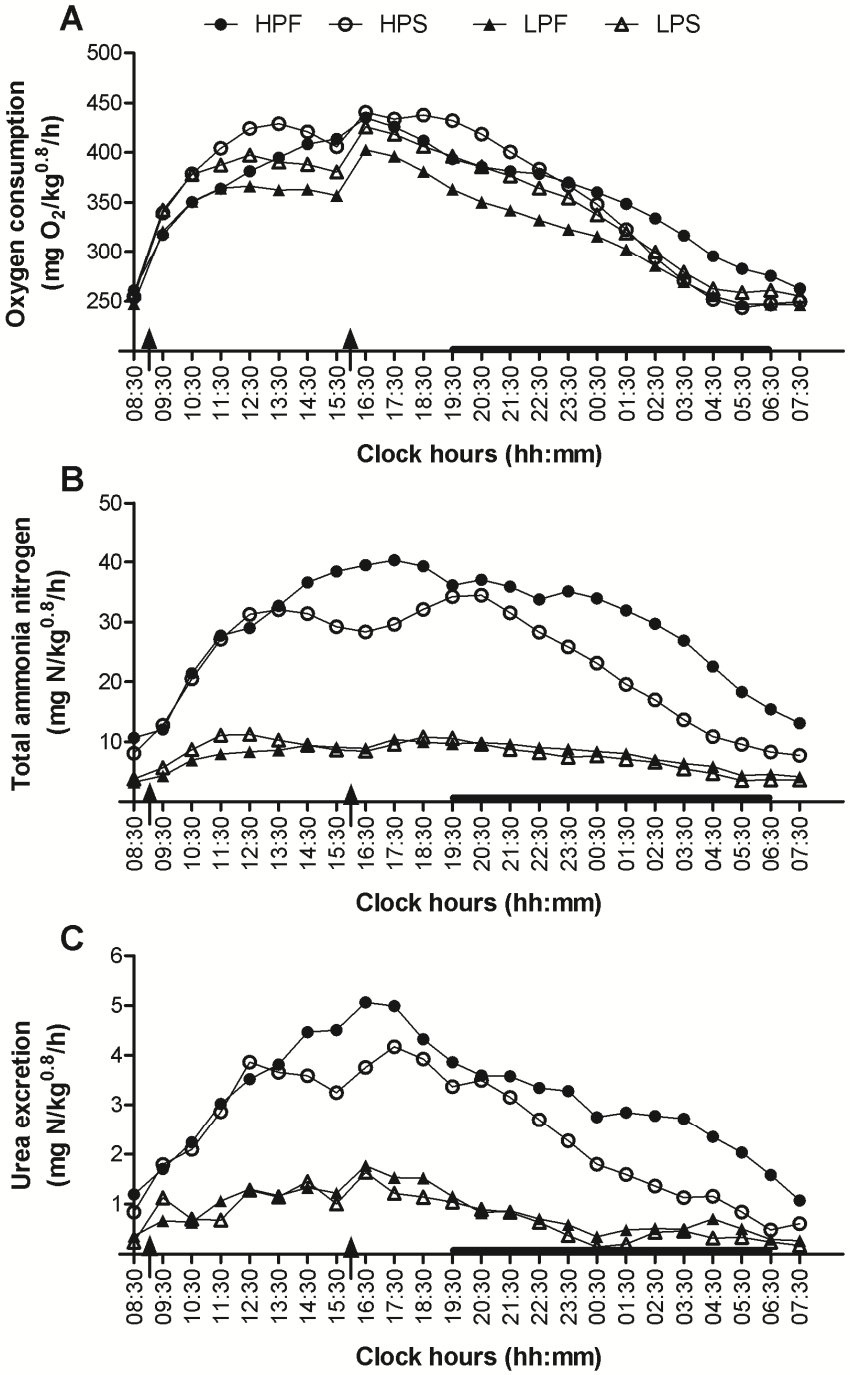

Figure 6.2 Mean hourly oxygen consumption (mg O_2/kg$^{0.8}$/h) (A), total ammonia nitrogen excretion (TAN; mg N/kg$^{0.8}$/h) (B), and urea excretion (mg N/kg$^{0.8}$/h) (C) pattern over 24 hours of Nile tilapia fed diets with varying amounts of protein and non-protein energy source (fat *vs.* starch). HPF – Diet with high protein and fat as NPE source; HPS – Diet with high protein and starch as NPE source; LPF – Diet with low protein and fat as NPE source; LPS – Diet with low protein and starch as NPE source. In X-axis, the two vertical arrows correspond with feeding time and the horizontal black bar represent night hours. The value measured over an hour is represented as the mean value in that respective hour; for e.g. the mean value between 08.00 and 09.00 hrs is represented at 08.30 hrs.

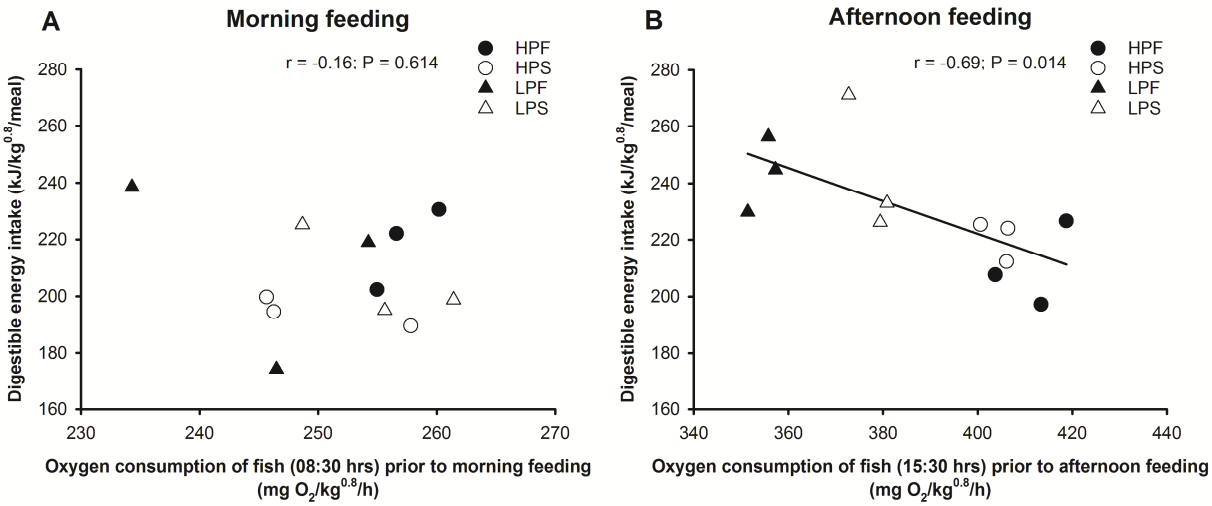

Figure 6.3 Relationship between pre-feeding oxygen consumption and digestible energy intake of Nile tilapia during morning (A) and afternoon (B) feeding.

Discussion

Under captive conditions, Nile tilapia consumes significantly more feed in the afternoon (second meal) compared to the morning (first meal of the day) feeding (Tran-Duy et al., 2008). In agreement, tilapia in the present study also had a higher feed intake at afternoon than at the morning feeding in three out of the four diet groups. The higher intake of tilapia during afternoon might reflect an inherent feeding behavior in line with the diel feeding rhythms. However, Nile tilapia under self-feeding condition shows active diurnal (Toguyeni et al., 1997; Tran-Duy et al., 2008) and nocturnal feeding behavior (Fortes-Silva et al., 2010). Besides, a large variety of diel changes in external factors such as light, temperature and dissolved oxygen (DO) levels of water are likely to synchronize the feeding rhythm of fish (Aranda et al., 1999; Kestemont, 2001). For instance, at a low DO level (i.e., below normoxia) tilapia did not exhibit differences in feed intake between morning and afternoon feeding, but at a high DO level (normoxia), the feed intake was greater in the afternoon than in the morning feeding (Tran-Duy et al., 2008). In the present study, fish were kept under a relatively high DO level which allowed them to exhibit the differences in feed intake between feeding sessions. In addition, fish were reared under constant rearing conditions (temperature, photoperiod, etc.) which are considered optimal for Nile tilapia. Therefore, the effect of these external factors on the differences in feed intake between feeding session is not only expected to be minimal, but also to be similar between the four diet groups. As a consequence, the observed differences in feed and digestible energy intake of tilapia between feeding sessions (morning vs. afternoon) are likely to be provoked by the applied contrast in the dietary protein level and NPE source. In the present study, the ingested macronutrients

influenced metabolic status of the tilapia as indicated by the difference in within-day pattern of oxygen consumption and nitrogen excretion.

Despite the similar digestible energy content of diets, the digestible energy intake of tilapia during morning feeding was higher in fish fed fat diets compared to starch diets. These differences in the digestible energy intake between diets groups at the morning feeding does not relate to the pre-feeding oxygen consumption of tilapia. This implies a lesser impact of pre-feeding metabolic status of the fish on feed intake at the morning meal/feeding, probably related with the 17 h inter-meal interval which allowed the strong decline in levels of oxygen consumption and nitrogen excretion. The stomach capacity (fullness) can determine the maximum feed intake of a fish at a single meal (Brett, 1971). This implies that the feed intake might be affected by volume of the diets (Jobling, 1980; Ruohonen et al., 1997). However, in the present study the feed intake of tilapia at the morning meal/feeding is less likely to be affected by the dietary volume. Since, compared to morning feeding the feed intake (in g dry matter) of tilapia was higher during afternoon feeding in three out of the four diet groups, which suggests that the feed intake of tilapia at morning feeding was not limited by their stomach capacity. Altogether, based on the present results it is difficult to explain the differences in intake of tilapia at the morning feeding.

