

Aus dem Institut für Tierernährung und Stoffwechselphysiologie
der Christian-Albrechts-Universität zu Kiel

**„Studies on adequate supply of Methionine + Cystine in diets of
juvenile turbot (*Psetta maxima*)“**

Dissertation

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ESZTER KINGA

&

JÁKOB FRIEDRICH

„Pax optima rerum“

Leitspruch der Christian-Albrechts-Universität zu Kiel

***"Wissenschaftlich ist grenzenlose Kritik und Selbstkritik;
unwissenschaftlich ist die Besorgnis, der Zweifel könne läh-
men"***

Karl Jaspers

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LIST OF ABBREVIATIONS*

*Commonly used abbreviations for nutrients, energy and chemical compounds, statistical parameters, as well as for dietary treatments are not included. Formulas for some definitions are given in the respective chapters.

| | |
|----------|---|
| ANF..... | anti-nutritional factor |
| FCF..... | Fulton's condition factor |
| FGR..... | feed to gain ratio (commonly known as feed conversion ratio – FCR) |
| FM..... | fish meal |
| HSI..... | hepatosomatic index |
| LCC..... | lower critical concentration (of Met+Cys) |
| LW..... | live weight |
| PER..... | protein efficiency ratio |
| RCP..... | retained crude protein [$\text{mg kg}^{-0.8} \text{d}^{-1}$] |
| RE..... | retained energy [$\text{kJ kg}^{-0.8} \text{d}^{-1}$] |
| RFI..... | relative feed intake [% d^{-1} in DM] |
| RG..... | relative growth [% LW d^{-1}] (commonly known as specific growth rate – SGR) |
| RPC..... | rapeseed protein concentrate |
| SI..... | splenic index |
| UCC..... | upper critical concentration (of Met+Cys) |
| WG..... | weight gain [in % or g] |

GENERAL INTRODUCTION

Fish is a high quality protein source and in some parts of the world essential for human nutrition. For more than two decades European decreasing and worldwide stagnating fishery captures increase the relevance of fish produced in aquaculture (FAO 2014; World Bank 2013). This global trend has remained uninterrupted. In 2012, 42.2 % of total fish productions were contributed by farmed food fish, compared with 13.4 % in 1990 worldwide (FAO 2014).

A relevant flatfish species in marine aquaculture is turbot (*Psetta maxima*) (Brown 2002; FAO 2016). In the 1970s farming of turbot started in Scotland, and developed in particular within the last decades in European countries like France, Portugal and most strongly in Spain (FAO 2016). After fluctuations of the production volume of turbot in Europe in the previous years, especially China developed its turbot production significantly (FAO 2014, 2016; Hu et al. 2015; Ma et al. 2013; Li et al. 2011). Globally, in 1993 the amount of farmed turbot was 1725 t per year, 10799 t were produced in 2003 and in 2013 the yearly amount increased to 76997 t (FAO 2016). As a high quality product preferred by consumers a high market value can be considered (FAO 2016; Lee et al., 2003) and its production is the highest among flatfishes (Brown 2002). This suggests that the role of turbot farming makes this species increasingly important.

Thus, providing high quality feeds represent a big challenge in turbot nutrition. Several studies investigated the nutrient requirements for turbot, like for vitamins (Stéphan et al. 1995), minerals (von Danwitz et al. 2016), lipids (Bell et al. 1995; Cho et al. 2005) and crude protein (CP). Specific attention was paid to the latter nutrient (Cho et al. 2005; Lee et al. 2003; Kaushik 1998; Kroeckel et al. 2013, 2015; Li et al. 2011; Ma et al. 2013; Peres and Oliva-Teles 2008). With a determined requirement between 49.4 % and 55.0 % CP in the diet (Cho et al. 2005; Lee et al. 2003; Li et al. 2011) for turbot juveniles, feed sources rich in qualitatively high CP, meeting the essential amino acid (EAA) requirements, are necessary (Fournier et al. 2002; Kaushik 1998; Kaushik and Seiliez 2010; Peres and Oliva-Teles 2008). Fish meal (FM) is a source which meets these demands to a high extent and was therefore used to a large extent in the past decades (Cowey 1975; FAO 1996, 2014). Nevertheless, the low sustainability of FM production in terms of high prices and inferior impacts regarding ecological traits (FAO 2014; Martínez-Llorens et al. 2012; Samuel-Fitwi et al. 2013; World Bank 2013) increased the relevance of alternative protein sources to replace FM. For this purpose proteins from lupin, rapeseed, soy, hermetia, corn and wheat glutens are increasingly used and investigated for turbot (Bonaldo et al. 2011, 2015; Burel et al. 2000; Day and Plascencia González 2000; Fournier et al. 2004; Kroeckel et al. 2012; Nagel et al. 2012b, 2014; Regost 1999; von Danwitz et al. 2016). Especially the economic pressure induced by high feed costs forces the fish feed industry to use higher amounts of protein sources which increase the risks of amino acid (AA) imbalances (Kaushik and Seiliez 2010; Martínez-Llorens et al. 2012).

Due to the lack of precise knowledge about AA requirements in turbot, research has been intensified to allow adequate dietary formulation and to avoid impaired growth performance (Dietz et al. 2012; Fournier et al. 2004; Kroeckel et al. 2015). This is one reason for higher CP concentrations in the feed (Wilson and Halver 1986). Higher CP concentration is not necessarily combined with a higher growth, as it is shown in studies which investigated the relevance of CP concentrations on ammonia excretion, for rainbow trout (Cheng et al. 2003) and red drum (*Sciaenops ocellatus*) (Webb and Gatlin 2003). In both studies higher ammonia excretions at higher CP concentration in the diets were identified, whereas lower CP concentrations led to less strong ammonia excretions. However, Bonaldo et al. (2011) found for turbot higher ammonia excretions for diets high in plant protein but with similar CP concentrations. This is most probably due to the lower protein utilization caused by a more unfavorable AA composition.

A less efficient protein utilization leads to higher feed costs and higher nitrogen excretion, which deteriorates the welfare and performance of turbot (van Bussel et al. 2012), and increases the costs of water purification in recirculating systems (Aubin et al. 2006; Samuel-Fitwi et al. 2013).

On the contrary, reduced CP concentrations in diets may result in deficiencies of specific EAAs, especially when higher proportions of protein sources other than FM are used. Beside Lysine (Lys), especially sulfur containing amino acids, methionine (Met) and the semi-EAA Cyst(e)ine (Cys), might become growth limiting factor in fish nutrition (Kaushik and Seiliez 2010). For instance the performance for fish fed diets with high soy protein inclusions could be improved by Met supplementation, as shown for rainbow trout (*Oncorhynchus mykiss*) (Mambrini et al. 1999) and hybrid Tilapia (*Oreochromis niloticus* × *O. mossambicus*) (Figueiredo-Silva et al. 2015). Similarly, when using poultry by-products with Met and Lys supplementation, the growth performance for rainbow trout is enhanced (Steffens 1994).

The relevance of Met+Cys for protein accretion and growth was demonstrated for several fish species by dose-response trials (Liao et al. 2014; Niu et al. 2013; Tulli et al. 2010; Zhou et al. 2011). In addition, studies detected cataracts in Arctic charr (*Salvelinus alpinus*) (Simmons et al. 1999) or yellowtail (*Seriola quinqueradiata*) (Ruchimat et al. 1997) when fed Met+Cys deficient diets. Met is essential for several biochemical pathways, and is a precursor for S-Adenosylmethionine, which is the most important origin for trans-methylation processes (Kwasek et al. 2014; Regina et al. 1993; Waterland 2006).

The total Lys requirement for turbot was determined by Peres and Oliva-Teles 2008, and the maintenance requirement for Lys, as well as the availability in wheat gluten by Kroeckel et al. (2013, 2015). Whereas only one study determined the requirement for L-Met and the efficiency of the Met-analogue 2-hydroxy-4-(methylthio) butanoic acid (HMTBa) for turbot (Ma et al. 2013).

Therefore, it was one target of the study conducted in chapter 1 to determine the lower critical concentration (LCC) of sulfur containing amino acids (Met+Cys). The LCC is defined as the breakpoint from an increase of performance to the plateau and determines the requirement.

Beside deficiencies, oversupplies with Met+Cys can occur and were found to be relevant. Sauberlich (1961) showed in a study with weanling rats that Met is the most toxic AA and Cys is the seventh when compared to 17 other proteinogenic AAs. A negative impact on growth and health parameters of sulfur containing amino acids were frequently shown for terrestrial animals like young pigs, mice, dogs, rats, humans or ducks (Baker 2006; Edmonds and Baker 1987; Merino et al. 1975; Regina et al. 1993; Rees et al. 2006; Xie et al. 2007). As well, for fish, negative impacts of Met+Cys oversupplies on growth were observed, however, not investigated in detail (Ma et al. 2013; Murthy and Varghese 1998; Zhou et al. 2006, 2011). The reasons of toxic effects of Met+Cys are multifactorial and can be partly due to hepatic coma and metabolic acidosis caused by catabolized Met+Cys, as shown for rats (Wamberg et al. 1987) and dogs (Merino et al. 1975). For aquatic species, like Atlantic salmon (*salmo salar*) or seabass (*Dicentrarchus labrax*), high Met supplementation led to increasing concentrations of intermediate products in Met metabolism, like S-Adenosylhomocysteine and L-Homocysteine, which can impair fish growth performance (Espe et al. 2008; Tulli et al. 2010). Hence, it will be shown in chapter 1 how hepatic S-Adenosylhomocysteine and Met+Cys concentration are related.

Potential toxic effects of Met+Cys in fish diets might occur when high levels of rapeseed proteins (Nagel et al. 2014; Slawski et al. 2011; von Danwitz et al. 2016), wheat gluten and supplementation of free Met+Cys (Fournier et al. 2004; Kroeckel et al. 2015) or feather meals (Kikuchi et al. 1994) are included in the diets.

The emphasis of the present thesis was to elucidate the relevance of Met+Cys oversupplies in diets of juvenile turbot (*P. maxima*). Therefore, the study in chapter 1 determined beside the LCC, the upper critical concentration (UCC) for Met+Cys. Thus, a range for Met+Cys concentration is intended to be defined where maximum performance can be expected.

Whereas the experiment in chapter 2 is reevaluating solely the UCC of chapter 1 within a range of Met+Cys oversupply which can be reached in practical dietary formulations with high inclusion levels rich in Met+Cys (Fournier et al. 2004; Kroeckel et al. 2013, 2015; Kikuchi et al. 2007; Nagel et al. 2012a, 2012b, 2014; Slawski et al. 2011; von Danwitz et al. 2016).

In two experiments presented in chapter 3 (trial 1 and 2) the relevance for Met+Cys oversupply was exemplarily evaluated with rapeseed protein concentrate (RPC). RPC was used in diets for common carp (*Cyprinus carpio*) (Slawski et al. 2010), rainbow trout (Teskeredžić et al. 1995), and turbot (Nagel et al. 2014; Slawski et al. 2011; von Danwitz et al. 2016). Therefore trial 1 investigated the intestinal availability of Met+Cys in RPC in comparison to FM. In

trial 2 the potential toxic effects alone of Met+Cys were evaluated and compared to other depressing anti-nutritional factors from RPC towards FM (Francis et al. 2001).

The current study will provide new information about the range of concentrations (LCC, UCC) within which Met+Cys can be included into diets for turbot. Furthermore, it is the aim to evaluate the protein source RPC regarding optimum sulfur containing amino acid supply.

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CHAPTER 1

DETERMINATION OF THE LOWER AND UPPER CRITICAL CONCENTRATION OF METHIONINE + CYSTINE IN DIETS OF JUVENILE TURBOT (*PSETTA MAXIMA*)

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Abstract

In a 56 days lasting growth trial the lower and the upper critical concentration (LCC, UCC) of sulfur containing amino acids (Methionine, Met; Cystine, Cys) in diets for juvenile turbot (*Psetta maxima*) were determined. Nine different isonitrogenous diets (crude protein (CP) = 55.3 % on dry matter (DM) basis) were fed in threefold repetition to juvenile turbot (mean live weight 25.8 g ± 3.0 s.d.) once daily until apparent satiation. Met+Cys concentrations of diet 1 to 7 ranged between 0.8 % and 2.0 % in DM, realized by 0.2 % increments of L-Met supply. Cys concentration were steady at 0.3 % of DM. Diets nos. 8 and 9 contained 4.0 and 6.0 % Met+Cys, respectively to cover the range of strong oversupply. A segmental linear regression model was applied to determine the LCC and UCC of Met+Cys for relative growth (RG), relative feed intake (RFI), feed to gain ratio (FGR), protein efficiency ratio, metabolic retained CP and metabolic retained energy. The LCC for these parameters, except FGR, lies within a small range of 1.23 – 1.28 % in diet DM (2.22 – 2.31 % in CP). Whereas the range of the UCCs is larger (2.10 – 3.82 % Met+Cys in diet DM; 3.80 – 6.91 % in CP). The LCC derived for RG is 1.25 % (± 0.02 s.e.) in diet DM (2.26 % in CP) and for RFI 1.24 % (± 0.03 s.e.) Met+Cys in diet DM (2.24 % in CP). The UCC for RG is 2.68 % (± 0.21 s.e.) (4.85 % in CP) and 2.10 % (± 0.41 s.e.) Met+Cys in diet DM (3.80 % in CP) for RFI. The chemical body composition is significantly influenced by the Met+Cys concentration, especially at deficiencies. The apparent digestibility (organic matter, DM, CP, crude ash and energy) for 3 diets (nos. 3, 5, 7) did not show significant differences, neither among the diets nor between the feces collection methods (stripping vs. dissection). At a strong oversupply of Met+Cys the concentration of hepatic S-Adenosylhomocysteine is negatively correlated to growth performance and can be used as one indicator of Met+Cys toxicity. Based on these values the amount of necessary Met supplementation in deficient diets or maximum inclusion rates of protein sources rich in Met+Cys can be derived for turbot.

1. Introduction

The European as well as the worldwide aquaculture production of turbot (*Psetta maxima*) becomes increasingly important due to its high commercial value. Albeit there were fluctuations in the yearly production volume within the last decade, a significant increase could be observed (FAO 2014). Hence, the need for scientifically based recommendations for energy and nutrient supply to optimize diet composition and to use the expensive feed resources more efficiently increases.

Several studies regarding the nutrient requirements of turbot were conducted (Bell et al. 1995; Lee et al. 2003; Li et al. 2011a; Regost et al. 2003). Specific attention was paid on crude protein (CP) requirement of fish (Wilson and Halver 1986). Especially turbot require high amounts of CP between 49.4 % and 55.0 % CP (Cho et al. 2005; Lee et al. 2003; Li et al. 2011a).

Few information is available for the optimum dietary amino acid pattern. Until now, it is derived from the whole body protein essential amino acid (EAA) pattern (Kaushik 1998). Due to the lack of precise knowledge about the amino acid requirement in turbot the allowances of EAA in practical dietary formulations of turbot are relatively high to avoid impaired growth performance. This could also be one reason for a high estimated quantitative protein requirement (Wilson and Halver 1986) and to a less efficient protein utilization. This in turn has a strong impact on feed costs and also led to higher nitrogen emissions in production water which could deteriorate the fish welfare as well as the sustainability of turbot production (Aubin et al. 2006). To date, the requirements and utilization efficiencies of lysine (Kroeckel et al. 2013, 2015; Peres and Oliva-Teles 2008) and arginine (Fournier et al. 2002, 2003) were experimentally determined for juvenile turbot.

However, the requirement of the sulfur containing EAA Methionine (Met) and the semi-EAA Cyst(e)ine (Cys) is less precisely known for turbot. Nevertheless, Ma et al. (2013) investigated the L-Met requirement in comparison to the Met analogue 2-hydroxy-4-methylthio butanoic acid (HMTBa).

Depending on the finfish species (hybrid striped bass (*Morone saxatilis* × *M. chrysops*); channel catfish (*Ictalurus punctatus*); rainbow trout (*Oncorhynchus mykiss*); red drum (*Sciaenops ocellatus*)), Cys can replace Met up to 40 – 60% (Griffin et al. 1994; Harding et al. 1977; Kim et al. 1992; Moon and Gatlin 1991). Therefore, Cys reduces the required amount of Met for maximum growth and Met as Cys is present in all natural protein sources. Hence, the recommendations for supply should be given as the sum of Met+Cys (Wilson and Halver 1986). Due to the mentioned limited replacement rate of Met by Cys their proportion should be additionally given.

The increased use of protein sources other than fish meal can elevate the risk of unbalanced Met+Cys supply. High inclusion levels of legume proteins (e.g.: soy, pea, faba bean) can lead

to Met+Cys deficiencies. For instance Figueiredo-Silva et al. (2015) showed in diets with high inclusion levels of soy in hybrid Tilapia (*Oreochromis niloticus* × *O. mossambicus*) an improved growth performance due to the supplementation of Met. Øverland et al. (2009) detected a lower digestibility of Cys in soy compared to fishmeal and pea protein in Atlantic salmon (*Salmo salar*). This also reduces the available amount of Met+Cys and increases the requirement of total Met+Cys.

Met is predominantly needed for protein synthesis (Niu et al. 2013; Tulli et al. 2010). Beside the need for Met as an essential component of body protein, Met is required for maintenance and health. Some studies detected cataracts due to Met deficiencies in Arctic charr (*Salvelinus alpinus*) (Simmons et al. 1999) or yellowtail (*Seriola quinqueradiata*) (Ruchimat et al. 1997). Met is also essential for several biochemical pathways, such as a precursor for S-Adenosylmethionine (SAM), which is the most important source of trans-methylation processes (Kwasek et al. 2014; Regina et al. 1993; Waterland 2006). On the other hand several substances like Choline or Taurine have Met+Cys sparing effects, (Baker 2006; Duan et al. 2012; Wang et al. 2014).

An oversupply of Met+Cys can occur due to high inclusion levels of the protein sources corn gluten, feather meal or rapeseed protein products (Li et al. 2011b; Nagel et al. 2012), which are all rich in sulfur containing amino acids. A distinct oversupply of Met+Cys can lead to toxic effects, like metabolic acidosis in weanling rats (Wamberg et al. 1987) or a hepatic coma in dogs (Merino et al. 1975). Several dose–response studies of Met in finfish (turbot; Indian major carp (*Labeo rohita*); Cobia (*Rachycentron canadum*); black sea bream (*Sparus macrocephalus*)) indicated a decreasing growth performance due to Met oversupply (Ma et al. 2013; Murthy and Varghese 1998; Zhou et al. 2006, 2011). Hu et al. (2015) recommended a HMTBa level below 5 % in diets for turbot. But in none of these studies a specific upper critical concentration (UCC) of Met+Cys was determined.

Therefore it was the purpose of the current study to determine the lower critical concentration (LCC) and the UCC of Met+Cys for juvenile turbot.

2. Materials and methods

2.1. Experimental diets

All nine experimental diets consisted of the same basal ingredients, except additional L-Met and a mixture of non-essential amino acids (NEAA) (Table 1.1). Based on the Met+Cys deficient basal diet (no.1), all diets were within a range of 5.2 % (on dry matter (DM) basis), with varying L-Met and NEAA (50 % L-Glutamic acid, 30 % Glycine, 20 % L-Alanine) concentration (Tables 1.1 and 1.2). Met+Cys concentration of seven diets ranged between 0.8 % and 2.0 % (DM), realized by addition of 0.2 % increment steps of L-Met to the basal diet, respectively. For the remaining two diets increment steps of 2.0 % L-Met were made, to cover the range of strong oversupply. Due to the same amounts of protein sources in all dietary mixtures, the

Cys concentration remained at a constant level of 0.3 % in DM. To keep all diets iso-nitrogenous (CP = 55.3 % DM), diets were balanced with the appropriate amount of NEAA (Tables 1.1 and 1.2). All diets were formulated to be also, isolipidic and isoenergetic (crude lipid = 15.3 % DM; gross energy = 21.8 MJ/kg DM) (Table 1.1). A mixture of 10 % of EAA (free of Met+Cys) was included in every diet to ensure the sufficient supply of turbot with all EAA (Kaushik 1998; Peres and Oliva-Teles 2008), except Met+Cys (Table 1.1). This helped to boost the Met+Cys deficiency. The amino acid composition of all diets is given in Table 1.2. The diets were manufactured by a stepwise addition of all ingredients, an intense mixing and pelletized by a 4 mm matrix (*L 14-175, AMANDUS KAHL, Reinbeck, Germany*). During the pelletizing process, the temperature never exceeded 50°C.

2.2. Experimental setup

The trials were conducted at the *Gesellschaft für Marine Aquakultur mbH (GMA Büsum, Germany)*, with juvenile turbot provided by *Maximus A/S (Gudnaesstrandvej 17, 7755 Bedsted Thy, Denmark)*.

The fishes were stocked in rectangular rearing tanks with a water volume of 60 L and a bottom surface of 0.17m², respectively. The trial took place in a small recirculating aquaculture system (RAS) with a mechanical cleaning filter (hamburger mats), a nitrifying moving-bed biofilter, a protein-skimmer with moderate ozonation (*Ozone 200, 200mg O₃/h, Aqua Medic, Bissendorf Germany*) and a UV light unit. The photoperiod was adjusted to a light:dark cycle of 12:12 h. During the experimental period, water quality parameters were analyzed once a day. Water temperature was maintained at 17.9 ± 0.4°C and salinity level at 18.8 ± 0.4 ppt (*HI 96822 Seawater Refractometer, Hanna Instruments Inc., Woonsocket-RI-USA*) (Imslund et al. 2001). The mean oxygen level was 8.1 ± 0.3 mg L⁻¹ with a saturation of 95.8 ± 3.5 % (*Handy Polaris, Oxy-Guard International A/S, Birkerød, DK*). Average pH was 7.4 ± 0.2 (*GMH 3530, Digital pH-/ mV-/ Thermometer, Greisinger electronic, D*), total NH₄-N and NO₂-N levels were 0.2 ± 0.1 mg L⁻¹ and 0.1 ± 0.04 mg L⁻¹ (*Microquant test kits for NH₄⁺ and NO₂⁻, Merck KGaA, Darmstadt, Germany*), respectively.

Twenty-seven rectangular rearing tanks were stocked with 12 juvenile turbot each. The adaption period lasted 14 days and diet no. 6 (Tables 1.1 and 1.2) was fed to all experimental groups at a feeding level of 0.9% of initial live weight (LW) per day.

At the start of the growth trial (day 0), individual mean LW was 25.8 g (± 3.0 s.d.), resulting in an average tank iLW of 310.0 g (± 3.0 s.d.). The turbot were fed once a day until apparent satiation for 56 days. Pellets which still remained in the tank 15 min after feeding were siphoned and counted. Correct feed intakes were determined by taking the average dry pellet weight. Every diet was fed to three random tanks to achieve a triplicate repetition for statistical analysis.

Consecutively, a digestibility trial for the diets no. 3, 5 and 7 were conducted where diets were also fed until apparent satiation. Due to the recommendation of Vandenberg and De La Noüe (2001), titanium dioxide (TiO₂) was used as an inert marker to determine the apparent digestibility coefficients (ADC) (Table 1.1).

2.3. Sampling

At the beginning of the growth trial 2 * 6 turbot with a similar weight were randomly selected, chopped and deep-frozen to be analyzed for initial total body composition (day 0). Prior to the final sampling after 56 feeding days, fishes were starved for 48 h to ensure a depleted gut.

For every tank the total fish weight, the individual final live weight (fLW) and length were determined, to calculate the Fulton's condition factor (FCF).

To determine the proximate total body composition 3 randomly selected fishes per tank were chopped, pooled and stored (-18°C) in a plastic bag until analyses. Blood samples were taken from 3 fishes with heparinized syringes (*Injekt 5 mL, Sterican 21G * 1.5, Braun, Melsungen, Germany*). Blood pH was determined individually directly during sampling via a pH probe (*Ionode IJ44, TPS Pty Ltd., Brisbane, Australia*), which remained in the blood sample until the pH has stabilized.

To determine hepatosomatic index individual liver weight was measured from 4 additional fishes per tank. Hepatic tissue were pooled per tank and put into 40mL water/methanol (H₂O (bi-dest.):CH₃OH; 1:1) solution until subsequent analysis for S-Adenosylhomocysteine (SAH).

For feces collection two different methods were used, consecutively. Final guts of the fishes were stripped by an abdominal massage for a period of 10 days every second day and 7 h after feeding. All samples of one replicate were pooled in one plastic beaker and were deep-frozen immediately at - 18°C. After a recreation period of 14 days all fishes were killed 7 h after feeding. The gut contents were sensitively dissected from the final gut, as described by Dietz et al. (2012, 2013).

