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Effects of the thermal environment on the bioenergetics of rainbow trout (*Oncorhynchus mykiss*) using group respirometry

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Für meine Mutter Renate, meine Frau Natalie und unsere Tochter Antonia.

Euch widme ich dieses Werk

…

“You did not kill the fish only to keep alive and to sell for food, he thought. You killed him for pride and because you are a fisherman. You loved him when he was alive and you loved him after.”

Ernest Hemingway, The old man and the sea

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ADC	Apparent Digestibility Coefficient
ADP	Adenosine Diphosphate
AIA	Acid Insoluble Ash
AQ	Ammonia Quotient
ATP	Adenosine Triphosphate
BW	Live Body Weight
DEI	Digestible Energy Intake
E_n	Non-Fecal Nitrogen Losses
FCR	Feed Conversion Ratio
FE	Fecal losses
FI	Feed Intake
GEI	Gross Energy Intake
IPCC	Intergovernmental Panel on Climate Change
MBW	Metabolic Body Weight
MEI	Metabolizable Energy Intake
NPES	Non-Protein Energy Substrate
NRC	National Research Council
OC	Oxygen Consumption
OCLTT	Oxygen and Capacity-Limited Thermal Tolerance Hypothesis
OE	Oxcalorific Equivalent
RARS	Recirculating Aquaculture Respirometric System
RAS	Recirculating Aquaculture System
RE	Retainable Energy
RQ	Respiratory Quotient
SDA	Specific Dynamic Action
SDA_{coef}	Specific Dynamic Action coefficient
SDA_{dur}	Specific Dynamic Action duration
SMR	Standard Metabolic Rate
TAN	Total Ammonia Nitrogen
VA	Voluntary Activity

GENERAL INTRODUCTION

1. Farming of fish as ectothermic animals in the era of global climate change – current and future challenges

The Intergovernmental panel on climate change (IPCC) is a body installed by the United Nations (UN), to address the impacts of climate change on the environment, economics, and the human population. It recognizes climate change as a major threat to human health well-being, and livelihood (Barange et al., 2018). At the 2015 United Nations Climate Change Conference, 196 countries agreed to limit the increase in mean global temperature to well below 2 °C, compared to the pre-industrial era, in a treaty known as the Paris Climate Agreement (UN, 2015). Sophisticated models however predict an increase in global temperature of up to 3 °C by 2100, even if all current agreements are met (Liu & Raftery, 2021). Air Currents like the polar Jetstream, which have a major role in modulating the local weather in Central Europe, are changing in speed and direction, due to a decline in arctic sea ice and changes in evapotranspiration patterns. This has already led to several extreme weather phenomena in central Europe that have been directly linked to climate change including the persistent cold spells in the winter of 2008/2009 and the heat wave in 2018 (Stendel et al., 2021).

Climate change as a global event has distinct regional effects that can pose a challenge for the areas affected (Teng et al., 2013). Extreme weather events, like heavy rain following floods or heat waves with droughts, are predicted to increase in frequency and duration. Agriculture is projected to be among the business sectors most affected by climate change (Rosenzweig et al. 2014). Especially the farming of ectothermic animals like fish may be substantially affected as the entire metabolism of the animal is determined by environmental conditions (Sae-Lim et al., 2017; Adhikari et al., 2018). Hence it is of utmost importance for aquaculture research to gain detailed insight into the temperature-dependent biology of farmed fish species.

Every species of fish holds a certain range of tolerated temperatures, which it can survive (Chen et al., 2015). The farming of rainbow trout (*Oncorhynchus mykiss*), a popular aquaculture species in Germany, relies to a large degree on the technology of flow-through or semi-flow-through systems (FIZ, 2021). These systems use water from natural flowing water bodies and redirect certain amounts of it from the original water body to a farming site. In these systems, the water temperature on the farming site relies heavily on the water temperature of the original water body. In these systems, water temperatures of up to 20 °C are not uncommon in the summer (Weirup et al., 2021). However, literature suggests that the optimal temperature for the growth of rainbow trout is located somewhere between 15 - 17 °C (Hokanson et al., 1977; Mellery et al., 2016). Therefore, studies investigating the effects of a volatile thermal environment, that may further increase in volatility due to an unstable global climate, should ideally cover temperatures towards the upper range of that optimum and possibly above it to account for the general increase of global temperatures.

2. Direct and indirect measurements of energy metabolism in fish

The dependency of metabolic processes on the temperature in poikilothermic animals like fish makes the use of bioenergetic research an adequate tool when targeting the impacts of climate change on farmed fish species (Clark et al., 2013). Bioenergetic research assesses energetic losses to calculate energy gains as a benchmark of potential growth and relative fitness of an animal. The basic energy balance equation of a bioenergetic approach has been described by Jobling (1994) as E(P) = E(In) - E(Out) where E(P) is energy potentially available for growth, E(In) is energy ingested and E(Out) is energy losses by metabolism and other processes like fecal excretion. Over the years several other terms have been established and used to accurately describe the factors of the equation. The energy potentially available for growth is often termed recovered energy or retainable energy (RE) as it is the remainder of energy still in the fish after all measurable energetic losses have been accounted for (NRC, 1981). Several energy losses can be measured but the by far most relevant is energy lost as heat (Blaxter, 1989). All animals liberate heat as a consequence of tissue turnover, the transformation of feed into body tissue, the immune system, or for activity and reproduction.

Two basic principles have emerged to assess the energetic losses as heat in fish. The direct approach measures the heat dissipated by the animal into the environment (Smith et al., 1978; McCollum et al., 2006). However, the far more common approach is the indirect method based on the measurement of oxygen consumption of the animals (Gnaiger, 1983). This methodology requires the use of so-called respirometers in an approach termed respirometry. The use of respirometry approaches is especially proliferating in the context of the thermal environment and its effects on fish biology (Fry & Hart, 1948; Brett, 1964; Claireaux & Lefrançois, 2007; Pörtner & Knust, 2007; Farrell et al., 2008; Pörtner et al., 2008; Pörtner & Farrell, 2008; Munday et al., 2009; Nilsson et al., 2009; Pörtner, 2010; Pörtner & Peck, 2010; Clark et al., 2011; Eliason et al., 2011; Donelson et al., 2012; Munday et al., 2012; Pörtner, 2012; Clark et al., 2013, Adams et al., 2018, 2022).

3. Respirometry and energy partitioning

The method of respirometry is based on the key metabolic pathways that combust nutrients into usable energy in the form of adenosine triphosphate (ATP). All classes of macronutrients, namely carbohydrates, lipids, and protein can be used as so-called metabolic fuel to generate ATP from adenosine diphosphate (ADP) and inorganic phosphate in several steps. Although a surplus of energy is generated by these steps, the transformation is in itself an energy-consuming process. The aerobic combustion of nutrients will yield ATP in the presence of oxygen. The reaction will be explained briefly by using three reference nutrients being combusted according to Jobling (1994). The combustion of carbohydrates can be described by the example of glucose in the following equation:

$$C_6H_{12}O_6 + 6\ O_2 \rightarrow 6\ CO_2 + 6\ H_2O$$

The combustion of lipids can be described by the reaction of palmitic acid as an example of a saturated fatty acid:

$$CH_3(CH_2)_{14}COOH + 23\ O_2 \rightarrow 16\ CO_2 + 16\ H_2O$$

The combustion of protein will yield nitrogen end products such as ammonia (NH_3) in addition to carbon dioxide and water and can be described with the exemplary reaction of alanine:

$$NH_2CHCH_3COOH + 3\ O_2 \rightarrow 3\ CO_2 + 2\ H_2O + NH_3$$

All of the mentioned reactions require O_2 and will produce heat and ATP. While the exact stoichiometry of these reactions is complex, three basic conclusions have been drawn for practical purposes (Gnaiger et al., 1983):

1. The respiration of 1 g of O_2 results in an oxicaloric equivalent of 14.06 kJ g^{-1} dissipated as heat into the environment and therefore being lost for the animal.
2. The respiratory quotient or RQ (ratio of moles of CO_2 liberated per moles O_2 consumed) allows for an estimation of the primary non-protein nutrient being combusted. The combustion of carbohydrates liberates the same amount of CO_2 as it requires O_2 (RQ = 1) while the combustion of lipids requires less O_2 than it liberates CO_2 (RQ = 0.72). The closer the RQ is to one of the two references, the more of the respective nutrient was being combusted as metabolic fuel.
3. The ammonia quotient or AQ (ratio of moles NH_3 excreted per moles of O_2 consumed) gives insight into the proportion of the energy metabolism being fuelled by the combustion of protein. An AQ value of 0.27 corresponds with 100 % of the energy metabolism being fuelled by the combustion of protein.

Oxygen is therefore the central molecule in all of the mentioned reactions and measuring its consumption by the animal is the key element of respirometry. Measuring either or both quotients, respiratory- or ammonia-, may be adequate depending on the hypothesis being tested.

The measuring of ammonia may also be advantageous for another practical reason. Ammonia excreted is not free of energy and may constitute energy losses that are important to asses to obtain a realistic RE value as a benchmark for potential growth. This thesis uses the practical assumption by Elliot & Davidson (1975) that ammonia equals an energetic loss of 24.9 kJ g^{-1}. We furthermore used the

assumption that teleost fish may excrete between 10 - 20 % of their nitrogen compounds as urea, a molecule with an energetic equivalent of 23.0 kJ g^{-1}.

Ammonia is however not the only excretory product still containing energy but constitutes a rather small fraction when compared to the energy voided as faces. With knowledge of all measurable energetic losses, it is possible to calculate the RE as a remainder of the gross energy intake (GEI) by use of an energy partitioning scheme developed by the NRC (1981) (Fig. 0-1).

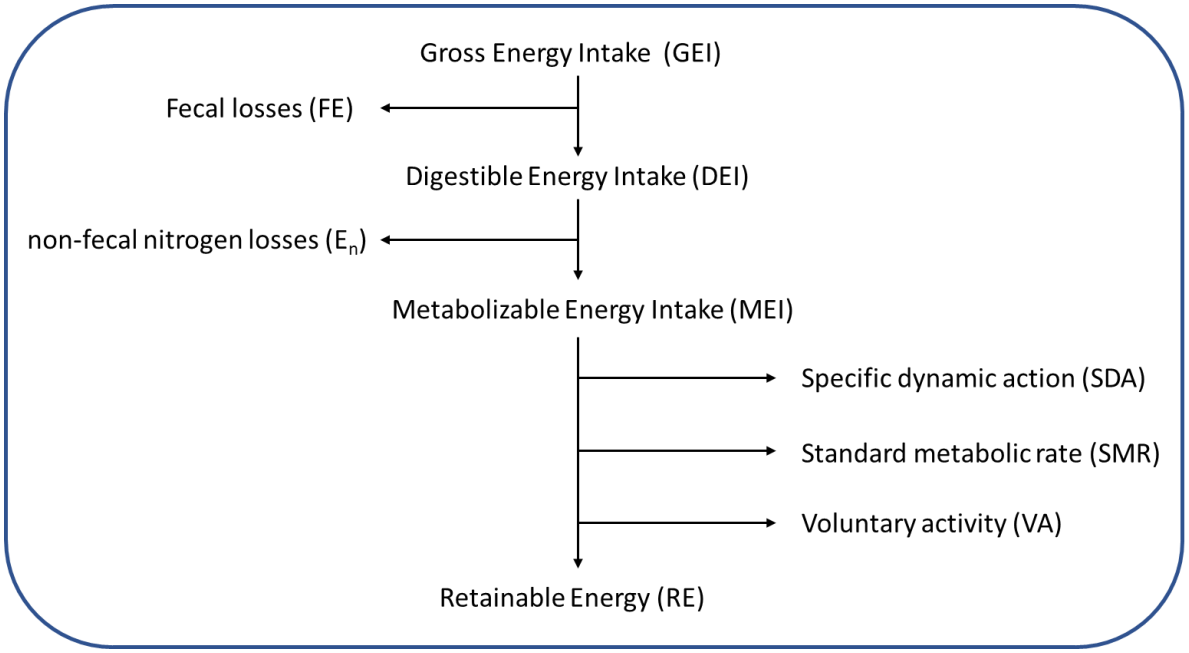

Fig. 0-1: Energy partitioning scheme as measured in this thesis, describing the energy flow through an animal (changed to NRC, 1981). Arrows pointing to the right indicate losses measurable by oxygen consumption. Arrows to the left indicate losses by excretory products.

The approach of energy partitioning according to the NRC (1981) distinguishes between three different types of oxygen-requiring processes: the standard metabolic rate (SMR), the specific dynamic action (SDA), and voluntary activities by the animal (VA). The SMR is the energy loss calculated from the oxygen consumption of an unfed and resting fish (Fry & Hart 1948; Chabot et al., 2016). It accounts for the sheer energetic expense of maintaining basic body functions such as heart rate and the upkeep of the immune system.

The VA results in an energetic expense that may be quite difficult to quantify. Therefore, many researchers have used respirometers specially designed to restrict free movement of the fish or force the animals to swim in an artificial stream at a fixed speed (Blažka et al. 1960; Brett, 1964; Nelson, 2016; Hvas & Oppedal, 2019). These systems usually test a single fish. Respirometry with groups of fish under culture-like conditions in tanks that allow free movement is rare (Lupatsch et al., 2009; Remen et al., 2016; Hvas & Oppedal, 2019). The original energy partitioning scheme by the NRC (1981) included the net energy as the remainder of MEI after losses by SMR and SDA have been accounted for. However, the assessment of net energy requires the fish to either exhibit little to no VA by restriction of free movement or quantify its energy expenses. While the research of Bureau et al. (2002) assumes the calculation of an energy budget is affected by the voluntary movement of the fish to a neglectable degree, the NRC notes that this assumption may be an oversimplification according to Cooke et al. (2000). Therefore, careful consideration of the applicability of group respirometry is required, since VA is not restricted and cannot be accurately distinguished from SMR and SDA thus not allowing for an accurate assessment of net energy.

The SDA is the postprandial metabolic response directly associated with a feeding event. It accounts for the energetic expenses of ingestion, digestion, absorption, biosynthesis, nutrient turnover, and assimilation of a meal (Jobling 1981, 1994). The SDA is measured as the excess oxygen consumption above SMR following a meal (Fig. 0-2). For practical reasons the SDA is frequently measured over 24 h following a meal but the duration of SDA responses in fish is highly variable and may range between 3.5 up to 390 h, depending on the environmental temperature, tested species, and the diet (Smith et al. 1978; Boyce & Clarke, 1997; Secor, 2009, Steinberg et al., 2017, 2018).

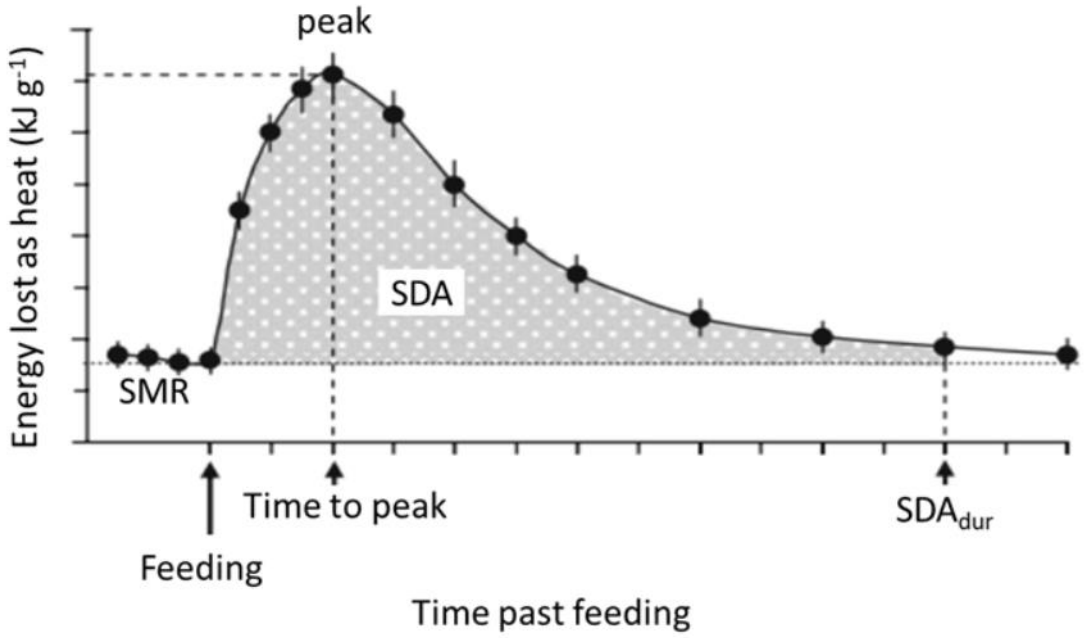

Fig. 0-2: Theoretical SDA reaction following a meal (changed to Secor, 2009).

4. Energy substrate combustion for metabolic fuel

The diet is generally known to be a highly potent modulator of the partitioning of energy. The nutrient composition of a diet may influence its digestibility, nitrogen losses, and associated protein combustion rate and the magnitude and duration of SDA (Kleiber, 1962; Beamish et al., 1986; Kieffer et al., 1998; Stone, 2003; Khan et al., 2014; Welengane et al., 2019). In general, it appears advantageous to minimize the combustion of protein for energy demands as it is associated with a higher energetic loss than other nutrients, since amino acids need to undergo costly deamination, before entering the energy metabolism (Secor, 2009). Furthermore, is protein among the highest cost drivers in aquaculture feeds and protein sources often produce a large carbon footprint due to long transportation chains and production methods, especially for plant-based protein sources like soybean meal (Sauer & Leite, 2012; Oliveira, 2016; Jones et al., 2020). To increase economic and ecological sustainability it is therefore highly beneficial if dietary protein is efficiently converted by the fish into body protein and therefore sellable yield for the producer. However, to satisfy the energy demands of the animal, alternative energy-yielding sources like carbohydrates and lipids need to be efficiently combusted by involved metabolic pathways (Carter, 2008 Guerreiro et al., 2012).

As mentioned before all classes of nutrients can be combusted to produce ATP. The central metabolic pathway for this combustion is the citric acid cycle, also known as the Krebs cycle (Krebs & Johnson, 1937). The different nutrient classes enter the citric acid cycle over different routes according to Müller & Frings (2009) as shown in Fig. 0-3. Regulation of these routes is complex but two important modulators are the temperature at which the respective reaction takes place and the concentration of nutrients (Kieffer et al., 1998; Stone, 2003). It is therefore possible to modulate the combustion of the different nutrient classes by special design of the diet. A good example of this diet-induced modulation is the so-called protein-sparing effect (Shiau & Peng, 1993; Mohanta et al., 2007; Welengane et al., 2019). When a surplus of carbohydrates and lipids are present in the diet, these nutrients will become the primary energy source for combustion, especially when the dietary protein content is limited (Kaushik & Médale, 1994). Especially the combustion of carbohydrates may be of special interest to the industry since it is a cheap, available, and sustainable source of energy.

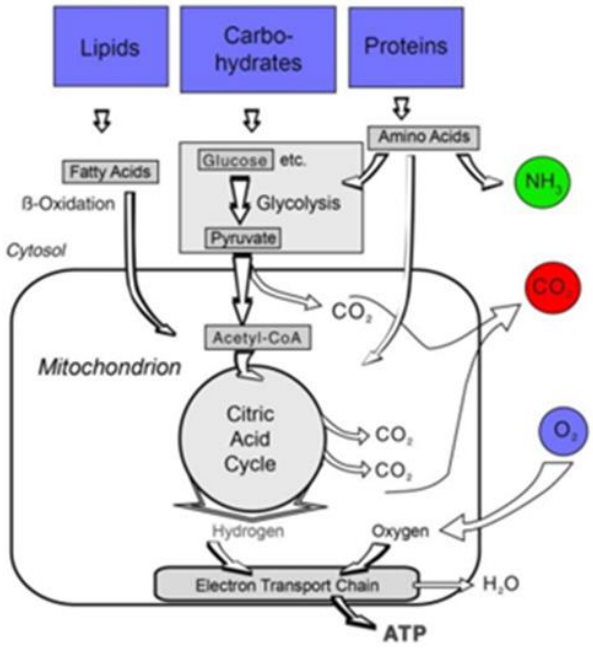

Fig. 0-3: Citric acid cycle (Krebs cycle) as the central metabolic pathway combusting nutrients in an exothermic reaction to yield ATP as the universal energy storage (changed to Müller & Frings, 2009).

5. Aim of this thesis

This thesis follows the overall aim to investigate the energy partitioning and relative combustion of nutrients over a range of temperatures towards the upper thermal optimum for growth of rainbow trout and above. The goal is to further the understanding of the interactions between the environmental temperature and the energy metabolism to maximize the potential for the growth of the fish and the efficient use of resources, especially protein.

The first chapter (Chapter I) of this thesis addresses the following research questions with special recognition of the SDA as a large fraction of the total energy losses as heat and the group respirometer as a highly complex methodology:

> *Is the SDA coefficient (the sum of energy lost as SDA over its entire course as a fraction of GEI) in rainbow trout dependent on the environmental temperature in a bioenergetic approach using group respirometry?*
>
> *Does group respirometry yield results that are comparable to the common approach of single fish respirometry with restricted movement or does it need to be recognized as a unique method?*

The trial was designed to assess metabolic data at a range of three temperatures (14; 17; 20 °C) that were sequentially applied to the entire system and each temperature was held for six days. The study was designed to test the controversially discussed hypothesis by McCue (2006) and Secor (2009) on the temperature independency of the SDA coefficient (SDA_{coef}) (Fig. 0-4). We further aimed to compare the data of RE with the model by Elliot & Hurley (2002) who have designed a sophisticated and broadly accepted model predicting RE values at a range of different temperatures while feeding different rations. At each tested temperature we fed three different rations in relation to the live body weight (BW) of the fish (0.65; 0.975; 1.3 %) in an effort to yield comparable results with Elliot & Hurley (2002) and therefore provide evidence for the practicability of group respirometry.

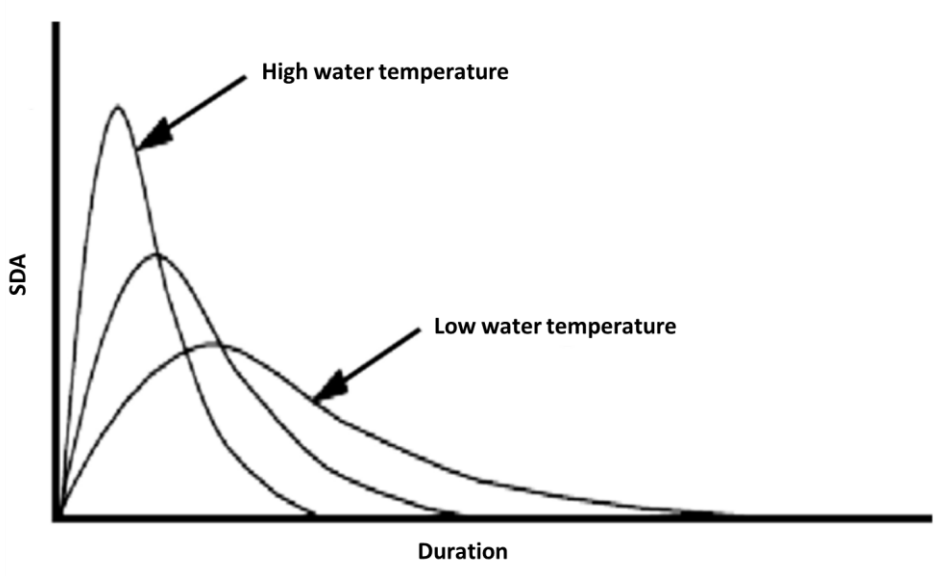

Fig. 0-4: Hypothetical SDA curves in relation to environmental temperature according to McCue (2006; changed to Luo & Xie, 2008).

Since the SDA is the energetic expense fraction most responsive to the diet it appeared relevant to continue with the focus on this variable. Early research showed that carbohydrates and lipids as dietary energy-yielding sources may result in quite similar SDA values at an environmental temperature of 8 °C but at 18 °C the SDA values in a high carbohydrate diet were significantly higher than in a high lipid diet (Gulliaume et al., 1999). Since these early findings, many advancements in the use of carbohydrates for fish and especially carnivorous species like the rainbow trout have been made. However, the impact of novel carbohydrate sources in relation to temperature on the SDA has not been re-evaluated. With increasing prices for protein and lipid sources and rising sustainability concerns, we deemed it appropriate to investigate the role of SDA in regard to the use of modern carbohydrate sources for trout feeds.

The second chapter (Chapter II) of this thesis will therefore focus on answering the following research question:

> *Do carbohydrates induce a higher SDA than lipids when used as a primary non-protein energy source and is this mechanism temperature dependent?*

The experiment was designed as a restricted ration experiment with a ration of 1.3 % body weight to all treatment groups. A total of five temperatures (12; 14; 16; 18; 20 °C) were sequentially applied to the whole system and each temperature was held for five days per measurement cycle.

The second chapter already targets the protein-sparing effect as an increasingly important topic in aquaculture nutrition. The interactions between the protein-sparing effect and the environmental temperature are still poorly understood but decreased fish meal availability and rising global temperatures have increased the necessity for adequate research on the topic. The third chapter (Chapter III) of this thesis will therefore focus on answering the following research question:

> *What are the ideal thermal conditions for a maximized visibility of the protein-sparing effect regarding temperature, dietary protein content, and protein-to-energy ratio?*

Chapter III concludes this thesis. The experiment was also designed as restricted ration experiments with a ration of 1.3 % body weight fed to each treatment group. A total of five temperatures (12; 14; 16; 18; 20 °C) were sequentially applied to the whole system and each temperature was held for five days for a measurement cycle.