The digestible energy intake at afternoon feeding was inversely related to the one and two hour pre-feeding oxygen consumption of tilapia. This suggests that the changes in metabolic status (oxygen consumption) of fish induced by the composition of the morning meal/feeding influences the feed intake at the subsequent afternoon feeding. This is in line with evidence in eel (*Anguilla anguilla*), which shows feed intake was related to the metabolic status, as the level of minimum oxygen consumption prior to feeding was negatively correlated to the amount of feed intake (Heinsbroek et al., 2008).. In the present study, the tilapia fed high protein diet not only had a higher magnitude of oxygen consumption, but oxygen consumption also plateaued more slowly than in tilapia fed the low protein diets. The latter perhaps suggests a slow rate of gut passage in tilapia fed high protein diet as seen previously in other fish species (e.g., Fris and Horn, 1993). Thus, a slower gut emptying rate may have contributed to the reduced feed intake during the afternoon feeding in these groups (Jobling, 1980; 1981;Riche et al., 2004; Seginer, 2008).

In mammals, consumption of different macronutrients (protein, fat, starch) is suggested to elicit different levels of satiety (Blundell et al., 1993; Rolls and Hammer, 1995). 'Satiety' is the state of fullness between two meals or feeding in animals (Blundell et al., 1996; Blundell and Tremblay, 1995). This non-instantaneous inter-meal state begins after the ending of a meal/feeding (i.e., after food ingestion), and is believed to impart its

effect on the amount of food consumed during the subsequent meal/feeding. For each unit energy (caloric) intake, protein is suggested to generate a greater satiety followed by starch and then by fat, thus suggested to affect food (energy) intake in mammals (Potier et al., 2009; Westerterp-Plantenga et al., 1999). In the field of fish nutrition, little is known on the potential differences in the satiety effect of different macronutrients on feed intake. In line with the above studies, our data indicate that the tilapia fed high protein diet in the morning reduced their digestible energy intake at afternoon feeding. This effect can be ascribed to the satiety effect of the morning protein intake on the subsequent digestible energy intake at afternoon feeding, as no effect of protein level was seen at the morning feeding. Also in mammals, high protein preloads (e.g. at breakfast) reduce food intake in the subsequent *ad libitum* (e.g. at lunch) meal (Stubbs et al., 1996; Weigle et al., 2005). The intake of a high dietary protein increases the overall metabolic energy expenditure (heat production) in mammals which is perceived to elicit satiety (Westerterp, 2004), and is analogous to oxygen consumption of tilapia in the present study. The dietary protein also exerts its satiety effect in mammals through mechanisms related to circulating plasma amino acid level (Mellinkoff et al., 1956), gut emptying (Jahan-Mihan et al., 2011), stimulation of satiety hormones like cholecystokinin and glucagon-like peptide-1 (Anderson and Moore, 2004), but these mechanisms need to be elucidated in fish.

In summary, the macronutrient composition of the diet affects the within-day feed intake of Nile tilapia and the effect of macronutrients differed between feeding sessions (morning vs. afternoon). The relation between digestible energy intake and pre-feeding oxygen consumption at morning and afternoon feeding suggests that the pre-feeding metabolic status influences the within-day feed intake in Nile tilapia.

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CHAPTER 7

General Discussion

Introduction

This thesis investigates the concept of '*oxystatic*' control of feed intake in fish, which states that at non-limiting water oxygen levels, the long term (weeks) voluntary feed intake in fish can be constrained by a set point value of oxygen consumption (heat production). This chapter reflects on the results from previous chapters to assess the validity of this concept, describes the perspectives and limitations of the concept, explores the possible mechanism involved and provides methodological considerations. Finally the implications of this research outcome are given in the context of fish farming and fish biology.

Validation of the oxystatic concept

Even at non-limiting water oxygen levels, the digestible energy intake (DEI) of rainbow trout and Nile tilapia was affected by the diet composition, as was shown in respectively chapters 2, 4 and 5 and in chapter 3. This difference in digestible energy intake was expected to be caused by the limitation of oxygen consumption (heat production) as proposed in the oxystatic concept. In rainbow trout (chapter 2) we found similar heat production and different DEI as seen previously in Nile tilapia (Tran-Duy et al., 2008). However, unlike in endothermic animals the relevance of heat production in control of feed intake in fish is questionable. Although heat production and oxygen consumption are interrelated, they are not identical. This was observed in tilapia (chapter 3). At the high digestible protein to digestible energy ratio diets the oxygen consumption of tilapia was similar while their heat production was different between the dietary non-protein energy sources (chapter 3). Further, irrespective of the oxygen consumption, the heat production of fish was affected by the dietary non-protein energy source (chapter 3 and 4) and amino acid balance (chapter 5, data not shown). All this confirms that the heat production is not a proper proxy for oxygen consumption. Hence it is impossible to draw conclusions regarding the oxystatic control of feed intake in fish based on the heat production values from chapter 2. In addition the fact that in chapter 2, we found different DEI and similar heat production values (chapter 2) suggests that the actual oxygen consumption of trout was likely to be different between the diet groups. However, this cannot be confirmed without the measurement of oxygen consumption. Therefore we wanted to validate the oxystatic concept by direct measurement of oxygen consumption in fish.

In Nile tilapia similar oxygen consumption was observed in three (HPF, HPS and LPS) diet groups which supports the proposed oxystatic concept (fig. 7.1A). The DEI of tilapia in these three diet groups might have been constrained by the set-point oxygen consumption (chapter 3). The treatment group (LPF) which did not conform to the

oxystatic concept consumed the lowest amount of oxygen but showed also the highest DEI and growth. Based on this observation, we hypothesize that the voluntary DEI can also be limited by other factors (e.g., fat/protein gain ratio) than the set-point oxygen consumption. This might occur when an optimal diet is given in terms of oxygen demand and the animal's requirement for lean body growth as determined by the genetic potential.

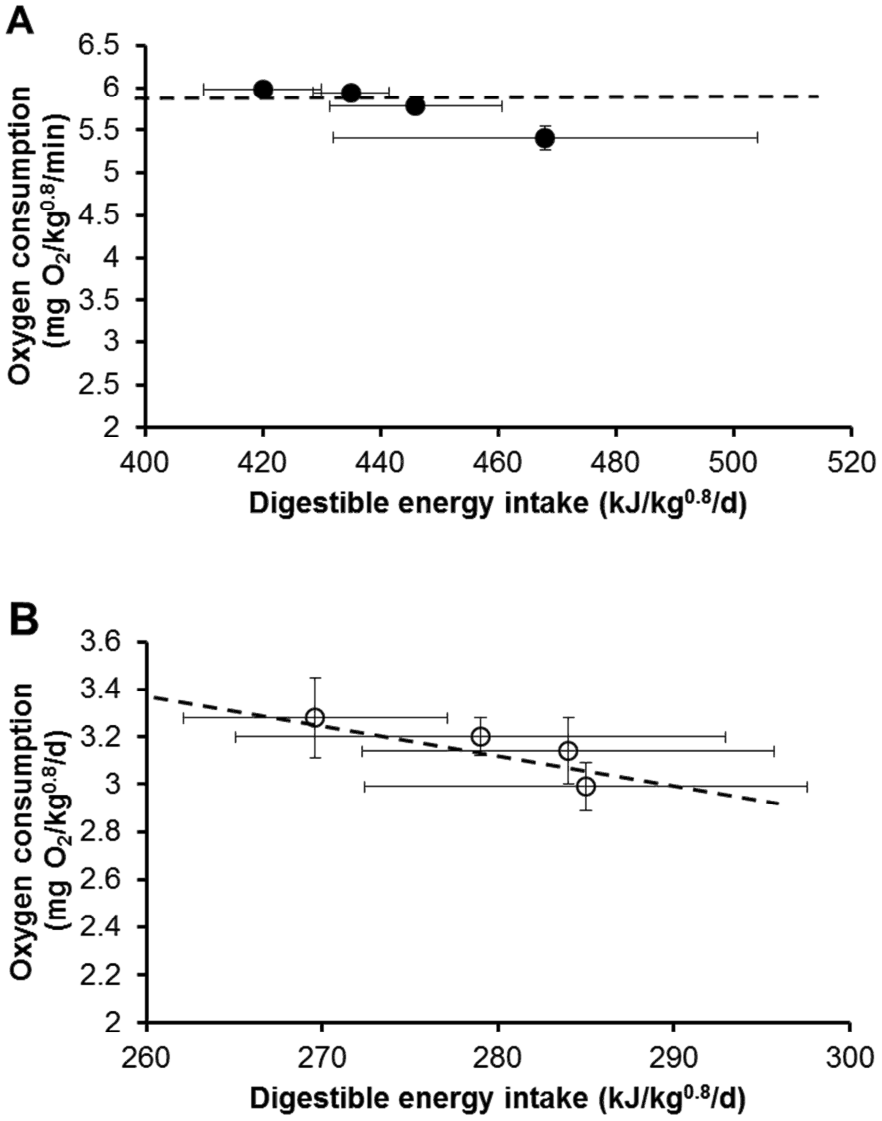

Figure 7.1 In relation to the proposed oxystatic concept, the observed digestible energy intake and oxygen consumption of Nile tilapia (A) and rainbow trout (B) from chapter 3 and 4, respectively.