For both feces collection methods apparent digestibility coefficient (ADC) was determined for dry matter (DM), organic matter (OM), crude ash (CA), crude protein (CP) and energy.

2.4. Chemical analyses

Prior to analyses the fish and feces were freeze-dried. Subsequently, the whole samples were minced ($\leq 1\text{mm}$) to be homogenized by a centrifugal mill (*ZM 100, Retsch GmbH & Co. KG, Haan, Germany*). Pelletized feeds and feces were pulverized by a mortar and a pestle.

DM, CA, CP, crude lipids (CL) and the energy content for all diets as well as for the chemical body compositions of the fish were analyzed in duplicates according to Weender Analysis

(European Union, 2009). CP, DM, CA and total energy content were analyzed to determine ADCs.

Table 1.1: Dietary formulations and proximate nutrient compositions.

| diet no. | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
|--|------------|------------|------------|------------|------------|------------|------------|------------|------------|
| Met+Cys [g 100 g ⁻¹ in diet DM] | 0.8 | 1.0 | 1.2 | 1.4 | 1.6 | 1.8 | 2.0 | 4.0 | 6.0 |
| Ingredients [g 100 g ⁻¹] | | | | | | | | | |
| fish meal (<i>Clupea harengus</i>) ^a | 14.0 | 14.0 | 14.0 | 14.0 | 14.0 | 14.0 | 14.0 | 14.0 | 14.0 |
| pea protein ^b | 12.8 | 12.8 | 12.8 | 12.8 | 12.8 | 12.8 | 12.8 | 12.8 | 12.8 |
| porc protein hydrolysate (Prolimat P) ^c | 10.0 | 10.0 | 10.0 | 10.0 | 10.0 | 10.0 | 10.0 | 10.0 | 10.0 |
| hemoglobin powder (porc) ^d | 8.5 | 8.5 | 8.5 | 8.5 | 8.5 | 8.5 | 8.5 | 8.5 | 8.5 |
| wheat gluten ^e | 4.0 | 4.0 | 4.0 | 4.0 | 4.0 | 4.0 | 4.0 | 4.0 | 4.0 |
| fish oil ^a | 12.0 | 12.0 | 12.0 | 12.0 | 12.0 | 12.0 | 12.0 | 12.0 | 12.0 |
| wheat starch ^e | 14.0 | 14.0 | 14.0 | 14.0 | 14.0 | 14.0 | 14.0 | 14.0 | 14.0 |
| Sodium-Carboxymethylcellulose ^f | 3.5 | 3.5 | 3.5 | 3.5 | 3.5 | 3.5 | 3.5 | 3.5 | 3.5 |
| Dicalciumphosphorous ^g | 4.0 | 4.0 | 4.0 | 4.0 | 4.0 | 4.0 | 4.0 | 4.0 | 4.0 |
| Marker (TiO ₂) ^h | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 |
| Vitamine/mineral premixture ⁱ | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 |
| essential amino acid (EAA) mixture ^{j*} | 10.0 | 10.0 | 10.0 | 10.0 | 10.0 | 10.0 | 10.0 | 10.0 | 10.0 |
| L-Methionine ^k non-essential amino acid (NEAA) mixture ^{l**} | 0.0 5.2 | 0.2 5.0 | 0.4 4.8 | 0.6 4.6 | 0.8 4.4 | 1.0 4.2 | 1.2 4.0 | 3.2 2.0 | 5.2 0.0 |
| Nutrients [g 100g ⁻¹ in diet DM] | | | | | | | | | |
| Moisture | 5.7 | 5.6 | 5.5 | 5.7 | 5.6 | 5.7 | 5.6 | 5.7 | 5.9 |
| Crude protein | 55.5 | 55.6 | 55.8 | 55.4 | 55.5 | 55.0 | 55.3 | 55.2 | 54.3 |
| Crude lipid | 15.5 | 15.3 | 15.6 | 15.3 | 15.3 | 15.7 | 14.8 | 15.0 | 15.2 |
| Crude fiber + NfE *** | 18.7 | 18.7 | 18.3 | 19.0 | 18.9 | 19.0 | 19.6 | 19.4 | 20.1 |
| Crude Ash | 10.3 | 10.5 | 10.3 | 10.3 | 10.3 | 10.3 | 10.3 | 10.4 | 10.4 |
| Gross Energy [kJ g ⁻¹ in DM] | 21.6 | 21.6 | 21.6 | 21.7 | 21.7 | 21.8 | 21.8 | 22.0 | 22.2 |
| Calcium | 2.1 | 2.2 | 2.1 | 2.2 | 2.1 | 2.1 | 2.1 | 2.1 | 2.2 |
| Phosphorus | 1.5 | 1.5 | 1.5 | 1.5 | 1.4 | 1.5 | 1.5 | 1.4 | 1.5 |
| Choline [mg kg ⁻¹ in DM] | 1314 | - | - | - | - | - | - | - | - |
| Taurine | 0.09 | - | - | - | - | - | - | - | - |

^a Vereinigte Fischmehlwerke Cuxhaven GmbH & Co KG, Cuxhaven, Germany; ^b Emsland-Stärke GmbH, Emlichheim, Germany; ^c Knochen- und Fett Union, Marl, Germany; ^d Günther Badenhop Fleischwerke KG, Verden-Aller, Germany; ^e Kröner Stärke GmbH, Ibbenbüren, Germany; ^f Enorica GmbH, Norderstedt, Germany; ^g Lehmann & Voss & Co. KG, Hamburg, Germany; ^h Kronos International Inc., Leverkusen, Germany; ⁱ Vitamin and Mineral mixture 517158 and 508240, Vitfoss, Grasten, Denmark; ^j Carl Roth GmbH & Co. KG, Karlsruhe, Germany; ^k VWR International GmbH, Darmstadt, Germany; * EAA mix. = 21.35g 100g⁻¹ L-Lysine (HCl) + 16.9g 100g⁻¹ L-Arginine + 13.75g 100g⁻¹ L-Isoleucine + 11.0g 100g⁻¹ L-Leucine + 9.9g 100g⁻¹ L-Threonine + 8.4g 100g⁻¹ L-Tyrosine + 7.1 g 100g⁻¹ L-Phenylalanine + 6.4g 100g⁻¹ L-Valine + 3.1g 100g⁻¹ L-Tryptophane + 2.1 g 100g⁻¹ L-Histidine; ** NEAA mix. = 50g 100g⁻¹ L-Glutamic acid + 30g 100g⁻¹ Glycine + 20g 100g⁻¹ L-Alanine; *** CF + NfE = crude fiber + nitrogen free extracts = 100-(crude protein + crude lipid + crude ash)

Table 1.2: Proximate amino acid composition of experimental diets.

| diet no. | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
|--|-------|-------|-------|-------|-------|-------|-------|-------|-------|
| Met+Cys [g 100 g ⁻¹ in diet DM] | 0.8 | 1.0 | 1.2 | 1.4 | 1.6 | 1.8 | 2.0 | 4.0 | 6.0 |
| EAA (semi-EAA)* [g 16 g N ⁻¹] | | | | | | | | | |
| Lysine | 8.40 | 8.33 | 8.35 | 8.50 | 8.60 | 8.70 | 8.61 | 8.66 | 8.83 |
| Arginine | 7.20 | 7.19 | 7.21 | 7.37 | 7.31 | 7.51 | 7.28 | 7.35 | 7.39 |
| Methionine | 0.84 | 1.14 | 1.52 | 1.72 | 2.16 | 2.51 | 2.82 | 6.28 | 10.14 |
| (Cyst(e)ine) | 0.55 | 0.63 | 0.57 | 0.61 | 0.65 | 0.64 | 0.65 | 0.63 | 0.69 |
| Threonine | 4.16 | 4.16 | 4.16 | 4.15 | 4.10 | 4.32 | 4.22 | 4.40 | 4.42 |
| Valine | 4.53 | 4.52 | 4.50 | 4.48 | 4.50 | 4.59 | 4.48 | 4.55 | 5.00 |
| Isoleucine | 4.70 | 4.71 | 4.74 | 4.78 | 4.79 | 4.92 | 4.79 | 4.78 | 4.89 |
| Leucine | 7.85 | 7.76 | 7.80 | 7.81 | 7.76 | 7.95 | 7.72 | 7.78 | 8.02 |
| Phenylalanine | 4.76 | 4.79 | 4.50 | 4.84 | 4.81 | 4.80 | 4.87 | 4.86 | 4.89 |
| (Tyrosine) | 3.38 | 3.03 | 3.11 | 3.04 | 3.19 | 3.03 | 3.10 | 3.03 | 3.08 |
| Histidine | 2.65 | 2.61 | 2.58 | 2.62 | 2.65 | 2.74 | 2.66 | 2.65 | 2.69 |
| Tryptophan | 1.05 | 1.01 | 1.01 | 0.98 | 1.07 | 1.04 | 1.05 | 1.06 | 1.10 |
| NEAA** | | | | | | | | | |
| Aspartic acid | 6.34 | 6.35 | 6.38 | 6.39 | 6.39 | 6.52 | 6.36 | 6.57 | 6.81 |
| Serine | 3.09 | 3.03 | 3.07 | 2.99 | 2.98 | 3.01 | 2.99 | 3.11 | 3.17 |
| Glutamic acid | 15.72 | 15.39 | 15.18 | 14.97 | 14.82 | 14.88 | 14.24 | 12.64 | 11.06 |
| Proline | 4.45 | 4.48 | 4.42 | 4.34 | 4.29 | 4.59 | 4.58 | 4.42 | 4.64 |
| Glycine | 8.46 | 8.35 | 8.25 | 8.23 | 8.11 | 8.24 | 7.95 | 7.07 | 5.87 |
| Alanine | 6.40 | 6.35 | 6.36 | 6.33 | 6.14 | 6.16 | 5.94 | 5.30 | 4.69 |

*EAA = essential amino acids; **NEAA = non essential amino acids

DM content was determined by drying the samples for 4 h at 103°C. The CA content was analyzed by remaining the sample for 4 h in a combustion oven at 550°C. CP (N * 6.25) was determined by the Kjeldahl method and the CL was analyzed by with a Soxhlet extractor, without hydrolysis. The residual fractions in the diets were determined by summarizing the content of crude fiber (CF) and nitrogen free extracts (NFE) by calculation.

The energy was measured by an adiabatic bomb calorimeter (*C200, IKA-Werke GmbH & Co. KG, Staufen, Germany*).

To determine TiO₂ concentrations in feed and in feces, each sample was solubilized by 4 h Kjeldahl procedure using 0.96 g * g⁻¹ sulfuric acid. Afterwards 0.35 g * g⁻¹ hydrogen peroxide was laced to the filtered TiO₂ solution to form a yellow complex. Its intensity was measured by extinctions of a spectrophotometer (*visible spectrophotometer 6300, Barloworld Scientific Ltd. T/AS Jenway, Dunmow, Essex, U.K.*) at 405 nm. The extinction values were recorded and corrected by a standard to calculate the TiO₂ concentration.

The AA composition (Table 1.2) of the diets were analyzed by *LUFA-ITL GmbH (Kiel, Germany)* according to the method *VO(EG) 152/2009, L54/23-32*. Tryptophan was analyzed by the method *EU 152/2009 (A)(SP)*. Calcium and Phosphorus were determined via *VDLUFA*

VII 2.2.2.6. Choline (*mikrob. Akt., QMP_KL_51_27*) and Taurine (*VO(EG) 152/2009, III, F*) were analyzed for diet no. 1 (Table 1.1).

The determination of the SAH concentration in fish liver was analyzed via high performance liquid chromatography (*HPLC/MS*) by *Labor Dr. Helle für Lebens- und Arzneimittelforschung GmbH & Co. KG, Bremerhaven, Germany*.

2.5. Definitions, calculations and statistical analyses

The following parameters were defined as follows:

Relative growth (RG) = $(\ln(\text{final live weight (g)}) - \ln(\text{initial live weight (g)})) * 100 / \text{feeding days}$

Relative feed intake (RFI) = $\text{feed intake (DM) in \% of average live weight} / \text{feeding days}$

Feed to Gain Ratio (FGR) = $\text{g feed intake (DM)} / \text{g live weight gain}$

Protein efficiency ratio (PER) = $\text{g live weight gain} / \text{g crude protein intake}$

Geometric mean live weight (GMLW) in g = $(\text{initial live weight} * \text{final live weight})^{0.5}$

Metabolic live weight (MLW) in kg = $(\text{GMLW}/1000)^{0.8}$

Retained crude protein (RCP) in $\text{mg kg}^{-0.8} \text{ d}^{-1}$ = $(\text{mg retained crude protein} / \text{mGMLW}) / \text{feeding days}$

Retained energy (RE) in $\text{kJ kg}^{-0.8} \text{ d}^{-1}$ = $(\text{kJ retained energy} / \text{mGMLW}) / \text{feeding days}$

According to the statements of Dietz et al. (2012) and the studies of Lupatsch et al. (2003), where several fish species were compared, the metabolic weight exponent of 0.8 was used.

Fulton's condition factor (FCF) = $(\text{live weight in g} / (\text{total length in cm})^3) * 100$

Hepatosomatic index (HSI) = $(\text{g liver weight} / \text{g live weight}) * 100$

ADC of nutrients, energy, DM or OM (x) of diets in % = $100 * (1 - (\% \text{ feed TiO}_2 / \% \text{ fecal TiO}_2) * (\% \text{ fecal}_x / \% \text{ feed}_x))$, where the subscript x stands for a nutrient, energy, DM or OM, respectively.

The statistical analyses were realized by the software R (R Core team 2014). To determine the LCC and the UCC for RG, RFI, FGR, PER, RCP and RE, a segmental linear regression analysis was used. For the variables iLW and final individual LW, FCF, HSI and blood pH (several values per tank) the data evaluation started with the definition of an appropriate statistical mixed model (Laird and Ware 1982; Verbeke and Molenberghs 2000). This model included all treatments (diet nos. 1 – 9) as fixed factor. Tank was regarded as random factor. For the variable SAH, all variables for total body composition, and all ADC (one value per tank) a simple linear model was used, including the treatments as fixed factor. The data were assumed to be normally distributed and to be homoscedastic, based on a graphical residual analysis. Analyses of variances (ANOVA) and multiple contrast tests (e.g.: Bretz et al. 2011) were conducted in order to compare the treatment effects. Effects of sampling method or diet on the ADCs were conducted via 2-way ANOVA. All other parameters were analyzed via 1-way ANOVA. $P \leq 0.05$ was defined as the level of significance.

The LCCs and the UCCs, plateau and slopes were classified by \pm standard error (s.e.). All remaining data are presented as arithmetic mean values \pm standard deviation (s.d.).

3. Results

No fish died or was removed during the experiment.

3.1. Growth performance, feed utilization and physiological parameters

All performance parameters (Relative growth – RG, Relative Feed Intake – RFI, Feed to Gain Ratio – FGR, Protein Efficiency Ratio – PER, Retained Crude Protein – RCP and Retained Energy - RE) are given as arithmetic mean values (\pm s.d.) in Table 1.3.1. In Table 1.3.2 the LCC and UCCs of Met+Cys in % diet DM and in % of CP, as well as the regression coefficients and the plateau are presented. LCC and UCCs, regression coefficients, as well as plateaus for RG, RFI, FGR, PER, RCP and RE are significant ($P < 1 \cdot 10^{-4}$). The smallest range among the LCC and the UCCs was found for RFI (1.24 ± 0.03 s.e. – 2.10 ± 0.41 s.e. Met+Cys in diet DM), whereas the highest range for FGR (1.04 ± 0.04 – 3.82 ± 0.18 s.e. Met+Cys in diet DM). The values for the LCC of all evaluated parameters, except for FGR, lay in a small range of 1.23 – 1.28 Met+Cys % in diet DM (2.22 – 2.31 Met+Cys % in CP DM), whereas UCCs show larger variations (2.10 – 3.82 Met+Cys % in diet DM; 3.62 – 6.91 Met+Cys % in CP).

The impact of the Met+Cys concentration (% diet DM) on RG is exemplarily illustrated in Figure 1.1. The breakpoints reflect the LCC with 1.25 % (± 0.02 s.e) Met+Cys (2.26 % in CP) and the UCC at 2.68 % (± 0.21 s.e) Met+Cys (4.85 % in CP).

Initial and final individual LW, Fulton's Condition Factor (FCF), hepatosomatic index (HSI), as well blood pH are given in Table 1.4. A Met+Cys level < 1.2 % in diet DM led to a significantly lower final individual LW and concentrations of 4.0 and 6.0 % (nos. 8 and 9) to significantly decreases compared to the concentrations of 1.2 – 2.0 % in diet DM. Similar findings were received for the FCF. Shortages and oversupplies of Met+Cys are leading to lower HSI. Especially a strong oversupply (diet no. 9) reduces the HSI most, in tendency stronger than by a shortage of Met+Cys. Blood pH was not affected by Met+Cys concentrations, but a slight decrease with increasing Met+Cys concentration could be observed.

A strong oversupply with Met+Cys (diet no. 9) led to a significant increase of the S-Adenosylhomocysteine (SAH) concentration in the liver (Figure 1.2). A slight negative correlation between growth performance and SAH concentration could be observed within the range of undersupply up to a moderate oversupply (diet nos. 1 – 7) (Figure 1.2; Table 1.3.1).

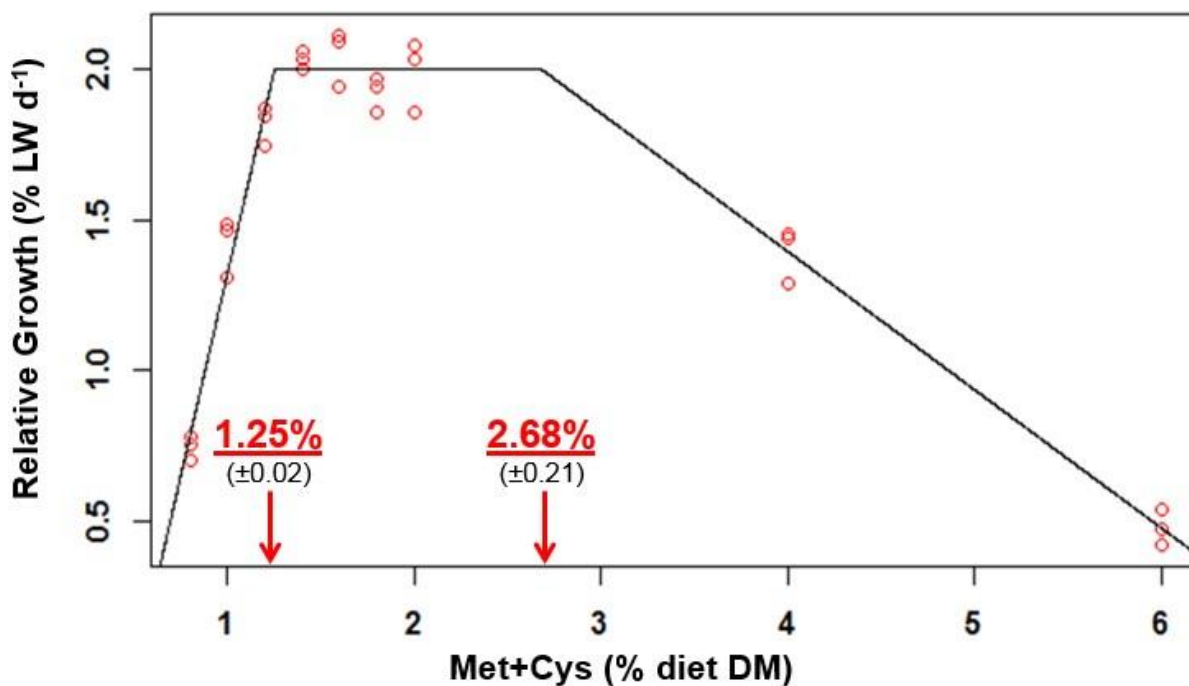


Figure 1.1: The relationship between the Met+Cys concentration in the diets and the relative growth [RG, % LW d⁻¹] of juvenile turbot (*Psetta maxima*), fed with 9 different diets for 56 days.

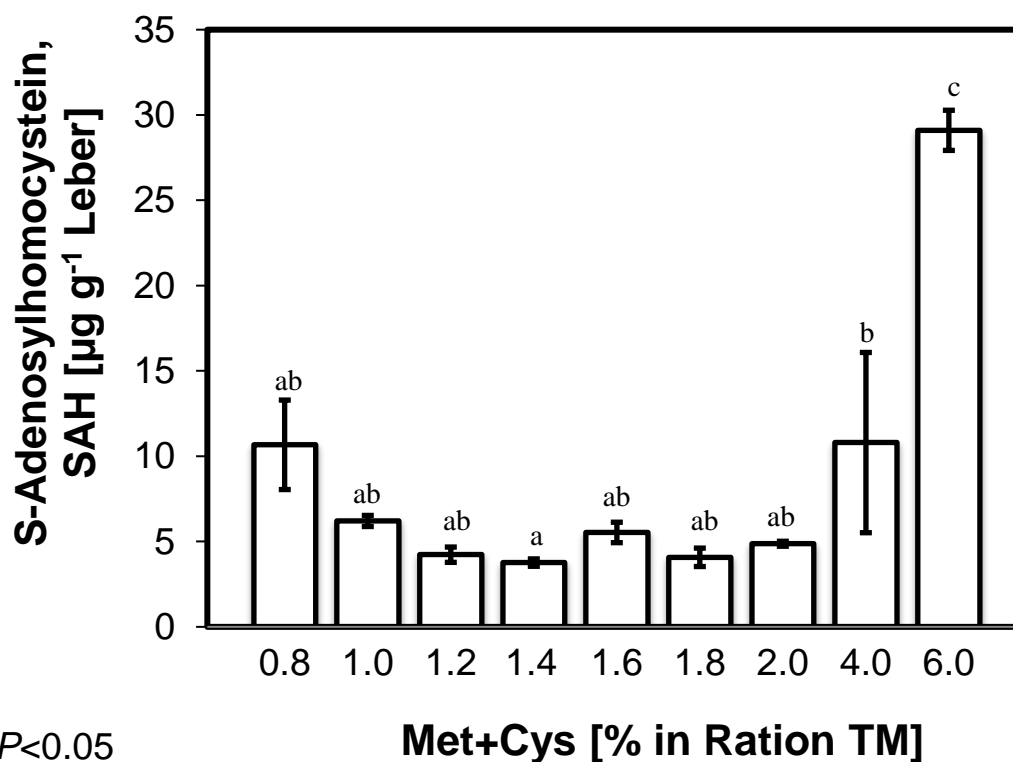


Figure 1.2: The concentration of hepatic S-Adenosylhomocysteine (SAH), in relation to Met+Cys concentration in diets of juvenile turbot (*Psetta maxima*), different letters represent significant differences (Tukey test, $P \leq 0.05$) with pooled samples from $n = 3$ tanks.