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Chapter I

SDA coefficient is temperature dependent in rainbow trout (*Oncorhynchus mykiss*, Walbaum 1792) in a practical approach using group respirometry

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Abstract

Rising global temperatures have raised the need for detailed knowledge of the effects of rising temperatures on the physiology of animals used in aquaculture. Here we used a multifactorial bioenergetic approach using groups of rainbow trout (*Oncorhynchus mykiss*) with an average single fish weight of 183.75 g ± 0.65 g to investigate the interactions of feeding and temperature with key metabolic variables. We used a recirculating aquaculture respirometric system (RARS) to test three ration sizes (0.65; 0.975; 1.3 % of live body weight (BW)) over a range of three consecutive temperatures (14; 17; 20 °C). The fish were fed once per day for six days at each temperature and subsequently starved for five days to return to the standard metabolic rate (SMR). This study aimed to answer the highly discussed topic of the temperature dependency of the specific dynamic action (SDA)-variables SDA-coefficient (SDA_{coef}) and SDA-duration (SDA_{dur}). We were able to provide evidence, that in rainbow trout the SDA_{coef} is highly dependent on the environmental temperature in the first-ever approach to assess these variables in a group respirometer with this species. We compared the results of this study with a sophisticated bioenergetic model by Elliot & Hurley (2002) and thereby provide evidence for the practicability of group respirometry as a method to assess bioenergetic data under culture-like conditions.

Keywords: *Rainbow trout, SDA, group respirometry, temperature, ration size*

1. Introduction

To this day rainbow trout (*Oncorhynchus mykiss*) remains one of the most used fish in freshwater aquaculture in central Europe. While the species can tolerate a broad thermal spectrum with a critical thermal limit of up to 29 °C, growth is maximized when temperatures are at an optimum between 15 - 17 °C (Jobling, 1994; Hokanson et al., 1977; Chen, et al., 2015; Mellery et al., 2016). As all fish are poikilotherm animals it means that their body temperature and metabolic processes are highly dependent on the environmental temperature. Consequently, temperature is among the most important production limiting factors. Controlling temperature, to not exceed the optimum, can be very cost-intensive, especially but not exclusively during the summer, and is not profitable for flow-through systems or impossible, due to the origin of production water (Galbreath et al., 2004; Kankainen et al., 2014). With rising global temperatures and changes in weather patterns, research regarding the physiological responses of fish at a broad range of environmental temperatures has become increasingly important over the last years (Jobling, 1981; Handeland et al., 2008; Paschke et al., 2018; Jutfelt et al., 2021).

At temperatures above the optimum fish usually decrease their feed intake, most likely due to limited oxygen supply while oxygen demand for feeding is increased. The so-called oxy-static control of feed intake has led to the common practice to reduce rations upon above optimal temperatures but scientific background is scarce (Galbreath et al., 2004; Saravanan et al., 2012; 2013, Jutfelt et al., 2021). In general, it is important for an aquaculture enterprise to adjust feeding management so that ration sizes support the rapid and efficient growth of the animals. While animal growth is important for aquaculture enterprises it is also important to optimize the feeding efficiency described as animal growth per feed intake (Brett & Groves, 1979). Several studies suggest that the feeding efficiency is maximized when rations are below satiation, a ration size associated with maximum animal growth per se. Hardy & Kaushik (2022) describe as a general rule for feeding that maximum feeding efficiency is achieved when rations are just below satiation. Azevedo et al. (1998), however, found that feeding levels as low as 70 % of satiation do not result in a reduced nutrient retention in rainbow trout.

Alongside the practical reason of oxygen limitation, there may be another practical reason to reduce feeding rations when temperatures are above optimum. Research by Forseth & Johnson (1994) suggested that a decrease in feed intake may actually increase the optimal temperature for growth of fish. This assumption is supported by a model created by Elliot & Hurley (2002) that calculated that rations as low as 60 % of satiation may result in a higher feeding efficiency at above optimum temperatures, compared to a maximum feed intake when oxygen satiation in the water is kept at 100 %.

When considering the feeding efficiency and environmental temperatures it is also important to assess the so-called specific dynamic action (SDA), describing the energy expenditure above maintenance that can be directly associated with the ingestion, digestion and absorption, biosynthesis, nutrient turnover and assimilation of a meal (Jobling, 1994). The energy allocated to SDA may constitute a large fraction of the energy uptake and subsequent feeding efficiency but its interactions with the environmental temperature are highly controversial (Soofiani & Hawkins, 1982; Tirsgaard et al. 2015). While reviews by McCue (2006) and Secor (2009) have considered SDA variables such as the SDA coefficient (SDA_{coef}), a variable describing the percentage of energy allocated to SDA over its entire duration divided by the full energy content of a meal, to be unaffected by environmental temperatures, other studies have found a significant interaction (Soofiani & Hawkins, 1982; Guinea & Fernandez, 1997; Luo & Xie, 2008; Tirsgaard et al., 2015). Usually, respirometers used for such approaches are designed to restrict free movement in order to assess accurate measurements of sole metabolic responses. However, the use of a respirometer allowing free movement has been considered advantageous as it may measure the energy metabolism of fish under culture-like conditions (Stiller et al. 2016; Hvas & Oppedal, 2019). Although some of those group respirometers have already been used and yielded appropriate data output, a call for special carefulness when working with complicated methodologies such as respirometric systems is frequently issued and it is suggested to evaluate if the methodology is widely applicable (Clark et al., 2013; Hardy & Kaushik, 2022).

This study uses a multifactorial, bioenergetic approach using a group respirometer in order to test the highly discussed hypothesis that the SDA variable SDA_{coef} is responsive to environmental temperatures in an aquaculture-relevant species. The goal of this study was assessed in a single experimental approach using rainbow trout. The data of this study were compared to the model described by Elliot & Hurley (2002) to scale out potential limitations and advantages and make a reliable evaluation of comparability with the common respirometric systems that intentionally restrict social interaction and voluntary activity of the fish.

2. Material & Methods

2.1 Respirometric system

The trial was conducted at the Fraunhofer Research Institution for Individualized and Cell-Based Medical Engineering in Büsum, Germany, and approved by the ethics committee of the state of Schleswig Holstein. The experimental system was a flow-through respirometric system with semi-continuous detection of key water variables (Fig.1-1). The system consisted of ten housing tanks arranged in a circle and an additional reference tank each of 250 L volume respectively. The tanks were incorporated into one water body of a recirculating aquaculture system (RAS). The tanks were supplied with water by a circular pipeline with a flow rate of 360 L/h respectively. The tanks were closed and the water body had no water surface except a 7 cm^2 overflow prohibiting gas exchange with the atmosphere. The total volume of the system was 5 m^3. The system was based on the RARS described by Stiller et al. (2013). The system was modified, by adding a moving bed biofilter and a UV chamber as well as an airlift system, to saturate the water with oxygen. The system's water treatment also consisted of a 1 m^3 trickling filter, for CO_2 stripping, and a total of three sedimentation units and two particle filters. Water temperature was regulated by two flow-through coolers (Titan-4000, AB, Aqua Medic GmbH, Bissendorf, Germany) and a power switch control system (One-A2-13-60, Senect GmbH & Co. KG, Landau, Germany). Measuring of oxygen concentration in the water was performed with an oxygen optode 4330 (Aandera data instruments, Serial No. 1557, Bergen, Norway). Measurement of

pH was conducted with an intermediate junction electrode (IJ44A, TPS Pty Ltd, Brisbane, Australia). Total ammonia nitrogen (TAN) was measured manually with a loop flow ortophtaldehyde fluorometric autoanalyzer (Systea S.P.A. Anagni, Italy). All measurements besides TAN were performed automatically every second hour.

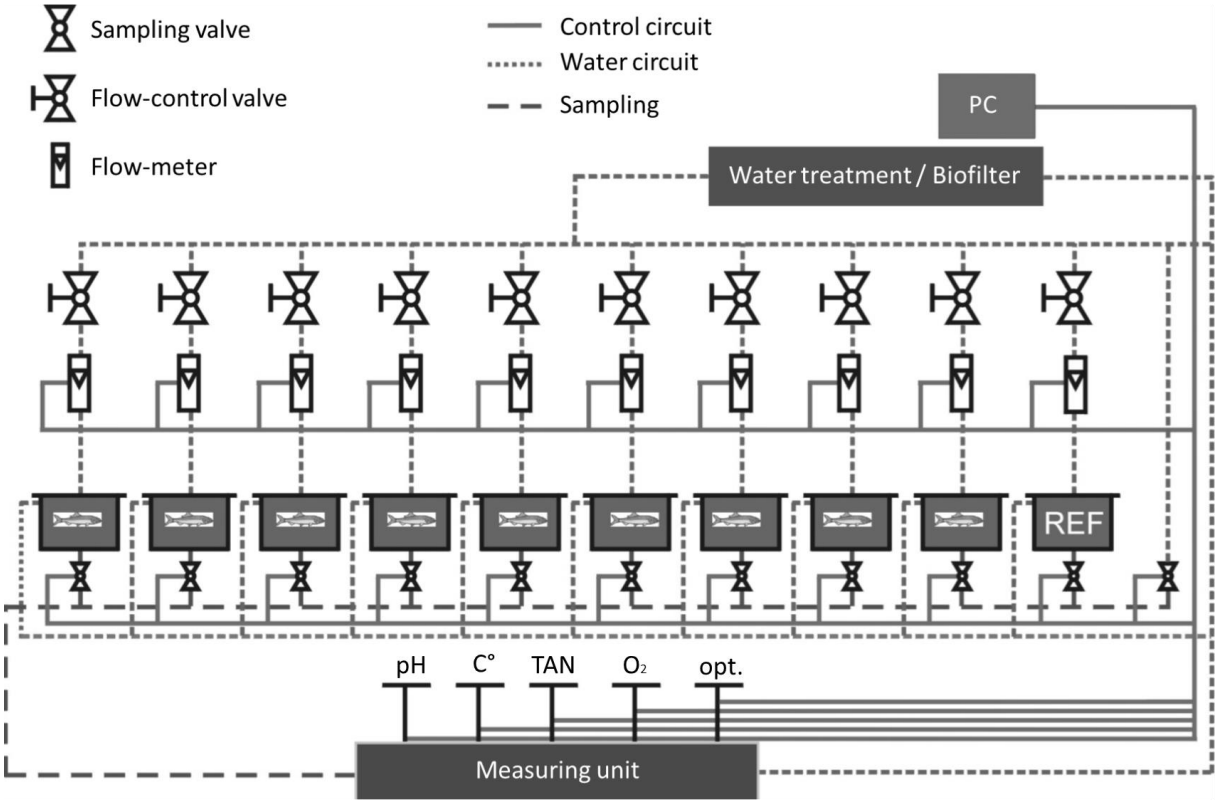

Fig. 1-1: Schematic setup of the respirometric system. (Image changed to Marxen et al. (2010). Displayed with the courtesy of the Forschungs- und Technologiezentrum Westküste).

2.2 Fish husbandry

We obtained an all-female batch of rainbow trout with an average live body weight (BW) of 183.75 g ± 0.65 g from Forellenzucht Trostadt GmbH & Co. KG at Reurieth, Germany. Upon arrival, the fish were transferred into a RAS with a volume of 1500 L with a 1000 L housing unit and a 500 L water treatment unit. We acclimated the fish to the system for a total of one week at a temperature of 12 °C. During this time, they were fed a commercial diet (ALLER Gold, 3mm, ALLER Aqua, Christiansfeld, Denmark). One week prior to the experimental start, twelve fish were transferred to nine of the

respirometer chambers respectively. The tenth chamber was kept empty of fish to assess reference measurements of the system. The stocking density per tank was 9.55 kg/m^3 ± 33.69 g/m^3. We fed the fish with 0.4 % of their respective live body weight (BW) and subsequently starved them for a total of three days prior to the experimental start. This was done to ensure that the metabolic rate of the animals would not be influenced by the last meal ingested in accordance with Ming Tang & Boisclair (2011). The fish were bulk-weighed directly before the experimental start to assess the current BW for calculations of rations and metabolic body weight (MBW) which is further described in the following sections. Oxygen levels were kept well above 70 % of saturation to ensure unobstructed respiration. Nitrogen compounds were kept below 0.05 mg/L. Ammonia was tested bihourly in each tank with the integrated autoanalyzer and nitrite twice weekly with a color indicator test kit (McColortest™, Merk KGaA, Darmstadt, Germany). Salinity remained at 3 ppm and pH of the water body at approximately 7.5. The light regime was set to a 12-h day and night cycle. Critical water parameters ammonia, nitrite, pH, turbidity, and salinity remained well within safe ranges for rainbow trout according to Hamers & Schreckenbach (2002). The temperature was kept within a range of ± 0.2 °C of the respective testing temperature.

2.3 Temperature setup and feeding

The temperature of the water body was increased from 14 °C to 17 °C, to 20 °C in three steps, over a total of 33 days. Each temperature was kept constant for a temperature sequence of 11 days. We bulk-weighed each group in between each temperature sequences to determine the current BW for rations and calculations of MBW. A total of six days were given for acclimation to each new temperature sequence. During this time the fish were fed with three different ratios of a commercial diet (ALLER Gold, 3 mm, ALLER Aqua, Christiansfeld, Denmark) daily at 10.00 a.m. The rations corresponded with the feeding recommendation by the producer at 18 °C. Rations were chosen to be 100 % of the recommendation (Standard ration (S-ration: 1.3 % BW)), 75 % of the recommendation (medium-low ration (M-ration: 0.975 % BW)) and 50 % of the recommendation (Low-ration (L-ration: 0.65 % BW)). The proximate nutrient composition of the diet is provided in Tab. 1-1. In a preliminary

test we tested 5 groups of trout at 14 °C and found that more feeding days did not lead to a further increase of postprandial oxygen consumption. In an effort to prevent group internal competition for feed, we applied the rations to the tank so that sufficient pellets were distributed in the water column at each time for each fish to ingest several pellets. New pellets were given into the experimental tanks once all supplied pellets were ingested by the group. This was done until the complete ration was ingested. All dietary treatments were performed as triplicates. During the feeding period, a total of 2 h was used for daily maintenance tasks. During the maintenance, fecal matter was removed from the tanks, the ammonia analyzer was recalibrated and the reference tank was cleaned to avoid the formation of oxygen-consuming biofilm.

Tab. 1-1: Analysed nutritional composition (% DM, after desiccation) of the test diet.

Diet	**Aller Aqua Gold (3 mm)**
Moisture (% DM)	6.6
Crude protein (% DM)	43.2
Crude lipid (% DM)	29.1
Crude ash (% DM)	7.8
NfE + crude fiber (% DM)	13.4
Gross energy (MJ/kg)	23.5

2.4 Measuring of metabolic response

Measuring of the metabolic data was performed for 24 h following the last feeding of each temperature sequence except for SDA variables SDA duration (SDA_{dur}) and SDA_{coef}, which were assessed over 100 h following the last meal. No maintenance tasks were performed during this time to leave the fish undisturbed. Oxygen, TAN, pH, and temperature were measured continuously for 100 h. Each tank was measured for 12 min at one time and a full measuring cycle of all nine stocked

tanks plus the reference was completed and logged every 2 h. We standardized the data set by applying the metabolic weight exponent of 0.8 according to Clarke & Johnston (1999) to calculate the MBW. The oxicalorific equivalent (OE) of 14.06 kJ energy loss per g O_2 consumed (Gnaiger, 1983) was applied to convert measured oxygen consumption into an energetic value. We calculated key metabolic values from the oxygen uptake and ammonia excretion and combined them into an energy budget. To conclude a temperature sequence the fish were starved for 100 h, to ensure a return to SMR according to Clark et al. (2013) and assess SDA variables SDA_{dur} and SDA_{coef}. The temperature was then increased and the next temperature sequence began.

Variables used for the calculations, if not mentioned in the calculation itself, were gross energy (GE), feed intake (FI), energy content in the diet (kJ g^{-1}) oxygen consumption (OC), and losses of nitrogen compounds via the gills (N-loss). The following formulae used are adapted from Steinberg et al. (2018).

Gross energy intake (GEI) was the total amount of energy consumed (unit: kJ) during a single meal divided by MBW.

$$\text{GEI}\ (\text{kJ kg}^{-0.8}\ \text{day}^{-1}) = \frac{\text{GE (kJ g}^{-1}\text{)} \times \text{FI (g day}^{-1}\text{)}}{\text{MBW (kg}^{0.8}\text{)}}$$

A separate trial, described below, was performed to account for fecal losses, to be able to describe the digestible energy intake (DEI) based on determined digestibility coefficients for dietary energy.

$$\text{DEI}\ (\text{kJ kg}^{-0.8}\ \text{day}^{-1}) = \frac{\text{DE (kJ g}^{-1}\text{)} \times \text{FI (g day}^{-1}\text{)}}{\text{MBW (kg}^{0.8}\text{)}}$$

Losses by non-fecal nitrogen (E_n) excretion were calculated as TAN divided by the MBW with the assumption, that finfish excrete approximately 15 % of nitrogen as urea, with an energetic equivalent of 23 kJ g^{-1} and 85 % of nitrogen as ammonia, with an energetic equivalent of 24.9 kJ g^{-1} (Elliott & Davison, 1975; France & Kebreab, 2008).

$$\text{E}_\text{n}\ (\text{kJ kg}^{-0.8}\ \text{day}^{-1}) = \frac{\text{N -loss}\ (\text{g day}^{-1}) \times \text{E}_\text{E}\ (\text{kJ g}^{-1})}{\text{MBW (kg}^{0.8}\text{)}}$$

The ammonia quotient (AQ) was calculated as the TAN extracted, divided by the oxygen consumed. An AQ value of 0.27 corresponds with a 100 % protein utilization (Gnaiger, 1983).

The difference between DEI and E_N was calculated as metabolizable energy intake (MEI).

$$\text{MEI (kJ kg}^{-0.8}\text{ day}^{-1}) = \text{DEI (kJ kg}^{-0.8}\text{ day}^{-1}) - \text{E}_\text{n}\text{ (kJ kg}^{-0.8}\text{ day}^{-1})$$

Specific dynamic action (SDA) is the energetic loss, measured as the postprandial elevated O_2 consumption calculated to the OE, above SMR.

$$\text{SDA (kJ kg}^{-0.8}\text{ h}^{-1}) = \text{heat (kJ kg}^{-0.8}\text{ h}^{-1}) - \text{SMR (kJ kg}^{-0.8}\text{ h}^{-1})$$

Heat was calculated as the sum of oxygen consumed during 24 h following the last meal, divided by MBW, and calculated to the OE.

$$\text{heat (kJ kg}^{-0.8}\text{ day}^{-1}) = \text{OC (g kg}^{-0.8}\text{ day}^{-1}) \times 14.06\text{ kJ g}^{-1}$$

SMR is determined as the mean of 10 % of the lowest oxygen consumption rates per hour measured over 100 h post-feeding following Clark et al. (2013) calculated to the OE.

The difference between MEI and heat was calculated as the retainable energy (RE), being the sum of energy of GEI subtracted by all accountable energetic losses. In this study, the RE functions as the main indicator, for potential growth of the fish.

$$\text{RE (kJ kg}^{-0.8}\text{ day}^{-1}) = \text{MEI (kJ kg}^{-0.8}\text{ day}^{-1}) - \text{heat (kJ kg}^{-0.8}\text{ day}^{-1})$$

The ammonia quotient (AQ) is calculated as moles of excreted ammonia divided by moles of consumed oxygen according to Gnaiger (1983).

SDA variables were measured according to Secor (2009) and Tirsgaard et al. (2015) over 100 h. Measured variables were SDA_{dur} defined as the time of elevated O_2 consumption above SMR until it converges with SMR + 5 % and SDA_{coef} defined as the calculated percentage of energy allocated to SDA over the entire course of SDA_{dur}.

2.5 Digestibility trial

To account for fecal losses a separate fecal collection trial was performed with the same diet, to determine their digestibility. Acid insoluble ash (AIA) was used as an inert marker. Parallel to the respirometry trial, a separate group of rainbow trout from the same batch was stocked into 1000 L round tanks with a 500 L RAS filtration unit. The temperature was increased from 14 °C to 17 °C and finally 20 °C within the same timeframe as in the respirometry trial. A digestibility was assessed for each of the three different temperatures. For technical reasons, the trial could not be performed as triplicates and serves only as an internal control. The tank was stocked with a total of 15.5 kg of fish, resulting in a stocking density of 15.5 kg/m^3. The tank was fed a daily ration of 1.3 % BW. For feces stripping, the fish were anesthetized with clove oil (1 mL per 40 L of water) until unconscious and then feces were stripped manually by slightly pressing the area around the lower gut. Digestibility was calculated using the apparent digestibility coefficient (ADC) after Cho et al. (1982) as:

$$\text{ADC of nutrient} = 1 - \left(\frac{F}{D} \times \frac{Di}{Fi}\right)$$

where D = nutrient (%) or energy (MJ/kg) concentration of diet; F = nutrient (%) or energy (MJ/kg) concentration of feces; Di = % digestion indicator (AIA) of diet; Fi = % digestion indicator (AIA) of feces. Energy content of the feces was determined via bomb calorimetry (C200, IKA, Staufen, Germany) and AIA content was assessed by the AGROLAB LUFA GmbH (Kiel, Germany).

2.6 Statistics

The statistical software R (2020) was used to evaluate the data. The data evaluation started with the definition of an appropriate statistical model, based on the generalized least squares following Box et al. (2015) for correlation and Carroll & Ruppert (2017) for heteroscedasticity. The model included the factors temperature and ration size as well as their interaction term as fixed factors. Also, the correlation of the measurement values due to the several time points (levels of temperature) was considered. The residuals were assumed to be normally distributed and heteroscedastic based on

graphic residual analysis. Based on this model, a pseudo R^2 was calculated according to Nakagawa & Schielzeth (2013) and an analysis of variances (ANOVA) was conducted, followed by multiple contrast tests in order to compare the several levels of the influence factors respectively according to Hothorn et al. (2008).

3. Results

All water conditions remained within safe levels for rainbow trout. The temperature had a fluctuation of < 0.5 °C. Neither turbidity nor high values of nitrate or ammonia occurred within the experimental time. No deaths occurred during the experiment. The fish grew from an average BW of 183.75 g ± 0.65 to an average BW of 188.34 g ± 0.87 over the entire experiment.

3.1 Digestibility of energy and nutrients

No relevant differences were detected between the different temperatures for the ADC of gross energy, crude protein, and crude lipid (Tab. 1-2).

Tab. 1-2: *Apparent digestibility coefficients of nutrients and energy in rainbow trout, tested at* three *different temperatures (n = 1).*

Temperature (°C)	Gross Energy	Crude Protein	Crude Lipid	NfE + Crude fiber
14	80,93	83,65	89,35	31,04
17	82,81	83,87	94,70	25,53
20	83,26	83,78	94,61	26,41

3.2 SMR, DEI, MEI, E_n

SMR was lowest at 14 °C and linearly increased at higher temperatures (0.65 %: p = 0.001; 0.975 %: p = 0.007; 1.3 %: p = 0.008). No statistically significant differences were detectable between the different temperatures for absolute values of DEI, MEI, and E_n (Tab .1-3).

Tab. 1-3: Energy budgets for rainbow trout reared at three different temperatures, and fed three different ration sizes. Values are cumulative values, measured over 24 h past feeding (En, heat, SDA, RE) or as a result of 100 h measuring post feeding (SMR), (n = 3).

Ration	GEI	DEI	MEI	SMR	En	heat	SDA	RE
	kJ $kg^{-0.8}$ day^{-1}							
14°C								
L	116.8 ± 0.1	94.5 ± 0.1	92.0 ± 0.7	24.8 ± 2.6	2.5 ± 0.7	36.7 ± 4.2	11.7 ± 1.6	55.4 ± 3.5
M	175.0 ± 0.6	141.6 ± 0.5	139.0 ± 0.7	24.7 ± 1.1	2.6 ± 0.4	42.7 ± 2.9	17.7 ± 2.6	96.3 ± 3.2
S	234.3 ± 1.0	189.6 ± 0.8	185.5 ± 0.7	29.3 ± 2.3	4.1 ± 0.1	47.8 ± 2.9	18.2 ± 2.1	137.7 ± 3.2
17°C								
L	117.8 ± 0.3	97.6 ± 0.3	95.6 ± 0.8	36.7 ± 1.8	2.0 ± 0.6	40.2 ± 1.7	6.5 ± 2.3	55.4 ± 1.8
M	178.0 ± 0.3	147.4 ± 0.2	144.1 ± 0.3	36.4 ± 1.9	3.3 ± 0.5	45.9 ± 4.2	8.8 ± 3.0	98.2 ± 3.9
S	234.1 ± 0.4	193.9 ± 0.3	189.9 ± 0.6	40.4 ± 1.9	4.0 ± 0.4	48.9 ± 0.6	10.3 ± 2.1	141.0 ± 1.0
20°C								
L	119.2 ± 0.3	99.3 ± 0.3	96.4 ± 0.4	35.6 ± 0.9	2.9 ± 0.2	53.0 ± 2.9	16.9 ± 2.0	43.4 ± 3.4
M	177.6 ± 0.5	147.9 ± 0.5	144.2 ± 0.9	33.6 ± 3.2	3.7 ± 0.4	54.2 ± 3.6	20.3 ± 2.5	90.0 ± 4.0
S	235.6 ± 0.5	196.2 ± 0.5	191.7 ± 0.5	38.0 ± 4.4	4.5 ± 0.1	62.2 ± 3.7	23.9 ± 2.4	130.0 ± 3.3

Values are mean ± SD, sample size is n = 3. GEI: gross energy intake; DEI: digestible energy intake; MEI: metabolizable energy; SMR: standard metabolic rate; E_n: energy of non-fecal nitrogen; heat: energy lost for heat production; SDA: specific dynamic action; RE: retainable energy. (n = 3)

3.3 SDA variables

3.3.1 Specific dynamic action duration (SDA_{dur})

The metabolic oxygen consumption post-feeding, converged with SMR +5 % within 100 h after the meal, across all groups. The duration of postprandial metabolic response increased with increasing temperatures. The duration of postprandial metabolic response increased above the lowest measured temperature. The duration of metabolic response was highest at 17 °C and lowest at 14 °C. Differences were not statistically significant ($p > 0.05$).