In rainbow trout, the replacement of dietary fat by starch decreased the DEI but increased the oxygen consumption (chapter 4). These differences in oxygen consumption are in contradiction with our hypothesis that the DEI of trout is constrained by a set-point oxygen consumption (fig. 7.1B). However, trout fed with diets varying in amino acid balance at normoxic conditions did show differences in DEI and at the same time consumed similar amounts of oxygen (chapter 5). This on its turn underlines again a possible set-limitation imposed by the oxygen consumption for feed intake in trout.

Overall, the oxygen consumption data in both Nile tilapia (chapter 3) and rainbow trout (chapter 4 and 5) both support and contradict the oxystatic concept, respectively. Apparently, feed intake is under control of multiple factors and there is no single factor which can be proposed to control voluntary feed intake under all conditions. Thus for all the existing theories of feed intake regulation, including the oxystatic theory, it is obvious that they are only valid in the absence of other constraints on feed intake. In addition, the set point oxygen consumption constrains particularly the voluntary feed intake in the long term. The experimental period of 6 weeks that we usually applied might have been insufficient to observe the set point oxygen consumption in all the diet groups. However, the similar oxygen consumption observed in the various studies of this thesis cannot be considered as a coincidence as it is observed in two very different fish species under different dietary regimes.

To test the oxystatic concept, we tried to influence the oxygen consumption of fish by changing the dietary oxygen demand (i.e., slope of line, chapter 1-fig. 1.4) in all the reported studies (chapter 3, 4 and 5). It is also possible to test the oxystatic concept by changing the oxygen consumption for maintenance without altering the dietary oxygen demand. In fish the maintenance can be influenced by several dietary (e.g., salt content) and environmental (e.g., water pH, salinity) factors through challenging the physiological homeostasis which in turn will affect the oxygen consumption.

Testing the oxystatic concept by altering maintenance

In growing fish, the total oxygen consumption consists of oxygen consumed for routine/basal metabolism (maintenance) and for growth (production). The oxygen consumption needed for the basal metabolism is essential to maintain homeostasis of several physiological processes in an organism. The acid-base homeostasis (pH) is one of the most important physiological processes maintained under homeostasis. Changes in water pH influence the acid-base homeostasis in fish (Janssen and Randall, 1975). An increase in oxygen consumption was observed in rainbow trout reared in low and high water pH compared to neutral water pH (Hargis, 1976). Hence, disturbance in the acid-base homeostasis of fish may alter the amount of oxygen consumption for maintenance.

Since, the oxystatic concept assumes set-point oxygen consumption as a constraint for voluntary feed intake, it is postulated that altering the maintenance oxygen consumption will also affect voluntary feed intake in fish (fig. 7.2).

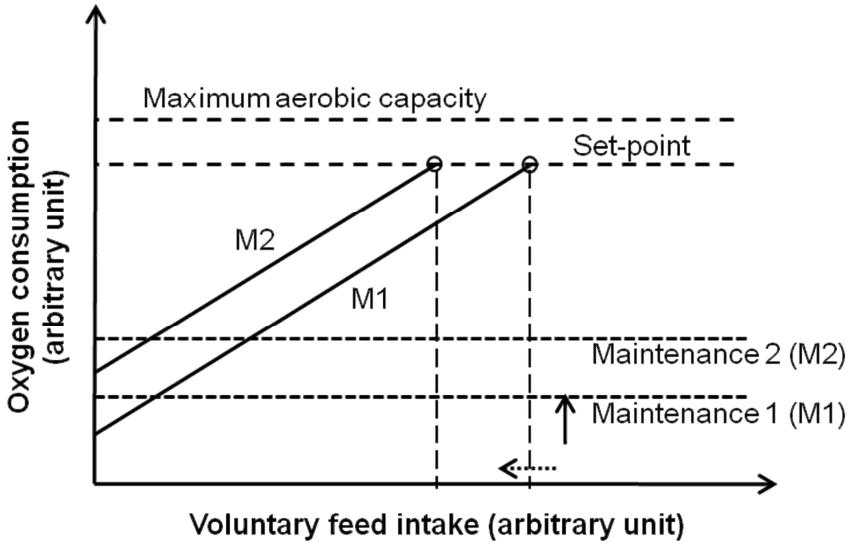

Figure 7.2 Hypothetical illustration of the influence of maintenance on the voluntary feed intake and oxygen consumption in fish

In other words, if the oxystatic concept holds true, an increase in the maintenance oxygen consumption will decrease the voluntary feed intake because of constraint in the oxygen consumption (fig 7.2). To test this, first a reliable and stable factor influencing maintenance is required. In fish the acid-base homeostasis can be affected by the dietary electrolyte balance (dEB) which in turn will alter the maintenance energy expenditure (Dersjant-Li et al., 2000). The dEB is defined as the sum of mineral cations minus the sum of mineral anions present in the diet. In animal nutrition, dEB is often simplified by restricting it to the difference in only the major ions of the diet i.e., as Na + K – Cl, in mEq/kg (Mongin, 1981). A preliminary study to assess the impact of different dEB levels (-200 to + 800 mEq/kg) on maintenance energy expenditure in Nile tilapia at a restricted feeding showed a lowest and highest maintenance energy in fish fed +200 dEB and +800 dEB, respectively (Saravanan et al., 2013).

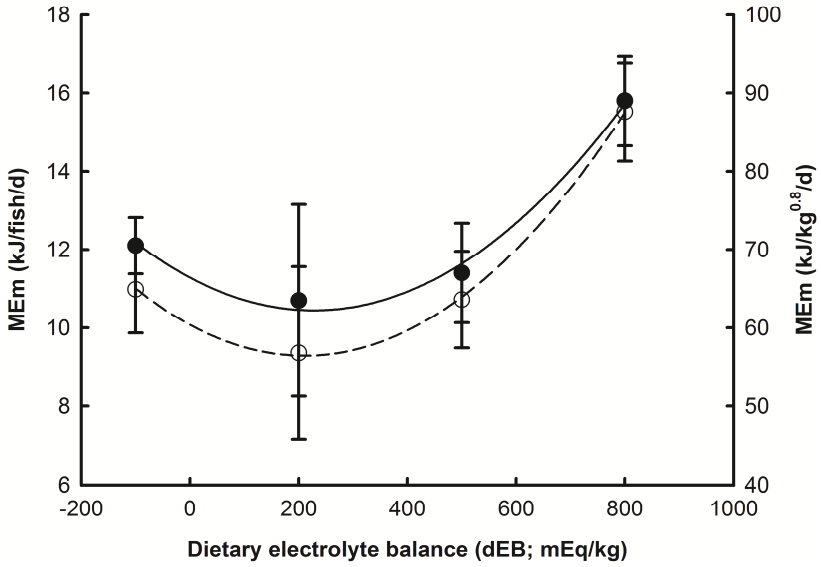

Figure 7.3 Relation between dietary electrolyte balance (dEB) and metabolisable energy expenditure for maintenance (MEm) in Nile tilapia (from Saravanan et al., 2013) .

Subsequently a study was conducted in the aquatic metabolic unit of Wageningen University with Nile tilapia (30 fish/ tank) to assess the impact of altering maintenance on the voluntary feed intake and oxygen consumption (Saravanan et al., unpublished data). Triplicate groups of fish were fed to satiation with one of the four diets contrasting in amino acid (methionine and lysine) balance (balanced vs. imbalanced) and dEB levels (+200 vs. +700 mEq/kg). The contrast in the dietary amino acid (AA) balance is expected to alter the dietary oxygen demand, being lower at a balanced AA than at the imbalanced AA diet. Similarly, the fish fed 200 dEB diets will have lower maintenance compared to 700 dEB diets. The oxygen consumption of fish was not expected to be affected by diets whereas an effect on DEI was expected at both AA balance and dEB levels. As shown in table 7.1, dEB affected the metabolisable energy expenditure for maintenance in agreement with our previous observation (Saravanan et al., 2013).

The DEI of tilapia was significantly affected by the dietary AA balance, but contrary to the expectation the DEI was unaffected by the dEB levels (P=0.102). We did not avail over the oxygen consumption data because of a technical failure in the oxygen measurement system during the experiment. Nevertheless, the similar DEI of tilapia between 200 and 700 dEB levels within the balanced AA and imbalanced AA diet group provide evidence against the postulated oxystatic concept. Furthermore, with increasing maintenance energy requirement the DEI was expected to decrease, but we found numerically higher DEI in tilapia, especially at the imbalanced AA diet groups. In line with this, rainbow trout kept at a low water pH consumed more feed possibly to

compensate for the higher maintenance cost (Dockray et al., 1996; Reid et al., 1997). Together these evidences raise doubts about the general validity of the oxystatic concept under conditions of altered maintenance in fish. However, before coming to the final conclusion regarding the validity of the oxystatic concept other factors (both dietary and environmental) affecting maintenance should be studied.

Table 7.1. Metabolisable energy for maintenance (MEm), digestible energy intake (DEI) and heat production (H) in Nile tilapia fed experimental diets to satiation for 6 weeks.