Table 1.3.1: Performance parameters of juvenile turbot fed varying Met+Cys concentrations [g 100 g⁻¹ in diet DM] (arithmetic mean ± standard deviation).

| diet no. | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
|------------------|---------------|----------------|---------------|---------------|----------------|---------------|----------------|---------------|--------------|
| Met+Cys | 0.8 | 1.0 | 1.2 | 1.4 | 1.6 | 1.8 | 2.0 | 4.0 | 6.0 |
| RG ¹ | 0.75 ± 0.03 | 1.42 ± 0.08 | 1.82 ± 0.05 | 2.03 ± 0.02 | 2.05 ± 0.08 | 1.92 ± 0.05 | 1.99 ± 0.10 | 1.39 ± 0.07 | 0.48 ± 0.05 |
| RFI ² | 1.12 ± 0.01 | 1.40 ± 0.02 | 1.71 ± 0.05 | 1.72 ± 0.03 | 1.80 ± 0.01 | 1.78 ± 0.03 | 1.75 ± 0.02 | 1.39 ± 0.11 | 0.99 ± 0.08 |
| FGR ³ | 1.51 ± 0.06 | 0.99 ± 0.07 | 0.94 ± 0.02 | 0.85 ± 0.02 | 0.88 ± 0.04 | 0.93 ± 0.01 | 0.89 ± 0.05 | 1.00 ± 0.06 | 2.10 ± 0.36 |
| PER ⁴ | 1.20 ± 0.04 | 1.83 ± 0.12 | 1.91 ± 0.04 | 2.13 ± 0.06 | 2.05 ± 0.09 | 1.96 ± 0.03 | 2.05 ± 0.11 | 1.83 ± 0.11 | 0.91 ± 0.17 |
| RCP ⁵ | 66.17 ± 11.11 | 140.32 ± 10.25 | 208.36 ± 5.92 | 237.02 ± 4.81 | 255.82 ± 15.53 | 223.96 ± 2.24 | 233.51 ± 10.01 | 160.11 ± 9.94 | 54.94 ± 5.35 |
| RE ⁶ | 2.33 ± 0.32 | 5.25 ± 0.43 | 7.48 ± 0.55 | 8.77 ± 0.12 | 9.03 ± 0.50 | 7.94 ± 0.31 | 8.82 ± 0.49 | 5.45 ± 0.65 | 1.39 ± 0.18 |

¹ RG = Relative growth in % LW d⁻¹ = $(\ln(\text{final live weight (g)}) - \ln(\text{initial live weight (g)})) * 100 / \text{feeding days}$; ² RFI = Relative feed intake = feed intake in % of live weight d⁻¹; ³ Feed to Gain

Ratio (FGR) = g feed intake (DM) / g live weight gain; ⁴ PER = Protein efficiency ratio = g live weight gain / g crude protein intake; ⁵ RCP = Recovered crude protein per fish [mg kg^{0.8} d⁻¹]; ⁶ RE = Recovered energy per fish [(kJ kg^{0.8} d⁻¹)]

Table 1.3.2: Lower and upper critical concentrations of Met+Cys (in diet DM and protein), Regression coefficients (Slope 1, Slope 2) and the plateau for growth performance parameters (\pm standard error).

| | Lower critical concentration of Met+Cys in diet DM [%] | upper critical concentration of Met+Cys in diet DM [%] | Lower critical concentration of Met+Cys in CP [%] | upper critical concentration of Met+Cys in CP [%] | Regression coefficient 1 (Slope 1) | Plateau | Regression coefficient 2 (Slope 2) |
|------------------|--|--|---|---|------------------------------------|-------------------|------------------------------------|
| RG ¹ | 1.25 \pm 0.02 | 2.68 \pm 0.21 | 2.26 \pm 0.04 | 4.85 \pm 0.38 | 2.67 \pm 0.18 | 2.00 \pm 0.03 | -0.46 \pm 0.04 |
| RFI ² | 1.24 \pm 0.03 | 2.10 \pm 0.41 | 2.24 \pm 0.05 | 3.80 \pm 0.74 | 1.47 \pm 0.13 | 1.76 \pm 0.02 | -0.20 \pm 0.02 |
| FGR ³ | 1.04 \pm 0.04 | 3.82 \pm 0.18 | 1.88 \pm 0.07 | 6.91 \pm 0.33 | -2.59 \pm 0.59 | 0.90 \pm 0.04 | 0.55 \pm 0.59 |
| PER ⁴ | 1.23 \pm 0.05 | 3.51 \pm 0.24 | 2.22 \pm 0.09 | 6.35 \pm 0.43 | 1.78 \pm 0.29 | 2.05 \pm 0.04 | -0.46 \pm 0.06 |
| RCP ⁵ | 1.28 \pm 0.03 | 2.53 \pm 0.29 | 2.31 \pm 0.05 | 4.58 \pm 0.52 | 355.48 \pm 27.20 | 237.58 \pm 3.85 | -52.58 \pm 5.44 |
| RE ⁶ | 1.28 \pm 0.03 | 2.43 \pm 0.33 | 2.31 \pm 0.05 | 4.39 \pm 0.60 | 12.86 \pm 1.17 | 8.64 \pm 0.17 | -2.03 \pm 0.23 |

¹ RG = Relative growth in % LW d⁻¹ = (ln (final live weight (g)) - ln (initial live weight (g))) * 100 / feeding days; ² RFI = Relative feed intake = feed intake in % DM of live weight d⁻¹; ³ Feed to Gain Ratio (FGR) = g feed intake (DM) / g live weight gain; ⁴ PER = Protein efficiency ratio = g live weight gain / g crude protein intake; ⁵ RCP = Recovered crude protein per fish [mg kg^{0.8} d⁻¹]; ⁶ RE = Recovered energy per fish [kJ kg^{0.8} d⁻¹]; all values are significant (P < 1 * 10⁻⁴).

Table 1.4: Performance and physiological parameters of juvenile turbot, fed by diets varying in Met+Cys concentrations [$\text{g } 100\text{g}^{-1}$ in diet DM] (arithmetic mean \pm standard deviation)

| diet no. | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
|------------------|-------------------------------|--------------------------------|-------------------------------|--------------------------------|--------------------------------|--------------------------------|--------------------------------|--------------------------------|-------------------------------|
| Met+Cys | 0.8 | 1.0 | 1.2 | 1.4 | 1.6 | 1.8 | 2.0 | 4.0 | 6.0 |
| initial indiv. | | | | | | | | | |
| LW [g] | 25.83 \pm 3.01 | 25.83 \pm 3.02 | 25.89 \pm 2.61 | 25.83 \pm 3.31 | 25.86 \pm 3.20 | 25.76 \pm 3.34 | 25.83 \pm 3.07 | 25.84 \pm 2.65 | 25.87 \pm 2.88 |
| final indiv. | | | | | | | | | |
| LW [g] | 39.20 \pm 4.65 ^a | 57.20 \pm 7.29 ^b | 71.63 \pm 9.13 ^c | 80.46 \pm 12.72 ^c | 81.45 \pm 14.79 ^c | 75.57 \pm 12.46 ^c | 78.71 \pm 14.00 ^c | 56.47 \pm 7.59 ^b | 33.87 \pm 5.65 ^a |
| FCF ¹ | 1.47 \pm 0.13 ^a | 1.67 \pm 0.13 ^b | 1.92 \pm 0.10 ^{cd} | 1.99 \pm 0.21 ^d | 1.99 \pm 0.19 ^d | 1.92 \pm 0.24 ^{cd} | 1.95 \pm 0.24 ^{cd} | 1.77 \pm 0.16 ^{bc} | 1.59 \pm 0.16 ^{ab} |
| HSI ² | 1.06 \pm 0.22 ^{ab} | 1.41 \pm 0.28 ^{bcd} | 1.78 \pm 0.27 ^d | 1.75 \pm 0.24 ^{cd} | 1.81 \pm 0.30 ^d | 1.82 \pm 0.35 ^d | 1.74 \pm 0.44 ^{cd} | 1.34 \pm 0.19 ^{abc} | 0.86 \pm 0.26 ^a |
| blood pH | 7.68 \pm 0.10 | 7.67 \pm 0.08 | 7.62 \pm 0.08 | 7.63 \pm 0.06 | 7.62 \pm 0.08 | 7.61 \pm 0.09 | 7.64 \pm 0.06 | 7.62 \pm 0.05 | 7.59 \pm 0.07 |

different superscript letters within the same row represent significant differences (Tukey test, $P \leq 0.05$); samples from $n = 3$ tanks, arithmetic mean \pm s.d.; ¹ FCF = Fulton's condition factor = (live weight in g / body length in cm^3) * 100; ² HSI = hepatosomatic index (HSI) = (g liver weight / g live weight) * 100.

3.2. Chemical body composition

The chemical body composition of turbot is presented in Table 1.5. A strong shortage of Met+Cys and a strong oversupply (diet nos. 8 & 9) led to significantly higher concentrations of moisture and crude ash (CA). Correspondingly, concentrations of CP, CL and energy were significantly lower than those within the plateau phase (Figure 1.1).

A shortage in Met+Cys (diet nos. 1 & 2) decreased CP content stronger compared to an oversupply. Diet nos. 8 & 9 are not significantly different compared to values on the plateau phase (Figure 1.1). A similar effect can be observed in tendency in the CP concentration of fat free matter (Table 1.5).

The initial proximate body compositions did not differ compared to diets with a shortage (diet nos. 1 & 2) or a strong oversupply (diet nos. 8 & 9) of Met+Cys, except the energy content in diet no. 8. Whereas fish fed diets within or near by the LCC and the UCC of RG (Figure 1.1; Table 1.3.2), showed significantly or in tendency different values (Table 1.5). For the CP content it can be ascertained that a shortage in Met+Cys concentration (diet nos. 1 & 2) decrease the CP content stronger than an oversupply, e.g. significantly for diet no. 8 or in tendency for diet no. 9, compared to values on the plateau phase (Figure 1.1). This effect can be confirmed in tendency by the CP concentration in fat free matter (Table 1.5).

It can be stated out that the growth performance is influencing the proximate body composition of the fishes (Table 1.3.1 and 5). The initial proximate body compositions did not differ compared to diets with a shortage (diet nos. 1 & 2) or a strong oversupply (diet nos. 8 & 9) of Met+Cys, except the energy content in diet no. 8. Whereas fish fed diets within or near by the LCC and the UCC of RG (Figure 1.1; Table 1.3.2), showed significantly or in tendency different values (Table 1.5).

3.3. Apparent digestibility – comparison between feces collection methods

No significant differences for each ADC (DM, OM, CP, CA, energy) were detected, neither among the three different diets (nos. 3, 5 & 7), nor between feces collection methods (Table 1.6). However, ADC coefficients determined by the dissection method except for CA, tended to be lower than those by stripping. The ADC for CA tended to be higher for dissection than for stripping.

Table 1-5: Initial and final chemical body composition (arithmetic mean \pm standard deviation) of juvenile turbot, fed differing Met+Cys concentrations [g 100g⁻¹ in diet DM]

| diet no. | Initial | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
|------------------------------------|---------------------------------|--------------------------------|--------------------------------|---------------------------------|--------------------------------|--------------------------------|--------------------------------|--------------------------------|--------------------------------|----------------------------------|
| Met+Cys | 0.8 | 1.0 | 1.2 | 1.4 | 1.6 | 1.8 | 2.0 | 2.0 | 4.0 | 6.0 |
| | in g 100 g ⁻¹ | | | | | | | | | |
| Moisture | 80.20 \pm 0.27 ^a | 79.50 \pm 0.60 ^{ab} | 78.85 \pm 0.38 ^{bc} | 77.94 \pm 0.20 ^{cd} | 77.32 \pm 0.36 ^d | 77.19 \pm 0.44 ^d | 77.66 \pm 0.36 ^{cd} | 77.18 \pm 0.18 ^d | 78.01 \pm 0.41 ^{cd} | 79.38 \pm 0.27 ^{ab} |
| Crude Protein | 13.39 \pm 0.36 ^{abc} | 13.19 \pm 0.54 ^a | 14.45 \pm 0.07 ^{ab} | 14.48 \pm 0.33 ^{cd} | 15.17 \pm 0.27 ^d | 14.55 \pm 0.24 ^{cd} | 14.57 \pm 0.24 ^{cd} | 14.57 \pm 0.34 ^{cd} | 14.64 \pm 0.08 ^{cd} | 14.20 \pm 0.25 ^{abcd} |
| Crude Lipid | 2.92 \pm 0.06 ^{ab} | 3.52 \pm 0.16 ^{bc} | 4.30 \pm 0.15 ^{cde} | 4.48 \pm 0.42 ^{de} | 5.02 \pm 0.08 ^e | 4.83 \pm 0.12 ^e | 4.55 \pm 0.28 ^{de} | 5.03 \pm 0.21 ^e | 3.95 \pm 0.39 ^{bed} | 2.47 \pm 0.10 ^a |
| Crude Ash | 3.68 \pm 0.02 ^{abc} | 3.66 \pm 0.12 ^{ab} | 3.29 \pm 0.11 ^{bcd} | 3.10 \pm 0.05 ^{de} | 2.94 \pm 0.21 ^{de} | 2.88 \pm 0.09 ^e | 3.11 \pm 0.09 ^{cde} | 3.09 \pm 0.06 ^{de} | 3.32 \pm 0.15 ^{bed} | 3.85 \pm 0.05 ^a |
| Energy | 4.08 \pm 0.05 ^{ab} | 4.22 \pm 0.15 ^{ab} | 4.61 \pm 0.07 ^{bc} | 4.92 \pm 0.15 ^{cd} | 5.08 \pm 0.09 ^d | 5.15 \pm 0.14 ^d | 4.93 \pm 0.05 ^{cd} | 5.17 \pm 0.04 ^d | 4.75 \pm 0.21 ^{cd} | 4.11 \pm 0.07 ^a |
| CP to fat free matter ¹ | 13.79 \pm 0.36 ^{ab} | 13.69 \pm 0.58 ^a | 14.07 \pm 0.11 ^{ab} | 15.13 \pm 0.12 ^{bcd} | 15.29 \pm 0.33 ^{cd} | 15.92 \pm 0.31 ^d | 15.26 \pm 0.23 ^{cd} | 15.36 \pm 0.34 ^{cd} | 15.25 \pm 0.14 ^{cd} | 14.57 \pm 0.26 ^{abc} |

Different superscript lower-case letters in the same row indicate significant differences between dietary treatments (Tukey test, $P \leq 0.05$); n = 3 tanks; ¹ Crude Protein to fat free matter coefficient = (crude protein / (moisture + crude protein + crude ash))*100.

Table 1.6: Apparent digestibility coefficients (ADC)¹ (%) of energy and nutrients for 3 experimental diets (arithmetic mean \pm standard deviation), influenced by stripping and dissection as feces collection methods.

| diet no. | 3 | | 5 | | 7 | |
|---|------------------|------------------|------------------|------------------|------------------|------------------|
| | 1.2 | | 1.6 | | 2.0 | |
| Met+Cys (g 100 g ⁻¹ diet DM) | stripping | dissection | stripping | dissection | stripping | dissection |
| Dry matter | 64.34 \pm 1.42 | 62.16 \pm 3.82 | 64.83 \pm 0.75 | 62.41 \pm 2.95 | 63.99 \pm 1.56 | 63.75 \pm 0.83 |
| Organic matter | 70.38 \pm 1.43 | 67.56 \pm 3.76 | 70.88 \pm 0.38 | 68.07 \pm 2.65 | 69.95 \pm 1.33 | 68.86 \pm 0.97 |
| Crude protein | 80.43 \pm 1.28 | 79.58 \pm 2.03 | 81.09 \pm 0.90 | 78.72 \pm 2.98 | 81.01 \pm 1.21 | 78.14 \pm 2.29 |
| Crude ash | 12.08 \pm 1.40 | 15.42 \pm 4.48 | 12.24 \pm 4.52 | 13.21 \pm 5.58 | 12.41 \pm 3.41 | 19.55 \pm 0.98 |
| Energy | 74.48 \pm 1.09 | 72.52 \pm 2.77 | 75.09 \pm 0.83 | 73.02 \pm 2.20 | 75.16 \pm 0.88 | 73.76 \pm 0.78 |

For all ADC values, no significant differences between dietary treatment and faeces collection method were indicated (2way ANOVA, $P \leq 0.05$); pooled samples from $n=3$ tanks per treatment; ¹ apparent digestibility coefficient = ADC, ADC of DM of diet in % = $100 * (1 - (\% \text{ dietary TiO}_2 / \% \text{ fecal TiO}_2))$, ADC of nutrients, energy or OM of diets in % = $100 * (1 - (\% \text{ dietary TiO}_2 / \% \text{ fecal TiO}_2) * (\% \text{ fecal}_x / \% \text{ dietary}_x))$, where the subscript x stands for nutrient, energy or OM.

4. Discussion

A dose–response trial is frequently applied to determine the requirement of amino acids in several species (e.g.: Kampman-van de Hoek et al. 2013; Murthy and Varghese 1998; Wang et al. 2015). A precondition is a basal diet which ensures an adequate supply with all nutrients and energy except the substance to be evaluated. The target of the present study was to determine the LCC as well as the UCC for the sulfur containing AA (Met+Cys). To receive values which can be used as a basis for recommendations in practical feeding systems it is furthermore necessary, that a high performance level is reached at the plateau level in the experimental study. In the present study RG reached 2.00 (± 0.03 s.e.) in % LW d⁻¹ (Table 1.3.2). In several other growth trials with turbot, the maximum RG (frequently expressed as specific growth rate – SGR) was lower (Dietz et al. 2012; Kroeckel et al. 2012, 2013, 2015; Lee et al. 2003; Peres and Oliva-Teles 2005; 2008; Regost et al. 1999) or similar (Bonaldo et al. 2011; Ma et al. 2013; Nagel et al. 2012, 2014), compared to those in the current study (Table 1.3.2). In two studies (Bonaldo et al. 2015; Day and Plascencia Gonzalez 2000) a higher RG was reached. Therefore it can be assumed, that the other derived parameters in Tables 1.3.1 and 1.3.2 are also sufficient to estimate the LCC and the UCC of Met+Cys.

Table 1.7 provides an overview about the Met (+Cys) requirements of different aquaculture finfish species. Here, some studies determined the requirement only for Met, neglecting the fact that Cys can provide up to 40 – 60 % of the Met requirement depending on the fish species (Griffin et al. 1994; Harding et al. 1977; Kim et al. 1992; Moon and Gatlin 1991). Therefore, these recommendations are of limited practicability. Consequently, Wilson and Halver (1986) suggested that the requirement of sulfur containing amino acids for fish should be formulated on the basis of Met+Cys. Furthermore, it would be even more accurate to express Met+Cys on an equimolar-sulfur basis. However, due to the low Cys compared to Met concentration in the diets and the small difference of the molar weight of Cys and Met, the data in Table 1.7 are presented as the sum of total sulfur containing AA (Met; Cysteine; Cystine). Though, this might lead to overestimated recommendations for Met+Cys in studies with comparatively higher Cys concentrations (e.g.: Zhou et al. 2006; Table 1.7).

In the only study on the L-Met (+Cys) requirement for turbot, the total dietary requirement for Met+Cys was estimated for the RG at 2.00 % DM in diet (3.31 % in CP) (Ma et al. 2013; Table 1.7). This determined value is clearly higher compared to the LCC in this study, where the RG was determined at 1.25 % in diet DM (± 0.02 s.e.) (2.26 % in CP ± 0.04 s.e.) (Table 1.3.2). Similar differences can be observed for the RFI when comparing the recommendations of Ma et al. (2013) (Table 1.7) with LCC for the RFI (Table 1.3.2). The relatively strong difference to our findings might be caused by the different regression analyses applied. In the current study a segmental linear regression analysis approach was used to identify the LCC as well the UCC of Met+Cys. Whereas Ma et al. (2013) determined the maximum point of re-

sponse via second-order polynomial regression analysis, which does not necessarily reflect optimum supply representing the beginning of the plateau phase.

The strong impact of the type of the statistical analysis chosen on those values was shown in a recent study in gibel carp (*Carassius auratus gibelio*) (Wang et al. 2015). Broken-line regression analysis resulted in a clearly lower Met requirement than a polynomial regression analysis. The literature survey in Table 1.7 shows that those studies using broken-line regression analysis give a lower Met+Cys requirement than those which derived the requirement from polynomial or quadratic regression analyses.

Furthermore, it has to be considered that the critical concentrations are influenced even by different broken-line regression approaches. The segmental linear regression taking into account 3 phases (increase, plateau and decrease) and defining LCC and UCCs (Figure 1.1) results in lower values than the more simple 2-phase broken-line approach yielding in only one critical value. Thus the LCCs in this study are even lower (Table 1.3.2), as they would be with the broken-line approaches used in Table 1.7. Even if the approach in the current study of a segmental linear regression analysis (Figure 1.1) can lead to underestimated values, it allows a determination of the LCC and the UCC of Met+Cys. This is not possible e.g. by a polynomial regression analysis or a broken-line regression with one breakpoint.

It can be speculated that the lower initial LW in the study of Ma et al. (2013) ($5.59 \text{ g} \pm 0.02 \text{ s.d.}$) compared to the initial LW used in this study ($25.8 \text{ g} \pm 3.0 \text{ s.d.}$) might be the reason for the difference. However, similar RG was reached in both studies. Furthermore, no evident relationship between LW and recommended Met+Cys supply exists (Table 1.7).

Most of the studies in Table 1.7 are providing higher recommendation values for Met+Cys, except the lower values for Arctic charr (*S. alpinus*) (Simmons et al. 1999) or the similar for rainbow trout (*O.2 mykiss*) (Kim et al. 1992), compared to the own findings for turbot (Table 1.3.2). Regarding the recommended Met+Cys concentrations provided by NRC (National Research Council) (2011) and in Table 1.7, salmonids seem to have a lower Met+Cys requirement in protein than most of the other aquaculture finfish species.

The high variability in feeding days between the different experiments is shown in Table 1.7. Though, it can be stated that the feeding days in the current study (56 days) are close to the average median of the 15 evaluated studies. Due to the fact that gained growth data are also strongly influenced by the experimental time, the results in this study are comparable to most evaluated studies (Table 1.7).

Table 1.7: Literature overview about analyses of Met (+Cys) requirements of different aquaculture finfish species.

| species | individual initial mean live weight (g) | feeding days | dietary protein (% dry matter) | Methionine enantiomere | statistical regression model | performance parameter | Methionine require- ment (% protein) | Cyst(e)ine inclusion (% protein) | Met+Cys requirement (% protein) | Reference |
|---|--|-----------------|---|---------------------------|------------------------------------|---|---|--|---------------------------------------|--|
| Turbot (<i>Psetta maxima</i>) | 5.59 (±0.02s.d.) | 75 | 48.00 | L-Met | polynomial | relative growth (SGR) feed intake | 3.31 (r ² =0.93) 3.27 (r ² =0.998) | 0.85* 0.12* | 4.16* 4.12* | Ma et al. (2013) Alam et al. (2000) |
| Japanese flounder (<i>Paralichthys olivaceus</i>) | 2.80 | 40 | 50.00 | L-Met | broken-line | weight gain feed efficiency | 2.98 (2.9 - 3.06*) ¹ 2.88 (2.82 - 2.94*) ¹ | 0.12* 0.84* | 3.10* 3.00* | Alam et al. (2000) |
| European seabass (<i>Dicentrarchus labrax</i>) | 13.4 (±0.2s.d.) | 84 | 47.5* | L-Met | broken-line | weight gain N deposition | 2.0 (±0.27rmse*) 1.8 (±0.21rmse*) | 0.84* 0.54* | 3.00 2.70 | Tulli et al. (2010) |
| Grouper (<i>Epinephelus coioides</i>) | 14.21 (±0.24s.d.) | 56 | 48.00 | L-Met | broken-line | weight gain | 2.73 (r=0.94) | 0.54* | 3.27* | Luo et al. (2005) |
| Cobia (<i>Rachycentrom canadum</i>) | 11.61 (±0.16s.d.) | 56 | 44 | - | quadratic | relative growth (SGR) | 2.64 (r ² =0.82) | 1.52* | 4.16* | Zhou et al. (2006) |
| Large yellow croaker (<i>Pseudo- scaena crocea</i>) | 1.23 (±0.02s.d.) | 70 | 43.00 | L-Met | polynomial | relative growth (SGR) feed efficiency | 3.34 (r ² =0.93) 3.22 (r ² =0.98) | 0.67* 0.44* | 4.02 3.91 | Mai et al. (2006) Niu et al. (2013) |
| Golden pompano (<i>Trachinotus ovatus</i>) | 12.40 (±0.02s.d.) | 56 | 43.00 | DL-Met | broken-line | weight gain nitrogen retention efficiency | 2.46 (r=0.85) 2.95 (r=0.83) | 0.44* 0.34* | 2.9* 3.39* | Niu et al. (2013) Moon and Gat- lin (1991) |
| Red drum (<i>Sciaenops ocellatus</i>) | 0.90 | 56 | 35.00 | L-Met | broken-line | weight gain feed efficiency | 2.69 (±0.17s.e*) | 0.34* | 3.03 | Moon and Gat- lin (1991) |
| Yellowtail (<i>Seriola quinqueradi- ata</i>) | 23.30 | 30 | 43.30 | L-Met | broken-line | weight gain feed efficiency | 2.56 | 0.72* | 3.28* | Ruchimat et al. (1997) |
| Black sea bream (<i>Sparus macro- cephalus</i>) | 13.25 (±0.19s.d.) | 56 | 38.00 | L-Met | polynomial | relative growth (SGR) protein productive value | 4.5 (r ² =0.90, r ² =0.91) 4.53 (r ² =0.93) | 0.82* 0.65* | 5.32 5.66 (5.35*) | Zhou et al. (2011) |
| Blunt snout bream (<i>Maga- lobrama amblycephala</i>) | 3.34 (±0.03s.d.) | 63 | 34.00 | L-Met | broken-line | relative growth (SGR) protein productive value | 2.5 (r ² =0.90) 2.47 (r ² =0.997, r ² =0.87) | 0.65* 0.65* | 3.15 3.12 | Liao et al. (2014) |

Continuation(1) of Table 1.7

| species | individual initial mean live weight (g) | feeding days | dietary protein (% dry matter) | Methionine enantiomere | statistical regression model | performance parameter | Methionine require- ment (% protein) | Cyst(e)ine inclusion (% protein) | Met+Cys requirement (% protein) | Reference |
|---|--|-----------------|---|---------------------------|------------------------------------|---|--|--|---------------------------------------|-------------------------------|
| Arctic charr (<i>Salvelinus alpinus</i>) | 20.50 | 112 | 44.44 | DL-Met | quadratic | weight gain prevention of lens pathology | 1.76 2.67 | 0.23* | 1.99* 2.90* | Simmons et al. (1999) |
| Rainbow trout (<i>Oncorhynchus mykiss</i>) | 11.0-15.0 | 42 | 35.00 | D-/L-Met | broken-line | weight gain | 1.49 | 0.81* | 2.30 | Kim et al. (1992) |
| Indian major carp (<i>Labeo rohita</i>) | 2.4 (±0.05s.d.) | 60 | 40.00 | L-Met | broken-line | weight gain | 2.88 | 0.35* | 3.23 | Murthy and Varghese (1998) |
| Gibel carp (<i>Carassius auratus gibelio</i>) | 51.0 (±0.02s.d.) | 62 | 42.87 | DL-Met | broken-line polynomial | weight gain | 1.69 ($r^2=0.999$, $r^2=0.91$) 2.3 ($r^2=0.72$) | 0.93* | 2.62* 3.23* | Wang et al. (2015) |

*Calculated by the author.