3.3.2 Specific dynamic action coefficient (SDA_{coef})

The post-feeding integrated excess of metabolic oxygen consumption above SMR, relative to the GEI showed a significant response to a difference in temperature at L-rations (14 ~ 17 °C: $p = 0.031$; 17 ~ 20 °C: $p = 0.036$) and S-rations (14 ~ 17 °C: $p = 0.047$; 17 ~ 20 °C: $p < 0.001$). At the M-ration, the values did not significantly differ between the temperatures. Across all ration sizes, values for 17 °C were lowest (14 ~ 17 °C: $p < 0.001$; 17 ~ 20 °C: $p < 0.001$) (Fig 1-2). Temperature and ration size did not interact significantly ($p > 0.05$).

3.4 Ammonia quotient

At the L-ration, no differences between the temperatures could be detected. Values showed a high variance, with one value at 14 °C being a multitude higher than the lower values at the same treatment. At the M-ration, values were highest at 17 °C (Fig. 1-3). The S-ration resulted in a significantly lower AQ at 20 °C than at 17 °C ($p = 0.392$). Temperature and ration size did not interact significantly ($p > 0.05$).

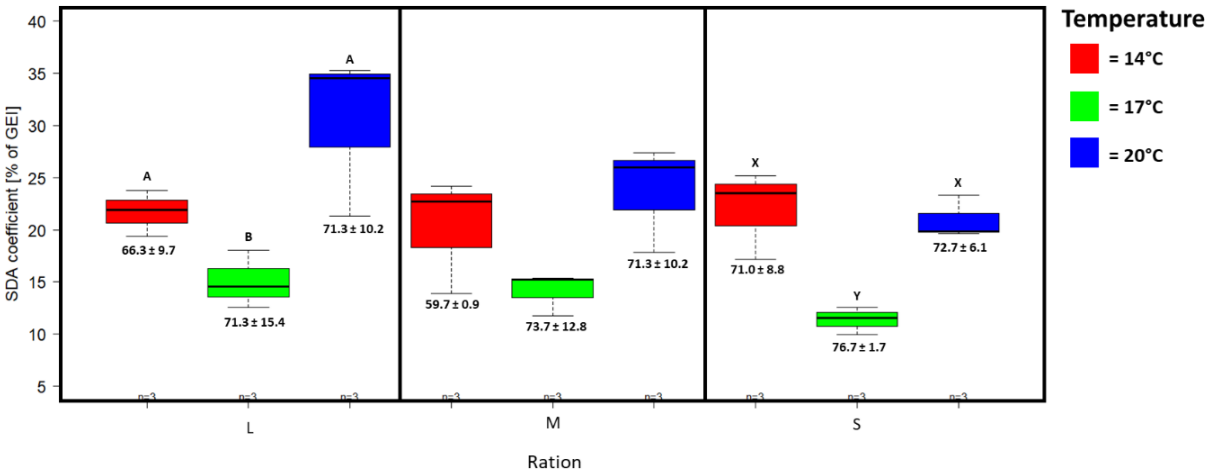

Fig. 1-2: SDA coefficient as post-feeding integrated excess of energy consumption above SMR, over the course of SDA duration, relative to the full energy content of the meal (GEI). Whiskers indicate the true values of maximum and minimum values. The boxes represent the calculated 25 and 75 percentiles. Capital letters indicate significant differences between temperatures within each ration ($p < 0.05$). Values beneath each box represent the SDA_{dur} with standard deviation.

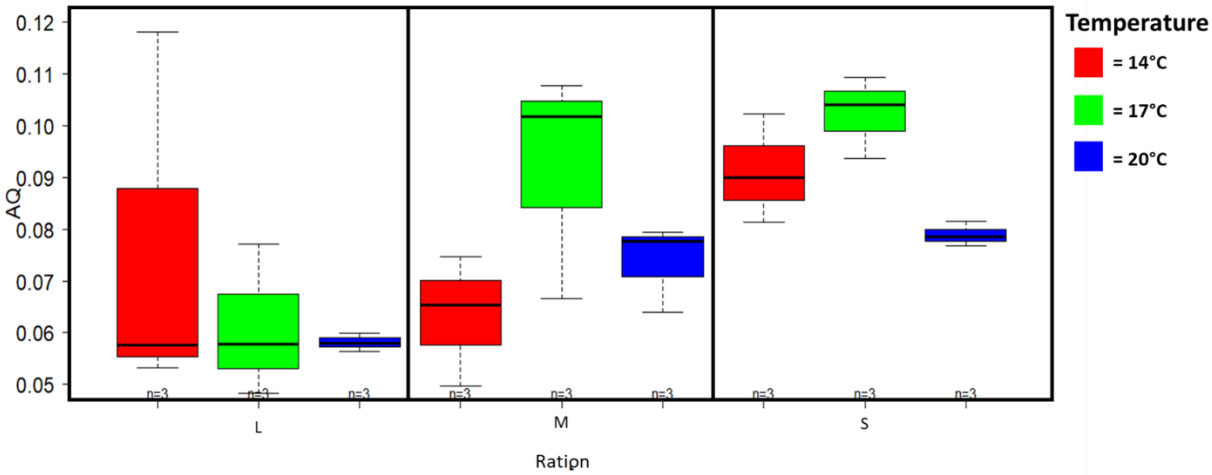

Fig. 1-3: Ammonia quotient at three different ration sizes, across three different temperatures. Whiskers indicate the true values of maximum and minimum values. The boxes represent the calculated 25 and 75 percentiles.

3.5 Retainable energy

Absolute values increased with increasing ration size across all temperatures (Tab. 1-3). Normalised to the relative percentage of GEI, the values were lowest for 20 °C regardless of the ration size (14 ~ 20 °C: $p < 0.001$;17 ~ 20 °C: $p < 0.001$; Fig. 1-4). An increase in temperature from 14 °C to 17 °C did not result in significant differences regarding the relative values of RE at L- and M-rations. The S-ration values were significantly higher at 17 °C than at 20 °C ($p = 0.001$). In accordance with absolute values, relative values also increased with increasing ration size, across all temperatures. Temperature and ration size did not interact significantly ($p > 0.05$).

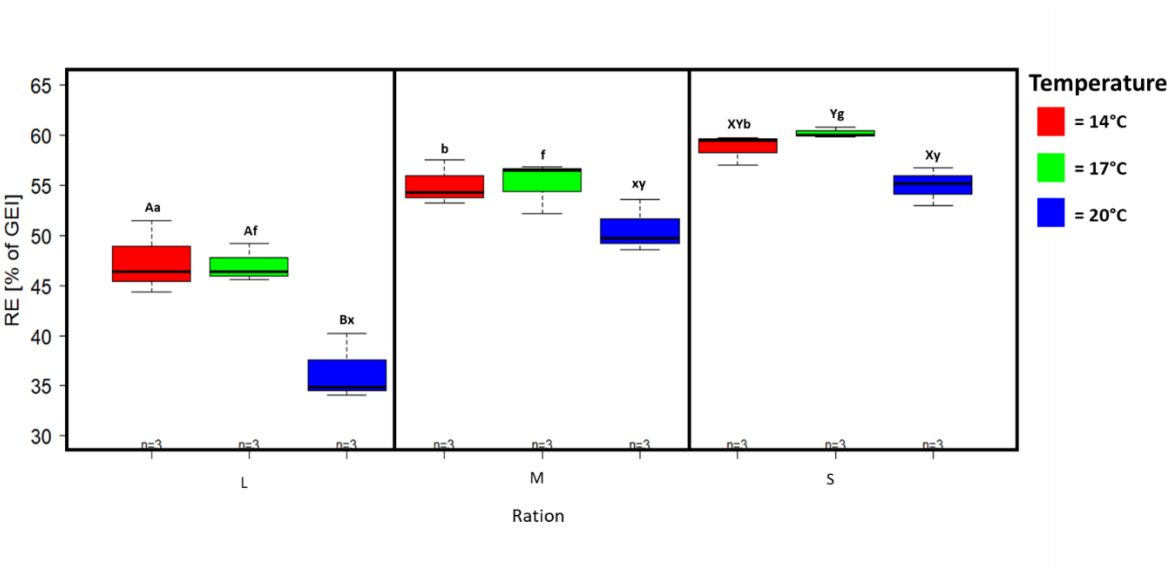

Fig. 1-4: Percentage of RE, relative to gross energy intake (GEI) of rainbow trout at three different ration sizes. The median is marked as a solid black line in the boxes. Whiskers indicate the true values of maximum and minimum values. The boxes represent the calculated 25 and 75 percentiles. Capital letters indicate significant differences between temperatures within each ration. Lowercase letters indicate significant differences between rations, within each temperature ($p < 0.05$).

4. Discussion

Aim of this study was to identify the interactions of ration size and temperatures and their interacting effects on the energy partitioning in rainbow trout. The temperatures were chosen to cover a range from below optimal temperatures for growth of rainbow trout at 14 °C via the optimal temperature of 17 °C according to Hokanson et al. (1977) to a deleterious temperature of 20 °C according to

Jobling (1994). These temperatures are all frequently found in flow through systems in Germany and are representative for the common thermal environment for rainbow trout farming in central Europe (Weirup et al., 2021). Feeding rations were chosen so that the S-ration was according to the recommended ration of the used commercial diet for trout of 100 - 200 g BW at 18 °C, as no recommendation was available for 17 °C. Lower rations corresponded with 50 - 75 % of the recommendation. The study was overall designed to test relevant conditions for trout production in central and northern Europe. We used a fixed ration in this study, as the voluntary feed intake rates may alter at different environmental temperatures. This mechanism is termed oxy-static control for feed intake or aerobic scope protection by reduction of food intake and is associated with limited oxygen supply in water at higher temperatures (Galbreath et al., 2004; Saravanan et al., 2012; 2013; Jutfelt et al., 2021).

Results of the apparent digestibility coefficient (ADC) in this study showed no effect of different environmental temperatures on the digestibility of energy or nutrients between 14 - 20 °C. We used the data as an internal validation and for the calculation of DEI. The assumption that digestibility may be unaffected by the environmental temperature at this thermal range is supported by the findings of Cho & Kaushik, (1990), who have investigated the effect on the apparent digestibility coefficient in rainbow trout and found no significant differences between 9 - 18 °C and Jobling (1994) who found digestibility of energy unaffected by a temperature increase from 11 - 15 °C.

The BW has been determined as a relevant factor that may alter the metabolic response of fish (Jobling, 1994). The feeding regime of six days of feeding and five days of fasting in combination with the given rations were chosen, to minimize actual growth and measure metabolic benchmarks for potential growth. Different BW due to growth might have resulted in misinterpretations of the results at different temperatures. The chosen feeding regime however, resulted only in a growth of 2.5 % and can be considered a minor factor.

We found standard metabolic rate (SMR) to increase linearly upon rising temperatures in this trial. SMR is mainly dependent on environmental factors and genetic and habitual background of the animals and unaffected by the diet since it describes by definition the losses of metabolic processes without the influence of feeding.

In this study, we assessed the specific dynamic action (SDA) over its entire course to determine the effects of temperature on SDA variables in rainbow trout, most importantly the duration until oxygen consumption returns to that of an unfed fish (SDA_{dur}) and total energy loss in relation to GEI termed SDA coefficient (SDA_{coef}).

The results of SDA_{dur} yielded results that contradict the findings of McCue (2006) and Secor (2009) that SDA_{dur} values would generally decrease upon increasing temperatures. In this study, the SDA_{dur} values were among the highest at an optimal temperature of 17 °C and lowest at the lowest tested temperature of 14 °C. When comparing the data with other studies it is necessary to consider the applied feeding strategy in terms of frequency and ration (Lo et al., 2022). Feeding to satiation may lead to different results as a fixed ration as the feed intake may also be dependent on the environmental temperature which may in turn substantially change the outcome of SDA_{dur} measurements (Jordan & Steffensen, 2007). However, several studies with a fixed ration fed once daily have found a low point of SDA_{dur} at the optimal temperatures for the respective tested species (Björnsson et al., 2001; Jordan & Steffensen, 2007; Behrens et al., 2012; Tirsgaard et al., 2015). Adams et al. (2018) found a similar response of increasing SDA_{dur} values when the water temperature was increased from 21 °C to 25 °C for a strain of rainbow trout specifically selected for high-temperature tolerance (H-strain, Pemberton Freshwater Research Centre, South Perth, Australia).

The values of SDA_{coef} appear to show a high level of responsiveness to environmental temperatures, contrary to the hypotheses of McCue (2006) and Secor (2009) as well as recent studies (Lo et al., 2022). At S- and L-rations, energetic losses at optimal temperatures (17 °C) were significantly lower than at

suboptimal temperatures (14; 20 °C). At the M-ration, the differences were non-significant but showed with a clear trend, that losses at 17 °C were lowest. Several studies have found similar results as the here presented study, stating, that the SDA_{coef} may respond to environmental temperatures in seabream (*Sparus aurata*; Guinea & Fernandez, 1997), cod (*Gadus morhua*; Soofiani & Hawkins, 1982; Tirsgaard et al., 2015), southern catfish (*Silurus meridionalis*; Luo & Xie, 2008) and several invertebrate species (Kalarani & Davies, 1994). Flikac et al. (2020) found however, that SDA responses at different temperatures may significantly vary among species and findings should not be generalized even if the species naturally share the same environment. The presented study is among the first to assess the SDA variables SDA_{dur} and SDA_{coef} using a group respirometer that allows free movement of the animals in the respiration chambers but was able to provide comparable findings to the common approach with single-fish respirometers.

Tirsgaard et al. (2015) have hypothesized that the digestibility of nutrients and energy may be responsible for an altered SDA_{coef} upon different environmental temperatures because protease activity appears to be lower at lower temperatures (Kofuji et al., 2005). Protein utilization is generally of special interest in research of SDA variables because several studies argue that up to 80 % of the magnitude of the SDA response could represent protein turnover and growth and that a larger SDA may indicate a higher growth potential (Coulson et al., 1978; Brown & Cameron, 1991; Khan et al., 2014). Since the ammonia quotient (AQ) reflects the relative proportion of the energy metabolism that is fueled by protein combustion, a higher SDA due to increased protein turnover for growth would consequently result in an increased AQ (Gnaiger, 1983). However, the AQ values in this study do not indicate such an effect as the lowest SDA values correspond with the highest AQ values. It is likely that in this study the ability to move “freely” is to a large degree responsible for the magnitude of the SDA_{coef}. In rainbow trout, it appears that the energy expenditure for movement is lower at optimal temperatures than beyond that range (Kieffer et al., 1998). It is therefore possible, that free movement would lead to a decreased SDA_{coef} at optimal temperatures, where movement is more inexpensive. The assumption is backed, as the SDA_{coef} and SDA_{dur} values appear to be independent of one another.

SDA_{dur} does not appear to be influencing the magnitude of SDA_{coef} but rather follows a circadian cycle, as the SDA response terminated during the night photoperiod in all treatments. It is known that temperature is a major modulator in fish behavior and that higher temperatures lead to a higher social interaction between individuals (Bartolini et al., 2014). This may explain the increased SDA_{dur} at higher temperatures in a system, where interaction and free movement are not restricted and can be modulated by thermal conditions.

As group respirometry allows for the voluntary activity of the animals, a trait that has often been viewed with concern since it may alter the oxygen uptake rates, it is important to assess whether the data of this study are overall comparable to other studies, using single respirometer chambers. We did so by in-depth comparison of the data of retainable energy (RE) with the established model by Elliot & Hurley (2002) which describes a similar approach of decreasing rations in rainbow trout at a range of different temperatures.

Absolute levels of RE levels were naturally higher at higher feeding rations based on a higher gross energy intake (GEI). The feeding efficiency which can be described as the relative share of RE on the GEI appeared to increase in a similar manner. We detected a low point of RE values at a temperature of 20 °C regardless of the feeding ration, which was significantly below that of the optimum at 17 °C in S and L rations. One key aspect of this study was to provide scientific background on the practicability and comparability of group respirometry with the common bioenergetic approach, which purposely restricts the voluntary movement of the animals. We found a high resemblance of our RE data with data provided by Elliot & Hurley (2002) who described a detailed model of energetic gains in relation to temperature and ration size in rainbow trout but measuring single fish in small compartment respiratory chambers. Elliot & Hurley (2002) found that RE in relation to increasing temperatures would result in a peak at the optimal temperature but that decreasing rations would lead to a generally lower curve with reduced slopes but in all rations an abrupt decline of RE at temperatures above a threshold of approximately 19 °C. At L-rations our RE results show little to no difference between 14 - 17 °C but an abrupt decline at 20°C indicating a flat slope without a true peak as described by the

model of Elliot & Hurley (2002). At the M-ration we observed a slightly higher RE at 17 °C than at 14 °C but without significance and again a decline at 20 °C, a pattern that might indicate a steeper slope towards the peak at the optimal temperature. At the S-ration the values of RE show a clear trend of a higher RE at 17 °C than at 14 °C indicating a steep slope towards a clear peak with a subsequent decline in RE at 20 °C. Overall the data of RE in this study fit the model of Elliot & Hurley (2002) very well. The model has been well established and is based on several previous studies and applies to a number of salmonid species tested in both growth and bioenergetic trials (Elliott & Hurley, 1995; Elliot & Hurley, 1997; Connor & Burge, 2003; Brown, 2004; Marine & Cech, 2004; Larsson et al., 2005; Forseth et al., 2009; Clark et al., 2011).

Although some studies have recognized group respirometry that allows free movement of the fish as a more natural approach that allows measurement of metabolic changes under culture-like conditions the by far more common bioenergetic approach is still to measure the fish in a single chamber that does not allow the fish to move freely (Remen et al., 2016; Stiller et al. 2016; Steinberg et al., 2018; Hvas & Oppedal, 2019). By providing a dataset that was assessed in a group respirometer but still largely resembles a well-established model assessed in a movement-restricting single fish respirometer we provide strong evidence that group respirometry produces data output that is highly comparable to common bioenergetic approaches and once again provides evidence, that bioenergetic approaches may yield results, that depict culture like conditions and are not as artificial as previously indicated.

5. Conclusion

This chapter followed the goal to test the controversial hypothesis, that SDA_{coef} is independent of environmental temperatures in poikilotherm animals. We found that the SDA_{coef} is in fact responsive to environmental temperatures in rainbow trout, a finding aligning with a number of recent studies addressing a variety of teleost fish. The study uses the first-ever approach to test this hypothesis in a group respirometer that allows for voluntary activity of the fish. By a detailed comparison of our results of RE values with the model created by Elliot & Hurley (2002) we could show that group respirometry

is an applicable method that yields comparable results to the common approach of inhibiting voluntary activity. Group respirometry may therefore be more applicable and relevant than initially indicated.

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Chapter II

Dietary carbohydrates induce a higher SDA than lipids in rainbow trout (*Oncorhynchus mykiss*) based on environmental temperature

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Abstract

As global temperatures increase so does the need to investigate how the energy metabolism of fish responds to a broad range of thermal conditions. Limited resources make it additionally important to use them sustainably in the feeds for aquaculture. Here we investigated the use of three different lipid-to-carbohydrate ratios (1 : 1; 0.6 : 1; 1.4 : 1) as non-protein energy substrates (NPES) in diets for rainbow trout (*Oncorhynchus mykiss*) under five different thermal regimes (12; 14; 16; 18; 20°C) in a bioenergetic approach using a group respirometer. The results showed that the diet with carbohydrates as the main NPES resulted in a quadratic relationship of the specific dynamic action (SDA) values to temperature while diets with lipids as the main NPES or a balanced ratio did not show such a response. SDA values in the diet with carbohydrates as the main NPES were significantly higher at temperatures around the optimum (15 - 17 °C) than in the diets with lipids as the main NPES or with a balanced lipid-to-carbohydrate ratio. The retainable energy (RE) was highly dependent on the standard metabolic rate (SMR) and SDA values did not carry over onto them. The protein utilization for energy combustion was significantly lower at 12 °C in the diet with carbohydrates as the main NPES than in the diet with lipids as the main NPES thus indicating that carbohydrates hold a relevant nutritional value, especially at lower temperatures.

Keywords: *respirometry, specific dynamic action, energy partitioning, energy substrates*

1. Introduction

Rainbow trout (*Oncorhynchus mykiss*) is still among the most used fish species for freshwater aquaculture in Europe. Although the species can tolerate a wide range of different temperatures, growth is known to be optimized at temperatures between 15 – 17 °C (Hokanson et al., 1977; Mellery et al., 2016). Temperatures above that optimum have long been discovered to have deleterious effects for the animals in terms of growth. In subtropical regions, the water temperatures on production sites already frequently exceed that optimum and with developments in global climate change similar events are forecasted even in more temperate regions (Battaglene et al., 2008; Clark et al., 2013; Lorentzen, 2009). The high costs or lack of possibility to control water temperature, especially in open system production, raise the need to investigate how to fish metabolism reacts to a broad range of environmental temperatures and how producers can cope with these effects (Galbreath et al., 2004; Kankainen et al., 2014).

As all fish are poikilothermic, temperatures are known to have significant influences on their energy metabolism (Cho et al., 1982; Cho & Kaushik, 1990). Low temperatures reduce the standard metabolic rate (SMR) thus resulting in lower metabolic maintenance costs (Jobling, 1994). Moreover, the environmental temperature appears to have a considerable impact on the metabolic fate of energy-yielding macronutrients (Enes et al., 2006; Kieffer et al., 1998; Moreira et al., 2008).

A key target of aquaculture research is to spare protein from being combusted for energy yield and make it available for growth as it is still among the largest cost factors (Jones et al., 2020). The provision of a surplus of available energy from non-protein energy substrates (NPES), mainly lipids and carbohydrates, has been proven to be able to spare protein from combustion in order to yield energy and induce the so-called protein-sparing effect (Kaushik & Médale, 1994).

While protein and lipids have long been known to supply fish with a large fraction of their energy demands, carbohydrates have only more recently been discovered to have good nutritional properties even for carnivorous species. In general, fish have no need to ingest and metabolize carbohydrates

because energetic requirements can be met solely by protein and lipids and carbohydrates required for anabolism can be synthesized by the fish themselves from amino- and fatty acids (NRC, 2011). The use of carbohydrates has therefore long been neglected in the use of aquafeeds, especially for the use in carnivorous fish like rainbow trout. However, the development of feed source processing and research have led to carbohydrate sources that are highly digestible and provide metabolizable energy to the animal (Kamalam et al., 2017). The use of pregelatinized starches is among the most promising methods to provide metabolizable energy from carbohydrates as a cheap and available source (Bergot, 1993; Honorato et al., 2010; Hua & Bureau, 2009).

Few studies have tested the metabolic fate of metabolizable energy from carbohydrates regarding the macronutrient composition or the interactions of environmental temperatures on the energy metabolism of fish. In general energy metabolism of fish can be assessed in bioenergetic approaches, that estimate the energetic gains of a meal by measuring energetic expenses (Jobling, 1994). Using such an approach Beamish et al. (1986) found that a diet with carbohydrates as the primary NPES lead to a significant increase in postprandial energy expenditure above SMR termed specific dynamic action (SDA) compared to a diet with lipids as the primary NPES at 15 °C. Further studies found that the effect may possibly be temperature-dependent (Gulliaume et al., 1999). The effects may well be dependent on secretagogue mechanisms in the insulin system but its complexity is still poorly understood (Polakof et al., 2011).

In general, the SDA is considered a key value to be assessed in bioenergetic approaches. It is the integrated postprandial excess in energy consumption above the metabolic baseline following a meal. It is considered to be the postprandial metabolic response directly associated with a feeding event and accounts for the energetic expenses of ingestion, digestion, absorption, biosynthesis, nutrient turnover, and assimilation of the diet (Jobling, 1994).

An efficient method to assess energetic expenditures is by measuring metabolism via oxygen consumption rates with a respirometer. Although the combustion of different macronutrient classes

may yield different amounts of energy, the stoichiometry of the chemical reactions allows for the practical simplification that respiration of 1 g of O_2 results in an oxicalorific equivalent (OE) of 14.06 kJ g^{-1} dissipated as heat into the environment and therefore being lost for the animal (Gnaiger, 1983; Jobling, 1994). The approach also allows the measurement of ammonia excretion which can be calculated via the oxygen consumption to determine the instantaneous protein turnover rate. This method of instantaneous metabolic fuel use has been considered more accurate than the depletion of nutrients over time from the tissues as it is done in compositional approaches (Brett, 1995; Lauff & Wood, 1996).