	Balanced AA		Imbalanced AA			P-value		
dEB (mEq/kg)	200	700	200	700	SEM	AA balance	dEB	AAxdEB
MEm ($kJ/kg^{0.8}/d$)	64	82	67	87	3.4	0.287	<0.001	0.808
DEI ($kJ/kg^{0.8}/d$)	357	360	235	254	6.0	<0.001	0.102	0.215
H ($kJ/kg^{0.8}/d$)	161	174	121	141	1.7	<0.001	<0.001	0.258

Perspectives and limitations of the oxystatic concept

Influence of temperature

The oxystatic concept assumes a 'fixed oxygen budget' that a fish can use for voluntary feed intake in the long term. However, when feeding fish with similar diets but at different environmental conditions, the oxygen consumption may differ. For instance, even at normoxia the changes in the water temperature affect oxygen consumption of fish by altering maintenance (basal metabolism) and aerobic capacity (active metabolism) (fig. 7.4).

As in all poikilothermic animals, in fish, temperature acts as an important steering factor for metabolism (Hansen and Falk-Petersen, 2001). With increasing water temperature the total oxygen consumption of fish increases, whereas the metabolic scope for feed intake increases steadily and then decreases at supra-optimal temperature (Brett, 1979). Therefore fish reared at different water temperature will have 'different set-point oxygen consumptions' according to the oxystatic concept. In addition, water temperature influences the rate of metabolism, hence the time within which the fish regulates its function including feeding may vary. Fish decreases voluntary feed intake at high temperature (i.e., above thermal preferendum of fish) due to the reduction in the metabolic scope (Mallekh and Lagardère, 2002).

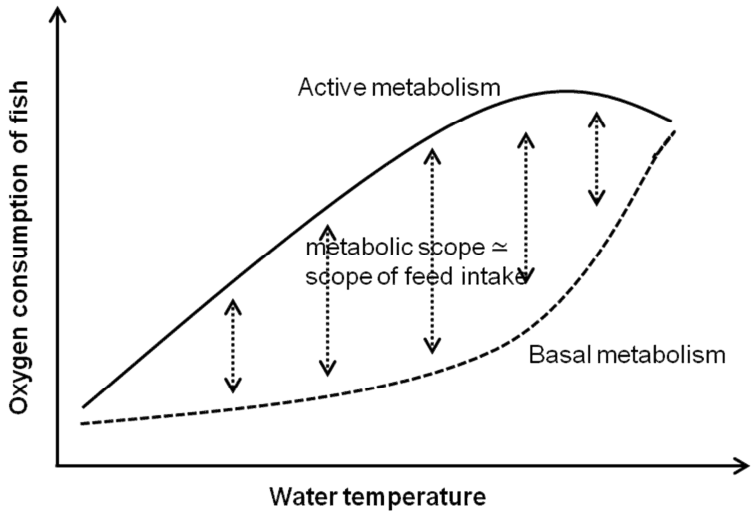

Figure 7.4 Relation between the water temperature and oxygen consumption in fish (modified from Wieser, 1985).

This reduction in metabolic scope reflects the limitation in the capacity for the uptake of oxygen at the gills and to deliver oxygen by the circulatory system to the respiring tissues under conditions of high oxygen demand (Jobling, 1997; Pörtner and Knust, 2007). In addition, the solubility of oxygen (thus the concentration of oxygen) in water decreases with increasing temperature and this might further affect the balance between oxygen demand and -supply of fish (Jensen et al., 1992). Therefore, a pronounced limitation of oxygen consumption on voluntary feed intake is foreseen at high sub-optimal water temperature.

In this thesis we always measured the feed intake and oxygen consumption of fish at the optimal temperature for Nile tilapia (27°C) and for rainbow trout (14°C). However, as discussed above and illustrated in fig 7.4, the oxystatic concept is more likely to be valid at high water temperature. In other words, the oxygen consumption will be a constraint for feed intake in fish at high water temperatures. Therefore, the contradicting outcome from the studies of this thesis might confer to the oxystatic concept at high temperature. On the other hand at low water temperature, constraint of oxygen consumption *per se* is not expected to control feed intake. At low temperature also limitations such as low enzyme activity might control the feed intake in fish.

Oxystatic concept in short term control of feed intake

The oxystatic concept proposed in this thesis considers the long term feed intake regulation. The outcome of long term feed intake can be due to the short term feed intake response of fish. As shown in chapter 6, the variation in the DEI of Nile tilapia at afternoon feeding was inversely related to the pre-feeding oxygen consumption. This

variation in the feed intake response of fish can also be related to limitation in oxygen consumption. Specifically, after the morning feeding the oxygen consumption of fish increases steadily in all diet groups and more or less attains a peak after 4 hours. During the peak hour of oxygen consumption the concentration of water oxygen concentration decreased dramatically from an average value of 5.6 mg/L to values close to 3.0 mg/L. Under such conditions, e.g., a reduced oxygen concentration in water combined with the peak oxygen demand for metabolism, the maximum rate of gill ventilation might be a limitation for oxygen consumption in fish. However, this needs to be verified using automated systems for measuring ventilation rates in fish, such as have been used by Altimiras and Larsen (2000).

Fish after feed deprivation showed an increased compensatory feed intake (hyperphagia) above their normal voluntary feed intake (Ali et al., 2003), which implies that the oxystatic concept may not be applicable in the short term feed intake regulation. It is also hypothesized that if oxystatic limitation is expected to control feed intake, then the hyperphagia in fish will not be sustained and therefore must level-off to the normal level of voluntary feed intake in long term. Therefore, studying the voluntary feed intake and oxygen consumption of fish under conditions of feed deprivation and re-feeding in long term will also help to further verify the validity of the oxystatic concept.

Overall the oxystatic concept combines dietary, environmental and fish factors to explain the long term control of feed intake. The results from this thesis suggest that its validity is limited to long term and under certain conditions. In addition, the oxystatic concept does not sufficiently explain the short term (within-day) variation in feed intake of fish. Nevertheless, it serves as an useful explanatory concept towards a better understanding of feed intake regulation in fish.

Dietary oxygen demand and oxygen efficiency in feed intake of fish

In this thesis, irrespective of the differences in the diet composition, the DEI of Nile tilapia (chapter 3) and rainbow trout (chapter 4) was inversely related to dietary oxygen demand (DOD). However there was a clear difference in the rate of decrease in DEI per unit increase in DOD between Nile tilapia (~14.4 kJ/kg$^{0.8}$/d/DOD) and rainbow trout (~7.1 kJ/kg$^{0.8}$/d/DOD). We initially suggested this to be due to the differences in handling macronutrients between species (see chapter 4). However, the combined data of DOD and DEI in rainbow trout from chapter 4 and chapter 5 at normoxic conditions indicates a slope of ~16.1 kJ/kg$^{0.8}$/d/DOD which is comparable to the slope in Nile tilapia (fig. 7.5).

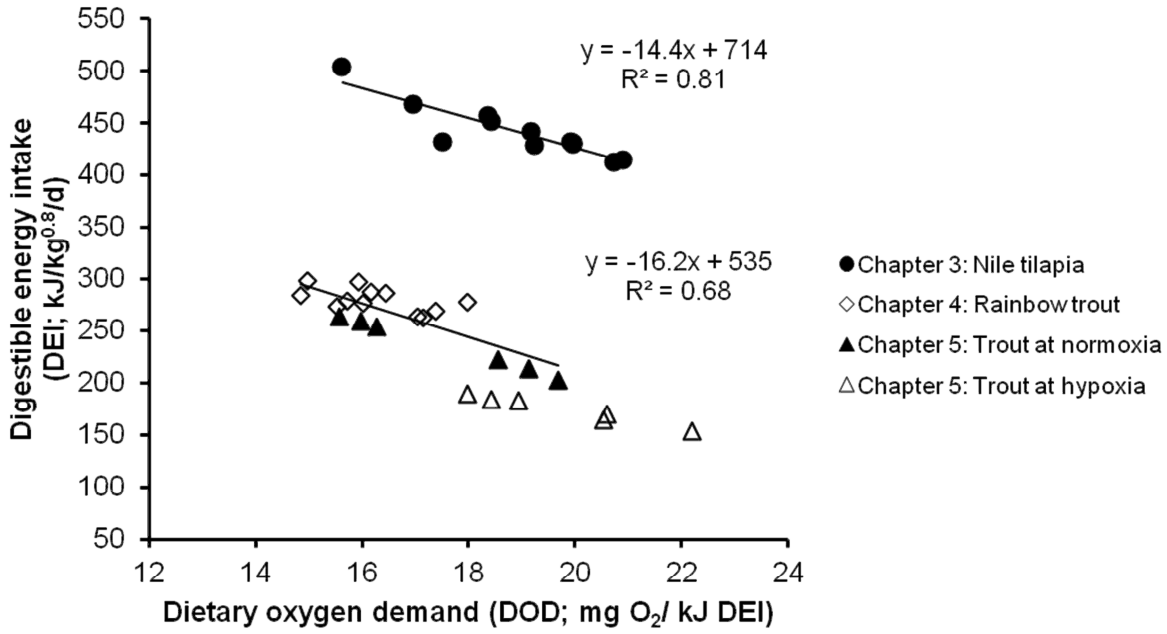

Figure 7.5 Relation between the dietary oxygen demand (DOD) and digestible energy intake (DEI) in Nile tilapia and rainbow trout.