†95% confidence interval.

Regarding utilization efficiencies enantiomers of L-Met (natural enantiomer) vs. DL- and D-Met the overview given in Table 1.7 does not provide a sufficient number of studies necessary for a solid comparison. Several mammals (rat, mouse, pig, dog, human) and chicken are able to use D-Met or DL-Met less, or similar towards the natural L-Met (Baker 2006). However, studies with Atlantic salmon (*S. salar*) of Sveier et al. (2001) indicated a similar growth, but also an improved protein utilization of D-Met towards L-Met, but in general with low growth rates independently from the treatment. Whereas Kim et al. (1992) did not find significant differences among D- and L-Met for growth. However, in the current study L-Met, as the natural enantiomer was utilized, to avoid a bias on the utilization efficiency of Met.

The supplementation of Taurine (2-aminoethanesulfonic acid) can improve fish growth due to several essential metabolic functions (Gaylord et al. 2007; Qi et al. 2012; Yun et al. 2012). Juvenile turbot (Wang et al. 2014) as well as e.g. rainbow trout (Gaylord et al. 2007) have the capability to synthesize taurine to a certain extent from sulfur containing amino acids (Met+Cys). Hence, it can be concluded that Taurine similar to Cys is semi-essential in turbot. This, beside other factors, makes it difficult to estimate Taurine requirement precisely (Baker 2006; Qi et al. 2012; Wang et al. 2014; Waterland 2006; Yun et al. 2012). Therefore, the level of taurine in the diet can be an important factor for the LCC of Met+Cys, and the high variability in the Met+Cys recommendations (Table 1.7) can be partially caused by variations of the Taurine level. Taurine content of the diets in dose response studies in Table 1.7 is not given, however it can be assumed that most basal diets were short in Taurine due to low levels of fish meal or other animal by-products. Results of Yun et al. (2012) and Qi et al. (2012) showed significant growth improvements due to extra taurine supplementations, higher than in the current study (Table 1.1). Hence it can be assumed that the relatively low Taurine supply might have led to some extent to elevated Met+Cys requirements.

Met and also Cys are known to be toxic at high level of oversupply. For this reason the UCC of Met+Cys in diets of turbot was determined. In young pigs and rats this negative impact of Met-oversupply is well documented and more pronounced than in other AA (Edmonds and Baker 1987; Sauberlich 1961). Toxicity of sulfur containing AA has also been showed in dogs (Merino et al. 1975), rats (Meng et al. 2013; Regina et al. 1993; Wamberg et al. 1987) and sheeps (Doyle and Adams 1980). In some dose-response studies of Met in different fish species a plateau-phase of the performance was observed (Alam et al. 2001; Luo et al. 2005; Niu et al. 2013; Simmons et al. 1999; Tulli et al. 2010). This indicates the irrelevance of a moderate oversupply. However, the upper critical level seems to be variable, above which a further supply decreases the performance (Ma et al. 2013; Liao et al. 2014; Mai et al. 2006; Murthy and Varghese 1998; Zhou et al. 2006, 2011). Hu et al. (2015) investigated the effect of tremendous oversup-

ply of the Met analogue HMTBa in turbot, which is similar efficient than L-Met (Ma et al. 2013) and recommended a supplementation of HMTBa below 5 % in the diet. In none of the cited studies in Table 1.7 the UCC of Met+Cys was determined by the far more precise regression analysis.

However, when comparing the LCC and the UCC among the different evaluated parameters, it can be clearly stated out that the variation of the LCC is smaller than of the UCC. The LCCs are rather similar for all investigated parameters (RG, RFI, PER, RCP, RE), except for FGR, within the range of 1.23 – 1.28 % in diet DM (2.22 – 2.31 % in CP), whereas the range of the UCCs is larger (2.10 – 3.82 % Met+Cys in diet DM; 3.80 – 6.91 % Met+Cys in CP). Therefore, the s.e. for the UCCs are clearly higher than for the LCCs (Table 1.3.2). Two reasons might be responsible: (i) turbot responded less sensitive to an oversupply of Met+Cys than to a deficiency, which is illustrated for RG in Figure 1.1, and (ii) oversupply was realized by much larger Met increments (2.0 %-unit, diet no. 7 and 8) than in diets no. 1 – 7 (0.2 %-units). Therefore, it seems to be necessary to more precisely determine the UCC in further studies with smaller increments of Met.

Strong oversupply of Met+Cys leads to severe negative impact of the metabolism (Tables 1.3.1 and 1.3.2; Figure 1.1). The reasons for this toxicity are very complex and depending on several factors (Baker 2006; Hu et al. 2015; Regina et al. 1993). Oversupplied Met has to be catabolized leading to an excess of sulfur, which cannot be completely eliminated, increasing the concentration of sulfuric acid, which was proven to induce metabolic acidosis in weanling rats (Wamberg et al. 1987). This effect was also observed by a small, non-significant decrease of the blood pH with rising concentration of Met+Cys in our study (Table 1.4). Furthermore, a significantly higher level of SAH in diet no. 9 compared to the levels in the other diets indicates the Met-toxicity. SAH is an intermediate product in the Met metabolism, a metabolite of S-Adenosylmethionine (SAM) and a precursor of L-Homocysteine (HCys) (Waterland 2006). SAM, as well as SAH concentrations in the liver are positively correlated to the Met toxicity in rats (Regina et al. 1993). This accumulation in hepatic tissue can partly be explained by a dysfunction of the liver due to non-enzymatic methylation of macromolecules by SAM, as the most important donator of methyl groups in the organism (Kwasek et al. 2014; Regina et al. 1993). The response of hepatic SAM and SAH to Met supplementation in rats is given in more detail by Waterland (2006).

The concentration of the H-Cys level relates to its precursor SAH. It has been shown by Tulli et al. (2010) that H-Cys in plasma of seabass (*Dicentrarchus labrax*) increased by increasing dietary Met concentrations. Hence, it can be concluded that the H-Cys level in the plasma in the current study might be increased at higher Met level, due to its relation to SAH (Figure 1.2) and according to the observations of Tulli et al. (2010).

Furthermore, SAH is known as a strong oxidant in metabolism related to several disorders like vascular diseases in humans (Durand et al. 2001; Roybal et al. 2004). The tendency of increased SAH levels in the range of Met+Cys deficiency (Figure 1.2) can be explained by the fact that Cys as a semi-EAA can be converted into Met, but Met cannot be converted to Cys (Lewis 2003) and that diet nos. 1 and 2 Cys was not deficient in relation to Met. Whereas (*Salmo salar*) Espe et al. (2014) did not observe different hepatic SAH concentration between a Met deficient and a Met supplemented diet in salmon, however hepatic SAM was higher in the Met deficient diet.

It has to be mentioned that blood pH and SAH in liver were measured approximately 48h post feeding. It can be assumed that the responses might have been more pronounced at shorter fasting. Pursuant to these observations, it can be stated that Met toxicity is related to metabolic effects of its precursors and, therefore due to this multifactorial situation difficult to explain.

Regarding the relation between HSI and Met+Cys deficiency, the literature findings are not consistent. Espe et al. (2014) observed decreasing HSI with Met supplementation in salmon. Other studies in gibel carp (*Carassius auratus gibelio*) (Wang et al. 2015) or blunt snout bream (*Megalobrama amblycephala*) (Liao et al. 2014) could not show any differences for HSI at varying Met+Cys concentrations. Studies of Niu et al. (2013) in golden pompano (*Trachinotus ovatus*) and Zhou et al. (2011) in black sea bream (*Sparus macrocephalus*) confirmed the findings of the current study that Met+Cys deficiency leads to a decreased HSI. The number of studies on the influence of strong oversupplies on the HSI is small. In the study of Hu et al. (2015) a strong oversupply with the L-Met analogue HMTBa leads to a non-significant decreased HSI in turbot which is confirmed by the own study. The own results show decreasing HSI at Met+Cys deficiency (diet nos. 1 and 2) and at strong oversupply (diet nos. 8 and 9).

FCF values in this study show a similar distribution to fLW (Table 1.4), RCP, and RE (Table 1.3.1). The increasing CP and decreasing CA contents of the fish bodies (Table 1.5) correspond to the increasing FCF (Table 1.4). In comparison to trials testing specific feed ingredients in diets with balanced AA composition (Nagel et al. 2012; Regost et al. 2003), FCF in turbot is affected to a smaller extent than in the current study. The significantly higher FCF by turbot fed with diets of balanced Met+Cys supply (diet nos. 3-7) can be explained by a lower proportion of skeletal bones and a higher proportion of muscle. Furthermore, FCF is also influenced by the genotype. Oesau (2012) detected a significantly higher fillet yield in Norwegian turbot strains with higher FCF than in strains from Iceland. These differences in the genotype can be confirmed in growth data by Dietz et al. (2012). This superior Norwegian strain (*Maximus A/S*) was used in the current study.

A distinct negative impact of Met+Cys imbalances on RFI was observed. Reducing the intake of unbalanced diets can be seen as a strategy to mitigate the negative effects of those diets on the metabolism. Clear influences of Met+Cys deficiencies on the feed intake were also shown by Simmons et al. (1999), Tulli et al. (2010), and Zhou et al. (2011).

The negative effect of Met deficiency on RG is in agreement with the effects on RFI, PER, RE, RCP, and FGR (Table 1.3.2). The influence of a shortage in Met+Cys can directly be seen in RCP, which leads to the similar distributions of RG, PER and RE, due to the efficiency of protein synthesis (Liao et al. 2014; Tulli et al. 2010; Zhou et al. 2011). The higher UCC for PER is in accordance with the high UCC for FGR.

One specific reason for the growth depression due to Met+Cys deficiency can be seen in the chemical body composition, like in the lower CP content (Table 1.5) and in the lower RCP (Tables 1.3.1 and 1.3.2). It can be seen that higher CA contents in the total body composition are related to poor growth of turbot and subsequently lower concentrations of CL, CP and Energy (Table 1.6), confirmed by Dietz et al. (2012) and Nagel et al. (2012).

Correspondingly, CP concentration of fat free matter is lower in deficient diets than at strong Met-oversupply (Table 1.5). Due to the high constancy of fat free matter composition (Kroeckel et al. 2013) it can be assumed that changes in its composition reflects severe impacts on the metabolism. Consequently, CP retentions are low (Tables 1.3.1 and 1.3.2), which is confirmed in other aquaculture species (Simmons et al. 1999; Tulli et al. 2010; Zhou et al. 2011).

ADC in diet no. 3, 5 and 7, which are near or within the plateau for RG (Figure 1.1), did not differ (Table 1.6). Whereas Zhou et al. (2011) detected a significant influence of Met-concentration on the ADC for all nutrients and energy, except for CL in black sea bream (*Sparus macrocephalus*). These findings are contradicting the results of the current study. Irrespective of the observations of Zhou et al. (2011) which are more related to the feed intake (Jansman et al. 2002), there are no evident reasons for any effect of L-Met concentration on digestibility. Especially, when the different Met levels are on or near by the same level of performance like in the current study (diet nos. 3, 5, 7).

It is well known, that feces collection method strongly influence ADC in aquaculture finfish. In rainbow trout stripping (abdominal massage) led to lower ADC than column (decantation) and collecting (collection devices) (Vandenberg and De La Noüe 2001). ADC received by collection feces by dissection and by stripping were not compared until now. In this study no differences between the two methods were found, however both led to relatively low values (Table 1.6). In other studies feces collection by dissection led to higher ADC (Dietz et al. 2012, 2013; Kroeckel et al. 2012) than by stripping (Nagel et al. 2012, 2014). However, it cannot be excluded that sampling time post feed-

ing might also have influenced ADC and might be responsible for differences between studies.

5. Conclusion

The LCC and UCC of Met+Cys in diets for turbot and a range for maximum performance could be determined in this study. Depending on the parameter used (RG, RFI, FGR, PER, RCP, RE), these ranges define optimum Met+Cys concentrations showing feeding situations where Met supplementation is necessary and maximum inclusion rates of feed protein sources rich in Met+Cys. Exemplarily, the LCC for RG is 1.25 % in diet DM (± 0.02 s.e.) (2.26 % in CP) and the UCC is 2.68 % (± 0.21 s.e.) Met+Cys in diet DM (4.85 % in CP). It seems to be necessary to take into account Met+Cys bioavailability when differences between protein sources occur. Further studies are required to more precisely determine the UCC.

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CHAPTER 2

EVALUATION OF THE UPPER CRITICAL CONCENTRATION (UCC) OF METHIONINE + CYSTINE IN DIETS OF JUVENILE TURBOT (*PSETTA MAXIMA*)

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Abstract

The upper critical concentration (UCC) of sulfur containing amino acids (Methionine, Met; Cystine, Cys) was determined in diets for juvenile turbot (*Psetta maxima*). In a growth trial of 56 feeding days, five iso-nitrogenous diets (crude protein, CP = 59.1 % DM) differing in the Met+Cys composition were fed once a day until apparent satiation to three tanks per diet. Each tank was stocked with 20 juvenile turbot (mean arithm. live weight 30.9 g \pm 3.2 s.d.). Range of possible oversupply were between 1.8 % and 3.0 % (in diet DM), and were realized by a four times addition of 0.2 % L-Met and 0.1 % L-Cys per increment. Segmental linear regression analyses was used to determine UCC for relative growth (RG), individual weight gain (iWG), relative feed intake (RFI), feed to gain ratio (FGR) and retained crude protein (RCP). The UCCs for these parameters ranged between 2.23 % (\pm 0.44 s.e.) for iWG and 2.56 % (\pm 0.45 s.e.) for RFI in diet DM (3.77 % and 4.33% in CP). Biometrical parameters and final individual live weight were not significantly influenced by Met+Cys concentration. Concentrations of chemical body compositions are only significantly affected for CP due to Met+Cys. Based on the evaluated UCCs in diets of juvenile turbot, maximum inclusion levels of protein sources rich in Met+Cys can be derived more precisely.

1. Introduction

Turbot (*Psetta maxima*) is a high value species and is increasingly used in commercial aquaculture in the last decade, especially in China and Europe (FAO 2014). It is a carnivorous aquaculture species with high requirement of crude protein (CP), which could range up to 49.4 to 55.0 % in diet (Cho et al. 2005; Lee et al. 2003; Li et al. 2011). To reduce the amount of fish meal in the diets by substituting with alternative protein sources, negative economic and environmental consequences could occur to some extent (Bonaldo et al. 2011, 2015; Fournier et al. 2004; Samuel-Fitwi et al. 2013; von Danwitz et al. 2016). Therefore a special focus on the amino acid requirement has become increasingly important. Whole essential amino acid (EAA) requirement for turbot was estimated according to the amino acid composition of the whole body protein (Kaushik 1998; Peres and Oliva-Teles 2008). The requirements and availabilities in protein sources of turbot for specific EAA were determined for Lysine (Kroeckel et al. 2013, 2015; Peres and Oliva-Teles 2008), Arginine (Fournier et al. 2002, 2003) and Methionine (Met) and Cystine (Cys) (semi-EAA) (Klatt et al. 2016, chapter 1).

High inclusion levels of protein sources other than fish meal can bear the risk of EAA deficiencies leading to reduced growth and CP retention (Furuya et al. 2004; Kroeckel et al. 2013, 2015; Tulli et al. 2010). On the other hand, an oversupply with specific amino acids (AA) can impair performance as shown for Leucine in rainbow trout (*Oncorhynchus mykiss*) (Choo et al. 1991) and Met+Cys for turbot (Klatt et al. 2016, chapter 1). The adverse impact of an oversupply with Met+Cys on growth performance was observed e.g. for Cobia (*Rachycentron canadum*), Black sea bream (*Sparus macrocephalus*) (Zhou et al. 2006, 2011) and Indian major carp (*Labeo rohita*) (Murthy and Varghese 1998). High inclusion ratios of protein sources rich in Met+Cys increase the risk of oversupply with Met+Cys, which was shown for wheat gluten (Fournier et al. 2004), rapeseed protein concentrate (Nagel et al. 2014; von Danwitz et al. 2016) and feather meal (Kikuchi et al. 1994). In turbot, such oversupply has been shown to negatively affect growth performance and health (Klatt et al. 2016, chapter 1).

Likewise, Met+Cys is known to be toxic for terrestrial species. In a study with mice, which compared the toxicity of several AAs, Met showed the highest toxicity (Sauberlich 1961). When fed in excess, Met+Cys are also well known to reduce performance and health of young pigs, mice, dogs or rats (Baker 2006; Edmonds and Baker 1987; Merino et al. 1975; Regina et al. 1993). Excessive oversupply can lead to hepatic coma and metabolic acidosis in dogs and weanling rats, (Merino et al. 1975; Wamberg et al. 1987). Similarly, in finfish, such as Atlantic salmon (*salmo salar*), turbot or seabass (*Dicentrarchus labrax*), high Met supplementation can lead to rising concentrations of intermediate products of Met metabolism, such as S-Adenosylhomocysteine and L-

Homocysteine, which can impair fish growth performance (Espe et al. 2008; Klatt et al. 2016, chapter 1; Tulli et al. 2010).

Several studies have defined the lower critical concentration of Met+Cys for aquaculture finfish (e.g.: Alam et al. 2000; Moon and Gatlin 1991; Tulli et al. 2010; Zhou et al. 2006, 2011). However, studies which defined the specific upper critical concentration (UCC) of Met+Cys are rare (Klatt et al. 2016, chapter 1). In a previous experiment, Klatt et al. (2016) (chapter 1) determined the UCC of Met+Cys with 2.68 % in diet or 4.85 % in CP derived from the response in relative growth (RG), however, these values varied among performance parameter used and they show high standard errors (s.e.).

Therefore, the aim of this study was to determine a more precise UCC of Met+Cys in diets for turbot (*P. maxima*).

2. Materials and Methods

2.1 Experimental diets

Based on a basal diet, 5 diets differing in Met+Cys concentrations were created. Met+Cys concentration ranged between 1.8 % and 3.0 % (in diet DM), by a four times addition of 0.2 % L-Met and 0.1 % L-Cys on the basal diet (no.1) per increment step, respectively, resulting in a percentage Cys in Met+Cys between 35 – 36 %. According to the findings of Klatt et al. (2016) (chapter 1), these ranges of Met+Cys concentrations in the diets cover a range of potential oversupplies. In order to keep all diets iso-nitrogenous (crude protein, CP = 59.1 % DM), diets were balanced with appropriate amounts of a mixture of non-essential amino acids (NEAA) (60 % L-Glutamic acid, 20 % Glycine, 20 % L-Alanine) (Table 2.1). All diets were formulated to be iso-lipidic and iso-energetic (crude lipid, CL = 14.9 % DM; gross energy = 22.7 MJ/kg DM) (Table 2.1). Likewise, the amino acid composition of all diets is given in Table 2.1, where a sufficient supply with all other EAAs for turbot was realized according to the recommendations of Kaushik (1998) and Peres and Oliva-Teles (2008).

The diets were manufactured by a stepwise addition of feed components and an intense mixing of all ingredients. The mixtures were pelletized by a 4 mm matrix (*L 14-175, AMANDUS KAHL, Reinbeck, Germany*). The temperatures never exceeded 52 °C to avoid heat damages of nutrients during the pelleting process.

2.2 Experimental setup

The feeding experiment was conducted at the *Gesellschaft für Marine Aquakultur mbH (GMA Büsum, Germany)*. The juvenile turbot were provided by *France turbot (le Bon Port, 85740 L'Epine, France)*.

Fishes were stocked in 15 rectangular rearing tanks with a water volume of 150 L, a tank height of 0.52 m and a bottom surface of 0.29 m², respectively. Each tank was

stocked with 20 juvenile turbot. Fishes were adapted to the rearing conditions for 8 days prior the experimental start. Each diet was fed to three randomly selected tanks. At the start of the growth trial, individual mean live weight (LW) was 30.9 g (\pm 3.2 standard deviation (s.d.)), resulting in an average tank LW of 611.7 g (\pm 6.1 s.d.). The turbot were fed once a day until apparent satiation for 56 days. Pellets which remained 15 minutes after feeding in the tanks were siphoned out and counted to determine feed intake assuming the average pellet weight.

The experiment was conducted in a recirculating aquaculture system (RAS) with a total water volume of 20 m³, containing a drum-filter, a nitrifying moving-bed biofilter (4 m³), a protein-skimmer with ozonation and a UV light unit for disinfection. Beside this trial, other trials with turbot of the same origin were carried out in the same RAS. The photoperiod was adjusted to a light:dark cycle of 14:10 hours. During the experimental period, water quality parameters were analyzed once a day. The average oxygen concentration was 9.2 mg L⁻¹ \pm 0.3 s.d. with a saturation of 112.5 % \pm 4.3 s.d. (*Handy Polaris, Oxy-Guard International A/S, Birkerød, DK*) and the water temperature was maintained at 18.1 °C \pm 0.3 s.d., with a salinity concentration of 24 ppt \pm 1.2 s.d. (*HI 96822 Seawater Refractometer, Hanna Instruments Inc., Woonsocket-RI-USA*) (Imslund et al. 2001). The pH was 7.6 \pm 0.1 (*GMH 3530, Digital pH-/ mV-/ Thermometer, Greisinger electronic, D*), the total ammonia nitrogen (NH₄-N) and the nitrite nitrogen (NO₂-N) concentration were 0.1 mg L⁻¹ \pm 0.1 and 0.3 mg L⁻¹ \pm 0.1 s.d. (*Microquant test kits for NH₄⁺ and NO₂⁻, Merck KGaA, Darmstadt, Germany*), respectively.

2.3 Sampling

Before starting the growth trial (day 0) two samples of 8 turbot, respectively, were chopped and stored (-18°C) in a plastic bag to be analyzed for initial total body composition. These fishes had a similar weight distribution as the ones which were used for the growth trial.

Prior to the final sampling after 56 feeding days, fishes remained unfed in their tanks for 48 hours to ensure a depleted gut. For every tank total fish weight, individual weight and length were determined to calculate the Fulton's condition factor (FCF). The individual liver and spleen weight were measured from 6 fishes per tank to determine hepatosomatic index (HSI) and splenic index (SI).

The chemical body composition was determined by a sample of 6 randomly selected fishes per tank, which were chopped, pooled and stored (-18°C) in a plastic bag until analyses.