This study investigated the hypothesis, that isoenergetic and isonitrogenous diets with different lipid-to-carbohydrate ratios induce a similar SDA response at lower temperatures but higher lipid ratios result in a lower SDA at higher temperatures. The second tested hypothesis is that a surplus of NPES at a constant dietary protein level results in a similar rate of protein utilization for energy, meaning carbohydrates and lipids have similar protein-sparing properties regardless of the environmental temperature. By using a respirometer which allows the fish to show a regular social and swimming behavior we aim to provide a link between the bioenergetic approach using small container measuring tanks and trials using a compositional approach that holds the fish under culture-like conditions.

2. Material & Methods

2.1 Respirometric system

The trial was conducted using a recirculating aquaculture respirometric system (RARS) described by Stiller et al. (2013) at the Fraunhofer-Fraunhofer Research Institution for Individualized and Cell-Based Medical Engineering in Büsum, Germany (Fig. 2-1). The described system was expanded by a UV chamber and a 700 L moving bed biofilter. A total of ten tanks with an individual volume of 250 L were arranged in a circle and supplied with water at a rate of 360 L/h per tank. The tanks were closed and the water body had no water surface exposed to air except a 7 cm^2 overflow to prohibit gas exchange with the atmosphere. Temperature control was achieved using two flow-through coolers

(Titan-4000, AB, Aqua Medic GmbH, Bissendorf, Germany) and a power switch control system (One-A2-13-60, Senect GmbH & Co. KG, Landau, Germany). Key water parameters were measured semi-continuously with high-resolution oxygen optode (O_2) (Aandera data instruments, Model: 4330, Serial No. 1557, Bergen, Norway), an intermediate junction electrode (pH; IJ44A, TPS Pty Ltd, Brisbane, Australia) and an automatized loop flow ortophtaldehyde fluorometric autoanalyzer (total ammonia nitrogen (TAN; Systea S.P.A. Anagni, Italy). All tanks were measured with the same sensors to avoid measurement errors.

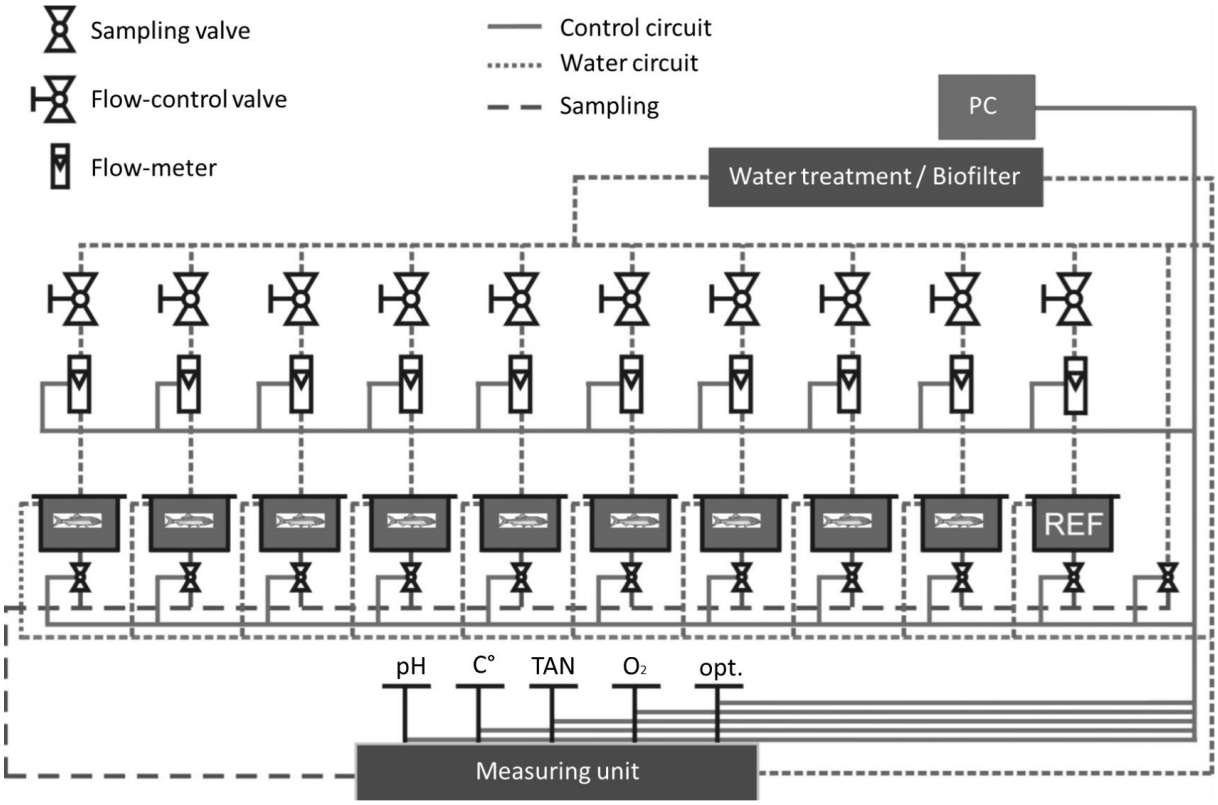

Fig. 2-1: Schematic setup of the respirometric system. (Image changed to Marxen et al. (2010). Displayed with the courtesy of the Forschungs- und Technologiezentrum Westküste).

2.2 Fish husbandry

An all-female batch of rainbow trout with an average live body weight (BW) of 234.58 g ± 3.46 g was obtained from Forellenzucht Trostadt GmbH & Co. KG at Reurieth, Germany. Upon arrival, the fish were transferred into a 1500 L recirculating aquaculture system (RAS) with a 1000 L housing unit and a 500 L water treatment unit. The fish were acclimated to the system at a temperature of 15 °C for a

total of two weeks. During this time, they were fed a commercial diet (ALLER Gold, 3 mm, ALLER Aqua, Christiansfeld, Denmark). One week prior to the experimental start, the fish were transferred to nine of the respirometric chambers, with the tenth chamber kept empty of fish to assess reference measurements of the system. Ten trout were stocked per tank, resulting in a stocking density of 9.38 kg/m^3. Each group of ten trout in a tank served as an individual replicate. Each dietary treatment was performed in triplicates (n = 3). The trout were starved for four days prior to the experimental start to ensure that the metabolic rate of the animals would not be influenced by the last meal ingested (Ming Tang & Boisclair, 1995). The fish were bulk-weighed directly before the experimental start to assess the current body weight (BW) for calculations of rations and metabolic body weight (MBW) which is further described in the following sections. To ensure unobstructed respiration, oxygen levels were kept well above 70 % of saturation. Ammonia and nitrite were kept below 0.05 mg/L. Ammonia was tested bihourly in each tank with the beforementioned autoanalyzer and nitrite twice weekly with a color indicator test kit (McColortest^{TM}, Merk KGaA, Darmstadt, Germany). Salinity was adjusted to 2 ppt by adding full marine sea water (23 ppt) to tap water during the initial filling of the tank and remained at that level without any further application. The pH of the water body remained at approximately 7.5. The light regime was set to a 12- h day and night cycle. Critical water parameters ammonia, nitrite, pH, turbidity, and salinity remained well within safe ranges for rainbow trout according to Hamers & Schreckenbach (2002). The temperature was kept within a range of ± 0.2°C of the respective testing temperature.

2.3 Diet formulation and processing

The diets were formulated as isoenergetic and designed to contain three different dietary lipid-to-carbohydrate energy ratios. The diets were formulated isonitrogenous to contain approximately 37 % crude protein and only carbohydrate energy to lipid energy were adjusted. The diets in this study will therefore be referred to by their primary source of NPES as the balanced diet (BAL), carbohydrate energy diet (COH), and lipid diet (LIP) with corresponding lipid to carbohydrate energy ratios of: BAL = 1 : 1; COH = 0.6 : 1; LIP = 1.4 : 1 (Tab. 2-1). Bentonite was used as a non-nutritive filler as wheat

starch and fish oil provide different volumes at the same energy content. Also, wheat gluten was added with the removal of wheat starch to account for the gluten content in this ingredient and therefore to keep the diet isonitrogenous.

Tab. 2-1: Ingredient composition (% of inclusion of original substance) of the test diets. Pellet size 4 mm.

	COH	**BAL**	**LIP**
Raw material	% of inclusion		
Fish meal (Clupea sp.)	42.00	42.00	42.00
Blood meal	5.00	5.00	5.00
Gelatine	5.00	5.00	5.00
Biolysine	0.30	0.30	0.30
DL_Methionine	0.10	0.10	0.10
L-Phenylalanine	0.36	0.36	0.36
Wheat starch	40.00	32.70	27.50
Wheat gluten	0.00	0.18	0.30
Fish oil	4.10	6.95	8.95
Vitamin premix	0.50	0.50	0.50
TiO_2	1.00	1.00	1.00
$Ca_3(PO_4)_2$	1.00	1.00	1.00
Bentonite	0.64	4.91	7.99

After homogenization of the ingredients, the feed was pelletized (4 mm; L 14-175, AMANDUS KAHL; Reinbeck, Germany). The diets were designed to meet the nutritional requirements of rainbow trout, according to NRC (2011), especially regarding amino acids and essential fatty acids. Titanium dioxide was added to each diet, with an inclusion level of 1 % of the total volume, to serve as an inert marker for assessment of digestibility. The diets were analyzed dry matter after desiccation at 103 °C, followed

by 4 h at 550 °C to determine ash content. Crude protein content was assessed via the Kjeldahl method (InKjel 1225 M, WD 30; Behr Düsseldorf, Germany)(Tab. 2-2). Gross energy content was determined via bomb calorimetry (Atwater & Snell, 1903; C200, IKA, Staufen, Germany) and crude lipid with HCl hydrolysis with subsequent extraction using petroleum ether with a Soxhlet extraction (R106, S; Behr, Düsseldorf, Germany).

Tab. 2-2: Analyzed nutritional composition (% of OS) of the test diets. Pellet size 4mm.

Feed composition	COH	BAL	LIP
Moisture (% DM)	9.54	7.74	8.74
Crude protein (% DM)	37.01	37.36	37.79
Crude lipid (% DM)	8.59	11.46	13.56
Crude ash (% DM)	11.11	14.84	17.32
NfE + crude fiber (% DM)	33.75	28.60	23.70
Gross energy (MJ/kg)	17.77	17.99	17.78

2.4 Temperature setup and feeding

The temperature of the water body was sequentially increased from 12 - 20 °C in 2 °C steps, over a total of 45 days. Each temperature was held for a nine-day temperature sequence. All groups were bulk-weighed in between each temperature sequence to determine the current BW for rations and MBW. Five days were given for acclimation to each new temperature sequence. During this time the fish were fed with 1.3 % BW of their respective diet daily at 10.00 a.m. Prior to the experiment, we tested three groups of trout at 12 °C and found that more feeding days did not lead to a further increase in postprandial oxygen consumption. We therefore determined five days to be sufficient for the animal to adapt to the applied feeding regime. During feeding, we applied the rations to the tank so that sufficient pellets were distributed in the water column at each time for each fish to ingest several pellets to decrease group internal competition. New pellets were given into the experimental

tanks once all supplied pellets were ingested by the group. This was done until the complete ration was ingested. During the feeding period, a total of two h was used for daily maintenance tasks. During the maintenance, fecal matter was removed from the tanks, the ammonia analyzer was recalibrated and the reference tank was cleaned to avoid the formation of oxygen-consuming biofilm.

2.5 Measuring of metabolic response

Measuring of the metabolic data was performed for 24 h following the last feeding of each temperature sequence. No maintenance tasks were performed during this time to leave the fish undisturbed. Oxygen, TAN, pH, and temperature were measured continuously during the 24 h. Each tank was measured for 12 min at one time and a full measuring cycle of all nine stocked tanks plus the reference was completed and logged every 2 h. We standardized the data set by applying the metabolic weight exponent of 0.8 according to Clarke & Johnston (1999) to calculate the MBW. The oxicalorific equivalent (OE) of 14.06 kJ energy loss per g O_2 consumed (Gnaiger, 1983) was applied to convert measured oxygen consumption into an energetic value. We calculated key metabolic values from the oxygen uptake and ammonia excretion and combined them into an energy budget. To conclude a temperature sequence the fish were starved for 96 h, to ensure a return to SMR according to Clark et al. (2013). The temperature was then increased and the next temperature sequence began.

Variables used for the calculations, if not mentioned in the calculation itself, were gross energy (GE), feed intake (FI), energy content in the diet (kJ g^{-1}) oxygen consumption (OC), and losses of nitrogen compounds via the gills (N-loss). The following formulae used are adapted from Steinberg (2018).

Gross energy intake (GEI) was the total amount of energy consumed during a single meal divided by MBW.

$$\text{GEI}\ (\text{kJ kg}^{-0.8}\ \text{day}^{-1}) = \frac{\text{GE (kJ g}^{-1}\text{)} \times \text{FI (g day}^{-1}\text{)}}{\text{MBW (kg}^{0.8}\text{)}}$$

A separate trial, described below, was performed to account for fecal losses, to be able to describe the digestible energy intake (DEI) based on determined digestibility coefficients for dietary energy.

$$\text{DEI (kJ kg}^{-0.8}\text{ day}^{-1}) = \frac{\text{DE (kJ g}^{-1}) \times \text{FI (g day}^{-1})}{\text{MBW (kg}^{0.8})}$$

Losses by non-fecal nitrogen (E_N) excretion were calculated as TAN divided by the MBW with the assumption, that finfish excrete approximately 15 % of nitrogen as urea, with an energetic equivalent of 23 kJ g^{-1} and 85 % of nitrogen as ammonia, with an energetic equivalent of 24.9 kJ g^{-1} (Elliott & Davison, 1975; Dosdat et al., 1996; Kajimura et al., 2004; France & Kabreab, 2008).

$$\text{E}_\text{n}\text{ (kJ kg}^{-0.8}\text{ day}^{-1}) = \frac{\text{N -loss (g day}^{-1}) \times \text{E}_\text{E}\text{ (kJ g}^{-1})}{\text{MBW (kg}^{0.8})}$$

The difference between DEI and E_N was calculated as Metabolizable energy intake (MEI).

$$\text{MEI (kJ kg}^{-0.8}\text{ day}^{-1}) = \text{DEI (kJ kg}^{-0.8}\text{ day}^{-1}) - \text{E}_\text{n}\text{ (kJ kg}^{-0.8}\text{ day}^{-1})$$

Specific dynamic action (SDA) is the energetic loss, measured as the postprandial elevated O_2 consumption calculated to the OE, above SMR.

$$\text{SDA (kJ kg}^{-0.8}\text{ h}^{-1}) = \text{heat (kJ kg}^{-0.8}\text{ h}^{-1}) - \text{SMR (kJ kg}^{-0.8}\text{ h}^{-1})$$

Heat was calculated as the sum of oxygen consumed during 24 h following the last meal, divided by MBW calculated to the OE.

$$\text{heat (kJ kg}^{-0.8}\text{ day}^{-1}) = \text{OC (g kg}^{-0.8}\text{ day}^{-1}) \times 14.06\text{ kJ g}^{-1}$$

SMR is determined as the mean of 10% of the lowest oxygen consumption rates per hour measured over 100 h post-feeding, calculated to the OE (Clark et al., 2013).

The difference between MEI and heat was calculated as the retainable energy (RE), being the sum of energy of GEI subtracted by all accountable energetic losses. In this study, the RE functions as the main indicator, for potential growth of the fish.

$$\text{RE (kJ kg}^{-0.8}\text{ day}^{-1}) = \text{MEI (kJ kg}^{-0.8}\text{ day}^{-1}) - \text{heat (kJ kg}^{-0.8}\text{ day}^{-1})$$

The ammonia quotient (AQ) is calculated as moles of excreted ammonia divided by moles of consumed oxygen according to Gnaiger (1983).

All metabolic losses (E_n, SDA, heat) were logged every 2 h for a period of 24 h. We calculated the losses to cumulative values to address the losses over time. RE was calculated as the remainder of energy from a single meal after the cumulated losses have been accounted for.

As SDA responses may alter in dynamics upon different temperatures we assessed a possible shift in the SDA peak response in a pilot trial. We found that the measured timepoint was appropriate to detect the SDA peak response and to measure metabolic responses at all tested temperatures.

2.6 Digestibility trial

To account for fecal losses, and confirm the correctness of the formulation, a separate fecal collection trial was performed with the same diets, to determine their digestibility. Parallel to the respirometry trial, a separate group of rainbow trout from the same batch was stocked into three 1000 L round tanks with a 500 L RAS filtration unit. The temperature remained at 15 °C throughout the digestibility trial, as the digestibility of energy and nutrients did not significantly change at a range from 14 - 20 °C in a previous test trial in the same system, with fish of the same origin and fed a commercial reference diet (ALLER Gold, 3mm, ALLER Aqua, Christiansfeld, Denmark, Chapter I). Jobling (1994) reported similar results in rainbow trout upon a temperature increase from 11 – 15 °C. The fish were marked as triplicates by colored staining of a skin fold behind the eye. Three triplicates, each consisting of 22 individuals, were stocked into one treatment tank. Each tank was stocked with a total of 15.8 kg of fish, resulting in an average stocking density of 15.8 kg/m^3. Each tank was fed the corresponding diet, received in the respirometer. The fish were fed a ration of 1.3 % BW daily. For feces stripping, the fish were anesthetized with clove oil (1 mL per 40 L of water) until the fish were unconscious and then feces were stripped manually by slightly pressing the area around the lower gut. Feeding in this group was performed at 9 a.m. and stripping of the feces at 1 p.m. Digestibility was calculated using the apparent digestibility coefficient (ADC) after Cho et al. (1982) as:

$$\text{ADC of nutrient} = 1 - \left(\frac{F}{D} \times \frac{Di}{Fi}\right)$$

where D = nutrient (%) or energy (MJ/kg) concentration of diet; F = nutrient (%) or energy (MJ/kg) concentration of feces; Di = % digestion indicator (TiO_2) of diet; Fi = % digestion indicator (TiO_2) of feces. Energy content of the feces was determined via bomb calorimetry (C200, IKA, Staufen, Germany) and TiO_2 content was assessed by the AGROLAB LUFA GmbH (Kiel, Germany).

2.7 Statistics

The statistical software R (2022) was used to evaluate the data. The data evaluation started with a definition of an appropriate statistical model based on generalized least squares (Box et al., 2015). The model included the factors temperature and diet, as well as their interaction term. Also, the correlations of the measurement values due to the several time points (levels of subsequently applied temperatures) were considered. The residuals were assumed to be normally distributed and to be homoscedastic. The assumptions were based on graphical residual analysis. Based on this model, a pseudo R^2 was calculated (Nakagawa & Schielzeth, 2012) and an analysis of variances (ANOVA) was conducted, followed by appropriate multiple contrast tests (Bretz, 2011; Hothorn & Hasler, 2008) in order to compare the several levels of the influence factors, respectively. All levels of diet were compared with each other, split for the several temperatures. Furthermore, all levels of temperature were compared with each other, split for the several diets. Additionally, a linear and a quadratic influence of the temperature was tested by appropriate contrasts. Also, a correlation analysis for the variables RE, MEI, SDA, and SMR was performed (note that the correlations considered are adjusted for the influence factors temperature and diet to avoid spurious correlations).

3. Results

3.1 Feeding

The trout ingested the complete rations within the 12 min that were allotted to feeding. No relevant internal competition for feed could be observed during the experiment.

3.2 Metabolic responses

3.2.1 Digestibility of energy and nutrients

The ADC of nutrients and energy of the diets are shown in Tab. 2-3. Upon analysis no statistically significant differences were detected between the different diets for the ADC of gross energy, crude protein, and crude lipid as well as nitrogen-free extracts and crude fiber ($p > 0.05$). Values of the ADC were further used to assess the DEI (Tab. 3).

Tab. 2-3: Apparent digestibility coefficient (%) for rainbow trout (Oncorhynchus mykiss) of three diets with different lipid-to-carbohydrate ratios (BAL = 1 :1; COH = 0.6 : 1; LIP = 1.4 : 1) at 15°C. n = 3.

Diet	Gross energy	Crude protein	Crude lipid	NfE + crude fiber
COH	85.9 ± 0.2	83.3 ± 0.3	90.8 ± 1.3	82.2 ± 0.8
BAL	83.7 ± 1.2	81.1 ± 1.9	85.7 ± 5.0	81.2 ± 1.9
LIP	86.0 ± 1.6	82.4 ± 1.0	89.2 ± 3.4	81.2 ± 0.6

3.2.2 Energy budget

Complete absolute values of all measured variables are summarized in a detailed energy budget (Tab. 2-4). The table shows complete energetic values as kJ $kg^{-0.8}$ day^{-1} of either energy intake (GEI), measured metabolic losses (E_n; SDA; heat) or remaining available energy after subtraction of the respective losses (DEI; MEI and RE) based on the formulae described above. The values were standardized to the percentage of GEI and further analyzed in the following by use of appropriate statistical tests.

Tab. 2-4: Energy budget for rainbow trout (Oncorhynchus mykiss) fed three isonitrogenous diets with different carbohydrate energy to lipid energy ratios(BAL = 1 :1; COH = 1 : 0.6; LIP = 1.4 : 1) at five temperatures.

Diet (Carbohydrate to lipid)	GEI	DEI	En	MEI	SDA	heat	RE
	kJ $kg^{-0.8}$ day^{-1}						
12°C							
COH	147.9 ± 0.1	123.7 ± 0.1	2.0 ± 0.4	121.7 ± 0.4	21.6 ± 5.3	36.0 ± 2.6	85.7 ± 2.9
BAL	146.5 ± 0.6	125.9 ± 0.5	1.5 ± 0.4	124.4 ± 0.2	16.1 ± 2.5	36.6 ± 4.8	87.8 ± 4.7
LIP	146.3 ± 0.3	125.9 ± 0.2	2.2 ± 0.2	123.7 ± 0.1	16.9 ± 3.7	35.7 ± 3.3	88.1 ± 3.4
14°C							
COH	148.9 ± 0.8	124.7 ± 0.1	2.5 ± 0.1	122.1 ± 0.6	22.2 ± 2.7	45.1 ± 7.1	77.0 ± 3.3
BAL	147.4 ± 0.4	126.7 ± 0.3	2.5 ± 0.6	124.2 ± 0.9	25.0 ± 2.5	52.1 ± 6.5	72.1 ± 6.8
LIP	148.0 ± 0.3	127.4 ± 0.2	2.6 ± 0.1	124.8 ± 0.2	20.3 ± 0.6	49.6 ± 3.4	75.2 ± 3.3
16°C							
COH	150.0 ± 0.7	125.5 ± 0.6	2.1 ± 0.1	123.4 ± 0.6	23.9 ± 3.2	55.2 ± 7.7	68.2 ± 7.3
BAL	148.0 ± 0.7	127.3 ± 0.6	2.0 ± 0.1	125.4 ± 0.8	32.3 ± 4.0	57.2 ± 2.4	68.1 ± 1.6
LIP	148.9 ± 0.2	128.1 ± 0.2	2.0 ± 0.2	126.1 ± 0.4	20.8 ± 0.8	51.7 ± 4.3	74.4 ± 3.9
18°C							
COH	150.8 ± 0.7	126.2 ± 0.6	2.6 ± 0.1	123.6 ± 0.6	25.1 ± 2.5	59.8 ± 3.4	63.8 ± 2.7
BAL	148.9 ± 0.7	128.0 ± 0.6	2.1 ± 0.2	125.9 ± 0.6	30.1 ± 5.1	58.4 ± 4.6	67.5 ± 4.2
LIP	149.8 ± 0.3	129.0 ± 0.2	2.4 ± 0.1	126.6 ± 0.2	22.6 ± 1.2	57.2 ± 1.5	68.2 ± 7.3
20°C							
COH	151.4 ± 0.9	126.7 ± 0.7	2.6 ± 0.2	124.2 ± 0.7	25.5 ± 2.4	62.5 ± 1.2	61.7 ± 0.7
BAL	149.7 ± 0.5	128.6 ± 0.5	2.3 ± 0.4	126.4 ± 0.7	26.9 ± 1.8	60.9 ± 2.3	65.5 ± 2.1
LIP	150.7 ± 0.3	129.7 ± 0.3	2.7 ± 0.1	127.0 ± 0.2	23.4 ± 6.9	60.5 ± 6.9	66.6 ± 7.0

Values are mean ± SD; GEI: Gross energy intake; DEI: digestibile energy intake; MEI: metabolizable energy intake; En: non-fecal nitrogen; heat: energetic losses of heat production; SDA: specific dynamic action; RE: retainable energy (n=3).

3.2.3 Standard metabolic rate

The standard metabolic rate increased linearly upon increasing temperatures (BAL: $p < 0.001$; COH: $p = 0.022$; LIP: $p = 0.003$)(Fig.2-2). The values showed no significant differences between the groups ($p > 0.05$). Average values ranged from 12.2 % of GEI at 12 °C to 23.9 % at 20 °C. Since the values display the metabolic rates without influence from the diet, diet is not considered a factor.