Despite the differences in the rearing temperature, 27°C for tilapia and 14°C for trout, the close similarity in the slope of the lines suggests that both species regulate the DEI in a similar pattern depending on the DOD. Therefore, regardless of the diet composition it is possible to use the DOD as a predictor of the DEI in fish at normoxic condition. However refinements in the determination of DOD for specific diets are required to avoid the confounding effect of basal oxygen consumption and DEI as mentioned below in the section on the methodological considerations.

Besides DOD, the findings from this thesis show that the DEI of Nile tilapia is directly related to the efficiency of oxygen utilization for energy retention (see chapter 3). From the studies performed with rainbow trout in chapter 4 and 5, a similar relation as in the equations below is shown.

DEI (kJ/kg$^{0.8}$/d)= 252 + 0.82 X; n=12, R^2=0.29, P=0.072 (chapter 4)

DEI (kJ/kg$^{0.8}$/d)= 75 + 6.0 X; n=6, R^2=0.95, P=0.001 (chapter 5 - normoxic condition)

DEI (kJ/kg$^{0.8}$/d)= 82 + 4.1 X; n=6, R^2=0.93, P=0.002 (chapter 5 - hypoxic condition)

Where, X is the efficiency of oxygen utilization for energy retention (retained energy in Joule per mg O_2 consumed). These results in both fish species comply with the concept of oxygen efficiency in feed intake regulation as proposed in ruminants (Ketelaars and Tolkamp, 1992). The oxygen efficiency theory assumes a cost-benefit model (oxygen consumption vs. net energy) rather than a constraint model thus 'no fixed oxygen budget' for an animal. Therefore, the oxygen consumption of animals fed different diets

might differ, which is opposite to the assumption of the oxystatic concept. Further the oxygen efficiency theory states that the voluntary feed intake of an animal is at the point where the efficiency of oxygen utilization for net energy is at its maximum. Thus, increasing the oxygen efficiency increases the voluntary digestible energy intake as shown in this thesis. However, at hypoxic conditions the involvement of oxygen efficiency in control of voluntary feed intake in rainbow is an obvious artefact, since feed intake of trout under hypoxia is clearly constrained by the water oxygen levels. Therefore in the presence of obvious constraints on the feed intake in fish (e.g., hypoxia, temperature in ectotherms) the validity of oxygen efficiency theory remains unclear. Further the availability of prey/food is sporadic in case of predatory fish species. Thus when food is available these species are likely to maximize the food intake subject to constraints instead of eating for a maximal efficiency as their main goal. Hence the feeding behaviour and feeding habit of fish has to be taken into consideration in feed intake theories.

Possible signalling mechanisms

The present thesis demonstrates the involvement of dietary oxygen demand and the limitation of oxygen consumption in the control of voluntary feed intake in fish. It is important to explore the signalling mechanisms through which the central feeding system (hypothalamus) senses the limitation in oxygen consumption to regulate feed intake in fish. Oxygen is vital for the aerobic metabolism in all tissues, as it functions as the final acceptor of electrons in the mitochondrial oxidative phosphorylation for ATP production (Goda and Kanai, 2012). Therefore to sustain the aerobic ATP production, cells have to tightly regulate the supply of oxygen to meet their oxygen demand. Compared to fish, the mechanism of oxygen homeostasis has been studied extensively in mammals (Nikinmaa and Rees, 2005). Under oxygen limitation (hypoxia) the adaptive response of an animal is mediated by several oxygen sensors (e.g., molecular oxygen, reactive oxygen species, cytochromes, ATP, pH) through different effector systems (e.g., hypoxia-inducible factor pathway) (Renshaw and Nikinmaa, 2007). The hypoxia-inducible factor (HIF) is the key transcription factor which regulates the adaptive response under hypoxia in mammals and fish (Nikinmaa and Rees, 2005). Therefore it is proposed that the changes in feed intake response due to environmental hypoxia and difference in DOD at normoxia might be regulated through the HIF pathway, but such a direct link between the HIF and central feeding system needs to be elucidated in fish and in mammals.

In mammals, the oxygen- and energy (ATP)-dependent signals from liver to the brain might regulate feed intake (Friedman, 1998). This concept is later proposed as the 'hepatic oxidation theory' in the control of feed intake (Allen et al., 2009). The changes in

the oxygen and energy status in liver due to nutrient oxidation provide signals to the feed intake regulating systems in the brain (Allen et al., 2009). In fish, the hypothalamic neuropeptide Y (NPY) and cocaine amphetamine regulator transcript (CART) are important orexigenic and anorexigenic peptides, respectively (Volkoff et al., 2005). In line with this, the reduced feed intake of rainbow trout fed starch compared to fat diets was associated with increased hepatic cytochrome oxidase (marker of oxidative phosphorylation) together with higher CART mRNA expression in hypothalamus (Figueiredo-Silva et al., 2012). In another study, rainbow trout fed at normoxic conditions with a balanced amino acid and imbalanced amino acid diet (same diets used as in chapter 5) showed a reduced feed intake in the group fed an imbalanced- compared to a balanced- amino acid diet (Figueiredo-Silva et al. our own unpublished data). However, the mRNA expression of markers of oxidative phosphorylation (e.g., cytochrome oxidase) in the liver was similar between the diet groups (Figueiredo-Silva et al., unpublished data), suggesting the existence of a possible threshold level in the hepatic oxidative metabolism in signalling the regulation of feed intake. The effector mechanism by which the hepatic oxidative status signals the brain is not yet clearly understood.

Methodological considerations

Dietary oxygen demand (DOD)

Like feed efficiency, the DOD is also a functional property of a diet and is affected by the dietary nutrient composition as shown in chapters 3, 4 and 5. The DOD in these chapters was calculated by dividing the mean total oxygen consumption and the mean voluntary DEI of fish over the experimental period expressed as mg O_2/kJ DEI. However, when calculating the DOD in this way, the difference in DEI may confound the outcome. The inverse relation between DOD and DEI implies that the DOD of a specific diet will be affected by the changes in the voluntary DEI of fish. In other words, an increased DEI will decrease the DOD. The relation between the weekly mean satiation DEI and DOD (calculated from weekly mean oxygen consumption and DEI) of Nile tilapia from the study of chapter 3, confirms that the DOD of the diet is affected by changes in the DEI of fish (fig. 7.6A). Further, the DOD of a diet can be calculated as marginal DOD or gross DOD depending on whether the total oxygen consumption of fish is corrected for maintenance. The total oxygen consumption of fish consists of oxygen consumed for maintenance and growth (production). In this thesis we calculated the gross DOD with total oxygen consumption (including maintenance) divided by the voluntary DEI. The contrast in the DOD between diets is mainly caused by the differences in oxygen consumption used for growth. Therefore, changing maintenance is expected to have minimal influence on the DOD. This is supported by the observation in Nile tilapia that

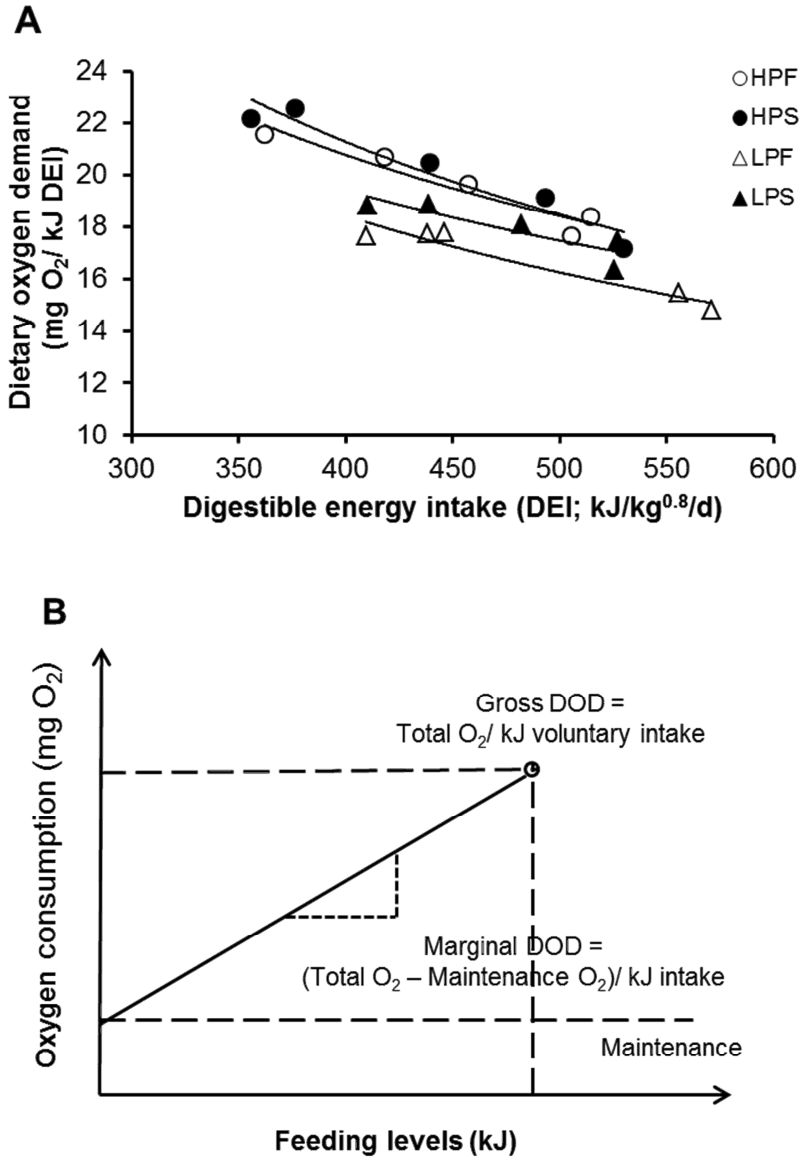

Figure 7.6 (A) The dietary oxygen demand as affected by the DEI in Nile tilapia (B) Approach to determine marginal dietary oxygen demand.

the differences in maintenance energy (MEm) between 200 and 700 dEB was almost equal to their differences in the heat production within the balanced- and imbalanced-amino acid diet groups (table 7.1). Overall, the gross DOD values of diets calculated in the previous chapters have less strength to be a real good predictor of feed intake. To overcome this problem the marginal DOD of a diet has to be determined from the slope of the line between the DEI and oxygen consumption of fish fed at different feeding levels as illustrated in fig. 7.6B.