Table 2.1: Dietary formulation and dietary nutrient and amino acid compositions.

| diet no. | 1 | 2 | 3 | 4 | 5 |
|---|-------|-------|-------|-------|-------|
| Met+Cys [g 100 g ⁻¹ in diet DM] | 1.8 | 2.1 | 2.4 | 2.7 | 3.0 |
| Ingredients [g 100 g ⁻¹] | | | | | |
| fish meal (<i>Clupea harengus</i>) ^a | 40.0 | 40.0 | 40.0 | 40.0 | 40.0 |
| hemoglobin powder (porc) ^b | 4.8 | 4.8 | 4.8 | 4.8 | 4.8 |
| wheat gluten ^c | 17.0 | 17.0 | 17.0 | 17.0 | 17.0 |
| fish oil ^a | 10.0 | 10.0 | 10.0 | 10.0 | 10.0 |
| wheat starch ^c | 11.5 | 11.5 | 11.5 | 11.5 | 11.5 |
| Calciumhydrogenephosphate ^d | 2.5 | 2.5 | 2.5 | 2.5 | 2.5 |
| Marker (TiO ₂) ^e | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 |
| Vitamine/mineral premixture ^f | 7.0 | 7.0 | 7.0 | 7.0 | 7.0 |
| essential amino acid (EAA) mixture ^{g,h*} | 10.5 | 10.5 | 10.5 | 10.5 | 10.5 |
| L-Methionine ^h | 0.15 | 0.35 | 0.55 | 0.75 | 0.95 |
| L-Cystine ^h | 0.075 | 0.175 | 0.275 | 0.375 | 0.475 |
| non-essential amino acid (NEAA) mixture ^{i,**} | 1.775 | 1.475 | 1.175 | 0.875 | 0.575 |
| Nutrients [g 100 g ⁻¹ in diet DM] | | | | | |
| Moisture | 5.3 | 5.8 | 5.6 | 5.5 | 5.3 |
| Crude protein | 59.4 | 59.3 | 58.8 | 59.6 | 58.5 |
| Crude lipid | 15.1 | 14.8 | 15.1 | 15.0 | 14.7 |
| Crude fiber + NfE *** | 14.2 | 14.9 | 15.1 | 14.3 | 15.9 |
| Crude Ash | 11.3 | 11.1 | 11.1 | 11.2 | 11.0 |
| Gross Energy (kJ g ⁻¹ in DM) | 22.7 | 22.7 | 22.7 | 22.7 | 22.6 |
| Calcium | 2.7 | 2.8 | 2.7 | 2.7 | 2.7 |
| Phosphorus | 1.8 | 1.8 | 1.8 | 1.7 | 1.7 |
| Amino acid composition [g 16 g ⁻¹ N] | | | | | |
| Lysine | 10.33 | 10.59 | 10.48 | 10.63 | 10.98 |
| Methionine | 1.90 | 2.16 | 2.57 | 2.77 | 3.13 |
| Cystine | 1.14 | 1.32 | 1.51 | 1.61 | 1.79 |
| Aspartic acid | 5.80 | 5.62 | 5.76 | 5.63 | 5.79 |
| Threonine | 4.54 | 4.52 | 4.71 | 4.50 | 4.65 |
| Serine | 3.47 | 3.44 | 3.52 | 3.43 | 3.55 |
| Glutamic acid | 16.42 | 16.28 | 16.12 | 15.67 | 15.72 |
| Proline | 5.94 | 5.50 | 5.71 | 5.44 | 5.77 |
| Glycine | 5.28 | 5.03 | 4.93 | 4.77 | 4.70 |
| Alanine | 4.58 | 4.39 | 4.34 | 4.18 | 4.10 |
| Valine | 4.90 | 4.77 | 5.05 | 4.99 | 5.12 |
| Isoleucine | 4.07 | 4.08 | 4.17 | 4.06 | 4.15 |
| Leucine | 6.80 | 6.78 | 6.90 | 6.68 | 6.87 |
| Tyrosine | 3.57 | 3.59 | 3.66 | 3.56 | 3.55 |
| Phenylalanine | 4.49 | 4.66 | 4.64 | 4.48 | 4.61 |
| Histidine | 2.59 | 2.61 | 2.69 | 2.60 | 2.65 |
| Arginine | 7.00 | 7.02 | 7.14 | 7.00 | 7.11 |
| Tryptophan | 1.06 | 1.06 | 1.12 | 1.09 | 1.08 |

Continuation of Table 2.1

^a Vereinigte Fischmehlwerke Cuxhaven GmbH & Co KG, Cuxhaven, Germany; ^b Günther Badenhof Fleischwerke KG, Verden-Aller, Germany; ^c Kröner Stärke GmbH, Ibbenbüren, Germany; ^f Enorica GmbH, Norderstedt, Germany; ^d Lehmann & Voss & Co. KG, Hamburg, Germany; ^e Kronos International Inc., Leverkusen, Germany; ⁱ Vitamin and Mineral mixture 517158 and 508240, Vitfoss, Grasten, Denmark; ^g Carl Roth GmbH & Co. KG, Karlsruhe, Germany; ^h VWR International GmbH, Darmstadt, Germany; * EAA mix. = 360 g kg⁻¹ L-Lysine (HCl) + 180 g kg⁻¹ L-Arginine + 72 g kg⁻¹ L-Isoleucine + 50 g kg⁻¹ L-Leucine + 100 g kg⁻¹ L-Threonine + 80 g kg⁻¹ L-Tyrosine + 60 g kg⁻¹ L-Phenylalanine + 60 g kg⁻¹ L-Valine + 13 g kg⁻¹ L-Tryptophane + 25 g kg⁻¹ L-Histidine; ** NEAA mix. = 600 g kg⁻¹ L-Glutamic acid + 200 g kg⁻¹ Glycine + 200 g kg⁻¹ L-Alanine; *** CF + NfE = crude fiber + nitrogen free extracts = 1000 - (crude protein + crude lipid + crude ash)

2.4 Chemical analyses

The total body of the fishes was freeze-dried. Subsequently, the whole samples were minced (≤ 1 mm) by a centrifugal mill (*ZM 100, Retsch GmbH & Co. KG, Haan, Germany*). Pelletized feeds were pulverized by a mortar and a pestle.

Dry matter (DM), crude ash (CA), CP, CL and energy content of all diets and fish bodies were analyzed in duplicates according to Weender Analysis (European Union 2009).

DM content was determined by drying the samples for 4 hours at 103°C. CA content was analyzed by remaining the sample for 4 hours in a combustion oven at 550°C. CP (N*6.25) was determined by the Kjeldahl method and the CL with a Soxhlet extractor without hydrolization. The residual fraction in DM (crude fiber – CF and nitrogen free extracts – NFE), was calculated by subtracting CP, CL and CA from 100. The energy was measured by an adiabatic bomb calorimeter (*C200, IKA-Werke GmbH & Co. KG, Staufen, Germany*).

AA composition (Table 2.1) of the diets was analyzed by *LUFA-ITL GmbH (Kiel, Germany)* according to the method *VO(EG) 152/2009, L54/23-32*. Tryptophan was analyzed by the method *EU 152/2009 (A)(SP)*. Calcium and Phosphorus were determined according to *VDLUFA VII 2.2.2.6*.

2.5 Definitions and calculations

The following parameters were defined:

Relative growth (RG) = $(\ln(\text{final live weight (g)}) - \ln(\text{initial live weight (g)})) * 100 / \text{feeding days}$

Individual weight gain (iWG) = final individual live weight (g) - initial individual live weight (g)

Relative feed intake (RFI) = feed intake in % of average live weight / feeding days

Feed to Gain Ratio (FGR) = g feed intake (DM) / g live weight gain

Geometric mean live weight (GMLW) in g = $(\text{initial live weight} * \text{final live weight})^{0.5}$

Metabolic live weight (MLW) in kg = $(\text{GMLW}/1000)^{0.8}$ (according to Dietz et al. 2012 and Lupatsch et al. 2003)

Retained crude protein (RCP) in $\text{mg kg}^{-0.8} \text{ d}^{-1} = (\text{mg retained crude protein} / \text{GMLW}) / \text{feeding days}$

Fulton's condition factor (FCF) = $(\text{live weight in g} / (\text{total length in cm})^3) * 100$

Hepatosomatic index (HSI) = $(\text{g liver weight} / \text{g live weight}) * 100$

Splenic index (SI) = $(\text{g spleen weight} / \text{g live weight}) * 100$

2.6 Statistical analyses

The statistical software R (R Core team 2015) was used to evaluate the data.

For the determination of the UCCs for RG, iWG, RFI, FGR and RCP a segmental linear regression analysis (plateau – decrease/increase) was used. The plateau, upper critical concentration (UCC) and slope \pm s.e., respectively, were given and evaluated via Akaike Information Criteria (AIC) (Yamaoka et al. 1978).

The statistical mixed model according to Laird and Ware (1982) and Verbeke and Mo-lenberghs (2000) were applied for the parameters with several values per tank: individual LW, final LW, FCF, HSI and SI. A linear model was applied for parameters of chemical body composition (one value per tank) (moisture, CP, CL, CA and energy). The data were assumed to be normally distributed and to be homoscedastic. These assumptions are based on a graphical residual analysis. The statistical model included treatment (Met+Cys concentration) as (fixed) factor, the tank was regarded as random factor if measurement values were available for individual fishes per tank. An analysis of variances (ANOVA) was conducted, followed by all-pair comparisons for the means of the treatments (e.g.: Bretz et al. 2011). This data is presented as arithmetic mean values \pm s.d..

$P < 0.05$ was defined as the level of significance.

3. Results

No fish died or was removed during the experiment.

3.1 Growth performance, biometrical parameters, feed intake and utilization

The arithmetic mean values (\pm s.d.) for the parameter individual weight gain (iWG), relative growth (RG), relative feed intake (RFI), feed to gain ratio (FGR) and retained crude protein (RCP) for each diets are given in Table 2.2.1.

UCC of Met+Cys for all parameters (iWG, RG, RFI, FGR, RCP) in diet DM (\pm s.e.) and in dietary CP (\pm s.e.), as well the plateau (\pm s.e.), the regression coefficient (\pm s.e.) and the AIC are presented in Table 2.2.2. All UCCs and plateaus, except the regression coefficients ($P \geq 0.05$), are significant ($P < 1 * 10^{-4}$). Exemplarily, the relationship between Met+Cys concentration and RG is given in Figure 2.1.

The lowest UCC of the investigated parameter was determined for the parameter iWG with 2.23 % Met+Cys in diet DM (± 0.44 s.e) (3.77 % in CP). The highest value was detected for RFI with 2.56 % Met+Cys in diet DM (± 0.45 s.e) (4.33 % in CP). However, UCCs and regression coefficients showed high s.e..

Initial and final individual LW, Fulton's Condition Factor (FCF), hepatosomatic index (HSI) and the splenic index (SI) are shown in Table 2.3. Here, no significant differences could be found ($P \geq 0.05$).

3.2 Chemical body composition

Moisture, CL and energy concentrations of the bodies are not influenced by the Met+Cys concentration ($P \geq 0.05$), whereas initial concentrations of the fish bodies are significantly lower for CL and energy, and initial moisture concentration is significantly higher ($P < 0.05$) (Table 2.4).

CP concentration increased for all treatments compared to the initial value significantly, except for diet 1. The CA concentration is partly influenced by the Met+Cys concentration. The highest value can be found for diet 1, which is significantly different to the initial value and to diet 5.

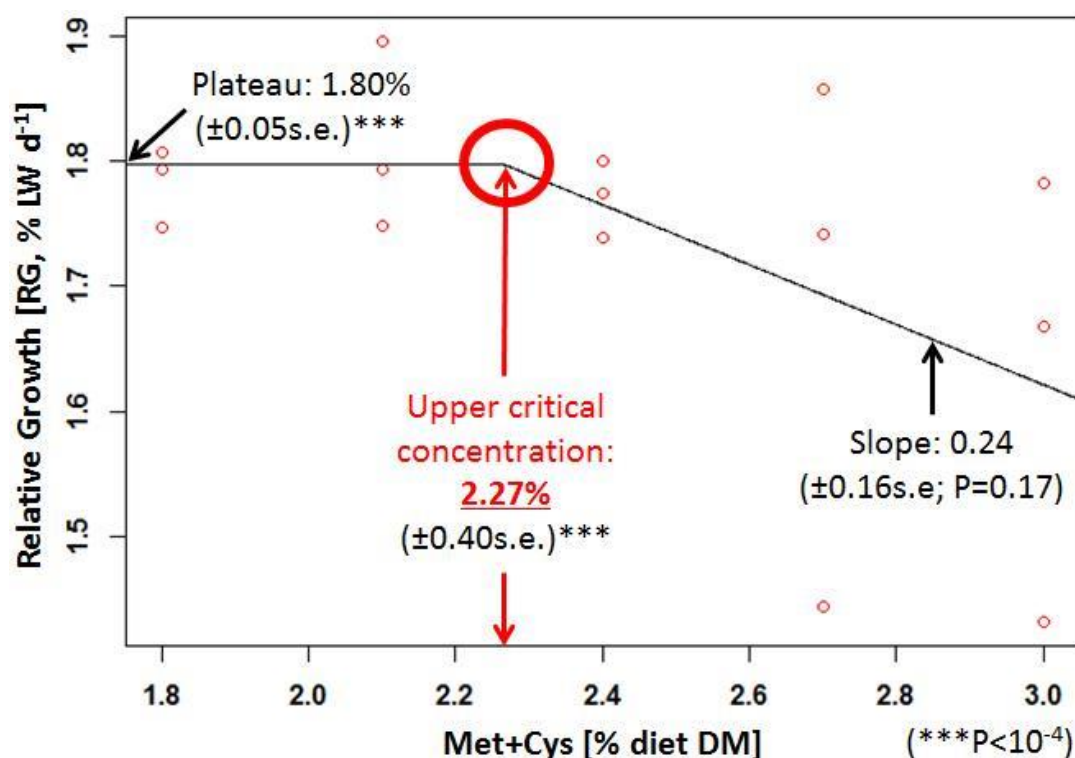


Figure 2.1: Relationship of Met+Cys concentration in the diets and relative growth [RG, % LW d⁻¹] of juvenile turbot (*Psetta maxima*), described by broken-line regression.

Table 2.2.1: Performance parameters of juvenile turbot fed varying Met+Cys concentrations (arithmetic mean \pm standard deviation)

| diet no. | 1 | 2 | 3 | 4 | 5 |
|---|------------------|------------------|------------------|------------------|------------------|
| Met+Cys [g 100g ⁻¹ in diet DM] | 1.8 | 2.1 | 2.4 | 2.7 | 3.0 |
| RG ¹ | 1.78 \pm 0.03 | 1.81 \pm 0.06 | 1.77 \pm 0.02 | 1.68 \pm 0.17 | 1.63 \pm 0.15 |
| iWG ² | 52.39 \pm 1.87 | 53.82 \pm 2.71 | 51.59 \pm 0.76 | 48.32 \pm 7.38 | 45.85 \pm 5.91 |
| RFI ³ | 1.13 \pm 0.02 | 1.14 \pm 0.03 | 1.14 \pm 0.00 | 1.09 \pm 0.11 | 1.10 \pm 0.07 |
| FGR ⁴ | 0.63 \pm 0.02 | 0.63 \pm 0.01 | 0.64 \pm 0.01 | 0.65 \pm 0.02 | 0.68 \pm 0.02 |
| RCP ⁵ | 80.64 \pm 1.76 | 87.59 \pm 0.56 | 83.11 \pm 1.25 | 80.62 \pm 7.57 | 77.26 \pm 7.66 |

¹ RG = Relative growth (% LW d⁻¹) = (ln (final live weight (g)) - ln (initial live weight (g))) * 100 / feeding days; ² iWG = individual weight gain (g) = individual final live weight (g) - individual initial live weight (g); ³ RFI = Relative feed intake = feed intake in % DM of live weight d⁻¹; ⁴ Feed to Gain Ratio (FGR) = g feed intake (DM) / g live weight gain; ⁵ RCP = Retained crude protein per fish (mg kg^{-0.8} d⁻¹).

Table 2.2.2: Upper critical concentrations of Met+Cys [% in diet DM and protein], Plateau and the Regression coefficient (Slope) for growth performance parameters (\pm standard error)

| | upper critical concentration of Met+Cys in diet DM (%) | upper critical concentration of Met+Cys in CP (%) | Plateau | Regression coefficient (Slope) | AIC ⁶ |
|------------------|--|---|---------------------|--------------------------------|------------------|
| RG ¹ | 2.27 \pm 0.40*** | 3.84 \pm 0.68*** | 1.80 \pm 0.05*** | -0.24 \pm 0.16 | -16.44 |
| iWG ² | 2.23 \pm 0.44*** | 3.77 \pm 0.74*** | 53.10 \pm 2.06*** | -9.57 \pm 6.87 | 95.77 |
| RFI ³ | 2.56 \pm 0.45*** | 4.33 \pm 0.76*** | 1.14 \pm 0.02*** | -0.15 \pm 0.19 | -32.67 |
| FGR ⁴ | 2.48 \pm 0.32*** | 4.19 \pm 0.54*** | 0.64 \pm 0.01*** | 0.07 \pm 0.05 | -70.95 |
| RCP ⁵ | 2.31 \pm 0.46*** | 3.91 \pm 0.78*** | 84.12 \pm 2.46*** | -9.76 \pm 8.20 | 101.10 |

¹ RG = Relative growth (% LW d⁻¹) = (ln (final live weight (g)) - ln (initial live weight (g))) * 100 / feeding days; ² iWG = individual weight gain (g) = individual final live weight (g) - individual initial live weight (g); ³ RFI = Relative feed intake = feed intake in % DM of live weight d⁻¹; ⁴ Feed to Gain Ratio (FGR) = g feed intake (DM) / g live weight gain; ⁵ RCP = Retained crude protein per fish (mg kg^{-0.8} d⁻¹); ⁶ AIC = Akaike Information Criteria; *** P < 10⁻⁴; n.s. = P > 0.05.

Table 2.3: Performance and physiological parameters of juvenile turbot, fed by diets varying in Met+Cys concentrations (arithmetic mean \pm standard deviation)

| diet no. | 1 | 2 | 3 | 4 | 5 |
|---|-------------------|-------------------|-------------------|-------------------|-------------------|
| Met+Cys [g 100g ⁻¹ in diet DM] | 1.8 | 2.1 | 2.4 | 2.7 | 3.0 |
| initial individual LW ¹ [g] | 30.57 \pm 3.29 | 30.58 \pm 2.92 | 30.42 \pm 3.13 | 30.69 \pm 3.47 | 30.67 \pm 3.24 |
| final individual LW ¹ [g] | 82.96 \pm 22.42 | 84.40 \pm 19.10 | 82.00 \pm 21.35 | 79.01 \pm 25.45 | 76.52 \pm 21.65 |
| FCF ² | 2.10 \pm 0.19 | 2.10 \pm 0.17 | 2.07 \pm 0.16 | 2.09 \pm 0.21 | 2.04 \pm 0.19 |
| HSI ³ | 1.69 \pm 0.37 | 1.67 \pm 0.30 | 1.86 \pm 0.29 | 1.68 \pm 0.40 | 1.71 \pm 0.35 |
| SI ⁴ | 0.10 \pm 0.03 | 0.09 \pm 0.04 | 0.09 \pm 0.03 | 0.09 \pm 0.03 | 0.12 \pm 0.06 |

No significant differences within the same row were detected (Tukey test, $P \geq 0.05$); samples from $n = 3$ tanks, arithmetic mean \pm s.d.; LW = live weight; ²FCF = Fulton's condition factor = (live weight in g / body length in cm³) * 100; ³HSI = hepatosomatic index (HSI) = (g liver weight / g live weight) * 100; ⁴SI = splenic index (SI) = (g spleen weight / g live weight) * 100; (Tukey test, no significant differences within the same row).

Table 2.4: Initial and final chemical body composition (arithmetic mean \pm standard deviation) of juvenile turbot, fed differing Met+Cys concentrations [in g 100g⁻¹; Energy in kJ g⁻¹]

| diet no. | 1 | 2 | 3 | 4 | 5 |
|--|----------------------------|----------------------------|----------------------------|----------------------------|----------------------------|
| Met+Cys [g 100 g ⁻¹ in diet DM] | 1.8 | 2.1 | 2.4 | 2.7 | 3.0 |
| Initial | | | | | |
| Moisture | 77.5 \pm 4 ^b | 75.7 \pm 2 ^a | 75.6 \pm 3 ^a | 75.4 \pm 1 ^a | 75.6 \pm 4 ^a |
| Crude Protein | 13.9 \pm 1 ^a | 14.5 \pm 1 ^{ab} | 14.9 \pm 2 ^{bc} | 15.1 \pm 2 ^{bc} | 15.1 \pm 0 ^{bc} |
| Crude Lipid | 4.0 \pm 2 ^a | 5.0 \pm 1 ^b | 5.1 \pm 3 ^b | 5.0 \pm 1 ^b | 5.0 \pm 2 ^b |
| Crude Ash | 1.6 \pm 1 ^a | 2.8 \pm 3 ^b | 2.2 \pm 4 ^{ab} | 2.2 \pm 2 ^{ab} | 1.7 \pm 3 ^a |
| Energy | 4.7 \pm 0.1 ^a | 5.3 \pm 0.1 ^b | 5.4 \pm 0.1 ^b | 5.4 \pm 0.0 ^b | 5.4 \pm 0.2 ^b |

Different superscript lower-case letters in the same row indicate significant differences between dietary treatments (Tukey test, $P < 0.05$); $n = 3$ tanks.

4. Discussion

Several studies investigated the Met+Cys requirement by dose-response trials and analyzed the lower critical concentration by broken-line regression (Alam et al. 2000; Klatt et al. 2016, chapter 1; Tulli et al. 2010) or by identifying the maximum response by polynomial regression analyses (Simmons et al. 1999; Zhou et al. 2011). These studies contributed to determine the critical ratio of single EAA (ideal protein) for several aquaculture species (Alam et al. 2000; Furuya et al. 2004). Deficiencies of Met+Cys in diets of turbot can occur due to high inclusion levels of legume proteins (e.g.: soy, pea).

However, besides deficiencies oversupplies with AA can impair the performance of animals, especially sulfur containing AA (Met+Cys) are known to be toxic (Baker 2006; Klatt et al. 2016, chapter 1; Sauberlich 1961). Several studies evaluated the toxicity of Met+Cys for terrestrial animals like young pigs (Edmonds and Baker 1987), humans (Rees et al. 2006), dogs (Merino et al. 1975) or rats (Regina et al. 1993; Sauberlich 1961). However, studies in fish which investigated the effects of Met+Cys oversupplies are rare and inconsistent (Zhou et al. 2011).

Dose-response studies of Met+Cys with Japanese flounder (*Paralichthys olivaceus*), Grouper (*Epinephelus coioides*) or European seabass (*Dicentrarchus labrax*) showed a plateau-phase after reaching maximum performance (Alam et al. 2000; Luo et al. 2005; Tulli et al. 2010). These studies indicated the irrelevance of moderate oversupplies on growth performance for fish. However, higher sensitivities of the investigated species to Met+Cys was observed, where performance of fish has decreased at higher concentrations. Such observations were made in Indian major carp (*Labeo rohita*) (Murthy and Varghese 1998), Cobia (*Rachycentron canadum*) or Black sea bream (*Sparus macrocephalus*) (Zhou et al. 2006, 2011). Nevertheless, none of the mentioned studies evaluated the effects of Met+Cys oversupply in detail, from which a precise upper critical concentration (UCC) could be derived.

Nevertheless two studies investigated the effect of oversupply in diets of juvenile turbot by defining threshold values. A study of Hu et al. (2015) recommended the safe concentration below 5% in the diet supplemented with Met-analogue 2-hydroxy-4-(methylthio) butanoic acid (HMTBa). In a previous study, Klatt et al. (2016) (chapter 1) determined the UCC of sulfur containing amino acids (Met+Cys, L-enantiomer) by the far more precise regression analyses (segmental broken-line) for juvenile turbot. In this study the UCC of Met+Cys (L-enantiomers) for turbot was determined based on several parameters (RG, RFI, FGR, protein efficiency ratio – PER, RCP, retained energy – RE). It was pointed out that s.e. of the UCCs were much higher compared to s.e. of the lower critical concentrations. This was probably due to the following reasons: (i) turbot reacts less sensitive to an oversupply than to a deficiency, and (ii) the oversupply was realized by much larger increments in the experimental diets than within the range of deficiency which might lower the accuracy of determination.

Therefore, the present study was performed with smaller Met+Cys increments among the experimental diets (Table 2.1) to lower the effect of reason (ii). The range of Met+Cys between 1.8 – 3.0 % Met+Cys in diet DM (3.05 – 5.08 in % CP) (Table 2.1) covered the range of moderate to distinct oversupply and could be found in other studies (Kroeckel et al. 2015; von Danwitz et al. 2016).

As presented in Figure 2.1 and Table 2.2.2 the determined UCCs for all investigated parameters (RG, iWG, RFI, FGR, RCP) were within a narrow range of 2.23 % (± 0.44 s.e.) for iWG up to 2.56 % (± 0.45 s.e.) in diet DM for RFI. This range is smaller than that found by Klatt et al. (2016) (chapter 1). The UCC for RG in this study was 3.84 (± 0.68 s.e.), but the UCC with 4.85 (± 0.38 s.e.) Met+Cys in CP derived by Klatt et al. (2016) (chapter 1) is comparatively higher. Whereas for RFI the UCC given by Klatt et al. (2016) (chapter 1) is considerably lower than in the present study (3.80 ± 0.74 vs. 4.33 ± 0.76 Met+Cys in CP), which is in accordance to the UCC-values derived for FGR and RCP. However, the s.e. for the UCCs was as high as in our previous study (Klatt et al. 2016, chapter 1), mainly due to the high variation of the response parameters within diets leading to non-significant slopes for all investigated parameters (Figure 2.1; Tables 2.1 and 2.2). Therefore, it has to be concluded that UCCs for sulfur containing amino acids (Met+Cys) cannot be determined with a similar precision than the lower critical values.