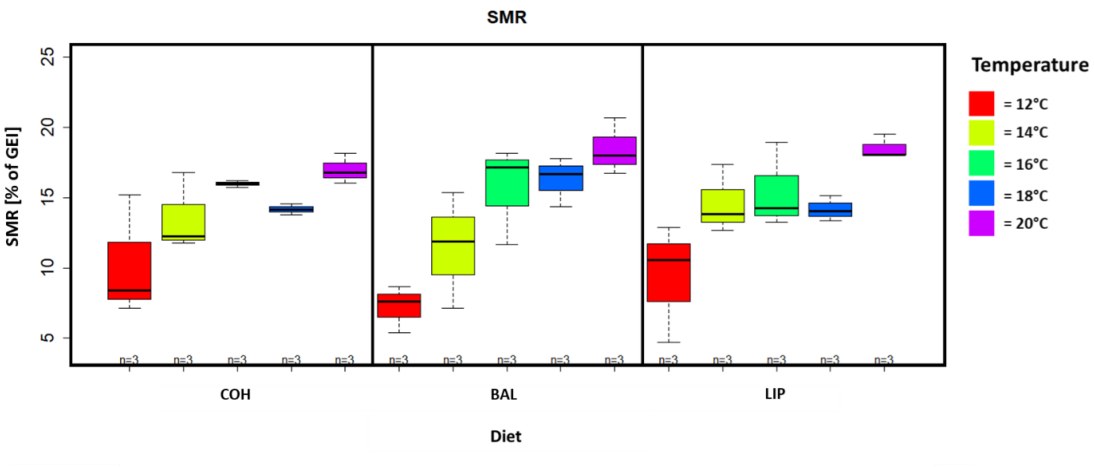

Fig. 2-2: Percentage of standard metabolic rate (SMR) relative to gross energy intake (GEI) of rainbow trout (*Oncorhynchus mykiss*) at three different isonitrogenous diets with different lipid energy to carbohydrate energy ratios (BAL = 1 : 1; COH = 0.6 : 1; LIP = 1.4 : 1) at five temperatures. The median is marked with a solid black line in the boxes. Whiskers indicate the true values of maximum and minimum values. The boxes mark 25 and 75 percentiles.

3.2.4 Specific dynamic action

Absolute values of SDA ranged from 16.1 kJ $kg^{-0.8}$ day^{-1} (COH diet at 12 °C) to 32.3 kJ $kg^{-0.8}$ day^{-1} (COH diet at 16 °C) corresponding with 10.9 % and 21.8 % of the GEI respectively. The values of SDA shown in Tab. 3-4 were standardized to their relative share of the GEI. The values of SDA showed no significant response, neither linear nor quadratic to temperature as a factor, when the diets BAL and LIP were fed ($p > 0.05$) (Fig. 2-3). When the COH diet was fed, values of SDA increased from 12 °C to 16 °C and declined again towards 20 °C. However, the difference in SDA values between the temperatures was only significant between 12 °C and 16°C ($p = 0.002$) and between 12 °C and 18 °C ($p = 0.011$). We found a significantly higher SDA at 16 °C when the COH diet was fed compared to the LIP diet ($p = 0.029$). For the COH diet statistical analysis revealed a linear ($p = 0.019$) and a quadratic relationship ($p = 0.005$) of the values to temperature as a factor. The calculated peak of SDA for the COH diet was at 17.05 °C.

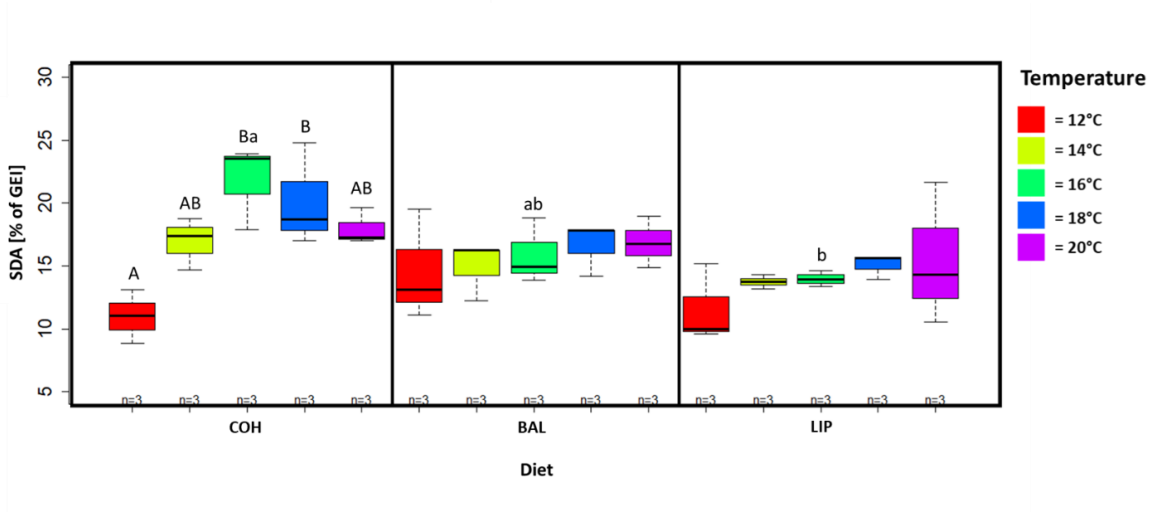

Fig. 2-3: Percentage of specific dynamic action (SDA) relative to gross energy intake (GEI) of rainbow trout (*Oncorhynchus mykiss*) at three different isonitrogenous diets with different carbohydrate energy to lipid energy ratios (BAL = 1 :1; COH = 0.6 : 1; LIP = 1.4 : 1) at five temperatures. The median is marked with a solid black line in the boxes. Whiskers indicate the true values of maximum and minimum values. The boxes mark 25 and 75 percentiles. Capital letters indicate significant differences between temperatures and lower-case letters significant differences between the diets.

3.2.5 Retainable energy

Absolute values of RE ranged from 61.7 kJ $kg^{-0.8}$ day^{-1} (BAL diet at 20 °C) to 88.1 kJ $kg^{-0.8}$ day^{-1} (LIP diet at 12 °C) corresponding with 40.7 % and 60.2 % of the GEI respectively. RE as a percentage of GEI showed no significant differences between the diets at each respective temperature ($p > 0.05$) (Fig. 2-4). Across all diets the values decreased linearly upon increasing temperatures (BAL: $p < 0.001$; COH: $p < 0.001$; LIP: $p < 0.001$). The values of RE were always highest at 12 °C across all diets. When the BAL diet was fed, the values of 12 °C were significantly higher than the values at 18 °C ($p < 0.001$) and 20 °C ($p < 0.001$). For the COH diet, values of 12 °C were significantly higher than the values of 16 °C ($p = 0.001$), 18 °C ($p < 0.001$), and 20 °C ($p < 0.001$). When the LIP diet was fed, values of 12 °C were significantly higher than values at 14 °C ($p = 0.024$), 16 °C ($p = 0.033$), 18 °C ($p = 0.002$), and 20 °C ($p < 0.001$). Values between 14 °C and 20 °C showed no significant differences across all diets ($p > 0.05$), but all values showed a decline toward higher temperatures regardless of the diet.

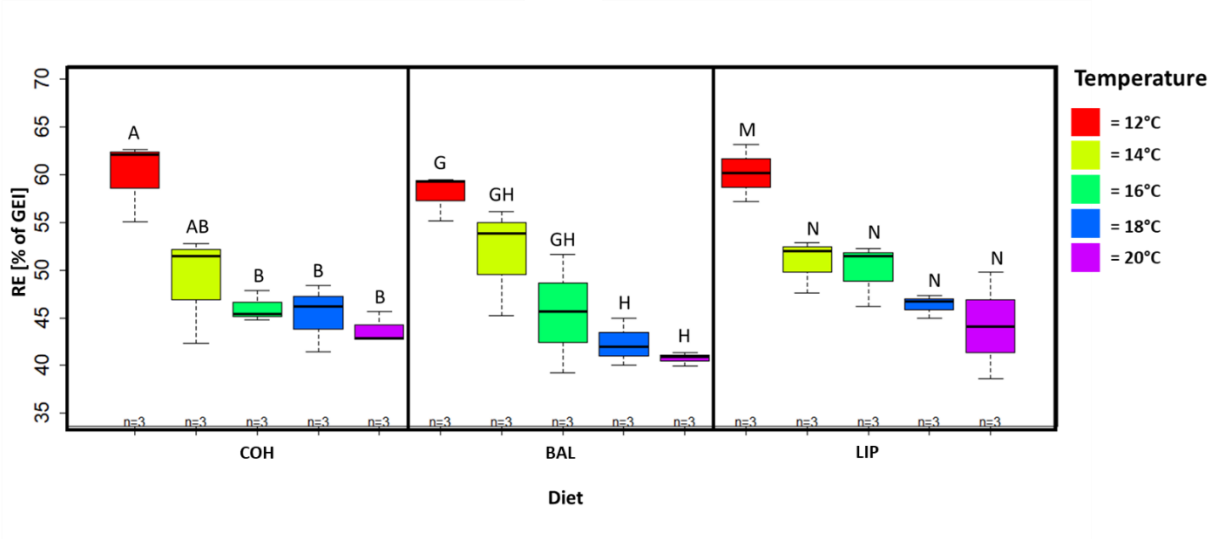

Fig. 2-4: Percentage of retainable energy (RE) relative to gross energy intake (GEI) of rainbow trout (*Oncorhynchus mykiss*) at three different isonitrogenous diets with different carbohydrate energy to lipid energy ratios (BAL = 1 :1; COH = 0.6 : 1; LIP = 1.4 : 1) at five temperatures. The median is marked with a solid black line in the boxes. Whiskers indicate the true values of maximum and minimum values. The boxes mark 25 and 75 percentiles. Capital letters indicate significant differences between temperatures and lower-case letters significant differences between the diets.

3.2.6 Ammonia quotient

Absolute values of AQ ranged from 33.1 × 10^{-3} (COH diet at 16°C) to 56.6 × 10^{-3} (LIP diet at 12 °C) corresponding with 12.3 % and 20.9 % of the energy metabolism being fueled from protein (Fig.2-5). For the BAL diet, values were significantly lower at 16 °C than at 14 °C (p = 0.031). For the LIP diet, values were significantly higher at 12 °C than at 16 °C (p = 0.044) and 18 °C (p = 0.049). We found a significantly higher AQ at 12 °C when the LIP diet was fed compared to the COH diet (p = 0.027).

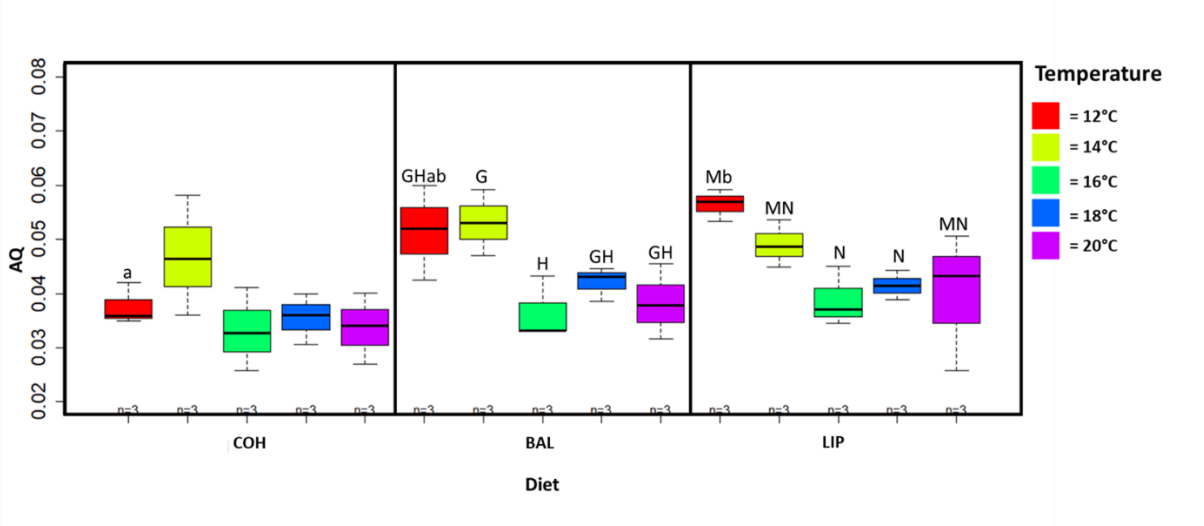

Fig. 2-5: Ammonia quotient of rainbow trout (*Oncorhynchus mykiss*) at three different isonitrogenous diets with different carbohydrate energy to lipid energy ratios (BAL = 1 :1; COH = 0.6 : 1; LIP = 1.4 : 1) at five temperatures. The median is marked with a solid black line in the boxes. Whiskers indicate the true values of maximum and minimum values. The boxes mark 25 and 75 percentiles. Capital letters indicate significant differences between temperatures and lower-case letters significant differences between the diets.

4. Discussion

This study addressed the energetic effects of diets with different primary NPES ratios and their interactions with different temperatures. The temperatures were specifically chosen to range from below optimal temperatures via the optimal temperature for growth according to Hokanson et al. (1977) and Mellery et al. (2016) to an above-optimal temperature of 20 °C. Although the growth per se was not measured in this study we believe that the findings of the mentioned studies provide a good benchmark. The COH diet was resembling the diets of Beamish et al. (1986) who found that a diet with carbohydrates as a main NPES leads to a significantly higher SDA than diets with lipids as a main NPES at an environmental temperature of 15 °C.

Since all diets in this study contained high inclusions of carbohydrates and/or lipids in relation to protein, it was necessary to assess the digestibility of both energy and nutrients, since especially carbohydrates are known to significantly decrease in digestibility at high inclusion rates (Schmitz et al., 1983; Tung & Shiau, 1991). Analysis of the data revealed no significant

differences between the diets regarding the digestibility of energy and macronutrients. We assumed the energy and nutrient digestibility to be largely unaffected by temperature, based on the studies of Cho & Kaushik (1990) who found no differences in the digestibility of nutrients or energy between 9- 18 °C. We also determined the ADC of nutrients and energy from a commercial diet (ALLER Gold, 3mm, ALLER Aqua, Christiansfeld, Denmark) between 14 – 20 °C in rainbow trout of the same origin as the ones used in this study and found no significant differences in nutrient and energy digestibility (Chapter I). Based on our findings we conclude that all diets contained an appropriate macronutrient composition and provided a sufficient amount of digestible energy and were therefore suitable candidates for assessing the role of dietary lipid-to-carbohydrate ratios and their interactions with temperature.

SDA values showed no statistically significant increase upon increasing temperatures when the BAL or LIP diet was fed. The SDA values for COH however responded to increasing temperatures with a quadratic relationship that peaked at 17.05 °C, close to the thermal optimum, and subsequently declined. In general, the findings are in accordance with the results presented by Guillaume et al. (1999) as the SDA showed a high resemblance between diets with either carbohydrate or lipid as the primary NPES at lower temperatures but higher SDA values at temperatures around the optimum. By testing metabolic responses at a broad spectrum of temperatures with small thermal increase steps we found that SDA values do not only increase upon increasing temperatures but rather decrease at temperatures above the optimum resulting in a parabolic relationship of SDA to environmental temperatures in diets with carbohydrate as the primary NPES. Beamish et al. (1986) hypothesized that the higher SDA of diets with carbohydrates as the primary NPES compared with diets with lipids as the primary NPES at 15 °C were a result of low combustion of carbohydrates as metabolic fuel inducing a higher rate of protein utilization both from diets and tissue to meet an elevated energy demand. This would result in a higher energy loss by SDA as the utilization of protein for combustion is associated with costly deamination processes (Beamish et al., 1986; Cho & Bayley, 1976; Kleiber, 1962). However, the energy-yielding combustion of protein is also associated with the

excretion of ammonia, and by measuring the ammonia excretion and oxygen consumption it is possible to calculate the AQ which can be calculated to the relative metabolic combustion rate of protein (Gnaiger, 1983). This study however could not show a significant difference in AQ between the diets at temperatures between 14 - 20°C. AQ values are discussed in detail in the following sections.

Since the significant differences in SDA between the LIP and the COH diet upon different temperatures cannot be explained by an increased incorporation of protein as metabolic fuel we hypothesize that carbohydrate fluxes and insulin levels in the bloodstream are responsible for an increase of SDA towards 16 °C in the COH diet. It is generally accepted, that feeding carbohydrate-rich diets promote the conversion of carbohydrates into lipids, a process termed lipogenesis (Hung & Storebakken, 1994; Polakof et al., 2011). Before lipids can be synthesized the carbohydrates need to enter the bloodstream in the form of glucose. Thermal conditions are known to delay the flux of glucose into the bloodstream of rainbow trout as a decrease in temperatures from 15 °C to 6 °C resulted in a 45 % lower glucose flux describing the rate of glucose appearance over disappearance in the bloodstream (Haman et al., 1997). Although it is known that elevated dietary carbohydrate levels do not necessarily result in a proportional increase in insulin levels, a higher glucose load upon optimal temperatures around 15 - 17°C is likely to still increase the insulin levels in the bloodstream (Blasco et al., 2004; Legate et al., 2001; del Sol Novoa et al., 2004). Increased insulin levels are also known to stimulate lipogenesis from carbohydrate precursors but the metabolic fuel use of lipids is on the other hand inhibited by high insulin levels (Polakof et al., 2011). This synthesis of lipids is usually associated with a high energetic cost. It appears likely that an increase of costly lipogenesis towards optimal temperatures, due to high dietary carbohydrate and insulin levels in the bloodstream is the main cause for an increase in SDA when carbohydrate is the main form of NPES. Several studies have found that carbohydrate and insulin levels in the bloodstream both increase the protein-sparing effect and promote the incorporation of dietary amino acids into body protein (Jobling et al., 1997; Choi & Weber, 2015). In this study, all diets contained relatively high levels of carbohydrates and we found the AQ to be lowest at 16 °C although the results were not always significant. We believe that the high levels of available carbohydrates that

enter the bloodstream at a higher flux at 16 °C and the subsequent increase of insulin levels are responsible for the optimal energy sparing at optimal temperatures. It is likely that the LIP diet in this study and the diets of Chapter I contained a more ideal dietary lipid-to-carbohydrate ratio that would spare protein from being combusted as metabolic fuel around optimal temperatures but would not overload the bloodstream with glucose and subsequent insulin to inhibit the metabolic combustion of lipids.

This study could not show a significant difference in AQ between the diets at temperatures between 14 - 20 °C. This is in accordance with studies of Brauge et al. (1994; 1995) who found no differences in protein retention, a value closely related to the AQ, in rainbow trout upon different dietary lipid-to-carbohydrate ratios. It has therefore been concluded that carbohydrates and lipids are equally effective to induce a protein-sparing effect. However, at 12 °C we found a significantly lower AQ for the COH diet than the LIP diet suggesting that the protein-sparing properties of carbohydrates and lipids may possibly be temperature dependent and especially dietary carbohydrates may be better metabolized upon colder temperatures. This hypothesis is supported by findings of Kieffer et al. (1998) who discovered that with an acclimation temperature of 5°C, protein, lipid and carbohydrate contributed to the metabolic fuel use at resembling rates (respectively 27, 35 and 42% of total oxygen consumed) but when acclimated to 15 °C the fuel use transitioned to contribution rates of 30 % from protein, 46 - 58 % from lipid but only 15 % from carbohydrates. This supports the hypothesis that dietary carbohydrates contribute to the energy metabolism at a larger degree at lower temperatures. The study of Kieffer et al. (1998) found protein as a metabolic fuel to be largely independent of temperature. However, high utilization of carbohydrates is known to suppress the conversion of amino acids into carbohydrates thus sparing protein usage as metabolic fuel (Cowey et al., 1977; Sanchez-Muros et al., 1998; Choi & Weber, 2015). It is generally known that low inclusions of dietary protein can increase the protein-sparing effect when a surplus of carbohydrates and/or lipids are present as energy substrates. The study of Kieffer et al. (1998) did not use a low dietary protein

content. It is therefore possible that when carbohydrate is the main NPES and protein levels are low, carbohydrates can induce a temperature-dependent protein-sparing effect at low temperatures.

We found a linear decrease of RE values towards higher temperatures. The RE values show a high correlation with the SMR values. The results of RE did not show significant differences between the different diets regardless of the temperature. This is contrary to the findings of Beamish et al. (1986) who found growth to be impaired when diets with carbohydrates as the main NPES were fed but the study was carried out with glucose as a source of dietary carbohydrate while this study used gelatinized starch. Studies found that growth in tilapia (Shiau & Chen, 1993; Shiau & Lin, 1993; Shiau & Peng, 1993; Shiau & Liang, 1995), channel catfish (Robinson & Li, 1995), sturgeon (Hung et al., 1989; Deng et al., 2000) and Atlantic salmon (Arnesen et al., 1995) was better when gelatinized starch was the source of dietary carbohydrates compared to pure glucose. The here presented study used gelatinized wheat starch as a source of dietary carbohydrates but found similar results as the ones by Beamish et al. (1986) in terms of SDA. We therefore conclude that dietary wheat starch as the main form of NPES results in an increase of SDA upon temperatures around the optimal temperature in a similar manner as pure glucose. It appears likely that the findings of the SDA values do not carry over to the values of RE upon COH diet feeding due to cumulative effects of the different energetic losses, mainly SDA and SMR, that consequently result in the RE. SDA and SMR showed high standard deviations in this study but are combined into heat which accounts for up to 45.7 % of the total energetic losses. We therefore propose that the high standard deviations superimpose on the interactions of SDA and RE values and the findings of the COH diet for SDA do not continue to the RE values.

A key limitation to the bioenergetic approach using a respirometer is that the fish are mostly held in small containers housing a single fish. These systems do usually not allow free movement or natural behavior of the animals (NRC, 2011). Also, the fish often need to be force-fed and handled intensely directly before measuring its metabolism thus inflicting stress on the fish that may impact the respiration, leading to an overestimation of energetic losses of the experimental treatments. This study

uses a group respirometer with large housing tanks, allowing free movement and a natural behavior of the fish under culture-like conditions (Stiller, 2016). Another advantage of the here used system is that the fish can be cultured over several weeks. Here we were able to use this advantage to test for several subsequent temperatures that were applied to the whole system, allowing for a broad thermal regime to be applied with small steps of temperature increases while feeding different lipid-to-carbohydrate ratios as NPES in the diets. This allows for a high-resolution depiction of the energy partitioning and metabolic fate of energy-yielding substrates. The data of this study show a high resemblance to the data provided by Beamish et al. (1986) as well as Gulliaume et al. (1999), both using a small compartment respirometer designed to house a single fish. By measuring similar results as the mentioned studies but using a system with large housing tanks that allow free movement and culture-like behavior, we provide evidence that respirometric approaches are suitable methods to measure SDA values under various environmental conditions despite previous concerns.

5. Conclusion

The dynamics of SDA of diets with different lipid-to-carbohydrate ratios at different temperatures suggest that a diet with carbohydrate as the primary NPES can induce a higher SDA than lipid-based and balanced diets but all show resembling SDA rates at temperatures below and above optimum. The increase is likely due to the fluxes of glucose and insulin and their properties to modulate the lipid metabolism. At a colder temperature of 12 °C, there appear to be similar RE and SDA values between carbohydrate and lipid-based diets but AQ values are significantly lower for the carbohydrate base diets. The results often show a high resemblance to similar bioenergetic approaches using a respirometer designed to hold one fish at a time with intense handling. The high resemblance of the data with the data from this study, that uses a group respirometer in which handling of the fish is avoided, shows that bioenergetic approaches produce reliable data that may be applicable to real practice conditions.

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Chapter III

Determining the optimal thermal conditions for a maximum protein-sparing effect in rainbow trout (*Oncorhynchus mykiss*)

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Abstract

Research on the protein-sparing effect is highly relevant and many studies have addressed this topic in the past. Studies should be designed so that environmental conditions like the temperature enable a maximized visibility of the induced protein-sparing effect. We performed an instantaneous bioenergetic study with rainbow trout (*Oncorhynchus mykiss*) of 206.30 g ± 2.86 g in a group respirometer with nine 250 L tanks at five different water temperatures (12; 14; 16; 18; 20 °C) in order to determine the optimal thermal condition for a maximized protein-sparing effect to set the ground for future research on the topic. Twelve fish per tank were tested at a stocking density of 2.47 kg/m^3 ± 3.4 g/m^3 and fed three low-protein/high-energy diets with a constant crude protein content of approximately 35% and three different energy contents (17.35; 18.76; 20.50 MJ/kg) once daily at a ration of 1.3 % body weight (n = 3). Energy levels were increased by adding gelatinized wheat starch as a carbohydrate source and fish oil, canola oil, and palmitin as lipid sources. Three different dietary digestible protein/digestible energy ratios (DP/DE; 20.38; 19.08; 18.09 mg/KJ) were achieved by replacing bentonite as a non-nutritive filler with carbohydrates and lipids. Oxygen consumption and ammonia excretion were assessed to obtain the potentially retainable energy (RE) and the ammonia quotient (AQ) as benchmarks for potential growth and protein-sparing effect. The results showed the lowest relative metabolic fuel use of protein at 16.9 °C ± 0.13 °C. We determined this temperature to set the optimal thermal condition for the induction of a maximum protein-sparing effect in juvenile rainbow trout with a body weight of approximately 200 g. We were able to reduce average metabolic fuel use of protein across diets from 16.2 % ± 2.3 % at 12 °C to 8.0 % ± 1.2 % at 16 °C providing relevant information for future researchers to increase the visibility of the protein-sparing effect in studies with rainbow trout.