Heat production (H)

In a biological system the heat liberates due to basal metabolism, heat increment of feeding and physical activity by the metabolic conversion of nutrients into energy (Kleiber, 1975). The H can be determined in three ways, direct calorimetry, indirect calorimetry and comparative slaughter technique. Unlike in homeotherms, the determination of H using direct calorimetry is difficult in fish. In terrestrial animals H is commonly determined by indirect calorimetry using the respiratory measurement of oxygen consumption and CO_2. However, in fish often the oxygen consumption alone is measured and the CO_2 is neglected due to the complexity in the analytical measurements (Bureau et al., 2002). In addition, heat production in fish is considered to be mainly due to the catabolism of amino acid and fats. Therefore a standard oxycalorific coefficient of 13.6 kJ/ g O_2 has been suggested to estimate H using oxygen consumption (Cho and Kaushik, 1990). However, the H estimated with the oxycalorific coefficient of 13.6 kJ/ g O_2 and the oxygen consumption of Nile tilapia from chapter 3 is lower than the H values obtained from the comparative slaughter technique (table 7.2). This suggests that when a standard oxycalorific coefficient is used, the H is underestimated.

Table 7.2. The comparison between heat production (H) values estimated from comparative slaughter technique and indirect calorimetry using the standard oxycalorific coefficient (13.6 kJ/ g O_2).

	Diets			
	HPF	HPS	LPF	LPS
Oxygen consumption (g/kg$^{0.8}$/d)	8.50	8.64	7.78	8.35
Comparative slaughter technique[1]				
H (kJ/kg$^{0.8}$/d)	163	180	157	175
Indirect calorimetry[2]				
H (kJ/kg$^{0.8}$/d)	116	118	106	114

[1] H= metabolizable energy intake – retained energy. [2] H= Oxygen consumption x 13.6

The main reason for the difference between the H values obtained from the two methods is possibly due to the type of nutrient oxidation. Compared to a conventional fish feed, in our study the fish were fed with diets having a large contrast in macronutrient composition. This would alter the type of nutrient oxidation thereby affecting the amount of heat produced per gram of oxygen consumption. Therefore in studies applying contrast in dietary nutrient composition, the measurements of both oxygen consumption and CO_2 production are required for good estimation of H using indirect calorimetry.

Experiments with groups of fish compared to individual fish

The oxystatic concept proposed the limitation in the oxygen consumption on the control of voluntary feed intake in fish. In other words an individual fish is supposed to have set-point oxygen consumption which controls its feed intake. In this thesis, however, the feed intake and oxygen consumption were measured in groups of fish (at tank level), representing the averages of all fish. As such, our data are only a proxy of the real relation between feed intake and oxygen consumption. However, in fish, there is not really a choice under group housing, measuring individual feed intake of fish on a daily basis is a real practical challenge. In addition, individual housing of fish in a tank will affect their voluntary feed intake, and won't provide realistic data either. In this thesis' studies, the only measurement made at individual level was body weight of fish at start and end of the experiments. For instance the coefficient of variation of final body weight was affected in Nile tilapia (study of chapter 3, data not shown). This variation in body weight of tilapia fed different diets can be due to the differences in feed intake and/or nutrient conversion efficiency. It is also likely that each individual have their own level of set point oxygen consumption value. Unfortunately, this will be very difficult to monitor in fish.

Implications of our research

Aquaculture

In aquaculture, the supply of oxygen is one of the most limiting environmental factors which determine the fish production capacity. A low water oxygen level limits the feed intake of fish thereby reducing the growth (Davis, 1975) as is shown also in chapter 5. Therefore, in intensive fish farming systems (e.g., recirculating aquaculture systems) it has been a common practice to maintain a continuous supply of oxygen through aeration and oxygenation. The minimum concentration of water dissolved oxygen (incipient DO) needed to meet the oxygen demand for feed intake depends on the fish size (Tran-Duy et al., 2012) and the nutrient profile of the diet as indicated by the differences in the feed intake of rainbow trout between amino acid-balanced and –imbalanced diets at hypoxic

conditions (chapter 5). The present results highlight the importance of diet composition in estimating the oxygen requirement for specific aquaculture production systems. Besides the water oxygen level, the accumulation of metabolites like CO_2 and nitrogenous compounds (e.g., nitrite, ammonia) in water of intensive aquaculture systems would affect the oxygen carrying capacity in fish (Basu, 1959; Lewis Jr and Morris, 1986), which in turn might affect the feed intake of fish. Further, at the non-limiting water oxygen levels and in the absence of other external factors affecting oxygen uptake, fish increase feed intake with decreasing DOD and as well with the increasing efficiency of oxygen utilization for energy retention. These parameters should be taken into consideration in the prediction models for a good prediction of feed intake in fish.

Most fish farming occurs in open culture systems like ponds and cages which are prone to seasonal changes in temperature and light which in turn might result in fluctuations in water dissolved oxygen levels. This variation in water dissolved oxygen level will affect feed intake of fish. However, it is possible to minimize the impact by adjusting the nutrient composition of feed (i.e., adjusting the dietary oxygen demand) depending on the specific culture condition.

Moreover, under farming conditions the daily feed ration is fed to the fish in distinct feeding sessions or alternatively, fed continuously. The results from this thesis show that when the ration is fed in two feeding sessions, the enhanced oxygen consumption of fish following the first meal/feeding affects feed intake during the subsequent second feeding. Therefore increasing the number of feeding sessions or feeding continuously will lower the peak oxygen consumption of fish which might reduce the impact of dietary macronutrients on the within-day variation in feed intake.

Aquatic ecosystem

Unlike in farming conditions, the food is not readily available for fish in their natural habitat. Therefore the fish have to actively forage on different types and amounts of food/prey items to fulfil their nutrient requirements. In addition, fish have to adjust their feeding behaviour depending on the prevailing environmental conditions and the availability of food. For instance, the increasing occurrence of hypoxia in marine waters and freshwater bodies due to eutrophication and climate change might shift the energy flow by disturbing the food web (Diaz and Rosenberg, 2008). Hypoxia in aquatic environments affects the abundance, diversity and catch of fish (Breitburg, 2002). It also induces changes in the diet of fish due to structural shifts in food availability (Pihl, 1994; Pihl et al., 1992; Rahel and Nutzman, 1994). However, it is also possible that the fish itself have different food preferences under hypoxia, which is not yet known. A study in fruit fly shows an altered diet preference under chronic hypoxia. Flies avoided yeast

(high in protein) which might be toxic under hypoxia compared to normoxia (Vigne and Frelin, 2010). As shown in this thesis, depending on diet composition the oxygen demand might differ. Therefore, under hypoxic conditions organisms might prefer to consume a diet which demands low oxygen. The latter may be true for certain species of fish as well.

Main conclusions

From this thesis the following main conclusions can be drawn on the feed intake control mechanism in fish

- Even at non-limiting water dissolved oxygen levels, the capacity to consume oxygen and the limitations in oxidative metabolism of fish should also be considered in the long term control of voluntary feed intake.
- The consistent inverse relation between digestible energy intake and dietary oxygen demand in Nile tilapia and rainbow trout at different dietary regime suggests the involvement of dietary oxygen demand in the regulation of feed intake in fish.
- The minimum water dissolved oxygen level at which fish reduces the feed intake depends on the dietary oxygen demand. In other words, even at hypoxic conditions the maximum feed intake of fish is determined by the diet composition.
- As in higher animals, the difference in the macronutrient composition of diet seems to alter satiety and within-day feed intake in fish, which is related to the pre-feeding metabolic status.
- Overall, the oxystatic concept i.e., the voluntary feed intake in fish is constrained by a set point value of oxygen consumption appears to be valid at certain conditions. However, its generic application remains questionable. Yet, the oxystatic concept is unique as it combines dietary, environmental and fish factors and provides a conceptual insight for better understanding of feed intake regulation in fish.

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APPENDICES

Summary

The voluntary feed intake of fish is affected by several dietary, environmental and physiological factors. Compared to mammals the underlying mechanisms of feed intake regulation in fish have been less intensively studied. It is widely accepted that fish eat to satisfy their energy requirements, just like most other animals. Similarly, fish reduce feed intake when there is a limitation in the water dissolved oxygen levels. However, even at non-limiting water oxygen levels, the digestible energy intake of fish is affected by the nutrient composition of the diet. Based on the observation of similar heat production together with different digestible energy intake in Nile tilapia, we thought that a limitation in heat production (oxygen consumption) might affect the voluntary feed intake. In this thesis we proposed the 'oxystatic' concept which states that at non-limiting water oxygen levels and in the absence of other constraints, the feed intake of fish as measured over a period of weeks, can be constrained by a set-point value of oxygen consumption. Therefore the aim of this thesis was to assess the validity of the oxystatic concept and to elucidate the role of oxygen consumption in the control of feed intake in fish.