The Cys concentration in our previous study was low and constant (Klatt et al. 2016, chapter 1). Therefore, in the current study a higher percentage of Cys in Met+Cys between 35 – 36 % (cp. 2.1) was used. Cys, as a semi-EAA, can replace Met up to 40 – 60 %, depending on species (hybrid striped bass (*Morone saxatilis* \times *M. chrysops*); channel catfish (*Ictalurus punctatus*); rainbow trout (*Oncorhynchus mykiss*); red drum (*Sciaenops ocellatus*)) (Griffin et al. 1994; Harding et al. 1977; Kim et al. 1992; Moon and Gatlin 1991). Even though this percentage of replacement is not investigated for turbot, it can be assumed that it is valid to use Cys concentrations below 40 % as in the current study for levels below strong Met+Cys oversupplies (Lewis 2003).

For the parameters final LW, Fulton's condition factor – FCF, hepatosomatic index – HSI, splenic index – SI presented in Table 2.3 no significant differences were detected. The FCF in this study is similar to values at the high performance level in other studies (Bonaldo et al. 2015; von Danwitz et al. 2016), independent of the Met+Cys concentration. In contrast to our findings HSI in other studies decreases at oversupplies realized by HMTBa or L-Met in turbot (Hu et al. 2015; Klatt et al. 2016, chapter 1), and DL-Met in Atlantic salmon (*Salmo salar*) (Espe et al. 2008). However, findings in literature regarding the relationship of Met+Cys oversupply and HSI are not consistent compared to own findings (Table 2.3) and the findings of Klatt et al. (2016) (chapter 1).

Spleen size as a key organ for hematopoiesis and blood cell storage could be a valid tool to evaluate potential toxic factors such as Met+Cys at oversupplies. The SI in this study tended

to increase with increasing Met+Cys concentration (Table 2.3). Van Bussel et al. (2012) found a twofold higher SI in turbot stocked at nitrate-N levels of 500 mg L⁻¹ than at 0 mg L⁻¹, as well as a negative correlation between the SI and the growth performance. The SI in the current study was clearly higher than the SI of fishes in a non-pathological state (van Bussel et al. 2012). Nevertheless, studies which reported effects on SI in turbot are rare. In a study with rainbow trout (*Oncorhynchus mykiss*) it was found that the size of the SI also relates to different genotypes and bacterial loads and is not automatically related to a pathological status (Hadidi et al. 2008). SI in the current study as an indirect parameter for potential metabolic toxicity of sulfur containing amino acids is limited. Physiological parameters associated with Met-metabolism and indicating a toxicity of sulfur containing AA (Met+Cys), like S-Adenosylhomocysteine concentration, are described for turbot (Klatt et al. 2016, chapter 1) and Atlantic salmon (Espe et al. 2008) and are stronger influenced by Met+Cys concentration than SI.

The chemical body composition presented in Table 2.4 is similar to the composition determined in other studies with turbot of a similar weight (Dietz et al. 2012; von Danwitz et al. 2016). Likewise, the parameters (moisture, CP, CL, CA, energy) are partly influenced by the Met+Cys concentration. Moisture, CL and energy concentrations are not influenced by the Met+Cys concentration in the diet. The differences in the CP and CA concentration (Table 2.4) between diets are inconsistently related to the growth performance (Figure 2.1; Tables 2.2.1 and 2.2.2). The low impact of the Met+Cys oversupply range (Table 2.1) on the chemical body composition is also shown by Klatt et al. (2016) (chapter 1).

Finally, the results of this study raise the question, whether the previously reported and discussed upper critical concentrations (Table 2.2.2; Figure 2.1) are relevant for practical dietary formulations. Feeding trials with finfish using high inclusion rates of protein sources rich in Met+Cys have shown similar UCCs of Met+Cys concentrations. Studies using turbot fed with high inclusion levels of rapeseed protein sources (Nagel et al. 2012b, 2014; von Danwitz et al. 2016), wheat gluten and free Met (Fournier et al. 2004; Kroeckel et al. 2015) realized Met+Cys concentrations in CP which are higher than some of the UCC-values of the current study. In studies with other finfish species and high amounts of protein fractions of rapeseed in diets for rainbow trout (Nagel et al. 2012a) or with feather meal in Japanese flounder (Kikuchi et al. 1994), Met+Cys could most likely have reached concentrations of excessive oversupplies. Even if the UCCs of Met+Cys for this species were not investigated, potential toxic effects cannot be excluded when exceeding the UCC for turbot. It can also be assumed that the decrease in performance in these studies may be partly related to Met+Cys oversupplies by exceeding the UCC of Met+Cys determined in the current study (Table 2.2.2; Figure 2.1).

Furthermore, it has to be considered that the UCC of Met+Cys determined in this study is influenced by the availabilities of Met+Cys of the protein sources used. Therefore, it is neces-

sary to investigate Met+Cys availabilities in turbot, especially for protein sources with high amounts of Met+Cys, (Fournier et al. 2004; Kikuchi et al. 1994; Øverland et al. 2009) to estimate their critical inclusion levels more precisely.

5. Conclusion

It was shown that the potential inclusion level of protein sources rich in Met+Cys (e.g.: rapeseed, wheat gluten, feather meal) should be restricted, due to its potential toxic effects. Furthermore, supplementation of free Met+Cys should take into account the risk of negative effects due to oversupplies and should be strictly limited for Met+Cys deficient diets. The large number of studies with fish, which exceeded the determined UCCs of Met+Cys, corroborates the relevance of the current data for scientific, as well as practical feed formulation for turbot aquaculture. Nevertheless, the availability of Met+Cys of different feed sources has to be taken into account and might have an impact on the UCC as well.

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CHAPTER 3

INTESTINAL AVAILABILITY AND TOXICITY OF METHIONINE + CYSTINE FROM RAPESEED PROTEIN CONCENTRATE IN DIETS OF JUVENILE TURBOT (*PSETTA MAXIMA*)

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Rapeseed protein concentrate; Methionine + Cystine; Availability; Oversupply; Antinutritional factors.

Abstract

As promising plant protein sources for several aquaculture species, rapeseed proteins were identified. In this study, two feeding trials in the intestinal availability (trial 1) and the toxicity (trial 2) of sulfur containing amino acids (Methionine, Met; Cystine, Cys) from rapeseed protein concentrate (RPC) were investigated for juvenile turbot (*Psetta maxima*).

In trial 1 the intestinal availability for Met+Cys of fish meal (FM) and RPC vs. free Met+Cys as 100 % control was determined after 15 feeding days. In addition, the effect of extra energy without Met+Cys vs. additional energy plus Met+Cys supplementation was analyzed. To ensure a linear response, all diets were deficient in Met+Cys and were fed restrictively. Each tank was stocked with 15 fishes with 42.12 g (\pm 4.2 s.d.) individual live weight (LW).

Trial 2 investigated the effect of Met+Cys oversupply at high inclusion level of RPC (66 % in dietary crude protein – CP) vs. FM control. Therefore, the range of 2.2 of up to 3.0 % Met+Cys in diet dry matter (DM), including four FM (2.2 %, 2.5%, 2.7 %, 3.0 % Met+Cys in diet DM) and two RPC diets (2.5 %, 3.0 %) were designed by L-Met and L-Cys supplementation. Fishes were fed for 56 days once a day until apparent satiation and each tank was stocked with 20 turbot with an individual mean LW of 30.7 g (\pm 3.1 s.d.).

In trial 1 the intestinal availabilities were calculated via slope ratio assay. For weight gain in % (WG) and retained crude protein in mg kg^{-0.8} d⁻¹ (RCP), values are 95.7 and 71.4 % for FM, as well as 65.3 and 58.0 % for RPC, respectively. The effect of additional energy with Met+Cys supplementation tend to result in higher RCP values than without Met+Cys supplementation.

In trial 2, the impact of Met+Cys oversupply or anti-nutritional factors (ANF) on relative growth (RG), relative feed intake (RFI) and feed to gain ratio (FGR) were evaluated via linear regression analyses. Accordingly, the whole range of oversupply or divided into oversupply areas (A = 2.2 – 2.5 %; B = 2.7 – 3.0 % Met+Cys in diet DM) for FM and RPC was analyzed. The results showed no significant decrease due to increased Met+Cys oversupply.

Thus, it can be concluded that the lower performance of RPC diets is mainly due to ANFs rather than to high Met+Cys concentrations. Furthermore, the low intestinal availability of Met+Cys in RPC decreases the risk of Met+Cys toxicity.

1. Introduction

In the fast growing aquaculture industry it is highly relevant to replace fish meal (FM) with alternative protein sources (FAO 2014), especially for carnivorous aquaculture species as turbot (*Psetta maxima*) with a high protein requirement (Cho et al. 2005; Lee et al. 2003; Li et al. 2011). Therefore, high amounts of plant proteins like gluten products (Kroeckel et al. 2013, 2015; Regost et al. 1999), soy (Bonaldo et al. 2015) or rapeseed products (Burel et al. 2000b; Nagel et al. 2014; von Danwitz et al. 2016) were used. Nevertheless, plant protein utilization capability for fish and in particular for turbot nutrition is still limited due to imbalanced nutrient and amino acid (AA) compositions, lower intestinal AA availabilities (Kaushik and Seiliez 2010) or due to the presence of a wide variety of anti-nutritional factors (ANF), such as phytate, tannins or glucosinolates (Burel et al. 2000a; Francis et al. 2001; von Danwitz et al. 2016).

For several aquaculture species, as rainbow trout (*Oncorhynchus mykiss*) (Teskeredžić et al. 1995), cobia (*Rachycentron canadum*) (Luo et al. 2012) or Japanese seabass (*Lateolabrax japonicus*) (Cheng et al. 2010) rapeseed proteins are promising plant protein sources. Especially highly purified products such as rapeseed protein concentrates (RPC) were successfully integrated in the diets of turbot in the recent years (Nagel et al. 2014; Slawski et al. 2011; von Danwitz et al. 2016). Even though, several factors in rapeseed products were suspected or identified to reduce turbot performance, such as thyroid status negatively correlated to glucosinolates concentration (Burel et al. 2000a), poor palatability (Nagel et al. 2014) or low availability of phosphorous and protein due to high phytate concentrations (Burel et al. 2000b; von Danwitz et al. 2016).

It is mentioned by several studies that the essential amino acid (EAA) concentration in RPC is comparable to pattern of high quality FM and therefore suitable as replacement protein (Cheng et al. 2010; Nagel et al. 2014; Slawski et al. 2011). Nevertheless, the concentration in RPC of sulfur containing EAA Methionine (Met) and the semi-EAA Cysteine (Cys) is high. Particularly high is the ratio of Cys:Met in RPC compared to FM. To avoid an unbalanced Met+Cys composition in the diets, additional information about the intestinal availability of Met+Cys is necessary (Kaushik and Seiliez 2010). However, Met+Cys availability in RPC for turbot is not yet determined. Until now for turbot only Lysine availabilities for wheat glens and FM were determined (Kroeckel et al. 2013, 2015).

Furthermore, besides reduced AA availability lower growth rates can also be caused by impairments of ANFs (Burel et al. 2000a, 2000b; von Danwitz et al. 2016). This might lead in practical diet formulation to an oversupply with sulfur containing amino acids, especially Cys, with potentially negative effects. For several terrestrial invertebrates as dogs (Merino et al. 1975), humans (Rees et al. 2006), pigs (Edmonds and Baker 1987) or rats (Regina et al. 1993; Sauberlich 1961) toxic effects of sulfur containing AAs were evaluated. Likewise the

upper critical concentrations of Met+Cys for growth performance and feed utilization were determined in diets for turbot (*P. maxima*) (Klatt et al. 2016, chapter 1; Klatt and Susenbeth 2016, chapter 2) and can be exceeded in diets with high RPC inclusion rates (Nagel et al. 2014; von Danwitz et al. 2016).

Therefore, the aim of the present study is to evaluate the intestinal Met+Cys availability of FM and RPC (trial 1). In addition, it was investigated how strong the effect of Met+Cys over-supply is at high inclusion level of RPC as compared to FM control (trial 2).

2. Materials and methods

The nutrient, energy and AA characteristics of the FM and RPC used in these trials are presented in Table 3.1.

2.1 Fishes and rearing facilities – Trial 1 and 2

For both trials (1 and 2) juvenile turbot were provided by *France turbot (le Bon Port, 85740 L'Epine, France)*. Trials were conducted at the *Gesellschaft für Marine Aquakultur mbH (GMA Büsum, Germany)* in a recirculating aquaculture system (RAS) with a total water volume of 20 m³, containing a drum-filter, a nitrifying moving-bed biofilter (4 m³), a protein-skimmer with ozonation and a UV light unit for disinfection. In this RAS other trials with turbot of the same origin were carried out at the same time.

The fishes were stocked in rectangular rearing tanks with a water volume of 150 L, a tank height of 0.52 m and a bottom surface of 0.29 m², respectively. The photoperiod was adjusted to a light:dark cycle of 14:10 hours.

2.2 Diets

2.2.1 Dietary design – Trial 1

The dietary ingredients, nutrients, energy and AA composition are presented in Table 3.2. Trial 1 was designed to induce strong (Basal(1)) up to moderate (2-FM(1); 2-RPC(1)) Met+Cys deficiency of <2.26 (± 0.04 s.e.) % Met+Cys in CP (Klatt et al. 2016, chapter 1). The diets containing the test components (FM, RPC, AA-mix. incl. free Met+Cys, AA-mix. excl. free Met+Cys) were included and were added via two additional increments. Hence, in all diets a 100% intake of the diet Basal(1) could be ensured. The two feed increments contain 122.5 % (increment 1) and 145 % (increment 2) were added containing only additional sulfur containing amino acids (Met+Cys) from the protein source to be examined. This leads to daily feed intakes of 0.735 % live weight (LW) in fresh matter for increment 1 and of 0.87 % LW in fresh matter for increment 2 (cp. 2.4.1.). Thus, the additional feed intake in both increments based only on the test components to be evaluated (Table 3.2). The dietary ingredients, nutrients, energy and AA composition are presented in Table 3.2.

Table 3.1: Composition of fish meal (FM) and rapeseed protein concentrate (RPC), (nutrient composition, amino acid profiles and anti-nutritional components (phytate, tannins) in RPC), utilized in the experimental diets in trial 1 and 2.

| Ingredients | Fish meal (FM) (<i>Clupea harengus</i>) ^a | Rapeseed protein concentrate (RPC) ^b |
|---|--|---|
| Nutrients [g 100 g ⁻¹ in DM] | | |
| Moisture | 6.4 | 6.4 |
| Crude protein | 64.4 | 73.8 |
| Crude lipid | 10.7 | 9.3 |
| Organic residues* | 1.9 | 8.5 |
| Crude Ash | 16.6 | 2.0 |
| Gross Energy [MJ kg ⁻¹] | 21.3 | 23.7 |
| Amino acid composition [g 16 g N ⁻¹] | | |
| essential and semi-essential amino acids | | |
| Lysine | 14.3 | 10.0 |
| Arginine | 12.1 | 15.3 |
| Methionine | 5.6 | 4.2 |
| Cyst(e)ine | 2.0 | 3.5 |
| Methionine+Cyst(e)ine | 7.6 | 7.8 |
| % Cyst(e)ine in Methionine+Cyst(e)ine | 26.5 | 44.3 |
| Threonine | 8.7 | 9.3 |
| Valine | 10.8 | 11.0 |
| Isoleucine | 8.8 | 9.0 |
| Leucine | 14.5 | 16.1 |
| Phenylalanine | 8.1 | 9.0 |
| Tyrosine | 5.7 | 6.0 |
| Histidine | 5.0 | 5.8 |
| Tryptophan | 2.3 | 3.1 |
| non-essential amino acids | 0.0 | 0.0 |
| Aspartic acid | 18.1 | 20.1 |
| Serine | 8.3 | 8.8 |
| Glutamic acid | 25.3 | 40.2 |
| Proline | 9.6 | 11.4 |
| Glycine | 15.1 | 12.0 |
| Alanine | 13.2 | 10.0 |
| Anti-nutritional factors [mg g ⁻¹ in DM] | | |
| Phytate | - | 10.3 |
| Tannins | - | 3.2 |
| Glucosinolates** | - | n.d. |

^a Vereinigte Fischmehlwerke Cuxhaven GmbH & Co KG, Cuxhaven, Germany;

^b Helm AG, Hamburg, Germany;

*Organic residues = CF + NfE = crude fiber + nitrogen free extracts = 100-(crude protein + crude lipid + crude ash).

**not determineable by *LUF A ITL GmbH* or *Öhmi Analytik GmbH*, due to the RPC-matrix.

The basal mixture Basal(1) was mixed and pelletized in combination with all extra used ingredients (Table 3.2). To ensure a linear increase as response to determine Met+Cys availability, all diets were designed to be deficient in Met+Cys concentration of $<2.26 (\pm 0.04 \text{ s.e.})$ % in CP (Klatt et al. 2016, chapter 1). The Met+Cys sources FM and RPC (Table 3.1) were included in increment 1 (FM = 1-FM(1), RPC = 1-RPC(1)) and increment 2 (FM = 2-FM(1), RPC = 2-RPC(1)). For the diets containing only free AAs in the incremental protein, only increment 1 could be realized (AA-mix. excl. Met+Cys = 0-Met(1), AA-mix. incl. Met+Cys = 1-Met(1)) (Table 3.2), due to acceptance problems of increment 2 in pre-tests. Within each increment (1 and 2) the amount of metabolic Met+Cys ($\text{mg kg}^{-0.8} \text{ d}^{-1}$) and metabolic gross energy (GE) intake ($\text{kJ kg}^{-0.8} \text{ d}^{-1}$) is similar, respectively (Table 3.4.1).

All diets were designed to be iso-nitrogenous (crude protein, CP = 56.1 % DM), iso-lipidic (crude lipids, CL = 14.2 % DM) and iso-energetic (GE = 22.0 kJ g^{-1} DM) (Table 3.2). Except Met+Cys, all other nutrients and EAAs were ensured to be sufficient (Cho et al. 2005; Kaushik 1998; Lee et al. 2003; Peres and Oliva-Teles 2008).

2.2.2 Dietary design – Trial 2

In trial 2, different diets were designed to divide potentially negative effects of RPC diets due to Met+Cys oversupply from other effects, related to further reasons (Table 3.3). Nutrient, energy and AA composition for all diets are given in Table 3.3. The diets C1(2), FM1(2), C2(2) and FM2(2) contain a constant concentration of FM (40 % in diet). In the diets RPC1(2) and RPC2(2), 66 % of FM CP were replaced by RPC (Tables 3.1 and 3.3), respectively. Two different Oversupply-areas (A+B) investigate ranges of slighter (Oversupply-area A = 2.2 – 2.5 % in diet DM) and of stronger (Oversupply-area B = 2.7 – 3.0 % in diet DM) Met+Cys oversupply (Klatt et al. 2016, chapter 1) (Table 3.3). In Oversupply-area A the diet C1(2) contain 2.2 % and the diets FM1(2) and RPC1(2) contain 2.5 % Met+Cys in diet DM. As in Oversupply-area B the diet C2(2) contain 2.7 % and the diets FM2(2) and RPC2(2) contain 3.0% Met+Cys in diet DM, respectively (Table 3.3).

All diets were balanced by non-essential amino acids (NEAA) and EAA to be iso-nitrogenous (CP = 59.1 % DM) and to meet all nutrients and required EAA concentration (Cho et al. 2005; Kaushik 1998; Lee et al. 2003; Peres and Oliva-Teles 2008), except Met+Cys oversupply. Likewise, diets were iso-lipidic (CL = 14.9 %) and iso-energetic (GE = 22.6 kJ g^{-1}) (Table 3.3).

2.3 Feed manufacturing – Trial 1 and 2

All diets in trial 1 and 2 were manufactured by an intense mixing and a stepwise addition of all ingredients (Tables 3.1, 3.2 and 3.3). Each mixture was pressed via a pelletizer (*L 14-175, AMANDUS KAHL, Reinbeck, Germany*), through a 4 mm matrix. Temperatures never exceeded 52 °C, in order to avoid heat damages of nutrients during the pelletizing process.

Table 3.2: Dietary formulations, nutrient and amino acid compositions in trial 1.

| Diet | Basal(1) | | 0-Met(1) | | 1-Met(1) | | 1-FM(1) | | 1-RPC(1) | | 2-FM(1) | | 2-RPC(1) | |
|---|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|
| | - | Basal(1) | no | free Met+Cys | fish meal | RPC | fish meal | RPC | fish meal | RPC | fish meal | RPC | fish meal | RPC |
| Basal(1) + additional Met+Cys source | increment 1 | | | | | | | | | | | | | |
| diet Basal(1) + increment step 1 or 2 | increment 2 | | | | | | | | | | | | | |
| additional amount of feed to the diet Basal(1) [%] | 100.0 | 122.5 | 122.5 | 122.5 | 122.5 | 122.5 | 122.5 | 122.5 | 122.5 | 122.5 | 122.5 | 122.5 | 122.5 | 122.5 |
| feed intake per tank [% fresh matter of initial LW] | 0.600 | 0.735 | 0.735 | 0.735 | 0.735 | 0.735 | 0.735 | 0.735 | 0.735 | 0.735 | 0.735 | 0.735 | 0.735 | 0.735 |
| feed intake per tank [g d ⁻¹ fresh matter] | 3.75 | 4.59 | 4.59 | 4.59 | 4.59 | 4.59 | 4.59 | 4.59 | 4.59 | 4.59 | 4.59 | 4.59 | 4.59 | 4.59 |
| intake of Met+Cys [mg kg ^{-0.8} d ⁻¹] (mean \pm s.d.) | 25.4 (\pm 0.2) | 25.6 (\pm 0.1) | 25.6 (\pm 0.1) | 37.4 (\pm 0.1) | 37.3 (\pm 0.2) | 37.1 (\pm 0.2) | 37.1 (\pm 0.2) | 37.1 (\pm 0.2) | 37.1 (\pm 0.2) | 37.1 (\pm 0.2) | 37.1 (\pm 0.2) | 37.1 (\pm 0.2) | 37.1 (\pm 0.2) | 37.1 (\pm 0.2) |
| intake of gross energy [GE, kJ kg ^{-0.8} d ⁻¹] (mean \pm s.d.) | 66.4 (\pm 0.6) | 80.6 (\pm 0.4) | 80.6 (\pm 0.4) | 80.8 (\pm 0.3) | 81.8 (\pm 0.5) | 81.4 (\pm 0.4) | 81.4 (\pm 0.4) | 81.4 (\pm 0.4) | 81.4 (\pm 0.4) | 81.4 (\pm 0.4) | 81.4 (\pm 0.4) | 81.4 (\pm 0.4) | 81.4 (\pm 0.4) | 81.4 (\pm 0.4) |
| Ingredients [g 100 g ⁻¹ in diet] | | | | | | | | | | | | | | |
| fish meal (<i>Clupea harengus</i>) ^a | 12.6 | 10.3 | 10.3 | 10.3 | 23.5 | 10.3 | 10.3 | 23.5 | 10.3 | 10.3 | 31.0 | 10.3 | 31.0 | 8.7 |
| rapeseed protein concentrate (RPC) ^b | - | - | - | - | - | 11.4 | 11.4 | - | 11.4 | 11.4 | - | 11.4 | - | 19.3 |
| L-Methionine ^c | - | - | - | 0.2 | - | - | - | - | - | - | - | - | - | - |
| L-Cystine ^e | - | - | - | 0.1 | - | - | - | - | - | - | - | - | - | - |
| essential amino acid (EAA) mix. (incl. Glycine) ^{d,*} | 16.6 | 20.2 | 20.2 | 20.2 | 13.9 | 14.5 | 14.5 | 13.9 | 14.5 | 14.5 | 12.1 | 14.5 | 12.1 | 13.1 |
| non-essential amino acid (NEAA) mix. ^{d,**} | 3.5 | 6.3 | 6.3 | 6.0 | 4.0 | 3.5 | 3.5 | 4.0 | 3.5 | 3.5 | 4.3 | 3.5 | 4.3 | 3.4 |
| pea protein ^e | 8.1 | 6.6 | 6.6 | 6.6 | 6.6 | 6.6 | 6.6 | 6.6 | 6.6 | 6.6 | 5.6 | 6.6 | 5.6 | 5.6 |
| porc protein hydrolysate ^f | 9.9 | 8.1 | 8.1 | 8.1 | 8.1 | 8.1 | 8.1 | 8.1 | 8.1 | 8.1 | 6.8 | 8.1 | 6.8 | 6.8 |
| hemoglobin powder (porc) ^g | 8.1 | 6.6 | 6.6 | 6.6 | 6.6 | 6.6 | 6.6 | 6.6 | 6.6 | 6.6 | 5.6 | 6.6 | 5.6 | 5.6 |
| blue mussel meal ^h | 5.0 | 4.1 | 4.1 | 4.1 | 4.1 | 4.1 | 4.1 | 4.1 | 4.1 | 4.1 | 3.4 | 4.1 | 3.4 | 3.4 |
| shrimp meal ^a | 1.5 | 1.2 | 1.2 | 1.2 | 1.2 | 1.2 | 1.2 | 1.2 | 1.2 | 1.2 | 1.0 | 1.2 | 1.0 | 1.0 |
| fish oil ^a | 12.4 | 12.9 | 12.9 | 12.9 | 11.4 | 11.8 | 11.8 | 11.4 | 11.8 | 11.8 | 10.8 | 11.8 | 10.8 | 11.4 |
| wheat starch ⁱ | 12.6 | 12.8 | 12.8 | 12.8 | 12.5 | 11.7 | 11.7 | 12.5 | 11.7 | 11.7 | 12.4 | 11.7 | 12.4 | 11.0 |
| Sodium-Carboxymethylcellulose ^j | 3.7 | 3.7 | 3.7 | 3.7 | 3.0 | 3.2 | 3.2 | 3.0 | 3.2 | 3.2 | 2.6 | 3.2 | 2.6 | 2.9 |
| Dicalciumphosphorous ^k | 4.5 | 5.9 | 5.9 | 5.9 | 3.7 | 5.7 | 5.7 | 3.7 | 5.7 | 5.7 | 3.1 | 5.7 | 3.1 | 6.5 |