Keywords: *carbohydrate-rich diets, energy substrate ratios, respirometry, metabolic fuel use*

1. Introduction

Fish are poikilothermic animals which means that their metabolism and coherent growth are to a large degree directly dependent on the environmental temperature. Species with a broad thermal niche like the rainbow trout (*Oncorhynchus mykiss* W., 1792) are therefore favorable candidate species for production in temperate and subtropical regions. This species can tolerate thermal conditions of up to 29 °C although such high temperatures severely limit the growth potential of the animals (Chen et al., 2015). A much higher growth potential can be reached at 15 - 17 °C which has been determined to be the optimal temperature for growth of this naturally cold-water dwelling species (Hokanson et al., 1977; Mellery et al., 2016). However, water temperatures of up to 20 °C are not uncommon in the summer months on production sites for rainbow trout and closely related species in Germany and may reach even higher levels in the southern hemisphere (Lorentzen, 2008; Pankhurst & King, 2010; Barnes et al., 2011; Weirup et al., 2021). Therefore, detailed research on the impacts of environmental temperatures on the biology of farmed fish is highly relevant (Galbreath et al., 2004; Kankainen et al., 2014).

Especially the combustion of nutrients as metabolic fuel in order to satisfy energy demands by the fish may hold a special relevance as it is known to be majorly influenced by the environmental temperature (Kieffer et al., 1998). However, the dietary composition of nutrients that fuel the metabolism have themselves long been discovered to play a key role in the energy utilization and are a potential tool to optimize the efficient use of nutrients for the growth of fish facing thermal stress (Kamalam et al., 2016; Kaushik & Médale, 1994; Kieffer et al., 1998; Glencross & Bermudes, 2010). A special emphasis lies on the metabolic use of protein. Ideally, dietary protein is not combusted by the animal to yield energy but rather incorporated into body mass during growth (de Silva et al., 1991). However, protein and energy requirements are known to increase at higher temperatures (Helland et al., 2010; Lupatsch, 2009). Up to day, protein remains the largest cost factor in feeds for aquaculture and it has been suggested to emphasize the protein metabolism when investigating the interactions between diet composition and environmental parameters (Carter, 2008;

Jones et al., 2020). A main aim of aquaculture is therefore to ensure that dietary protein is converted into body protein by the fish, rather than being combusted as metabolic fuel especially, since the deposition of protein in the body results in a higher water gain than carbohydrates and lipids, thus resulting in a higher yield for the producer (Azevedo et al., 1998).

A useful method to increase the efficiency of the use of dietary protein is inducing a so-called protein-sparing effect, meaning that other nutrient classes like carbohydrates or lipids are combusted to yield energy while protein remains potentially available for growth. Several studies have already aimed to induce a high protein-sparing effect in a variety of aquaculture-relevant species (Shiau & Peng, 1993; Mohanta et al., 2007; Welengane et al., 2019). Increasing the dietary carbohydrate and lipid levels appears to be an effective method for the induction of a protein-sparing effect in rainbow trout and the effect appears to be maximized when dietary protein content is limited (Kaushik & Médale, 1994). However, since the combustion of nutrients is temperature dependent it appears likely that there is a major thermal impact on the protein-sparing effect, which may reduce the visibility of differences in studies targeting the topic and may lead to an underestimation and misinterpretation of induced effects.

An easy and reliable tool to measure the interaction of protein and dietary energy with the environmental temperature are instantaneous bioenergetic approaches (Cho, 1987). In general, bioenergetic approaches with a respirometer are regarded as highly relevant and more accurate than compositional trials when targeting protein efficiency, especially because the same fish can be measured repeatedly, thus increasing the comparability of treatments (Lauff & Wood, 1996). Specific bioenergetic approaches measure metabolite output, as well as consumed oxygen to calculate an instantaneous metabolic rate as a benchmark for the growth potential. The approach also allows for the measuring of nitrogen excretion which makes it possible to calculate the ammonia quotient (AQ) which indicates to what degree the energy metabolism is fueled by protein (Gnaiger, 1983). Further, it is possible to assess many data points within a short period, as the approach measures instantaneous

metabolic effects. This makes it possible to quickly measure the effects of a given environmental condition allowing for a broad range of conditions to be measured sequentially.

We have conducted a multifactorial bioenergetic study using a group respirometer and assessed oxygen consumption and nitrogen excretion to calculate key metabolic variables as benchmarks for potential growth and relative protein usage as metabolic fuel. The main targets were to identify the optimal thermal conditions for a maximized protein-sparing effect, so that temperatures can be kept at optimal conditions for an efficient protein usage where possible (e.g. RAS) and to set the ground for future researchers to increase the visibility of potential effects thus improving the field of nutritional research.

2. Material & Methods

2.1 Respirometric system

The trial was conducted in a recirculating aquaculture respirometric system (RARS) described by Stiller et al. (2013). The system was located at the Fraunhofer Research Institution for Individualized and Cell-Based Medical Engineering in Büsum, Germany. It was based on a flow-through respirometric system with semi-continuous detection of key water variables. A total of ten tanks, each with a volume of 250 L, were arranged around the measuring unit and continuously supplied with water at a rate of 360 L/h for each tank from the water treatment unit within the RAS. The system was expanded by a UV-treatment unit, a 700 L moving bed biofilter, an airlift system for oxygen saturation of the water, and two flow-through coolers (Titan-4000, AB, Aqua Medic GmbH, Bissendorf, Germany) with a power switch control system (One-A2-13-60, Senect GmbH & Co. KG, Landau, Germany). A high-resolution oxygen measurement was performed with an oxygen optode (Aandera data instruments, Model: 4330, Serial No. 1557, Bergen, Norway). The pH was measured with an intermediate junction electrode (IJ44A, TPS Pty Ltd, Brisbane, Australia). Total ammonia nitrogen (TAN) was measured with an automatized loop flow ortophtaldehyde fluorometric autoanalyser (Systea S.P.A. Anagni, Italy).

2.2 Fish husbandry

Rainbow trout with an average live body weight (BW) of 206.30 g ± 2.86 g were obtained from Forellenzucht Trostadt GmbH & Co. KG at Reurieth, Germany. All animals were female. Upon arrival, the fish were transferred into a recirculating aquaculture system (RAS) with a 1000 L housing unit and a 500 L water treatment unit. The water was tempered to 14 °C during a two-week acclimation period. The animals were transferred into the respirometer system four days prior to the start of the trial. Twelve fish were stocked into nine of the respirometric chambers and a tenth tank remained without fish, as an internal reference. The average weight of the fish was 206.6 g ± 2.4 g and the stocking density in the tanks was 2.47 kg/m3 ± 3.4 g/m^3. During acclimation to the system the temperature was set to 14 °C and the fish were fed with a commercial diet (ALLER Gold, 3mm, ALLER Aqua, Christiansfeld, Denmark). The trout were starved for four days prior to the experimental start to ensure, that the metabolic rate of the animals would not be influenced by the last meal ingested (Ming Tang & Boisclair, 2011). Directly upon starting the experiment, the fish were bulk-weighed, to assess the correct BW for calculations of rations and metabolic body weight (MBW), which are further described in the following sections. Oxygen levels were kept well above 70 % saturation, to ensure unobstructed respiration. Nitrogen compounds in the water body were kept below 0.05 mg/l. Ammonia was tested bihourly in each tank with the beforementioned autoanalyzer and nitrite twice weekly with a color indicator test kit (McColortestTM, Merk KGA, Darmstadt, Germany). Salinity remained at 2 ppm and pH of the water body at approximately 7.5. The light regime was set to a 12 - h day and night cycle. Critical water parameters ammonia, nitrite, pH, turbidity, and salinity remained well within safe ranges for rainbow trout according to Hamers & Schreckenbach (2002). The temperature was kept within a range of ± 0.2 °C of the respective testing temperature.

2.3 Diet formulation and processing

The diets contained three different digestible protein-to-digestible energy ratios (DP/DE) of 20.38; 19.08 and 18.09 mg/kJ. Since the diets were designed to have the same level of protein, only the dietary energy levels were adapted by replacing bentonite as a non-nutritive filler with carbohydrates and lipids. For a complete ingredient list see Tab. 3-1. The diets in this study will be referred to their energy levels as high energy diet (HE), medium energy diet (ME), and low energy diet (LE) with correspondence of: HE = 20.5 MJ/kg, ME = 18.76 MJ/kg, LE = 17.35 MJ/kg (Tab. 3-2.). The diets were formulated isonitrogenous and contained approximately 35 % crude protein. The ratio of carbohydrate to lipid energy content was set fix for 1 : 1.27. Energy contents for carbohydrates (17.6 MJ/kg) and for lipids (39.8 MJ/kg) were used according to internal analysis of the individual ingredients prior to processing. After homogenization of the ingredients, the feed was pelletized to 4 mm pellets using an L 14-175, AMANDUS KAHL (Reinbeck, Germany). The diets were designed to meet the amino acid requirements of rainbow trout, according to Rodehutscord & Pfeffer (1999). Titanium dioxide was added to each diet, with an inclusion level of 1 % of the total volume, to serve as an inert marker for assessment of fecal losses. The diets were analyzed for dry matter after desiccation at 103 °C, followed by 4 h at 550 °C to determine ash content. Crude protein content was assessed via the Kjeldahl method (InKjel 1225 M, WD 30; Behr Düsseldorf, Germany). Gross energy content was determined via bomb calorimetry (C200, IKA, Staufen, Germany) and crude lipid with HCL hydrolysis with subsequent extraction using petroleum ether with a Soxhlet extraction (R106, S; Behr, Düsseldorf, Germany).

Tab. 3-1: Ingredient composition (% of inclusion of original substance) of the test diets. Pellet size 4mm.

	LE	ME	HE
Raw materials (% of inclusion)			
Fish meal (Clupea sp.)	35.00	35.00	35.00
Blood meal	5.00	5.00	5.00
Gelatine	2.00	2.00	2.00
Biolysine	1.20	1.20	1.20
DL_Methionine	0.28	0.28	0.28
L-Phenylalanine	0.60	0.60	0.60
Gelatinized wheat starch	29.70	33.80	38.74
Isoleucin	0.20	0.20	0.20
Palmitin	6.40	6.40	6.40
Canola oil	0.00	1.90	4.08
Wheat gluten	0.23	0.10	0.00
Fish oil	4.00	4.00	4.00
Vitamin premix	0.50	0.50	0.50
TiO_2	1.00	1.00	1.00
$Ca_3(PO_4)_2$	1.00	1.00	1.00
Bentonite	12.89	7.02	0.00

Tab. 3-2: Analysed nutritional composition (% DM, after desiccation) of the test diets. Pellet size 4mm.

Feed composition	LE	ME	HE
Moisture (% DM)	7.6	8.0	7.4
Crude protein (% DM)	34.5	35.2	34.7
Crude lipid (% DM)	17.5	19.5	21.3
Crude ash (% DM)	21.7	16.1	9.1
NfE + crude fiber (% DM)	28.1	31.6	36.4
Gross energy (MJ/kg)	18.8	20.3	22.2
DP/DE ratio (mg/KJ)	20.4	19.1	18.1

2.4 Temperature setup and feeding

The temperature of the water body was sequentially increased from 12 °C to 20 °C in 2 °C steps, over a total of 45 days. Each temperature was held for a nine-day temperature sequence. In between each temperature sequence, all groups were bulk-weighed to determine the current BW for rations and MBW. The fish were acclimated to each temperature for a total of five days. During this time the fish were fed with 1.3 % BW of their respective diet daily at 10.00 a.m. Prior to the experiment, we tested three groups of ten rainbow trout of a different batch but with comparable BW (208.46 g ± 3.78 g) per tank at 12 °C and found that more feeding days did not lead to a further increase of postprandial oxygen consumption. The rations were fed to each tank so that sufficient pellets were distributed in the water column at a time for each fish to ingest several pellets to decrease group internal competition. New pellets were given into the experimental tanks once all supplied pellets were ingested by the group until the complete ration was ingested. Three different diets were fed during the trial. All treatments were performed as triplicates. During the feeding period, a total of two hours was used for daily maintenance tasks. During the maintenance, fecal matter was removed from the tanks, the ammonia analyzer was recalibrated and the reference tank was cleaned to avoid the formation of oxygen-consuming biofilm.

2.5 Measuring of metabolic response

Measuring of the metabolic data was performed for 24 h following the last feeding of each temperature sequence. Oxygen, TAN, pH, and temperature were measured continuously during the 24 h. Each tank was measured for 12 min at one time and a full measuring cycle of all nine stocked tanks plus the reference was completed and logged every 2 h. To standardize the dataset, the metabolic weight exponent of 0.8 according to Clarke & Johnston (1999) was applied to calculate the MBW. The oxicalorific equivalent (OE) of 14.06 kJ energy loss per g O_2 consumed was applied to convert measured oxygen consumption into an energetic value (Gnaiger, 1983). The key metabolic values were calculated from the oxygen uptake and ammonia excretion and combined into an energy budget. To conclude a temperature period the fish were starved for 96 h, to ensure a return to standard metabolic rate (SMR). The time as a fasting period has been recommended by a number of studies as a valid method to assess SMR (Cho & Kaushik, 1990; Clark et al. 2013). Following the fasting period, the temperature was increased and the next temperature sequence began.

The following formulae used are adapted from Steinberg (2018). Variables used for the calculations, if not mentioned in the calculation itself, were gross energy (GE), feed intake (FI), energy content in the diet oxygen consumption (OC), and losses of nitrogen compounds via the gills (N-loss).

Gross energy intake (GEI) was the total amount of energy consumed (unit: kJ) during a single meal divided by MBW.

$$\text{GEI}\ (\text{kJ kg}^{-0.8}\ \text{day}^{-1}) = \frac{\text{GE (kJ g}^{-1}\text{)} \times \text{FI (g day}^{-1}\text{)}}{\text{MBW (kg}^{0.8}\text{)}}$$

A separate trial, described below, was performed to account for fecal losses, to be able to describe the digestible energy intake (DEI) based on determined digestibility coefficients for dietary energy.

$$\text{DEI}\ (\text{kJ kg}^{-0.8}\ \text{day}^{-1}) = \frac{\text{DE (kJ g}^{-1}\text{)} \times \text{FI (g day}^{-1}\text{)}}{\text{MBW (kg}^{0.8}\text{)}}$$

Losses by non-fecal nitrogen (E_N) excretion were calculated as TAN divided by the MBW with the assumption, that finfish excrete approximately 15 % of nitrogen as urea, with an energetic equivalent

of 23 kJ g^{-1} and 85 % of nitrogen as ammonia, with an energetic equivalent of 24.9 kJ g^{-1} (Elliott & Davison, 1975; France & Kebreab, 2008).

$$\mathrm{E_n\ (kJ\ kg^{-0.8}\ day^{-1})} = \frac{\mathrm{N\ -loss\ (g\ day^{-1}) \times E_E\ (kJ\ g^{-1})}}{\mathrm{MBW\ (kg^{0.8})}}$$

The difference between DEI and E_N was calculated as Metabolizable energy intake (MEI).

$$\mathrm{MEI\ (kJ\ kg^{-0.8}\ day^{-1}) = DEI\ (kJ\ kg^{-0.8}\ day^{-1})\ -\ E_n\ (kJ\ kg^{-0.8}\ day^{-1})}$$

Specific dynamic action (SDA) is the energetic loss, measured as the postprandial elevated O_2 consumption calculated to the OE, above SMR.

$$\mathrm{SDA\ (kJ\ kg^{-0.8}\ h^{-1}) = heat\ (kJ\ kg^{-0.8}\ h^{-1})\ -\ SMR\ (kJ\ kg^{-0.8}\ h^{-1})}$$

Heat was calculated as the sum of oxygen consumed during 24 h following the last meal, divided by MBW, and calculated to the OE.

$$\mathrm{heat\ (kJ\ kg^{-0.8}\ day^{-1}) = OC\ (g\ kg^{-0.8}\ day^{-1}) \times 14.06\ kJ\ g^{-1}}$$

SMR is determined as the mean of 10 % of the lowest oxygen consumption rates per hour measured over 100 h post-feeding following Clark et al. (2013) calculated to the OE.

The difference between MEI and heat was calculated as the retainable energy (RE), being the sum of energy of GEI subtracted by all accountable energetic losses. In this study, the RE functions as the main indicator, for potential growth of the fish.

$$\mathrm{RE\ (kJ\ kg^{-0.8}\ day^{-1}) = MEI\ (kJ\ kg^{-0.8}\ day^{-1})\ -\ heat\ (kJ\ kg^{-0.8}\ day^{-1})}$$

2.6 Digestibility trial

For internal reference, we assessed fecal losses and digestibility by a fecal collection sampling. The purpose was to assess digestibility for the calculation of an energy budget and confirm the correctness of the diet formulations. After completion of the respirometry trial, the rainbow trout were stocked into three 1000 L round tanks with a 500 L RAS filtration unit at 15 °C. The temperature was not

changed during the trial, as the digestibility of energy and nutrients did not significantly change at a range from 14 - 20 °C in a previous test trial in the same system, with fish of the same origin and fed a commercial diet (Chapter I). Similar results for rainbow trout were also reported by Jobling (1994) upon a temperature increase from 11 - 15 °C. The fish were marked as triplicates by colored staining of a skin fold behind the eye. Three triplicates were stocked into their respective treatment tank. Each tank was stocked with a total of 16.2 kg of fish resulting in a stocking density of 16.2 kg/m^3. Each tank was fed the corresponding diet received as treatment in the respirometer. The fish were fed a ration of 1.3 % BW daily. For feces stripping, the fish were anesthetized with clove oil (1 mL per 40 L of water) until unconscious and then feces were stripped manually by slightly pressing the area around the lower gut. Digestibility was calculated using the apparent digestibility coefficient (ADC) after Cho et al. (1982) as:

$$\text{ADC of nutrient} = 1 - \left(\frac{F}{D} \times \frac{Di}{Fi}\right)$$

where, D = nutrient (%) or energy (MJ/kg) concentration of diet; F = nutrient (%) or energy (MJ/kg) concentration of feces; Di = % digestion indicator (TiO_2) of diet; Fi = % digestion indicator (TiO_2) of feces. Energy content of the feces was via bomb calorimetry (C200, IKA, Staufen, Germany), and TiO_2 content was assessed by the AGROLAB LUFA GmbH (Kiel, Germany).

2.7 Statistics

The statistical software R (2022) was used to evaluate the data. The appropriate model was defined upon the generalized least squares after Box et al. (1994) and heteroscedasticity after Carroll & Ruppert (1988). For temperature, a quadratic dependency was modeled with the measurement values for different time points considered. Residuals were assumed to be normally distributed based on graphical residual analysis. Based on the model for AQ, an analysis of covariances (ANCOVA) was conducted (Cochran, 1957), followed by multiple contrast tests (Hothorn et al., 2008; see also Bretz et al., 2011) in order to compare the diet-specific intercepts (representing the constant

shifts between the quadratic functions). For the results of ADC, an analysis of covariances (ANOVA) was conducted, followed by a pairwise t-test. For analysis of SDA, heat, and E_n a pseudo R^2 was calculated according to Nakagawa & Schielzeth (2013) and an analysis of variances (ANOVA) was conducted, followed by multiple contrast tests in order to compare the several levels of the influence factors respectively (Hothorn et al., 2008).

3. Results

3.1 Feeding

No group internal competition or aggression was observed during feeding. All pellets of a respective ration were completely ingested within a time of 12 min.

3.2 Metabolic responses

3.2.1 Digestibility of energy and nutrients

No relevant differences were detected between the different diets for the ADC of gross energy, crude protein, and crude lipid. However, in the digestibility of nitrogen-free extracts and crude fiber, that add up to the carbohydrates in the diet, there was a significant decrease in digestibility between the HE diet and the LE and ME diet (Tab.3-3).

Tab. 3-3: Effects of dietary protein to energy ratio on apparent digestibility coefficient (%) for rainbow trout (Oncorhynchus mykiss).

Diet	Gross energy	Crude protein	Crude lipid	NfE + crude fiber
LE	76.90 ± 0.50	85.42 ± 0.25	70.32 ± 1.01	71.67 ± 0.39[a]
ME	75.41 ± 0.88	85.25 ± 0.31	73.91 ± 0.79	67.46 ± 1.00[a]
HE	73.78 ± 0.92	85.4 ± 0.10	71.86 ± 1.08	60.00 ± 1.89[b]

Tab. 3-4: Energy budget for rainbow trout (Oncorhynchus mykiss) fed three isonitrogenous diets with different energy contents (HE = 20.5 MJ/kg, ME = 18.76 MJ/kg, LE = 17.35 MJ/kg) at five temperatures.

Diet	GEI	DEI	En	MEI	SDA	heat	RE	SMR
(relative energy conent)	kJ $kg^{-0.8}$ day^{-1}							
12°C								
LE	164.3 ± 0.3	128.0 ± 0.7	1.9 ± 0.2	126.4 ± 0.4	27.9 ± 4.7	48.6 ± 0.6	77.4 ± 0.3	20.7 ± 4.3
ME	177.5 ± 0.5	135.6 ± 0.9	2.2 ± 0.1	133.6 ± 0.3	30.4 ± 3.6	48.0 ± 2.2	85.5 ± 2.0	17.6 ± 1.4
HE	194.4 ± 0.4	141.4 ± 0.8	2.3 ± 0.4	139.1 ± 0.4	25.2 ± 5.7	43.2 ± 7.2	95.9 ± 7.5	17.9 ± 1.5
14°C								
LE	166.1 ± 0.5	129.7 ± 0.8	1.5 ± 0.2	125.2 ± 1.2	27.2 ± 1.4	46.9 ± 1.5	76.9 ± 1.6	19.7 ± 0.7
ME	179.9 ± 0.8	137.6 ± 0.9	1.8 ± 0.1	133.9 ± 0.5	28.6 ± 4.8	48.5 ± 3.6	83.6 ± 3.3	19.9 ± 1.2
HE	196.5 ± 0.7	142.9 ± 0.7	2.1 ± 0.1	139.5 ± 0.9	28.0 ± 6.4	48.3 ± 5.2	89.7 ± 5.5	20.3 ± 1.5
16°C								
LE	167.7 ± 0.2	130.7 ± 0.9	1.0 ± 0.2	129.9 ± 0.7	25.6 ± 1.0	51.5 ± 1.2	78.0 ± 0.7	25.8 ± 0.7
ME	180.9 ± 0.2	138.4 ± 1.0	1.4 ± 0.1	136.7 ± 0.2	29.6 ± 3.4	58.2 ± 3.5	78.5 ± 3.6	28.2 ± 2.6
HE	198.0 ± 1.1	144.1 ± 1.0	1.6 ± 0.1	142.5 ± 0.9	28.1 ± 0.7	54.9 ± 1.4	87.6 ± 2.2	26.9 ± 1.3
18°C								
LE	168.9 ± 0.5	131.9 ± 0.9	1.4 ± 0.3	130.2 ± 0.7	31.4 ± 1.8	55.8 ± 1.1	74.4 ± 1.5	24.4 ± 2.9
ME	182.0 ± 0.3	139.2 ± 0.9	1.5 ± 0.1	137.5 ± 0.9	26.7 ± 2.7	52.6 ± 0.4	84.9 ± 0.1	25.9 ± 3.4
HE	199.3 ± 1.1	145.0 ± 0.8	1.9 ± 0.2	143.2 ± 0.9	33.2 ± 3.5	57.3 ± 5.3	85.8 ± 5.9	24.1 ± 2.0
20°C								
LE	170.2 ± 0.5	132.6 ± 0.9	1.6 ± 0.4	130.3 ± 0.8	31.1 ± 3.6	55.8 ± 3.8	75.1 ± 4.5	24.8 ± 3.4
ME	183.2 ± 0.4	140.0 ± 0.7	1.9 ± 0.1	138.2 ± 0.4	30.1 ± 1.8	54.7 ± 3.2	83.4 ± 3.6	24.6 ± 2.0
HE	200.7 ± 1.3	146.1 ± 0.8	2.0 ± 0.1	144.0 ± 1.0	27.9 ± 3.0	52.6 ± 2.5	91.3 ± 3.4	24.7 ± 0.9

Values are mean ± SD; GEI: Gross energy intake; DEI: digestibile energy intake; MEI: metabolizable energy intake; SMR: standard metabolic rate; En: non-fecal nitrogen; heat: energetic losses of heat production; SDA: specific dynamic action; RE: retainable energy (n=3).

3.2.2 Energy Budget

Complete absolute values of all measured variables are summarized in a detailed energy budget (Tab. 3-4). The table shows complete energetic values as kJ $kg^{-0.8}$ day^{-1} of either measured metabolic losses (E_n; SDA; heat and SMR) or remaining available energy after subtraction of the respective losses (DEI; MEI and RE) based on the formulae of 2.5. The values were not statistically analyzed, due to the differences in GEI as a result of higher energy levels in the diets. The absolute values shown in Tab. 3-4 were normalized to the GEI for appropriate analysis. The data in relation to the GEI are shown below (Tab.3-5).