Our first objective was to verify the previous observation of similar heat production under non-limiting water oxygen condition in other fish species than Nile tilapia. In **chapter 2**, we investigated the feed intake, digestible energy intake and heat production of rainbow trout reared in a water flow-through system. The fish were fed to satiation with four iso-energetic diets contrasting in protein to energy ratio (low vs. high) and non-protein energy source (starch vs. fat). The results showed that the feed and digestible energy intake of trout was altered by the macronutrient composition of the diet. Further the calculated heat production of trout was similar and unaffected by the diets as seen previously in Nile tilapia. The repeated observations of similar heat production in both species suggest the feed intake of fish to be controlled by a physiological limitation in oxygen consumption and oxidative metabolism.

Next we investigated the existence of 'oxystatic' control of feed intake, by direct measurement of oxygen consumption and feed intake in two species, Nile tilapia (Chapter 3) and rainbow trout (chapter 4 and 5). The nutrient compositions of the diets were altered to create a contrast in dietary oxygen demand (mg O_2/ g or kJ feed) in order to influence the oxygen consumption of fish. In **chapter 3**, the feed intake, digestible energy intake and oxygen consumption were monitored in Nile tilapia fed to satiation with four iso-energetic diets contrasting in protein to energy ratio (low vs. high) and the type of non-protein energy (NPE) source (starch vs. fat). Feed intake and digestible energy intake of tilapia was affected by the dietary NPE source and protein to energy ratio, respectively. The macronutrient induced changes in digestible energy

intake of tilapia was negatively related to dietary oxygen demand and positively related to oxygen efficiency for energy retention. Moreover, the oxygen consumption was similar in three out of four diet groups. This suggests that even at non-limiting water oxygen levels, the voluntary feed intake might be constraint by the set-point oxygen consumption of fish in line with the oxystatic concept.

In **chapter 4**, we assessed the feed intake and oxygen consumption of rainbow trout fed to satiation with four diets highly contrasting in non-protein energy (NPE) source (starch vs. fat) and with similar digestible protein to digestible energy ratio. The dietary oxygen demand decreased linearly with increasing replacement of starch by fat in the diet. The digestible energy intake of trout increased with increasing fat as NPE in the diet. Similarly, the oxygen consumption of trout decreased with increasing dietary fat, which contradicted the oxystatic concept. However, the digestible energy intake of trout was inversely related to dietary oxygen demand in accordance with our previous findings in Nile tilapia (chapter 3). Together, these results suggest a possible role of dietary oxygen demand in feed intake regulation.

In **chapter 5**, we investigated the oxystatic concept in rainbow trout fed two diets contrasting in amino acid (methionine and lysine) balance (balanced diet vs. imbalanced diet). The contrast in dietary amino acid balance was expected to alter the dietary oxygen demand, being high with imbalanced than with balanced amino acid diet. Both diets were tested at two water oxygen levels (hypoxia vs. normoxia). Compared to normoxia, trout under hypoxia consumed 29% less feed. When fed imbalanced diet the feed intake of trout were 11% and 16% lower than balanced diet at hypoxia and normoxia, respectively. The oxygen consumption of trout was similar under hypoxia but also under normoxia. The identical oxygen consumption of trout at normoxia together with differences in feed intake supports the oxystatic concept, that is the feed intake of fish can be constrained by a set-point oxygen consumption.

The feed intake response of fish observed over 6 weeks in the above studies might be a result of short-term feed intake regulation. Therefore in **chapter 6**, we assessed the impact of dietary macronutrient composition on within-day (morning vs. afternoon feeding) variation in feed intake and oxygen consumption of Nile tilapia using data from the study of chapter 3. Further the relation between the within-day oxygen consumption and variation in within-day feed intake was investigated. The within-day digestible energy intake of tilapia was affected by the macronutrient composition of diet and their impact differed between feeding sessions. At morning feeding, regardless of the dietary protein level the digestible energy intake was higher in fish fed the diets with fat as NPE source than starch. These differences in digestible energy intake at the morning feeding were not related to oxygen consumption of tilapia, which suggests the involvement of

feed intake control mechanisms other than the oxygen consumption. Compared to the low protein diet, fish fed the high protein diet in the morning reduced the digestible energy intake at afternoon feeding with 10%. The difference in digestible energy intake of fish at the afternoon feeding was inversely related to the two and one hour pre-feeding oxygen consumption. The results suggest that the pre-feeding metabolic status (oxygen consumption) might influence the within-day feed intake.

In the final **chapter 7**, the general validity of the oxystatic concept was discussed based on the results from the above chapters. Moreover the validity of oxystatic concept was addressed in the context of altered maintenance requirements of fish and changing water temperature. Further the implications of this thesis results for aquaculture and for a natural aquatic ecosystem were discussed.

Overall the following conclusions can be drawn from this thesis:

- Even at non-limiting water dissolved oxygen levels, the capacity to consume oxygen and the limitations in oxidative metabolism of fish should also be considered in the long term control of voluntary feed intake.
- The consistent inverse relation between digestible energy intake and dietary oxygen demand in Nile tilapia and rainbow trout at different dietary regime suggests the involvement of dietary oxygen demand in the regulation of feed intake in fish.
- The minimum water dissolved oxygen level at which fish reduces the feed intake depends on the dietary oxygen demand. In other words, even at hypoxic conditions the maximum feed intake of fish is determined by the diet composition.
- As in higher animals, the difference in the macronutrient composition of diet seems to alter satiety and within-day feed intake in fish, which is related to the pre-feeding metabolic status.
- Overall, the oxystatic concept i.e., the voluntary feed intake in fish is constrained by a set point value of oxygen consumption appears to be valid for certain conditions. However, its generic application remains questionable. Yet, the oxystatic concept is unique as it combines dietary, environmental and fish factors and provides a conceptual insight for better understanding of feed intake regulation in fish

Samenvatting

De vrijwillige voedselopname van vissen wordt beïnvloed door verschillende voedings-, omgevings- en fysiologische factoren. Vergeleken met zoogdieren, zijn bij vissen de onderliggende mechanismen van de voedselopnameregulatie minder intensief bestudeerd. Het is algemeen aanvaard dat, net als de meeste andere dieren, vissen eten om in hun energiebehoefte te voldoen. Ook verminderen vissen hun voedselopname wanneer er een limitatie is in de, in het water opgeloste zuurstof niveaus. De verteerbare voedselopname van vissen wordt echter, ook bij niet limiterende zuurstofniveaus in het water, beïnvloed door de nutriëntensamenstelling van het dieet. Gebaseerd op waarnemingen bij Nijltilapia, waarbij de warmteproductie gelijk bleef bij verschillende verteerbare energieopnames, dachten wij dat een beperking in de warmteproductie (zuursofconsumptie) van invloed zou kunnen zijn op de vrijwillige voedselopname. In deze dissertatie introduceren we het concept 'oxystatic', wat aangeeft dat bij niet-limiterende zuurstofniveaus in het water en in de afwezigheid van andere beperkingen, de voedselopname van vissen gemeten over een periode van een aantal weken, beperkt kan worden door een zuurstofconsumptie set-point waarde. Daarom is het doel van deze dissertatie om de validiteit van het begrip oxystatic te bepalen en om de rol van zuurstofconsumptie in de voedselopnameregulatie van vissen te verklaren.

Onze eerste doelstelling was om de eerder genoemde observaties van gelijke warmteproductie bij niet-limiterende zuurstofniveaus in het water, te verifiëren voor andere vissoorten dan Nijltilapia. In **hoofdstuk 2** onderzochten we de voedselopname, verteerbare energieopname en warmte productie van regenboog forel, welke gekweekt werden in een doorstroom systeem. De vissen werden tot verzadiging gevoerd met vier iso-energetische diëten, welke verschilde in eiwit - energie ratio (laag vs. hoog) en niet-eiwit energiebronnen (zetmeel vs. vet). De resultaten lieten zien dat de voeropname en verteerbare energieopname van forel veranderde afhankelijk van de macronutriënten samenstelling van het dieet. Verder was de berekende warmte productie van forel gelijk en werd niet beïnvloed door de diëten, zoals ook eerder gezien bij Nijltilapia. De herhaaldelijke observaties van gelijke warmteproductie bij beide vissoorten suggereert dat de voedselopname van vissen gecontroleerd wordt door een fysiologische limitatie in zuurstofconsumptie en een oxidatief metabolisme.