Continuation(1) of Table 3.2

| Diet | Basal(1) | | 0-Met(1) | | 1-Met(1) | | 1-FM(1) | | 1-RPC(1) | | 2-FM(1) | | 2-RPC(1) | |
|--|---------------------------------------|----------|----------|-------------|--------------|-------------|-----------|------|-----------|-----|-----------|-----|-----------|-----|
| | - | Basal(1) | no | increment 1 | free Met+Cys | increment 1 | fish meal | RPC | fish meal | RPC | fish meal | RPC | fish meal | RPC |
| Basal(1) + additional Met+Cys source | diet Basal(1) + increment step 1 or 2 | | | | | | | | | | | | | |
| Vitamine/mineral premixture ¹ | 0.6 | | 0.6 | 0.6 | 0.6 | 0.6 | 0.6 | 0.6 | 0.6 | 0.6 | 0.6 | 0.6 | 0.6 | 0.6 |
| Dextrose ⁿ | 0.9 | | 0.7 | 0.7 | 0.7 | 0.7 | 0.7 | 0.7 | 0.7 | 0.7 | 0.6 | 0.6 | 0.6 | 0.6 |
| Nutrients [g 100 g ⁻¹ in diet DM] | | | | | | | | | | | | | | |
| Moisture | 5.6 | | 5.5 | 4.9 | 5.8 | 5.4 | 5.7 | 5.1 | | | | | | |
| Crude protein | 55.9 | | 55.7 | 55.2 | 56.8 | 56.5 | 56.7 | 56.0 | | | | | | |
| Crude lipid | 14.2 | | 14.4 | 14.5 | 14.3 | 14.2 | 14.0 | 14.0 | | | | | | |
| Crude fiber + NfE *** | 21.0 | | 20.4 | 20.9 | 18.9 | 19.7 | 19.0 | 20.1 | | | | | | |
| Crude Ash | 9.0 | | 9.6 | 9.4 | 10.0 | 9.6 | 10.3 | 9.9 | | | | | | |
| Gross Energy [kJ g ⁻¹ in DM] | 22.2 | | 21.9 | 21.8 | 22.1 | 22.1 | 22.1 | 22.0 | | | | | | |
| Calcium | 2.3 | | 2.6 | 2.5 | 2.5 | 2.5 | 2.6 | 2.7 | | | | | | |
| Phosphorous | 1.6 | | 1.8 | 1.8 | 1.7 | 1.8 | 1.7 | 2.0 | | | | | | |
| Ca/P | 1.4 | | 1.4 | 1.4 | 1.5 | 1.4 | 1.5 | 1.4 | | | | | | |

Continuation(2) of Table 3.2

| Diet | Basal(1) | | 0-Met(1) | | 1-Met(1) | | 1-FM(1) | | 1-RPC(1) | | 2-FM(1) | | 2-RPC(1) | | |
|--|----------|-------------|----------|--------------|--------------|-----------|-----------|------|----------|-----------|-----------|-----|----------|-------------|--|
| | - | Basal(1) | no | free Met+Cys | free Met+Cys | fish meal | fish meal | RPC | RPC | fish meal | fish meal | RPC | RPC | | |
| Basal(1) + additional Met+Cys source | | increment 1 | | | | | | | | | | | | increment 2 | |
| diet Basal(1) + increment step 1 or 2 | | increment 1 | | | | | | | | | | | | increment 2 | |
| Amino acid composition [g 16 g N ⁻¹] | | | | | | | | | | | | | | | |
| essential and semi-essential amino acids | | | | | | | | | | | | | | | |
| Lysine | | 10.2 | 10.4 | 9.9 | 9.4 | 9.5 | 9.3 | 9.0 | | | | | | | |
| Arginine | | 8.7 | 8.8 | 8.6 | 8.0 | 8.2 | 7.8 | 8.2 | | | | | | | |
| Methionine | | 1.0 | 0.8 | 1.0 | 1.2 | 1.0 | 1.3 | 1.0 | | | | | | | |
| Cyst(e)ine | | 0.6 | 0.5 | 0.7 | 0.6 | 0.9 | 0.7 | 1.0 | | | | | | | |
| Methionine+Cyst(e)ine | | 1.6 | 1.2 | 1.7 | 1.8 | 1.8 | 2.0 | 2.0 | | | | | | | |
| % Cyst(e)ine in Methionine+Cyst(e)ine | | 37.6 | 38.5 | 39.6 | 34.7 | 48.0 | 34.3 | 48.6 | | | | | | | |
| Threonine | | 5.1 | 5.1 | 5.0 | 4.7 | 4.9 | 4.7 | 4.9 | | | | | | | |
| Valine | | 5.6 | 5.2 | 5.0 | 5.2 | 5.6 | 5.4 | 5.6 | | | | | | | |
| Isoleucine | | 5.4 | 5.5 | 5.3 | 4.9 | 5.2 | 4.9 | 5.2 | | | | | | | |
| Leucine | | 9.2 | 8.9 | 8.5 | 8.4 | 8.8 | 8.4 | 8.8 | | | | | | | |
| Phenylalanine | | 5.1 | 5.0 | 4.7 | 4.7 | 4.9 | 4.7 | 4.8 | | | | | | | |
| Tyrosine | | 3.6 | 3.4 | 3.5 | 3.2 | 3.5 | 3.2 | 3.5 | | | | | | | |
| Histidine | | 2.7 | 2.3 | 2.2 | 2.4 | 2.5 | 2.4 | 2.5 | | | | | | | |
| Tryptophan | | 1.1 | 1.1 | 1.1 | 1.1 | 1.3 | 1.1 | 1.3 | | | | | | | |
| non-essential amino acids | | | | | | | | | | | | | | | |
| Aspartic acid | | 6.2 | 4.9 | 4.8 | 6.1 | 6.1 | 6.4 | 6.6 | | | | | | | |
| Serine | | 3.9 | 4.3 | 4.2 | 4.2 | 3.9 | 4.4 | 5.3 | | | | | | | |
| Glutamic acid | | 11.5 | 11.5 | 11.0 | 11.5 | 12.1 | 12.2 | 13.1 | | | | | | | |
| Proline | | 4.0 | 3.3 | 2.9 | 3.8 | 3.5 | 3.6 | 4.0 | | | | | | | |
| Glycine | | 9.4 | 10.8 | 10.3 | 9.7 | 9.1 | 9.9 | 9.0 | | | | | | | |
| Alanine | | 4.9 | 4.6 | 4.3 | 5.0 | 4.7 | 5.4 | 4.7 | | | | | | | |

Continuation(3) of Table 3.2

^a Vereinigte Fischmehlwerke Cuxhaven GmbH & Co KG, Cuxhaven, Germany;

^b Helm AG, Hamburg, Germany;

^c VWR International GmbH, Darmstadt, Germany;

^d Carl Roth GmbH & Co. KG, Karlsruhe, Germany;

^e Emsland-Stärke GmbH, Emlichheim, Germany;

^f Knochen- und Fett Union, Marl, Germany;

^g Günther Badenhof Fleischwerke KG, Verden-Aller, Germany;

^h Royal Frysk Muscheln GmbH, Emmelsbüll-Horsbüll, Germany, processed by the Costal Research & Management GmbH, Kiel, Germany;

ⁱ Kröner Stärke GmbH, Ibbenbüren, Germany;

^j Enorica GmbH, Norderstedt, Germany;

^k Lehmann & Voss & Co. KG, Hamburg, Germany;

^l Vitamin and Mineral mixture 517158 and 508240, Vitfoss, Grasten, Denmark;

^m Euroduna Rohstoffe GmbH, Barmstedt, Germany;

* essential amino acid mix. = EAA mix. = 19.0 g 100 g⁻¹ L-Lysine (HCl) + 15.9 g 100 g⁻¹ L-Arginine + 10.65 g 100 g⁻¹ L-Isoleucine + 12.7 g 100 g⁻¹ L-Leucine + 8.9 g 100 g⁻¹ L-Threonine + 7.7 g 100 g⁻¹ L-Tyrosine + 7.1 g 100 g⁻¹ L-Phenylalanine + 6.5 g 100 g⁻¹ L-Valine + 2.15 g 100 g⁻¹ L-Tryptophan + 1.4 g 100 g⁻¹ L-Histidine + 8.0 g 100 g⁻¹ Glycine;

** non-essential amino acid mix. = NEAA mix. = 40 g 100 g⁻¹ L-Glutamic acid + 30 g 100 g⁻¹ Glycine + 20 g 100 g⁻¹ L-Serine + 10 g 100 g⁻¹ L-Alanine;

*** Organic residues = CF + NFE = crude fiber + nitrogen free extracts = 100-(crude protein + crude lipid + crude ash).

Table 3.3: Dietary formulations, nutrient and amino acid compositions in trial 2.

| Diet | C1(2) | FM1(2) | RPC1(2) | C2(2) | FM2(2) | RPC2(2) |
|---|--------------------------------|--------|---------|--------------------------------|--------|---------|
| Met+Cys (g 100g ⁻¹ diet DM) | 2.2 | 2.5 | 2.5 | 2.7 | 3.0 | 3.0 |
| | Oversupply-area A [§] | | | Oversupply-area B [§] | | |
| Ingredients (g 100g ⁻¹ in diet) | | | | | | |
| fish meal (<i>Clupea harengus</i>) ^a | 40.0 | 13.3 | 40.0 | 40.0 | 13.3 | 40.0 |
| rapeseed protein concentrate (RPC) ^b | - | 33.7 | - | - | 33.7 | - |
| L-Methionine ^c | 0.5 | 0.6 | 0.6 | 0.7 | 0.9 | 0.9 |
| L-Cystine ^c | 0.2 | - | 0.2 | 0.4 | 0.2 | 0.5 |
| wheat gluten ^d | 17.0 | 17.0 | 17.0 | 17.0 | 17.0 | 17.0 |
| hemoglobin powder (porc) ^e | 4.8 | 4.8 | 4.8 | 4.8 | 4.8 | 4.8 |
| essential amino acid (EAA) mix. ^{f*} | 10.5 | 3.5 | 10.5 | 10.5 | 3.5 | 10.5 |
| non-essential amino acid (NEAA) mix. ^{f**} | 1.4 | 1.4 | 1.1 | 0.9 | 0.9 | 0.6 |
| fish oil ^a | 10.0 | 10.0 | 10.0 | 10.0 | 10.0 | 10.0 |
| wheat starch ^d | 11.5 | 11.5 | 11.5 | 11.5 | 11.5 | 11.5 |
| Calciumhydrogenephosphate ^g | 2.5 | 2.5 | 2.5 | 2.5 | 2.5 | 2.5 |
| Marker (TiO ₂) ^h | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 |
| Vitamine/mineral premixture ⁱ | 0.7 | 0.7 | 0.7 | 0.7 | 0.7 | 0.7 |
| Nutrients (g 100 g ⁻¹ in diet DM) | | | | | | |
| Moisture | 5.5 | 5.7 | 5.9 | 5.6 | 5.5 | 5.7 |
| Crude protein | 59.9 | 59.0 | 58.9 | 59.2 | 58.5 | 59.1 |
| Crude lipid | 15.1 | 15.0 | 14.6 | 15.0 | 14.9 | 14.7 |
| Organic residues *** | 14.0 | 15.1 | 19.1 | 14.7 | 15.4 | 19.0 |
| Crude Ash | 11.1 | 10.9 | 7.3 | 11.0 | 11.1 | 7.2 |
| Gross Energy (kJ g ⁻¹ in DM) | 22.7 | 22.6 | 22.4 | 22.6 | 22.7 | 22.4 |
| Calcium | 2.6 | 2.7 | 1.6 | 2.7 | 2.6 | 1.6 |
| Phosphorous | 1.7 | 1.8 | 1.2 | 1.7 | 1.7 | 1.1 |
| Ca/P | 1.5 | 1.5 | 1.4 | 1.6 | 1.6 | 1.4 |
| Amino acid composition (g 16 g N ⁻¹) | | | | | | |
| essential and semi-essential amino acids | | | | | | |
| Lysine | 11.1 | 11.4 | 6.8 | 11.7 | 11.4 | 7.1 |
| Arginine | 7.4 | 7.3 | 6.7 | 7.4 | 7.3 | 6.7 |
| Methionine | 2.5 | 2.7 | 2.8 | 2.9 | 3.1 | 3.3 |
| Cyst(e)ine | 1.3 | 1.5 | 1.5 | 1.7 | 1.8 | 1.8 |
| Methionine+Cyst(e)ine | 3.8 | 4.1 | 4.4 | 4.6 | 4.9 | 5.2 |
| % Cystine in Methionine+Cystine | 35.4 | 35.5 | 34.7 | 36.7 | 36.7 | 35.6 |
| Threonine | 4.8 | 4.8 | 4.4 | 4.8 | 4.8 | 4.3 |
| Valine | 5.1 | 4.8 | 4.9 | 4.8 | 4.7 | 5.5 |
| Isoleucine | 4.3 | 4.1 | 4.0 | 4.2 | 4.3 | 3.9 |
| Leucine | 7.1 | 7.2 | 7.8 | 7.2 | 7.1 | 7.9 |

Continuation(1) of Table 3.3

| Diet | C1(2) | FM1(2) | RPC1(2) | C2(2) | FM2(2) | RPC2(2) |
|--|--------------------------------|--------|---------|--------------------------------|--------|---------|
| Met+Cys (g 100g ⁻¹ diet DM) | 2.2 | 2.5 | 2.5 | 2.7 | 3.0 | 3.0 |
| | Oversupply-area A [§] | | | Oversupply-area B [§] | | |
| Phenylalanine | 4.8 | 4.7 | 4.9 | 4.8 | 4.7 | 4.8 |
| Tyrosine | 3.8 | 3.7 | 3.3 | 3.8 | 3.7 | 3.3 |
| Histidine | 2.7 | 2.6 | 3.0 | 2.7 | 2.6 | 2.9 |
| Tryptophan | 1.2 | 1.1 | 1.3 | 1.1 | 1.1 | 1.3 |
| non-essential amino acids | | | | | | |
| Aspartic acid | 5.8 | 5.8 | 7.4 | 5.8 | 5.8 | 7.5 |
| Serine | 3.6 | 3.6 | 4.2 | 3.6 | 3.6 | 4.2 |
| Glutamic acid | 17.2 | 17.4 | 23.7 | 17.6 | 17.2 | 23.0 |
| Proline | 5.9 | 6.0 | 7.2 | 6.0 | 5.6 | 7.1 |
| Glycine | 5.2 | 5.1 | 5.9 | 5.1 | 4.9 | 5.7 |
| Alanine | 4.9 | 4.7 | 5.0 | 4.7 | 4.5 | 5.0 |

^a Vereinigte Fischmehlwerke Cuxhaven GmbH & Co KG, Cuxhaven, Germany;

^b Helm AG, Hamburg, Germany;

^c VWR International GmbH, Darmstadt, Germany;

^d Kröner Stärke GmbH, Ibbenbüren, Germany;

^e Günther Badenhof Fleischwerke KG, Verden-Aller, Germany;

^f Carl Roth GmbH & Co. KG, Karlsruhe, Germany;

^g Lehmann & Voss & Co. KG, Hamburg, Germany;

^h Kronos International Inc., Leverkusen, Germany;

ⁱ Vitamin and Mineral mixture 517158 and 508240, Vitfoss, Grasten, Denmark;

* essential amino acid mix. = EAA mix. = 36.0 g 100 g⁻¹ L-Lysine (HCl) + 18.0 g 100 g⁻¹ L-Arginine + 7.2 g 100 g⁻¹ L-Isoleucine + 5.0 g 100 g⁻¹ L-Leucine + 10.0 g 100 g⁻¹ L-Threonine + 8.0 g 100 g⁻¹ L-Tyrosine + 6.0 g 100 g⁻¹ L-Phenylalanine + 6.0 g 100 g⁻¹ L-Valine + 1.3 g 100 g⁻¹ L-Tryptophan + 2.5 g 100 g⁻¹ L-Histidine;

** non-essential amino acid mix. = NEAA mix. = 60 g 100 g⁻¹ L-Glutamic acid + 20 g 100 g⁻¹ Glycine + 20 g 100 g⁻¹ L-Alanine;

*** Organic residues = CF + NfE = crude fiber + nitrogen free extracts = 100-(crude protein + crude lipid + crude ash);

[§]Oversupply-area A: C1(2), MC1(2), RPC1(2), 2.2 - 2.5 % Met+Cys in diet DM;

[§]Oversupply-area B: C2(2), MC2(2), RPC2(2), 2.7 - 3.0 % Met+Cys in diet DM.

2.4 Experimental procedures

2.4.1 Experimental procedure – Trial 1

In trial 1 the fishes were fed for 15 days. Fishes were adapted to the rearing conditions for 8 days prior the experimental start. Diet Basal(1) was fed to five randomly selected tanks, all other diets (0-Met(1), 1-Met(1), 1-FM(1), 1-RPC(1), 2-FM(1), 2-RPC(1)) were fed to three tanks, respectively (Table 3.2). Tanks were stocked with 15 juvenile turbot, with an individual LW of 42.12 g (\pm 4.2 s.d.) resulting in an average tank LW of 631.9 g (\pm 3.4 s.d.). All diets were given restrictively. The diet Basal(1) was fed as 0.6 %, for increment 1 (0-Met(1), 1-Met(1), 1-FM(1), 1-RPC(1)) and 2 (2-FM(1), 2-RPC(1)) 0.735 and 0.87 % feed intake of initial average tank LW were given, respectively (Table 3.2). Feed was given in equal proportions twice daily and no feed remained un-eaten in the tank.

Water parameters were measured daily. Temperature and salinity were kept at 18.2 °C (± 0.5 s.d.) and 25 ppt (± 0.9 s.d.) (*HI 96822 Seawater Refractometer, Hanna Instruments Inc., Woonsocket-RI-USA*) (Imsland et al. 2001). Oxygen level was 9.3 mg L⁻¹ (± 0.4 s.d.) with a saturation of 114.1 % (± 4.5 s.d.) (*Handy Polaris, Oxy-Guard International A/S, Birkerod, DK*). The pH was 7.5 (± 0.1 s.d.) (*GMH 3530, Digital pH-/ mV-/ Thermometer, Greisinger electronic, D*). The total NH₄-N and the NO₂-N concentration were 0.2 mg L⁻¹ \pm 0.1 and 0.4 mg L⁻¹ \pm 0.1 s.d. (*Microquant test kits for NH₄⁺ and NO₂⁻, Merck KGaA, Darmstadt, Germany*), respectively.

3.2.2 Experimental procedure – Trial 2

In trial 2, the turbot were fed once a day until apparent satiation for 56 days. Pellets which remained 15 minutes after feeding in the tanks were siphoned out to correct the feed intake by the average pellet weight. The adaption to the rearing conditions lasted 6 days before the experiment started. Each diet in trial 2 (Table 3.3) was fed to three randomly selected tanks. Each tank was stocked with 20 turbot. At the start of the growth trial (day 0), individual mean LW was 30.7 g (± 3.1 s.d.), with an average tank LW of 614.7 g (± 6.2 s.d.).

Water parameters were measured once per day. The average oxygen level was 9.2 mg L⁻¹ (± 0.3 s.d.), with a saturation of 112.5 % (± 4.3 s.d.) (*Handy Polaris, Oxy-Guard International A/S, Birkerod, DK*) and the water temperature was maintained at 18.1 °C (± 0.3 s.d.), with a salinity level of 24 ppt (± 1.2) (*HI 96822 Seawater Refractometer, Hanna Instruments Inc., Woonsocket-RI-USA*) (Imsland et al. 2001). The pH was 7.6 (± 0.1 s.d.) (*GMH 3530, Digital pH-/ mV-/ Thermometer, Greisinger electronic, D*), the total NH₄-N and the NO₂-N concentration were 0.1 mg L⁻¹ (± 0.1 s.d.) and 0.3 mg L⁻¹ (± 0.1 s.d.) (*Microquant test kits for NH₄⁺ and NO₂⁻, Merck KGaA, Darmstadt, Germany*), respectively.

2.5 Sampling

2.5.1 Sampling – Trial 1

Before the start of trial 1 (day 0), for three initial pooled samples (n = 7 fishes per sample), the individual liver weight was determined to calculate the initial hepatosomatic index (HSI). Subsequently, these fishes were chopped and freeze-dried (-18°C) in a plastic bag per pooled sample (n = 3), until analyses of initial chemical body composition. All turbot had a similar initial LW distribution as fishes stocked in the tanks (Table 3.5).

Prior to final sampling after 15 feeding days, the fishes were not fed for 24 hours to ensure a depleted gut. For every tank the total LW, individual LW and length were determined for every fish to calculate the Fulton's condition factor (FCF). For the chemical body composition a sample of 7 randomly selected fishes per tank were chopped, pooled and stored (-18°C) in plastic bags prior to chemical analyses, similar to the initial samples. To determine the final HSI, individual liver weight were recorded from 7 fishes per tank (Table 3.5).

2.5.2 Sampling – Trial 2

At day 0 of trial 2, two pooled samples with 8 turbot, respectively, with a similar initial LW distribution as fishes stocked for the growth trial were chopped and stored (-18°C) in a plastic bag to be analyzed for initial chemical body composition (Table 3.7).

Before starting final sampling after 56 feeding days, fishes remained unfed in their tanks for 48 hours to ensure a depleted gut. For every tank the total fish LW, individual LW and length were determined for every fish to calculate FCF. For the chemical body composition a sample of 6 randomly selected fishes per tank were chopped, pooled and stored (-18°C) in a plastic bag prior chemical body analyses. To determine HSI and splenic index (SI), individual liver and spleen weight were recorded from 6 fishes per tank (Table 3.7).

After three more feeding days (until apparent satiation) of the diets FM1(2), RPC1(2), FM2(2) and RPC2(2), blood samples were taken from the remaining fishes with heparinized syringes (n = 3 per tank) from the caudal vein 5 hours after feeding. Blood was centrifuged to separate blood plasma, aliquoted and the plasma immediately stored on ice and transferred to a deep freezer (-80°C).

2.6 Chemical analyses – Trial 1 and 2

The fishes used for chemical analyses were freeze-dried. Subsequently, the whole samples were minced ($\leq 1\text{mm}$) to be homogenized by a centrifugal mill (*ZM 100, Retsch GmbH & Co. KG, Haan, Germany*). Pelletized feeds were pulverized by a mortar and a pestle.

The dry matter (DM), crude ash (CA), crude protein (CP), crude lipids (CL) and the energy content for all diets as well as for the total body compositions of the fishes were analyzed in duplicates according to Weender Analysis (European Union 2009).

The DM content was determined by drying the samples for 4 hours at 103°C. The CA content was analyzed by remaining the sample for 4 hours in a combustion oven at 550°C. CP ($N \times 6.25$) was determined by Kjeldahl method and CL was analyzed by Soxhlet extractor. The energy was measured by an adiabatic bomb calorimeter (*C200, IKA-Werke GmbH & Co. KG, Staufen, Germany*). The organic residues in DM (crude fiber – CF and nitrogen free extracts – NFE), were calculated by subtracting CP, CL and CA from 100 %.