3.2.3 Energy budget

Absolute values provided in Tab. 3-4 were normalized to relative values as a percentage of GEI. Values of RE and GEI are provided as a reference. RE was analyzed as a key metabolic value in detail in section 3.2.4. Values of relative energetic losses E_n were lowest at 16 °C, significantly lower than at 20 °C across all diets and significantly lower than at 12 °C when the LE and ME diet was fed. In general, the values were higher at 20 °C than at 12 °C, in the case of the ME diet, this was significant. The relative SDA values decreased with increasing energy content in the diet with exceptions at 16 °C and 18 °C. A significant difference in SDA values was only detected between 16 - 18 °C when fish were fed the LE diet. All other differences, between diets and between temperatures were not significantly different from one another. Relative heat values were generally highest when the LE diet was fed and lowest when the HE diet was fed although this difference was not significant. When HE and LE diets were fed, heat steadily increased with rising temperatures, peaked at 18 °C, and again decreased at 20 °C. When the LE diet was fed heat significantly increased at 18 °C compared to the lower temperatures. At 18 °C the heat values for the LE diet were significantly higher than for ME and HE diets. At 20 °C the heat values of the HE diet were significantly lower than for the LE diet.

Tab. 3-5: Key metabolic losses for rainbow trout (Oncorhynchus mykiss) fed three isonitrogenous diets with different energy contents (HE = 20.5 MJ/kg, ME = 18.76 MJ/kg, LE = 17.35 MJ/kg) at five temperatures as a relative of gross energy intake (GEI). Upper-case superscript letters indicate significant differences between temperatures within each diet. Lower-case superscript letters indicate significant differences between the diets at each temperature.

Diet	GEI	E_n	SDA	heat	RE
	kJ $kg^{-0.8}$ day^{-1}		% of GEI		
12°C					
LE	164.3 ± 0.3	1.2 ± 0.1	17.0 ± 2.9	29.6 ± 0.3	47.2 ± 0.2
ME	177.5 ± 0.5	1.2 ± 0.1	17.1 ± 2.0	27.0 ± 1.2	48.1 ± 1.2
HE	194.4 ± 0.4	1.2 ± 0.2	13.0 ± 2.9	22.2 ± 3.7	49.4 ± 4.0
14°C					
LE	166.1 ± 0.5	0.9 ± 0.2	16.4 ± 0.8	28.3 ± 0.9	46.3 ± 0.9
ME	179.9 ± 0.8	1.0 ± 0.1	15.9 ± 2.6	26.9 ± 1.9	46.5 ± 2.0
HE	196.5 ± 0.7	1.1 ± 0.1	14.3 ± 3.3	24.6 ± 2.7	45.6 ± 2.6
16°C					
LE	167.7 ± 0.2	0.6 ± 0.1	15.3 ± 0.6	30.7 ± 0.7	46.5 ± 0.5
ME	180.9 ± 0.2	0.8 ± 0.1	16.6 ± 1.9	32.2 ± 2.0	43.4 ± 2.0
HE	198.0 ± 1.1	0.8 ± 0.1	14.2 ± 0.4	27.8 ± 0.8	44.2 ± 0.9
18°C					
LE	168.9 ± 0.5	0.8 ± 0.2	18.6 ± 1.0	33.0 ± 0.7	44.0 ± 0.8
ME	182.0 ± 0.3	0.8 ± 0.1	14.7 ± 1.5	28.9 ± 0.2	46.7 ± 0.1
HE	199.3 ± 1.1	0.9 ± 0.1	16.7 ± 1.8	28.8 ± 2.7	43.1 ± 2.8
20°C					
LE	170.2 ± 0.5	0.9 ± 0.2	18.3 ± 2.2	32.8 ± 2.3	44.1 ± 2.6
ME	183.2 ± 0.4	1.0 ± 0.1	16.4 ± 1.0	29.8 ± 1.8	45.6 ± 1.69
HE	200.7 ± 1.3	1.0 ± 0.1	13.9 ± 1.6	26.2 ± 1.3	45.5 ± 1.4

Values are mean ± SD; GEI: Gross energy intake; En: non-fecal nitrogen; SDA: specific dynamic action heat: energetic losses of heat production; RE: retainable energy (n=3).

3.2.4 Retainable Energy

Values of RE ranged between 40.1 % of GEI (HE diet at 18 °C) to 53.9 % of GEI (HE diet at 12 °C)(Fig.3-1). RE values were generally highest at 12 °C when the HE or ME diet was fed to the trout. The values declined towards 16 °C and subsequently increased towards 20°C. However, upon analysis of the values using a quadratic dependency model, no significant dependency of the values regarding temperature could be detected.

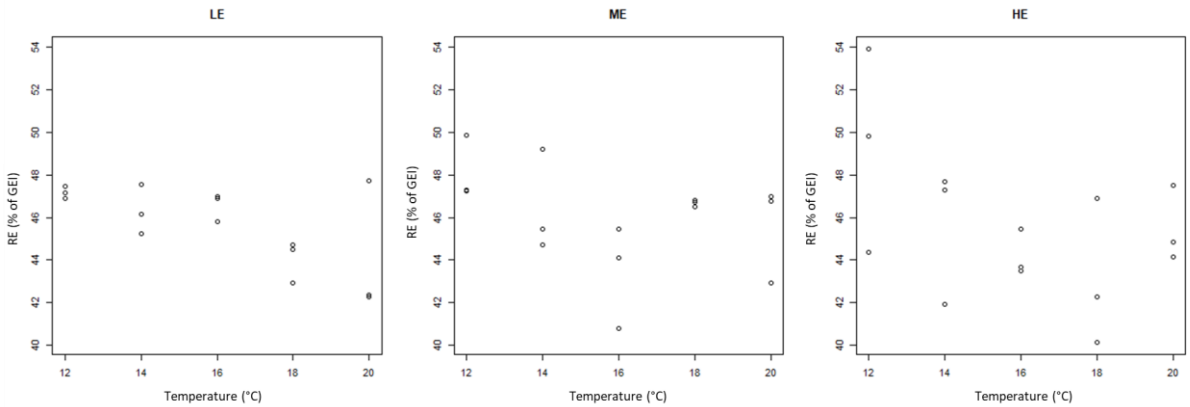

Fig. 3-1: Percentage of retainable energy (RE) relative to gross energy intake (GEI) of rainbow trout (*Oncorhynchus mykiss*) fed three isonitrogenous diets with different energy contents (HE = 20.5 MJ/kg, ME = 18.76 MJ/kg, LE = 17.35 MJ/kg) at five temperatures (12; 14; 16; 18; 20°C). Each data point represents a measurement of one tank with rainbow trout at each respective diet and temperature (n=3). No dependency of the data points to temperature as a factor could be detected using a quadratic dependency model.

3.2.5 Ammonia Quotient

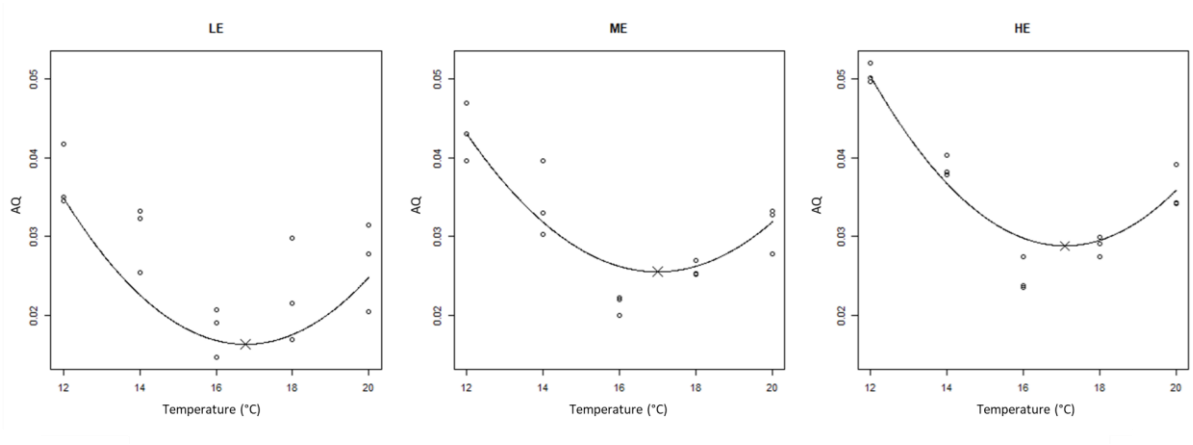

Fig. 3-2: Ammonia Quotient (AQ) of rainbow trout (*Oncorhynchus mykiss*) fed three isonitrogenous diets with different energy contents (HE = 20.5 MJ/kg, ME = 18.76 MJ/kg, LE = 17.35 MJ/kg) at five temperatures (12; 14; 16; 18; 20°C). A quadratic dependency model was used to analyze the data. Parabolas describe the quadratic dependency of AQ values on temperature. The lowest AQ values for each parabola are marked with a cross (×). Each data point represents a measurement of one tank with rainbow trout at each respective diet and temperature (n=3).

The following equations describe the parabolic relationship between AQ and temperature for the three tested diets (Fig. 3-2). While we used the mathematical symbol for multiplication in the material and methods, we used an asterisk here, to avert confusion with x as a variable.

LE: $y = 2.467*10^{-1} – 2.747*10^{-2}*x + 8.188*10^{-4}*x^2$

ME: $y = 2.286*10^{-1} – 2.390*10^{-2}*x + 7.034*10^{-4}*x^2$

HE: $y = 2.720*10^{-1} - 2.845*10^{-2}*x + 8.325*10^{4}*x^2$

The diet-specific linear and quadratic coefficients did not significantly differ from each other. We found a significant difference in the diet-specific intercepts between all diets (LE ~ ME: $p \leq 0.001$; LE ~ HE: $p \leq 0.001$; ME ~ HE: $p = 0.044$). We determined the calculated temperature at which y-values were minimal for each diet respectively and found for LE: 16.77 °C/AQ: 0.016, for ME: 16.99°C/AQ: 0.025 and for HE: 17.08 °C/ AQ: 0.028.

4. Discussion

The complex nature of the approach requires a careful and thorough evaluation of the design of the study, the tested diets, and the technical setup. The study assessed the use of low protein/high energy diets on the temperature-dependent catabolic fuel use of protein and the retainable energy from the diet. It is important to understand that the trial does not quantify actual growth or actually retained energy per se but estimates the potential for growth and relative protein combustion by quantifying the RE and AQ as benchmarks.

The LE diet was based on the diets used by Cho (1981) who found that the inclusion of 11 % crude lipid to a diet with 36 % crude protein for rainbow trout significantly decreased the consumed oxygen compared to diets with only 6 % lipid inclusion at the same protein content. Our LE diet was additionally formulated to contain high levels of carbohydrates (28.1 %) following the research of Kaushik et al. (1989) who found a 10 % reduction in ammonia nitrogen excretion upon inclusion of 30 % of dietary gelatinized starch. In order to exclude carbohydrate energy to lipid energy ratio as a factor, the achieved ratio was set fix for all tested diets. Our diet was intentionally designed to contain a low dietary protein-to-energy ratio, in order to induce a high protein-sparing effect and the design of the study targeted to determine the optimal thermal condition for a high visibility of this effect and a maximal efficient protein utilization in rainbow trout. The ration of 1.3 % was chosen as the lowest ration that would be completely be consumed by fish at a temperature of 12 °C within the 12 min allotted for measuring of each tank. We tested this assumption prior to the experiment with a different batch of rainbow trout of comparable size (208.46 g ± 3.78 g). Since the so-called oxy-static control for feed intake may significantly alter the feed intake at different temperatures, we fed a fixed ration to accurately compare the different temperature treatments (Galbreath et al., 2004; Saravanan et al., 2012; 2013). By doing so we created a highly relevant metabolic dataset to address the energy partitioning at different temperatures and set the base for future protein-sparing research. We are aware that this may hold certain limitations for the application for commercial trout farming although most commercial feeds are provided with a fixed ration recommendation (e.g. ALLER Gold,

ALLER Aqua, Christiansfeld, Denmark; AlltechCoppens Ultra, 3 mm, AlltechCoppens, Helmond, The Netherlands).

The design of the respirometer minimizes potential measurement errors by performing all measurements at the same test section (van Ginneken et al., 1994; Stiller et al., 2013). By additionally testing the same fish sequentially at different temperatures it was possible to further eliminate differences in the metabolic rates due to individual variation of the fish and make a reliable comparison of the metabolic response to the different temperatures. The sequential measuring also prevented an unequal distribution of thermal stress over an extended period since all individuals were kept in the same environmental conditions at each given time. This was required to include 18 – 20 °C as above optimal temperatures that may challenge the integrity of the used species that may lead to metabolic depression due to thermal stress beyond a dietary factor (Chen et al., 2015; Verleih et al., 2015; Akbarzadeh et al., 2018).

The main aim of the study was to induce a high protein-sparing effect by the high inclusion of carbohydrates and lipids as non-protein energy substrates. Values of E_n show a clear pattern that temperatures close to and at 16 °C lead to the lowest TAN excretion rates. We calculated the results of E_n and oxygen consumption to the AQ for proper analysis. Values of the AQ show a low relative metabolic combustion of protein with as little as 6.7 % ± 0.9 % of the energy metabolism being fueled from protein (LE diet at 16 °C). A comparable study by Stiller et al. (2016) using rainbow trout (BW: 64.9 g ± 6.0 g) in the same respirometric system at 16 °C showed, that approximately 19-21 % of the energy metabolism was being fueled from protein, when using an experimental diet, resembling the macronutrient composition of a commercial diet (Crude protein: 42.5 %; crude lipid: 27.3 %; starch: 8.6 %; energy: 23.4 MJ/kg, ALLER Gold, 3mm, ALLER Aqua, Christiansfeld, Denmark). Since we found a gradient increase in the AQ values upon further increasing the energy contents beyond the LE diet it is possible, that a higher protein-to-energy ratio would have further decreased AQ levels but all diets appear to be suitable for analyzing the interactions of different temperatures and the protein sparing

effect based upon the made comparison. Further reduction of protein might also hold the potential to not meet the amino acid requirements for rainbow trout (Rhodehutscourt & Pfeffer, 1998).

We found significant differences both between diets and temperatures in the AQ. In this study the metabolic combustion of protein decreased from an average of all diets from 16.2 % ± 2.3 % at 12°C to 8.0 % ± 1.2 % at 16 °C. A regression analysis revealed a high dependency of AQ values on temperature. We were able to calculate a minimum of AQ at an optimal temperature of 16.9 °C ± 0.13 °C. We found the effect to be very uniform in all tested diets, giving a high level of affirmation of the found results. We therefore conclude that 16.9 °C is the optimal temperature to induce a high protein-sparing effect in rainbow trout and we encourage it to be used in further studies investigating this mechanism.

Since the diets were formulated to contain very low protein-to-energy ratios, it was necessary to assess the digestibility of both energy and nutrients, since especially carbohydrates are known to significantly decrease in digestibility at high inclusion rates (Schmitz et al., 1983; Tung & Shiau, 1991). We found no significant differences in the digestibility of energy between the three different diets in the trial. This is in accordance with the study from Lanari et al. (1995) which found no difference in digestibility of energy upon decreasing dietary protein to energy ratios in rainbow trout. We assumed the energy and nutrient digestibility to be largely unaffected by temperature, based on the studies of Cho & Kaushik (1990) who found no differences in digestibility of nutrients or energy in rainbow trout (600 g) between 9 - 18 °C and Jobling (1994) between 11 - 15 °C. We also determined the ADC of nutrients and energy from a commercial diet (ALLER Gold, 3mm, ALLER Aqua, Christiansfeld, Denmark) between 14 - 20 °C in rainbow trout (183.75 g ± 0.65 g) of the same origin as the ones used in this study and found no significant differences in nutrient and energy digestibility (Chapter I). However, we did find a significant reduction of digestibility of carbohydrates between the diets LE and ME (ADC : 71.67 % and 67.46 % respectively) and the HE diet (ADC : 60.00 %)($p > 0.05$). This phenomenon is not uncommon and the findings of Stone (2003) linked the effect to an overload on involved digestive enzymes due to substrate saturation and subsequent discharge of excessive carbohydrates. The results

of the digestibility trial served mainly as a calculation base for the DEI and a detailed energy budget and should be considered with care.

The results showed no consistent pattern of the key metabolic losses SDA and heat. In this study the metabolic losses are calculated, together with the SMR into the value RE. The results do not show a significant dependency of RE values on environmental temperatures, regardless of the diet. The results of the RE values stand in contrast to the understanding that optimal temperatures lead to a maximized energy retention and growth (Dumas et al. 2010; Elliot, 1975; Ruyet et al., 2004; Sun et al., 2001, 2006). With studies often finding an increase of RE or related growth data at the thermal optimum, which we located between 15 – 17 °C according to Hokanson et al. (1977) and Mellery et al. (2016) it would suggest a negative correspondence between AQ and RE values. The lack of correspondence between the AQ and RE values is likely to be due to the role of carbohydrates and lipids as metabolic fuel that changes at different temperatures. This interaction between carbohydrates and lipids as metabolic fuel is usually assessed by the respiratory quotient. The respiratory quotient measures the carbon dioxide produced by a fish divided by the oxygen consumed and may be used to estimate whether carbohydrates or lipids are the predominant metabolic fuels being used apart from protein. Only few studies have performed such measurements with rainbow trout at rising temperatures.

The studies of Kieffer et al. (1998) found that with an acclimation temperature of 5 °C, protein, lipid and carbohydrate contributed to the metabolic fuel use at resembling rates (respectively 27, 35 and 42 % of total oxygen consumed) but when acclimated to 15°C the fuel use transitioned to contribution rates of 30 % from protein, 46 - 58 % from lipid but only 15 % from carbohydrates. Using this explanation, the high inclusion of carbohydrates would benefit the energy yield at temperatures below the optimum and the inclusion of lipids at and above the optimum, by effectively fueling the energy metabolism. Studies by Beamish et al. (1986) found that a fuel use of carbohydrates leads to higher energetic losses as heat when increasing the water temperature from 8 °C to 18 °C. Here we argue, that this effect, together with a decreased relevance of carbohydrates as metabolic fuel at higher

temperatures might superimpose the benefits of the optimal temperature on the RE when high levels of non-protein energy substrates are available.

In general, background information on the effect of different temperatures on the protein-sparing properties of non-protein substrates and the general metabolic fuel use is still scarce and this study might help identify possible mechanisms that will lead to a generally increased efficiency of protein for aquaculture diets in the future.

5. Conclusion

The study provided a high-resolution visualization of the dynamics of metabolic fuel use of protein at a broad spectrum of temperatures, ranging from below optimal (12 °C) to above optimal (20 °C). The results presented could reveal an optimal temperature for protein sparing at 16.9 °C ± 0.13 °C. We provide evidence that research on the protein-sparing effect might show a higher resolution and yield higher visibility of potential dietary effects at this water temperature in trout of approximately 200 g BW. Future research should additionally measure the RQ to accurately assess the relative combustion rates of carbohydrates and lipids in addition to protein. In systems where the temperature can be artificially altered (RAS) this temperature may result in a maximized efficiency of protein use as the combustion for energy demands is minimized.

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GENERAL DISCUSSION

1. Aerobic scope and energy budgeting

In a recent statement, the IPCC recognizes the special impact of climate change on respiration and bioenergetics in farmed fish and encourages researchers to perform studies on their interactions (Barange et al., 2018). Since the IPCC is itself composed of distinguished scientists some have followed their own call and performed respirometry research with fish in the context of climate change (Pörtner & Knust, 2007; Pörtner & Farrell, 2008; Pörtner et al. 2008; Pörtner, 2010; Pörtner & Peck, 2010; Pörtner, 2012; Paschke et al. 2018). From this context, the so-called oxygen and capacity-limited thermal tolerance hypotheses has evolved (OCLTT; Clark et al. 2013). The hypothesis states, that a researcher must only measure the difference between the minimum oxygen consumption of a resting fish and the maximum oxygen consumption after a chase-to-exhaustion protocol, to obtain a value termed aerobic scope. The aerobic scope may be used to determine the optimal temperature for an animal's general fitness and the thermal tolerance of a given species as well as its responses to changing temperatures (Eliason, et al., 2011; Donelson et al., 2012; Munday et al., 2012). However, the methodology is often complex and prone to errors and misleading interpretations. Especially chase protocols may be largely dependent on the individual condition, training status, life stage, and general habitus of the fish (Steffensen, 1989). The approach may additionally be unpractical for aquaculture purposes, as the method only allows quantifying the energetic expenses after intense exercise. However, aquaculture research mainly focuses on the effects of environmental conditions usually found during fish husbandry, the effect of feeding, or a combination of both, as tested by this thesis. However, using a chase-to-exhaustion protocol to measure aerobic scope, it is nearly impossible to obtain reliable data addressing the response to feeding, as they are likely to be superimposed by the effects of exercise (Alsop & Wood, 1997; Thorarensen & Farrell, 2006). Therefore, the OCLTT relates more to the general fitness of an animal and is therefore more appropriate for the research on the biology of wild fish populations.

For practical reasons the measuring of the SDA as a benchmark of postprandial response to a meal and the subsequent calculation of the RE via an energy budget is therefore a method frequently used to address aquaculture-related questions on the metabolic responses to the diet (Khan et al., 2014; Skov et al., 2017; Stiller et al., 2016). Approaches aiming to calculate an energy budget may use different experimental methods. Swim tunnel respirometers often consist of a tube in which the water body will be circulated by a propeller (Blažka et al. 1960; Brett, 1964; Nelson, 2016; Hvas & Oppedal, 2019). In these systems, the fish is forced to swim at a fixed but usually moderate speed to remain in place. The data of these studies may therefore represent a very artificial scenario, as the fish is forced to swim at a set velocity in a very confined environment. Another method to assess an energy budget is the so-called static respirometer. This approach does not require the fish to swim to stay in place and has been regarded advantageous to measure fish, that do not naturally swim much or to measure the minimum oxygen consumption of a resting fish (Clark et al., 2013). For static respirometers a fish mass to tank volume ratio (g/ml) between 1:20 and 1:100 has been suggested to be appropriate for moderate and low-speed swimming species and only for high-performance swimming species (e.g. *Thunnus spec.*) a ratio of up to 1:350 (Clark & Seymour, 2006; Blank et al., 2007; Steinhausen et al., 2008; Clark et al., 2011).

This thesis uses a novel group respirometer that was first described by Stiller et al. (2013) and has since been modified. The tanks of this respirometer have a volume of 250 L and are designed to allow for the free movement of the animals tested. The system has been used for experiments with fish mass-to-tank volume ratio (g/mL) in the respiration tanks ranging from 1:100 (Steinberg et al., 2017) up to 1:107 (Chapter II). The main novelty of the used respirometer is the ability to hold groups of fish over an extended period of weeks or even months. The individual fish tested during this thesis interacted naturally with other individuals in their group based upon a frequent visual evaluation of a trained staff member (Lina Weirup). Natural interaction however results in an increased oxygen consumption based on VA. As the oxygen consumption by VA cannot be quantified by the used approach it is difficult to distinguish them from the expenses by SDA and therefore correct assessment of SDA per se. However,

the system was introduced as a holistic approach that allows measuring metabolic responses to the diet in a culture-like condition (Stiller, 2016), and the system has since been regarded as such (Steinberg et al. 2018). Fish are known to increase activity following a meal and the ability to do so should be regarded as highly relevant as they show a realistic scenario even when the fish are being tested in a respiration chamber.

This thesis aimed to investigate the interactions of environmental temperatures towards the upper optimal range and above by assessment of key metabolic variables. The goal was to determine the temperature dependency of energetic losses and gains as a benchmark for growth and fitness of rainbow trout (*Oncorhynchus mykiss*) and to assess the relative combustion rates of protein for energy. With poikilothermic animals such as fish, it is crucial to find adequate mechanisms to modulate the metabolic response regarding changing temperatures. We, therefore, aimed to investigate the diet's temperature-dependent energy metabolism-modulating properties. The first experiment (Chapter I) focused on the dynamics of the SDA response during its entire duration, most notably its magnitude in dependency on the environmental temperature. The second experiment (Chapter II) focuses on the temperature-dependent differences in the SDA for the combustion of either dietary carbohydrates or lipids as energy-yielding nutrients. The third and concluding experiment (Chapter III) focuses on the protein-sparing properties of carbohydrates and lipids interacting with the temperature. All presented experiments contribute to the proliferating use of respirometry approaches in the research of the interactions of global climate change and fish biology and may be used to broaden the knowledge in future attempts using this methodology.

2. Respirometry – from basic research to practical solutions

Respirometry yields in-depth insight into key metabolic processes that focus on the biochemical turnover of nutrients. Although bioenergetic modeling is regarded as a practical method its main limitation lies in the used simplification that energy is allocated hierarchically and that growth is simply the surplus of energy after all energy losses have been accounted for (Elliot & Hurley, 2002). Therefore,

growth estimates by the assessment of RE should be regarded as scientific indicators rather than a predictor of actual growth (NRC, 2011). Studies addressing mainly the RE may therefore serve as basic research approaches that help to guide researchers to potential mechanisms that consequently need to be addressed in growth trials. However, the instantaneous nature of bioenergetic studies allows for a rapid sequence of several different environmental treatments that helps to easily identify what conditions should be targeted in the following studies. Respirometry can therefore be considered a powerful basic research tool.

When targeting losses either as energy or as the relative combustion of nutrients, bioenergetic research may hold the potential to be more than a basic research tool. When considering the energetic losses as SDA the approach assesses actual energetic expenses that reduce available energy regardless of the fate of the remainder (Chapter I & II). When targeting protein combustion, the approach becomes even more relevant for commercial applications because growth is to a large degree dependent on the available protein (Shearer, 1994; Dumas et al., 2010). In this case, it is the nature of the dietary treatment that may limit the commercial applicability of the study (Chapter III). In general, the bioenergetic approach is highly complex and may hold certain benefits for both basic research needs and commercial applications. However, its biochemical nature and the usage of simplifications make it more suitable for basic research. The core strength of the method lies in the assessment of relative nutrient combustion that may be highly relevant for the efficient use of dietary resources (NRC, 2011).