Vervolgens onderzochten we het bestaan van een 'oxystatic' gereguleerde voedselopname, door directe metingen van de zuurstofconsumptie en voedselopname in twee vissoorten; Nijltilapia (**hoofdstuk 3**) en regenboogforel (**hoofdstuk 4 en 5**). De nutriëntensamenstellingen van de diëten werden veranderd om een contrast te creëren in het zuurstofverbruik van de diëten (mg O_2 of kJ voer), om de zuurstofconsumptie van

de vissen te beïnvloeden. In **hoofdstuk 3** werden de voedselopname, verteerbare energieopname en zuurstofconsumptie gemonitord van Nijltilapia welke gevoerd werden tot verzadiging met vier iso-energetische diëten, welke verschilde in eiwit - energie ratio (laag vs. hoog) en het type niet-eiwit energiebron (NPE) (zetmeel vs. vet). Voedselopname en verteerbare energieopname van tilapia werden respectievelijk beïnvloed door de NPE bron van het dieet en de eiwit - energie ratio. De door de macronutriënten geïnduceerde verandering in verteerbare energieopname van tilapia was negatief gerelateerd aan de benodigde zuurstof voor het dieet en positief gerelateerd aan de zuurstof efficiëntie voor energiebehoud. Bovendien was de zuurstofconsumptie gelijk in drie van de vier dieet groepen. Dit suggereert dat zelfs bij niet-limiterende zuurstoflevels in het water, de vrijwillige voedselopname beperkt kan worden door de set-point zuurstofconsumptie van vissen, wat in line is met het oxystatic concept.

In **hoofdstuk 4**, hebben we de voeropname en zuurstofconsumptie bepaald van regenboogforellen welke gevoerd werden tot verzadiging met vier diëten die sterk verschilde in NPE bron (zetmeel vs. vet) en met vergelijkbare verteerbare eiwit – verteerbare energie ratio. De benodigde zuurstof voor het dieet nam lineair af met een toenemende vervanging van zetmeel door vet in het dieet. De verteerbare energieopname van forel nam toe met een toename van vet als NPE bron in het dieet. Ook verminderde de zuurstofconsumptie van forel bij een toename van vet in het dieet, wat tegenstrijdig is met het oxystatic concept. Echter, in overeenstemming met onze eerdere bevindingen in Nijltilapia, was de verteerbare energieopname van de forellen omgekeerd evenredig met de benodigde zuurstof voor het dieet (hoofdstuk 3). Gezamenlijk suggereren deze resultaten een mogelijke rol van de benodigde zuurstof voor het dieet in de voeropnameregulatie.

In **hoofdstuk 5**, onderzochten we het oxystatic concept door regenboogforellen te voeren met twee diëten, welke verschilde in aminozuur (methionine en lysine) balans (gebalanceerd dieet vs. niet-gebalanceerd dieet). Verwacht werd dat het verschil in aminozuurbalans in het dieet, de benodigde zuurstof voor het dieet zou veranderen, waarbij de benodigde zuurstof voor het dieet hoger is bij een niet-gebalanceerd dieet dan bij een gebalanceerd dieet. Beide diëten werden getest bij twee zuurstofgehaltes in het water (hypoxie vs. normoxia). Vergeleken met normoxia omstandigheden, consumeerde forellen 29% minder voer onder hypoxie omstandigheden. Wanneer de forellen gevoerd werden met een niet-gebalanceerd dieet, waren de voedselopnames 11% en 16% lager dan wanneer ze gevoerd werden met een gebalanceerd dieet onder respectievelijk hypoxie en normoxia condities. De zuurstofconsumptie van de forellen was gelijk onder zowel hypoxie als normoxia condities. De gelijke zuurstofconsumptie

van forellen onder normoxia omstandigheden welke gepaard gaat met verschillen in voedselopname ondersteunen het oxystatic concept, wat inhoudt dat de voedselopname van vissen beperkt kan worden door een set-point zuurstofconsumptie.

De voedselopname respons van de vissen, welke voor de bovengenoemde studies geobserveerd werden voor 6 weken, kan een gevolg zijn van de kortdurende voeropname regulatie. Daarom werd in **hoofdstuk 6** voor Nijltilapia, de impact van de dieet macronutriëntensamenstelling op de variatie in voedselopname en zuurstofconsumptie binnen een dag (ochtend vs. middag voeren) bepaald, door data te gebruiken uit de studie van hoofdstuk 3. Verder werd de relatie tussen de zuurstofconsumptie binnen een dag en de variatie in voedselopname binnen een dag bestudeerd. De verteerbare energieopname van tilapia werd binnen een dag beïnvloed door de macronutriënten samenstelling van het dieet en de impact verschilde tussen de voersessies. Tijdens de voersessie in de ochtend was de verteerbare energieopname hoger in vissen welke gevoerd werden met een dieet met vet in plaats van zetmeel als NPE bron, ongeacht het eiwitlevel van het dieet. Deze verschillen in verteerbare energieopname gedurende de ochtend voersessie waren niet gerelateerd aan zuurstofconsumptie van tilapia, wat, in plaats van de zuurstofconsumptie, de betrokkenheid van voeropname regulatie mechanisme suggereert. Vergeleken met vissen gevoerd met een dieet met een laag eiwit gehalte, verminderde vissen, welke in de ochtend gevoerd werden met een dieet met een hoog eiwit gehalte, hun verteerbare energieopname met 10%. Het verschil in verteerbare energieopname van de vissen in de middag voersessie was omgekeerd evenredig met de zuurstofconsumptie twee en een uur voordat het voeren begon. De resultaten suggereren dat de metabole status (zuurstofconsumptie) voorafgaand aan het voeren invloed zou kunnen hebben op de voedselopname binnen een dag.

In het slothoofdstuk, **hoofdstuk 7**, wordt, gebaseerd op de resultaten van de bovengenoemde hoofdstukken, de algemene validiteit van het oxystatic concept bediscussieerd. Bovendien wordt de validiteit van het oxystatic concept bepaald in de context van gewijzigde onderhoudsbehoeften en veranderende water temperatuur. Verder worden de implicaties van de resultaten van deze dissertatie voor aquacultuur en natuurlijke aquatische ecosystemen bediscussieerd.

De belangrijkste conclusies uit dit proefschrift zijn:

- Zelfs bij niet-limiterende zuurstofgehaltes in het water, moeten de capaciteit om zuurstof te consumeren en de beperkingen in oxidatief metabolisme ook beschouwd worden in de regulatie van de vrijwillige voedselopname over de lange termijn.

- De consistente omgekeerd evenredige relatie tussen verteerbare energieopname en de benodigde zuurstof voor het dieet van Nijltilapia en regenboogforel gezien bij verschillende dieet regimes, suggereert dat de benodigde zuurstof voor het dieet betrokken is bij de voedselopname regulatie van vissen.
- De minimum zuurstofconcentratie in het water waarbij vissen hun voeropname verminderen hangt af van de benodigde zuurstof voor het dieet. Met andere woorden, zelfs onder hypoxia condities wordt de maximale voeropname van vissen bepaald door de dieet samenstelling.
- Net als bij hogere dieren, lijkt het verschil in de macronutriëntensamenstelling van het dieet de verzadigdheid en de voedselopname van vissen binnen een dag te veranderen, wat gerelateerd is aan de metabole status voorafgaand aan het voeren.
- In het geheel genomen, lijkt het oxystatic concept i.e., de vrijwillige voeropname in vissen wordt beperkt door een set-point waarde van de zuurstofconsumptie, te gelden voor bepaalde condities. Echter, de generieke toepassing blijft twijfelachtig. Nochtans is het oxistatic concept uniek, want het combineert dieet, omgeving en vis factoren en biedt een conceptueel inzicht voor een beter begrip van voedselopname regulatie in vissen.

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About the Author

Saravanan Subramanian was born on the 15th of January in 1984 at Salem, India. In his youth, he was curious and fascinated about plant and animal life in his surrounding nature, which later helped to orient his secondary high school education in Biology. After completion of high school education in 2002, he enrolled in the same year as graduate student in the Bachelors of Fisheries Science at the Tamil Nadu Veterinary and Animal Sciences University, Tuticorin, India. He finished the four years bachelor's education with distinction. In 2006, he was awarded as one of the Best Fisheries Graduate of India (3rd position) by the Indian Professional Fisheries Graduate Forum. After bachelors, he continued his master's in Fish Nutrition and Biochemistry at Central Institute of Fisheries Education, Mumbai. There he was encouraged to work on crustacean nutrition for his thesis titled 'Physiometabolic responses of freshwater prawn, *Macrobrachium rosenbergii* to dietary carbohydrate'. His motivation and enthusiasm for research in fish nutrition directed him towards the collaborative project between INRA, st-pée, France and Aquaculture and Fisheries Group, Wageningen University (WU), the Netherlands on 'Nutritional and Environmental Control of Feed intake in Fish'. In 2009, he started his PhD where he was given the opportunity to conduct research both at WU and INRA. Four years of his research work led to the submission of this thesis. After PhD, he wishes to pursue research career in fish/shellfish physiology and nutrition.

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List of publications

Saravanan, S., Geurden, I., Figueiredo-Silva, A.C., Kaushik, S., Verreth, J., Schrama, J.W., 2013a. Voluntary feed intake in rainbow trout is regulated by diet-induced differences in oxygen use. The Journal of Nutrition. 143, 781-787.

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WIAS Graduate School Training and Supervision Program

Education and training 2009-2013	ECTS
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WIAS introduction course	
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Supervision of 2 MSc theses: "Effect of amino acid balance and dissolved oxygen level on feed intake of rainbow trout (*Oncorhynchus mykiss*)" – MSc thesis 2012, Suluh Nusantoro "Dietary amino acid- and electrolyte- balance on feed intake, growth and maintenance energy expenditure of Nile tilapia (*Oreochromis niloticus*)" – MSc thesis, Sebastian Marcus Strauch	
Total (1 ECTS credit equals 28 h study load)	**40.0**

Colophon

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