The findings of Chapter I show that the SDA per se is a temperature-dependent energetic loss. With group respirometry as a basic research tool, this finding may be of special interest when designing a growth trial at a fixed temperature. Temperatures during a trial should maintain stability to avert inequal metabolic expenses by dietary intake. Also, the growth effects of the treatments during a trial may be underestimated, if the thermal conditions induce a generally higher energetic expense that could be avoided.

Another finding of Chapter I is that group respirometry may be considered an adequate tool of bioenergetic research, indicated by the high resemblance with the classical study of Elliot & Hurley (2002). The applicability of group respirometry may hold special relevance for the commercial application of the findings used in such systems. The system shows in general a higher resemblance with culture-like production conditions than the conventional use of single-fish respirometers. The fish are not restricted in their movement and can socially interact with each other as they would during commercial production. Also, the fish are able to swim in schools which has been associated with a hydrodynamic advantage and increased welfare thus potentially reducing unnecessary energetic costs by swimming (Herskin & Steffensen, 2005; Marras et al., 2015; Svendsen et al., 2003).

This may hold a special interest for the improvement of fish welfare in animal testing. While ethical standards in scientific aquaculture research are generally high, it may sometimes be challenging to allow animal welfare according to the five freedoms model that is still widely applied (Johansen et al., 2006). The vast majority of aquaculture studies naturally allow for freedom from hunger and thirst as the fish are fed adequate test diets to increase growth in an aquatic environment. However, studies using a respirometry approach often require the fish to be placed individually in a confined, highly artificial space and usually after intense handling, especially when applying a chase protocol. In this case, some if not all of the other freedoms are potentially compromised.

Although the group respirometer is still a highly artificial system, it allows for a more natural behavior of the animals with social interaction (Stiller et al., 2016). Also, studies using such a system can be designed to hold the fish for a longer period than single respirometers thus reducing handling and leaving the fish in a more undisturbed environment. The group respirometer used in this study also applies a water current in the tank, that allows voluntary swimming but does not force it to swim at a fixed velocity. Group respirometry should not be considered a method that allows for a high degree of welfare, due to its artificial nature but it may lead to a reduction of welfare limitations compared with other systems.

3. Carbohydrates as a sustainable energy source for cold temperature-adapted diets

The SDA is a metabolic reaction with a long research history (Rubner, 1902; Wilhelmi, 1935; Brody, 1947; Kleiber, 1962; Jobling, 1981; McCue, 2006; Secor, 2009; Tirsgaard et al., 2015; Steinberg, 2018; Moffatt et al. 2022). For aquaculture, it is of great importance as it describes the metabolic response to the diet when environmental factors are constant. Therefore, we continued the detailed investigation of this highly relevant postprandial response in Chapter II where we tried to assess the energy expenses deriving from different dietary nutrient classes.

Chapter II targets the utilization of carbohydrates and lipids to saturate the metabolic fuel demand. The combustion of lipids and carbohydrates is however associated with an unequal energy expense upon combustion, relying on the environmental temperature (Gulliaume et al., 1999). The results of Chapter II may indicate that an adaptation of the dietary carbohydrate content may be a useful tool in the design of temperature-adapted feeds. Many feed producers already supply the market with seasonal adapted diets (e.g. ALLER Aqua, Christiansfeld, Denmark; Alltech Coppens, Helmond, Netherlands). The diets designed for winter months are mainly designed to improve feed intake or increase lipid and protein digestibility. The consideration of carbohydrate use during low-temperature periods has often been neglected mostly due to a suggested overall decreased digestibility of carbohydrates upon lower temperatures. However, the data of Cho & Kaushik (1990) suggest that the digestibility of nutrients and especially carbohydrates regarding the temperature may be highly dependent on the BW of the animal. In small rainbow trout with an individual BW of 20 g, the ADC of the three main nutrient classes plus energy differed significantly between the temperatures of 7°C, 11°C, and 15°C. In rainbow trout of 600 g, the ADC showed no significant differences at the same temperatures. This is in accordance with the results of Chapter I in which the ADC of carbohydrates did not substantially differ between 12-20°C in rainbow trout of 183.75 g ± 0.65 g. However, since the

results of the ADC cannot be statistically confirmed due to the small sample size (n = 1) the finding that the ADC is not affected by temperature in the here-tested rainbow trout remains an assumption.

Additionally, has the processing of carbohydrates substantially improved the digestibility and availability of carbohydrates in fish nutrition (Kamalam et al., 2017). When carbohydrates are digestible they become available to the metabolism of the animal. Research by Kieffer et al. (1998) suggested that carbohydrates may be the main substrate being combusted as metabolic fuel for energy in rainbow trout when the environmental temperature is low (5°C). At higher temperatures (15°C) carbohydrates substantially decrease in importance while the metabolic combustion of lipids increases. Considering these findings, it would appear highly relevant to further investigate if an increase of digestible carbohydrates in diets designed for low temperatures may be of special benefit since they are highly incorporated in the energy metabolism while holding similar properties as lipids regarding energy expenses as SDA.

In general, it is of great advantage to be able to use carbohydrates in animal nutrition, although fish can survive and grow without dietary carbohydrates (NRC, 2011). Carbohydrates derive from cheap and available resources that can be locally produced. Although the forms of carbohydrates are abundant only starches and sugars hold a nutritive value for fish. While early studies have predominantly used sugars, mainly in the form of glucose or glucose derivates, more recent studies have mainly used gelatinized starch as they hold a greater nutritional value for carnivorous fish (Hung et al., 1989; Shiau & Chen, 1993; Shiau & Lin, 1993; Shiau & Peng, 1993; Arnesen et al., 1995; Robinson & Li, 1995; Shiau & Liang, 1995; Deng et al., 2000).

The results of chapter II indicate that gelatinized starch may be a very good source of energy at colder temperatures, as they do not induce an elevated SDA compared to dietary lipids and literature shows an increased incorporation of carbohydrates into the energy metabolism (Kieffer et al., 1998). Also, the use of carbohydrates at lower temperatures can potentially reduce the relative protein combustion compared to lipids, making the use of them additionally interesting at lower temperatures. However,

there are several limitations to the use of carbohydrates in fish nutrition, especially in carnivorous species like the rainbow trout. A main concern is the induction of persistent hyperglycemia in fish after ingestion of carbohydrate-rich diets (Moon, 2001). Several factors are involved in this mechanism. For one the white muscle tissue, which is the largest tissue in fish, appears to hold a poor ability for the utilization of glucose and very low insulin receptor density (Navarro et al., 1999). Moreover, holds glucose a very low insulin secretagogue potency compared to protein (Mommsen & Plisetskaya, 1991). With such a complex mechanism involved the measuring the glucose flux, meaning the rate of appearance of glucose over the rate of disappearance in the bloodstream has evolved in the field and been regarded as a good method to measure glucose utilization and its impacts on the glycemic status of the animal (NRC, 2011). As discussed in chapter II it is likely that the flux of glucose may very well be temperature dependent as well.

In summary, at low temperatures (< 15°C) carbohydrates are highly digestible for carnivorous fish species, they are the main metabolic fuel used when available from the diet, they may not pose a high threat to induce hyperglycemia due to reduced glucose flux and they do not induce a higher SDA response than dietary lipids. They can additionally spare protein from metabolic combustion. In conclusion, it appears highly relevant to further investigate if higher amounts of carbohydrates should be included in seasonal adapted feeds. Chapter II could provide evidence that the inclusion of dietary carbohydrates in feeds for aquaculture holds a huge potential to increase the sustainability and the price of diets for trout during the winter. Future investigations should measure O_2 consumption and CO_2 liberation to calculate the RQ and thereby assess the proportion of carbohydrate and lipid combustion at different temperatures. Another consideration for future studies regarding the use of carbohydrates in cold temperature-adapted diets for rainbow trout is to measure the glucose flux in the bloodstream of the animals.

4. Respirometry as a powerful basic research tool for the development of protein efficient diets

While chapter II addresses the utilization and effects on SDA of carbohydrates and lipids at different levels, chapter III focuses on the protein-sparing properties of carbohydrates and lipids at a fixed ratio in relation to temperature. The chapter provides relevant basic research insights and determined the optimal temperature to induce a highly visible protein-sparing effect at 16.9°C in rainbow trout.

Aquaculture holds a huge potential for a sustainable method to produce animal protein for human consumption as fish have a very low feed conversion ratio (FCR) of 1.1-1.6 kg of feed input per kg seafood yield. The production of land animals tends to be associated with a much higher FCR with 1.4–1.8 for poultry, 2.6–4.4 for pork, and 3.5–9 for beef (Jones, 2020). However, contrary to most terrestrial livestock, the aquaculture sector is dominated by the production of carnivorous species like rainbow trout (Ritchie & Roser, 2019). The production of farmed fish therefore requires large quantities of fish meal from wild capture with 15,000,000 metric tons of fish processed into meal and oil for animal production in 2020 (FAO, 2022). Although alternative protein sources like soy or rapeseed have been proven to be good alternatives to replace fishmeal but a complete replacement of animal protein for aquaculture feeds is associated with growth retardations and animal health concerns (Kaiser et al. 2021). Over the last 30 years prices for fish meal increased by 237.46% (World bank) but the export price of a kg of gutted salmon decreased by 21.80% (International monetary fund). With the inevitability of fishmeal use for aquaculture but unequal development of resource and export prices, it is important to use protein efficiently and ensure that the largest fraction of protein is converted into body protein and therefore sellable muscle tissue rather than being combusted for energy demands. Since the findings are very uniformly across three diets with different DP/DE ratios it suggests that further research on the protein-sparing effect will yield highly relevant results at this temperature even when using a different dietary formulation. However, since the diets of Chapter III, all contain a very low dietary protein content, it would be preposterous to apply the findings to

commercial diets without further investigations. However, the design of the study may have set the ground for such investigations. In this experiment, the respirometer has proven to be a powerful tool because its use enabled for a high-resolution picture of the thermal dependency of the protein-sparing efficiency. A rapid following of thermal sequences with the same batch of fish minimized individual variances in metabolic rates thus decreasing potential sources of errors. If a similar trial would have been performed with fish held simultaneously at different temperatures for an extended period an unequal distribution of temperature as a stressor may have compromised the results, especially at upper temperatures above the optimum. The respirometer has therefore been proven to be a powerful tool for bioenergetic research and may guide research in the development of highly protein-efficient diets for commercial aquaculture applications. The further establishment of the group respirometer for thermal research during Chapter I and the potential for a sustainable resource use during Chapter II makes the third and last chapter the logical conclusion for this thesis as it uses a highly sophisticated and accurate experimental setup to determine the optimal conditions for research on the sustainable use of protein as a valuable resource.

5. Conclusion

This thesis provides valuable insights into the temperature-dependent metabolic fate of energy as well as nutrients. We provide evidence that the postprandial energy expense of feeding as the SDA (measured as its variable SDA_{coef}) may be in fact temperature dependent thus reducing the availability of energy for potential growth. We advance the research on temperature-adapted diets for commercial use by showing that carbohydrates have evolved to be potent energy substrates for carnivorous fish like rainbow trout at temperatures below the optimum. This may be of special interest in the future since carbohydrates are derived from cheap and sustainable resources that can be locally produced in central Europe. Research on the efficient use of protein will likely proliferate in the future and this thesis provides valuable information to be used in such studies. This may aid in understanding the exact mechanisms of protein utilization and the sustainable use of protein regarding economic and environmental applications. The respirometer has again been proven to be a powerful tool for research

on the impacts of the thermal environment of farmed fish. The use of a group respirometer is likely to be an adequate method that compares to the use of traditional-single fish respirometer while improving the comparability to commercial production methods by providing a more culture-like environment, thus potentially improving animal welfare of test animals in bioenergetic research. We hope that this thesis broadens the understanding of the thermal impacts on farmed fish biology in the perspective of an unstable and uncertain future of the global climate.

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SUMMARY

Sophisticated projections of the global climate situation predict a general increase in global temperatures of at least 1.5°C compared to the pre-industrial era. Such an increase does not simply lead to a rise in ambient temperatures in the local weather but rather to a complex situation of volatile local weather patterns across the globe. Since the environmental temperature is among the most relevant modulators of ectothermic animals' metabolism, aquaculture research targeting the effects of unstable thermal conditions has proliferated in the past. Temperature has complex effects on many relevant aspects of commercial aquaculture including growth, energy partitioning, and the relative metabolic combustion rates of different nutrient classes. It is further important for research to have knowledge of the interacting effects of environmental temperatures and targeted effects. It is therefore highly relevant for aquaculture research to gain an in-depth and high-resolution insight into the interactions of temperature and the metabolism of farmed fish species. This thesis aimed to provide such insight using respirometry as a powerful research tool. This method assessed energetic losses via oxygen consumption rates of the fish to calculate energy gains as a benchmark for relative growth potential using an established and widely accepted energy partitioning scheme. Additional measurements of ammonia excretion by the animals made it possible to calculate the relative protein combustion rate during metabolism. The thesis used a group respirometer that has recently been established as a method.

The first trial (Chapter I) was conducted to investigate the interactions of temperature on the energy lost during a postprandial response known as specific dynamic action (SDA). This variable is a direct response to a feeding event and accounts for the energetic cost of the ingestion, digestion, and the metabolic turnover of nutrients and is therefore highly relevant for aquaculture research and its practical applications. The SDA in relation to energy intake over its entire course is known as the SDA coefficient. This SDA variable has been suggested to be independent of the environmental temperature but opposing findings by many researchers have sparked a highly controversial discussion

on the topic. We conducted a restricted ration experiment feeding a commercial at three different rations (0.65; 0.975; 1.3 % feed per body weight) at three different temperatures (14; 17; 20°C) to groups of rainbow trout. The thermal treatments were applied sequentially since temperatures were increased over time. The results of Chapter I indicate that the SDA coefficient is likely to be highly influenced by the environmental temperature in rainbow trout. The design enabled a close comparison of the results with a classical study investigating the interactions of environmental temperatures and ration size. Close comparison was performed since the duration and magnitude of the SDA coefficient may be influenced by the voluntary activity of the animal which is not restricted in group respirometry but is in classical respirometry approaches. The results showed a high resemblance with the classical study providing evidence for the accuracy of our obtained data thus further establishing the group respirometer as an adequate and powerful research tool.

The second experiment (Chapter II) was performed in order to investigate the interactions of environmental temperature and the postprandial energetic expense of either dietary lipids or diets. Given the indication for the accuracy of the methods from Chapter I, we performed the second experiment using a similar study design. Three diets with different dietary lipid-to-carbohydrate ratios (1 : 1; 0.6 : 1; 1.4 : 1) were fed to groups of rainbow trout at a total of five environmental temperatures (12; 14; 16; 18; 20°C) that were sequentially applied. The results of the second experiment showed that carbohydrates and lipids result in similar postprandial energy loss as SDA at temperatures below and above the optimal thermal conditions for growth of rainbow trout between 15-17°C. At temperatures below that optimum, the lipid-rich diet also resulted in a significantly lower relative protein combustion compared to the carbohydrate-rich diet. At temperatures around the optimum for growth, the SDA response was significantly higher in groups fed the carbohydrate-rich diets compare to those fed the lipid-rich diet. The results indicated the high relevance of carbohydrates in diets for rainbow trout, especially at low temperatures. Not only were energetic losses comparable at below-optimal temperatures but also the relative protein combustion rate could be reduced. This is of special importance as the provision of dietary carbohydrates is cheaper and more available than sources of

lipids and especially protein. Sparing these valuable resources from combustion may be achieved in cold temperature adapted feeds for commercial application in rainbow trout. At temperatures at the thermal optimum, the use of carbohydrates appeared unfavorable since the energetic expense of feeding was higher in rainbow trout fed the carbohydrate-rich diet than in those fed the lipid-rich diet. At temperatures above the optimum, there was a decrease in the magnitude of the SDA response of fish fed the carbohydrate-rich diet and no significant difference could be detected between the groups. Since the relative protein combustion ratio was not significantly different at any temperature besides the ones below the optimum and given the high potential for fish to turn hyperglycemic, the use of carbohydrate-rich diets can in conclusion only be recommended at low temperatures.

The third experiment (Chapter III) concluded the thesis and aimed to use the evident practicability of the group respirometry in a highly sophisticated basic research approach. Goal was to gain a high-resolution depiction of the interactions of the relative protein combustion and the environmental interaction. Such interactions in rainbow trout have been suggested in the past but these studies only used either two or three temperatures to be compared. The study aimed to provide detailed insight for the use in future research on the protein-sparing effect, an effect that potentially minimizes the relative protein combustion during metabolism. The study used a total of five environmental temperatures (12; 14; 16; 18; 20°C) to determine the dynamics of the relative protein combustion for energy across a broad thermal range. In order to make a reliable statement of the thermal effect on the relative protein combustion, we used three diets with different digestible protein to digestible energy ratios (20.38; 19.08; 18.09 mg/kJ) to discriminate between the effects of the temperature and dietary effects. All diets yielded the result of a maximized protein sparing effect at a calculated temperature of 16.9°C ± 0.13°C. To our understanding the design of the study especially in combination with the highly sophisticated respirometric system creates a highly accurate depiction of the relative protein combustion in rainbow trout. Research on the protein-sparing effect will most likely proliferate in the future and by establishing the optimal thermal condition in rainbow trout we may have accelerated the research regarding the topic. Knowledge of the effect of thermal conditions on the

protein-sparing effect eliminated a potential source for incorrect interpretation of data due to an underestimation of the induced effects.

ZUSAMMENFASSUNG

Unterschiedliche Modelle zur Entwicklung des Klimawandels prognostizieren, selbst unter Einhaltung des Klimaabkommens von Paris, einen Anstieg der globalen Durchschnittstemperaturen von mehr als 1.5°C im Vergleich zur vorindustriellen Zeit. Dies stellt eine bedeutende Herausforderung auch für den gesamten Agrarsektor dar. Insbesondere die Haltung von wechselwarmen Tieren wird maßgeblich durch eine starke Veränderung des globalen Klimas und damit des lokalen Wetters beeinflusst. Die Haltungstemperatur stellt somit eine der bedeutendsten Einflussgrößen für den Energiehaushalt, die metabolische Umsetzung von Nährstoffen und letztendlich das Wachstum von Fischen in Aquakultur dar. Dies trifft auch für die Aquakultur in Mitteleuropa zu, eine Region in der die Regenbogenforelle (*Oncorhynchus mykiss*) von großer kommerzielle Bedeutung ist.

Die vorliegende Arbeit hat daher zum Ziel, die Interaktion von Temperatur und Energiehaushalt von Regenbogenforellen detailliert zu beschreiben und durch unterschiedliche Futterrationen und Fütterungsmethoden potentielle Mechanismen zu identifizieren, um energetische Verlustprozesse zu minimieren. Letztendlich sollen somit Fütterungsstrategien entwickelt werden, die ein optimales Wachstum von Fischen bei unterschiedlichen Haltungstemperaturen begünstigen.

Diese Arbeit bilanziert Energieaufnahme sowie Energieverluste mittels Respirometrie. Zur Erfassung der metabolischen Verlustprozesse des Energiestoffwechsels wurde ein Gruppenrespirometer nach Stiller et al. (2013) verwendet. Respirometrische Systeme sind darauf ausgelegt, metabolische Verluste mittels des Sauerstoffverbrauchs der Tiere zu errechnen. Drei Experimente wurden im Verlauf dieser Arbeit durchgeführt und stellen die einzelnen Kapitel dar. In allen Versuchen wurden Regenbogenforellen als Modellorganismen für die heimische Aquakultur verwendet.

In einem ersten Versuch (Kapitel I) wurde nachfolgend die Wassertemperatur von ursprünglich 12°C auf 17°C und schlussendlich auf 20°C erhöht. Je respirometrischer Kammer wurden 12 Fische à 183.75 g gehalten und ihre Energieverluste über die Sauerstoffkonzentration im Haltungswasser

ermittelt. Jede einzelne Temperatur wurde für 11 Tage gehalten. Insgesamt wurden drei unterschiedliche Rationen verfüttert (0.65; 0.975; 1.3 %/d*kg). Die Tiere wurden je Temperaturperiode für sechs der 11 Tage gefüttert und anschließend für 100 Stunden genüchtert bis sich der Sauerstoffverbrauch und somit die messbare Stoffwechselrate dem der ungefütterten Fische anglich. Ziel war es, die Temperaturabhängigkeit des Wärmezuwachskoeffizienten zu ermitteln. Dieser Wert beschreibt das Integral des Wärmezuwachses über dem Routinestoffwechsel. Der Versuch konnte die in der Literatur häufig dargestellte Ansicht, der Wärmezuwachskoeffizient wäre temperaturunabhängig, für Regenbogenforellen widerlegen.

Ein zweiter Versuch (Kapitel II) wurde mit einem vergleichbaren Versuchsaufbau durchgeführt. Insgesamt wurden fünf unterschiedliche Temperaturen zur Haltung der Fiche eingesetzt (12; 14; 16; 18; 20°C). Wie bereits im vorangegangen Versuch wurden die Temperaturperioden schrittweise appliziert und für eine Dauer von je 10 Tagen gehalten, wovon fünf als Fütterungs- und fünf Nüchterungstage durchgeführt wurden. Insgesamt wurden die Kammern mit je 10 Forellen à 234.58 g besetzt. Es wurden je Kammer eine von drei unterschiedlichen Experimentaldiäten mit unterschiedlichen Kohlenhydrat-Energie zu Lipid-Energie Verhältnissen gefüttert (1 : 1; 0.6 : 1; 1.4 : 1). Untersucht wurde die Annahme, dass Diäten mit einem hohen Kohlenhydrat-Energie zu Lipid-Energie Verhältnis zu einem ähnlich hohen Energieverlust durch den Wärmezuwachs führen, wie Diäten mit niedrigem Kohlenhydrat-Energie zu Lipid-Energie, wenn die Temperaturen unterhalb des Optimums (15-17°C) liegen. Gleichzeitig wurde angenommen, dass bei Optimaltemperaturen jedoch ein signifikant höherer Wärmezuwachs der Fische bei Diäten mit hohem Kohlenhydrat-Energie zu Lipid-Energie Verhältnis auftrete. Die Ergebnisse des Versuchs zeigten einen Kurvenverlauf des Wärmezuwachses mit einem Hochpunkt nahe der Optimaltemperatur, bei jenen Fischen denen eine Diät mit hohem Kohlenhydrat-Energie zu Lipid-Energie Verhältnis gefüttert wurde. Der Wärmezuwachs war bei optimalen Temperaturen zwischen 16-18°C signifikant höher als in den Gruppen, denen eine Diät mit niedrigem Kohlenhydrat-Energie zu Lipid-Energie Verhältnis gefüttert wurde. Zudem war der relative Protein Umsatz in Energie bei der Diät mit hohem Kohlenhydrat-Energie zu Lipid-Energie bei

Temperaturen unterhalb des Optimums geringer als in der Diät mit niedrigem Kohlenhydrat-Energie Anteil. Hieraus wurde geschlossen, dass Kohlenhydrate bei niedrigeren Temperaturen eine Nährstoffgruppe mit hohem nutritivem Wert sind und somit Potential als günstige und verfügbare Energiequelle für Futtermittel aufweisen.

Im dritten und letzten Versuch dieser Arbeit (Kapitel III) wurde ein hinsichtlich der Haltungstemperatur und der Fütterungsphasen identischer technischer Aufbau wie im zweiten Versuch gewählt. Erneut wurden je 10 Regenbogenforellen mit einem Durchschnittsgewicht von 206.30 g in den respirometrischen Kammern gehalten und untersucht. Es wurden insgesamt drei unterschiedliche Experimentaldiäten mit unterschiedlichen Protein-Energie zu Gesamt-Energie Verhältnissen gefüttert (20.38; 19.08; 18.09 mg/kJ). Zielsetzung des Versuchs war es, durch einen spezialisierten Versuchsaufbau, die optimalen thermalen Bedingungen für den sogenannten Protein-Spar-Effekt zu bestimmen. Hierbei wurde durch den Versuchsaufbau gezielt versucht, zwischen Effekten der Diäten und Effekten der Umgebungstemperatur auf den Proteinstoffwechsel zu differenzieren. Die Ergebnisse waren bei allen Testdiäten nahezu identisch und konnten mit einem hohen Maß an Bestimmtheit zeigen, dass bei Regenbogenforellen ein maximaler Protein-Energie-Spar-Effekt bei 16.9°C induziert werden konnte. Diese Erkenntnis birgt großes Potential für die weiterführende Forschung zum effizienten Einsatz von Protein in Futtermitteln. Zusätzlich bietet es die Möglichkeit, die Proteinverwertung von Regenbogenforellen zu maximieren, wenn die Wassertemperatur im Haltungssystem reguliert werden kann.

Zusammenfassend konnte diese Arbeit zum einen eine starke Interaktion von energiemetabolischen Schlüsselvariablen, im Besonderen dem Wärmezuwachs, mit variierender Haltungstemperatur und Futterration aufgezeigt werden. Durch die gewonnenen Erkenntnisse konnte für Kohlenhydrate ein immenses Potential für ökologisch und ökonomisch nachhaltige Futtermittel für Regenbogenforellen bei niedrigen Haltungstemperaturen erkannt werden. Bei optimalen Haltungstemperaturen konnte hingegen eine effizienteste Proteinnutzung erkannt werden.

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