The AA composition (Table 3.2) of the diets were determined by *LUFA-ITL GmbH (Kiel, Germany)* according to the method *VO(EG) 152/2009, III, F*. Tryptophan was analyzed by the method *VO(EG) 152/2009, III, G*. Calcium and Phosphorus were determined according to *VDLUF A III, 10.8.2*.

For trial 2 concentrations of thyroidal hormones (total thyroxine (T4), total triiodothyronine (T3)) in blood plasma were determined by using a solid phase enzyme-linked immunosorbent assay (ELISA) (*IBL International GmbH, Hamburg, Germany*). The ELISA based on a competitive binding, where hormone concentrations were measured photometrically at a wave-length of 450 nm.

2.7 Definitions and calculations – Trial 1 and 2

Following parameters are defined as follows:

Availability of Met+Cys sources = $(\text{slope}_{\text{Met+Cys source}} / \text{slope}_{\text{free Met+Cys}}) * 100$

Relative growth (RG) = $(\ln(\text{final live weight (g)}) - \ln(\text{initial live weight (g)})) * 100 / \text{feeding days}$

Weight gain (WG in %) = $((\text{final live weight (g)} / \text{initial live weight (g)}) - 1) * 100$

Relative feed intake (RFI) = $\text{feed intake in \% of live weight} / \text{feeding days}$

Feed to Gain Ratio (FGR) = $\text{g feed intake (DM)} / \text{g live weight gain}$

Geometric mean live weight (GMLW) in g = $(\text{initial live weight} * \text{final live weight})^{0.5}$

Metabolic live weight (MLW) in kg = $(\text{GMLW} / 1000)^{0.8}$ (according to Dietz et al. 2012 and Lupatsch et al. 2003)

Retained crude protein (RCP) in $\text{mg kg}^{-0.8} \text{d}^{-1}$ = $(\text{mg retained crude protein} / \text{GMLW}) / \text{feeding days}$

Fulton's condition factor (FCF) = $(\text{live weight in g} / (\text{total length in cm})^3) * 100$

Hepatosomatic index (HSI) = $(\text{g liver weight} / \text{g live weight}) * 100$

Splenic index (SI) = $(\text{g spleen weight} / \text{g live weight}) * 100$

2.8 Statistical analyses – Trial 1 and 2

The statistical software R (R Core team 2015) was used to evaluate the data. The graphical design of the Figures was done by Excel 2010 (MS Office).

The data were analyzed with a mixed model (Laird and Ware 1982; Verbeke and Molenberghs 2000) for the parameters with several values per tank (initial and final individual LW, FCF, HSI, SI, T₃, T₄) (Figure 3.4; Tables 3.5 and 3.7) and a linear model for chemical body composition (one value per tank) (Tables 3.5 and 3.7).

The slope-ratio approach e.g. described by Littell et al. (1997) was used to determine the intestinal availability of the sulfur containing amino acid sources.

All data-sets were assumed to be normally distributed and homoscedastic based on a graphical residual analyses. The statistical model included all treatments (trial 1: 0-Met(1), 1-Met(1), 1-FM(1), 1-RPC(1), 2-FM(1), 2-RPC(1), increment 1 and 2; trial 2: 0-Met(1), 1-Met(1), 1-FM(1), 1-RPC(1), 2 (2-FM(1), 2-RPC(1), Oversupply-area A and B) as fixed factors. The tank was regarded as random factor. The linear regression analysis, conducted for trial 2, was done for the parameter RG, RFI and FGR as response to Met+Cys concentration, respectively.

For the thyroid parameters T₃ and T₄ in trial 2 a 2-way ANOVA was used to evaluate Met+Cys concentration and feed source (FM, RPC) as impact factors. All other parameters were analyzed by 1-way ANOVA. $P < 0.05$ was defined as the level of significance. All-pair comparisons (Tukey) were conducted (Bretz et al. 2011) in order to compare the several levels of the influence factors.

The intercepts and slopes were classified by \pm standard error (s.e.). All remaining data are presented as arithmetic mean values \pm standard deviation (s.d.).

3. Results

No fish died or was removed before sampling.

3.1 Results – Trial 1

3.1.1 Growth efficiency, nutrient utilization and availabilities – Trial 1

In Table 3.4.1 mean values (\pm s.d.) are given for weight gain (WG) and retained crude protein (RCP) as response and for gross energy (GE) intake ($\text{kJ kg}^{-0.8} \text{d}^{-1}$) and Met+Cys intake ($\text{mg kg}^{-0.8} \text{d}^{-1}$) as impacts and were further evaluated by linear regression analyses (Table 3.4.2; Figures 3.1 and 3.2)

In Figure 3.1 it is illustrated that the increase of GE intake without additional Met+Cys has no relevant effect ($m = 3.93$). Whereas the addition of Met+Cys with the same GE intake shows an increase ($m = 18.67$). This shows that Met+Cys is the first limiting factor in trial 1. Thus, all slopes in Figure 3.2 and Table 3.4.2 are due to different Met+Cys availabilities.

Figure 3.2 illustrates the relation of metabolic Met+Cys intake ($\text{mg kg}^{-0.8} \text{d}^{-1}$) and WG. The impact of Met+Cys intake ($\text{mg kg}^{-0.8} \text{d}^{-1}$) from the sources free Met+Cys, fish meal (FM), and rapeseed protein concentrate (RPC) on WG and RCP are given in Table 3.4.2. Relative to free Met+Cys the intestinal availabilities of Met+Cys in FM for WG are 95.7 % and for RCP 71.4 % and higher than the availabilities in RPC for WG (65.3 %) and RCP (58.0 %) (Table 3.4.2).

3.1.2 Growth performance and biometrical parameters – Trial 1

As presented in Table 3.5 for trial 1, no significant differences between diets could be found between the final individual live weight (LW), the Fulton's condition factor (FCF) and the hepatosomatic index (HSI).

3.1.3 Chemical body composition – Trial 1

The parameter describing the chemical body composition (moisture, CP (crude protein), CL (crude lipid), energy), except the parameter CA (crude ash), are not influenced by Met+Cys concentration or source, as well not different to the initial values. Only CA concentrations are significantly higher for the treatment o-Met(1) compared to the initial concentration (Table 3.5).

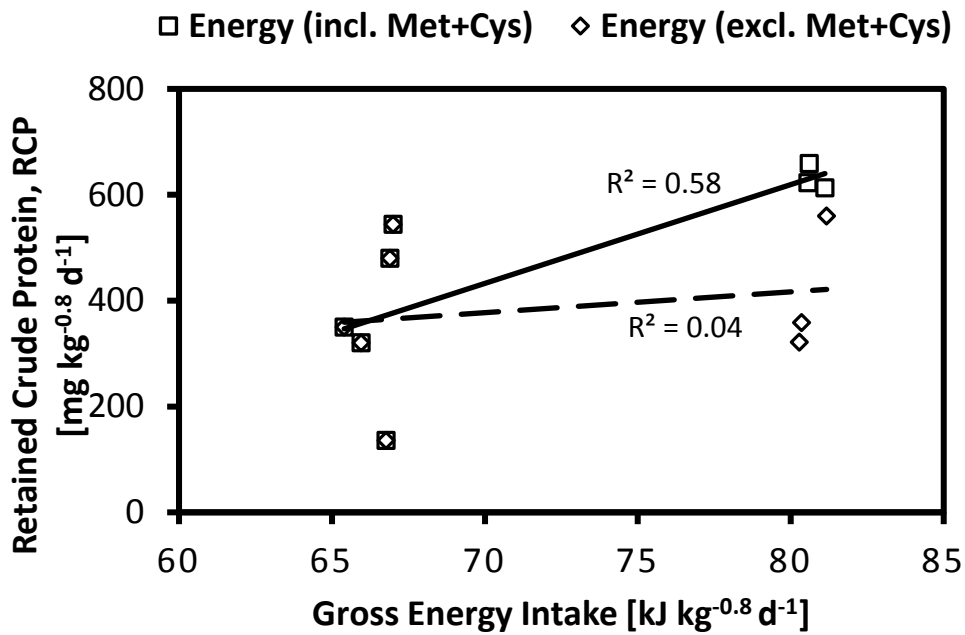


Figure 3.1 (Trial 1): Effect of gross energy (GE) intake [kJ kg^{-0.8} d⁻¹] with and without additional Met+Cys supplementation on retained crude protein (RCP) [mg kg^{-0.8} d⁻¹]. □ Energy (incl. Met+Cys) = $y = 18.7 \pm 9.2x - 874.1 \pm 662.1$; ◇ Energy (excl. Met+Cys) = $y = 3.9 \pm 9.3x + 101.8 \pm 668.5$; slopes and intercepts are not significant ($P \geq 0.05$); \pm standard error.

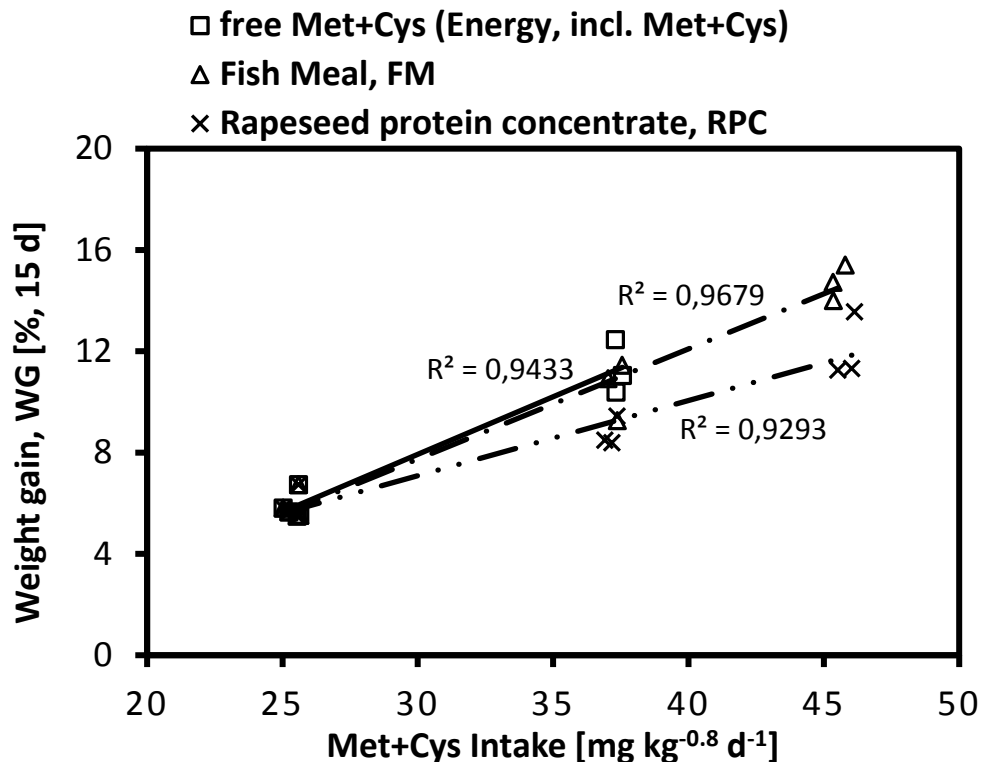


Figure 3.2 (Trial 1): Effect of Met+Cys intake [mg kg^{-0.8} d⁻¹] from different sources (free Met+Cys, fish meal, rapeseed protein concentrate), on weight gain (WG) [% 15 d]. □ free Met+Cys = $y = 0.45 \pm 0.06x - 5.7 \pm 1.9$; △ fish meal, FM = $y = 0.43 \pm 0.04x - 5.3 \pm 1.3$; × rapeseed protein concentrate, RPC = $y = 0.30 \pm 0.04x - 1.8 \pm 1.3$; slopes and intercepts are significant ($P < 0.001$); \pm standard error.

Table 3.4.1 (Trial 1): Arithmetic mean values (\pm standard deviation) of weight gain [WG in %] and retained crude protein [RCP in $\text{mg kg}^{-0.8} \text{d}^{-1}$] in turbot at different Met+Cys [$\text{mg kg}^{-0.8} \text{d}^{-1}$] and gross energy intakes [GE, $\text{kJ kg}^{-0.8} \text{d}^{-1}$] from free Met+Cys, fish meal (FM) and rapeseed protein concentrate (RPC) (arithmetic mean \pm standard deviation).

| Diet | Basal(1) | 0-Met(1) | 1-Met(1) | 1-FM(1) | 1-RPC(1) | 2-FM(1) | 2-RPC(1) |
|---|-------------------|-------------------|------------------|------------------|------------------|------------------|------------------|
| intake of Met+Cys (mean \pm s.d.) | 25.4 \pm 0.2 | 25.6 \pm 0.1 | 37.4 \pm 0.1 | 37.3 \pm 0.2 | 37.1 \pm 0.2 | 45.5 \pm 0.2 | 45.9 \pm 0.3 |
| intake of gross energy (GE) (mean \pm s.d.) | 66.4 \pm 0.6 | 80.6 \pm 0.4 | 80.8 \pm 0.3 | 81.8 \pm 0.5 | 81.4 \pm 0.4 | 96.0 \pm 0.4 | 96.8 \pm 0.6 |
| WG ¹ | 5.8 \pm 0.5 | 5.2 \pm 0.6 | 11.3 \pm 0.9 | 10.5 \pm 0.9 | 8.8 \pm 0.5 | 14.7 \pm 0.6 | 12.0 \pm 1.1 |
| RCP ² | 366.4 \pm 141.4 | 413.6 \pm 104.8 | 632.0 \pm 19.8 | 477.4 \pm 72.2 | 442.2 \pm 58.2 | 706.2 \pm 35.1 | 647.6 \pm 81.8 |

¹WG = weight gain (%) = ((final live weight (g) / initial live weight (g)) - 1) * 100; ²RCP = Retained crude protein per fish ($\text{mg kg}^{-0.8} \text{d}^{-1}$).

Table 3.4.2 (Trial 1): Regression coefficients¹ for Met+Cys intake ($\text{mg kg}^{-0.8} \text{d}^{-1}$) in turbot fed different Met+Cys sources (free Met+Cys, fishmeal (FM) rapeseed protein concentrate (RPC)) (\pm standard error).

| Parameter | Met+Cys source | b (intercept) | m (slope) | availability ⁴ | | |
|------------------|----------------|--------------------|---------------------|---------------------------|------|-------|
| WG ² | free Met+Cys | -5.7 \pm 1.9 | *** 0.45 \pm 0.06 | *** 100.0 | | |
| | FM | -5.3 \pm 1.3 | *** 0.43 \pm 0.04 | *** 95.7 | | |
| | RPC | -1.8 \pm 1.3 | *** 0.30 \pm 0.04 | *** 65.3 | | |
| RCP ⁴ | free Met+Cys | -200.5 \pm 335.0 | n.s. | 22.3 \pm 11.0 | n.s. | 100.0 |
| | FM | -53.6 \pm 224.8 | * | 15.9 \pm 6.4 | * | 71.4 |
| | RPC | 21.7 \pm 222.0 | n.s. | 12.9 \pm 6.3 | n.s. | 58.0 |

¹y = b + mx, describing the Met+Cys utilization efficiency as a function of intake ($\text{mg kg}^{-0.8} \text{d}^{-1}$);

²WG = weight gain (%) = ((final live weight (g) / initial live weight (g)) - 1) * 100;

³RCP = Retained crude protein per fish ($\text{mg kg}^{-0.8} \text{d}^{-1}$);

⁴availability of FM and RPC (relative to free Met+Cys) = (slope_{FM} or RPC / slope_{free Met+Cys}) * 100;

n.s. = not significant; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Table 3.5 (Trial 1): Growth performance, biometrical parameter and chemical body composition in turbot, fed different Met+Cys sources (free Met+Cys, fish meal (FM), rapeseed protein concentrate (RPC)) and feed amounts (arithmetic mean \pm standard deviation).

| diet | initial | Basal(1) | 0-Met(1) | 1-Met(1) | 1-FM(1) | 1-RPC(1) | 2-FM(1) | 2-RPC(1) |
|--|----------------------------|-----------------------------|----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|
| Met+Cys (g 100g ⁻¹ diet DM) | | 0.600 | 0.735 | 0.735 | 0.735 | 0.735 | 0.870 | 0.870 |
| initial individual LW ¹ | 42.0 \pm 5.2 | 42.2 \pm 4.0 | 42.2 \pm 4.2 | 42.1 \pm 3.9 | 42.0 \pm 4.2 | 42.2 \pm 4.8 | 42.1 \pm 4.6 | 42.1 \pm 4.5 |
| final indi- vidual LW ¹ | - | 44.6 \pm 4.3 | 44.4 \pm 4.9 | 46.9 \pm 5.0 | 46.5 \pm 4.7 | 45.9 \pm 5.2 | 48.3 \pm 5.1 | 47.1 \pm 5.4 |
| FCF ² | - | 1.8 \pm 0.1 | 1.8 \pm 0.1 | 1.8 \pm 0.1 | 1.8 \pm 0.1 | 1.8 \pm 0.1 | 1.8 \pm 0.1 | 1.8 \pm 0.1 |
| HSI ³ | 1.1 \pm 0.3 | 0.9 \pm 0.2 | 1.0 \pm 0.3 | 0.9 \pm 0.2 | 1.0 \pm 0.2 | 0.9 \pm 0.2 | 0.9 \pm 0.3 | 1.1 \pm 0.3 |
| moisture Crude Protein Crude Lipid | 78.0 \pm 0.1 | 77.9 \pm 0.4 | 77.7 \pm 0.5 | 77.8 \pm 0.2 | 78.0 \pm 0.3 | 77.9 \pm 0.2 | 78.1 \pm 0.1 | 77.5 \pm 0.1 |
| | 14.0 \pm 0.0 | 14.2 \pm 0.4 | 14.4 \pm 0.4 | 14.2 \pm 0.1 | 13.9 \pm 0.3 | 14.0 \pm 0.1 | 14.0 \pm 0.1 | 14.2 \pm 0.2 |
| | 4.0 \pm 0.2 | 3.6 \pm 0.2 | 3.6 \pm 0.3 | 3.6 \pm 0.0 | 3.6 \pm 0.1 | 3.8 \pm 0.1 | 3.7 \pm 0.2 | 4.0 \pm 0.1 |
| Crude Ash | 3.5 \pm 0.1 ^a | 3.8 \pm 0.1 ^{ab} | 3.9 \pm 0.1 ^b | 3.7 \pm 0.1 ^{ab} | 3.7 \pm 0.1 ^{ab} | 3.7 \pm 0.2 ^{ab} | 3.6 \pm 0.1 ^{ab} | 3.8 \pm 0.1 ^{ab} |
| Energy | 4.7 \pm 0.1 | 4.6 \pm 0.1 | 4.6 \pm 0.1 | 4.7 \pm 0.1 | 4.6 \pm 0.1 | 4.7 \pm 0.1 | 4.6 \pm 0.1 | 4.8 \pm 0.0 |

Different superscript letters within the same row represent significant differences (Tukey test, $P < 0.05$); ¹LW = live weight; ²FCF = Fulton's condition factor = (live weight in g / body length in cm³) * 100; ³HSI = hepatosomatic index = (g liver weight / g live weight) * 100.

3.2 Results – Trial 2

3.2.1 Growth performance, feed utilization, physiological and biometrical parameters – Trial 2

In Table 3.6.1 the mean values (\pm s.d.) are given for RG, RFI and FGR and in Table 3.6.2 the linear regression equations (Table 3.6.2; Figures 3.3.1 and 3.3.2). In Figure 3.3.1, the two different Oversupply-areas (A+B) (cp. 2.2.2.) are illustrated, which cover different areas of Met+Cys oversupply, which is exemplarily illustrated for RG as response. All presented parameter in Table 3.6.2 (RG, RFI and FGR) show no significant difference between the intercepts of FM as well as for RPC, hence Oversupply-area A and B are not significantly different. Likewise can be observed for the slopes within the Oversupply-areas (Figure 3.3.1; Table 3.6.2), which means that the treatments FM and RPC within both Oversupply-areas (A+B) are not significantly different.

Figure 3.3.2 illustrates the effect of Met+Cys concentration on RG. The intercepts of the regression equation for Met+Cys-total and RPC-total of RG, RFI and FGR are significant, whereas the slopes within the whole investigated Met+Cys range are not significant, neither for FM nor for RPC (Figure 3.3.2; Table 3.6.2). The intercepts for FM present superior intercepts compared to RPC for RG, RFI and FGR (Table 3.6.2). Differences in the regression equations between FM and RPC can be observed especially for the parameter RG (Figure 3.3.2).

The thyroidal hormones triiodothyronine (T₃) and thyroxine (T₄) in blood plasma show no significant difference between the feed source (FM vs. RPC) and the Met+Cys concentration (Figure 3.4).

The final individual LW is significantly lower for the rapeseed diet RPC₁₍₂₎ compared to the fish meal diets C₁₍₂₎, C₂₍₂₎ and FM₂₍₂₎, the biometrical parameter FCF, HSI and SI are not significantly different between treatments (Table 3.7).

3.2.2 Chemical body composition – Trial 2

Moisture and CA concentrations in the body indicate no significant differences and for energy only the initial concentration is significantly lower (Table 3.7). The CP of the chemical body composition shows no significant differences for the rapeseed diets (RPC₁₍₂₎, RPC₂₍₂₎) compared to the initial value, whereas the FM diets (C₁₍₂₎, FM₁₍₂₎, FM₂₍₂₎) are significantly higher. For the CL only the diets C₁₍₂₎ and C₂₍₂₎ were significantly different to the initial concentration. However, no significant differences between all RPC and FM diets were found for CP and CL, as well (Table 3.7).

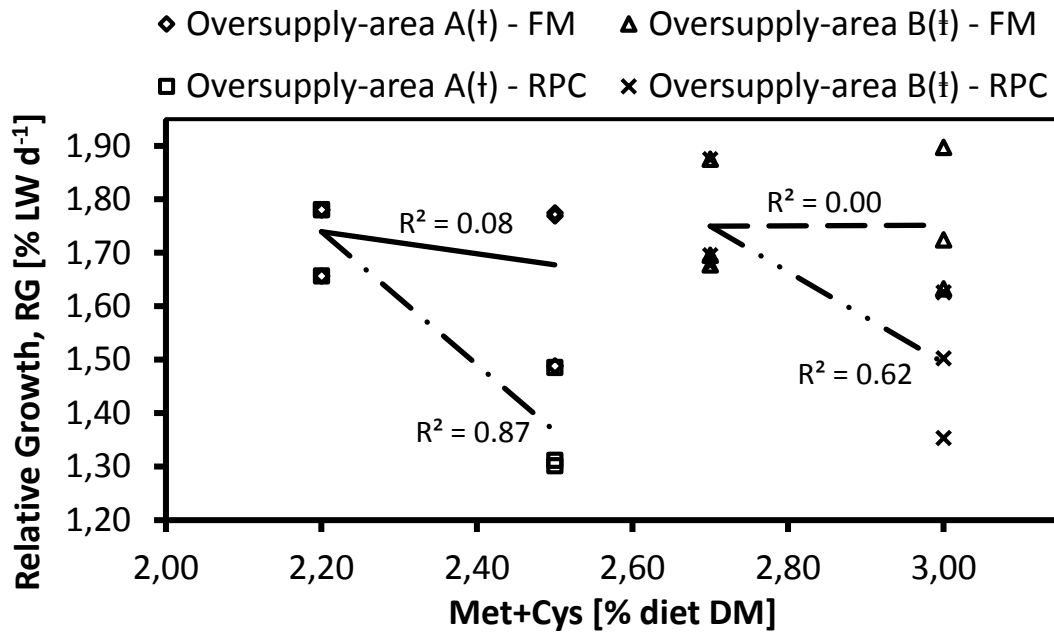


Figure 3.3.1 (Trial 2): Effect between Met+Cys concentration [% diet DM] from fish meal (FM) and rapeseed protein concentrate (RPC) and relative growth (RG) [% LW d⁻¹] on different Oversupply-areas (A+B). † Oversupply-area A: 2.2 - 2.5 % Met+Cys in diet DM; ‡ Oversupply-area B: 2.7 - 3.0 % Met+Cys in diet DM; ◆ Oversupply-area A – FM = $y = -0.2 \pm 0.4x + 2.2 \pm 1.0$; □ Oversupply-area A – RPC = $y = -1.2 \pm 0.4x + 4.5 \pm 1.0$; ▲ Oversupply-area B – FM = $y = 0.0 \pm 0.4x + 1.8 \pm 1.2$; × Oversupply-area B – RPC = $y = -0.9 \pm 0.4x + 4.1 \pm 1.2$; no significant differences within and between Oversupply-areas ($P \geq 0.05$); ± standard error.

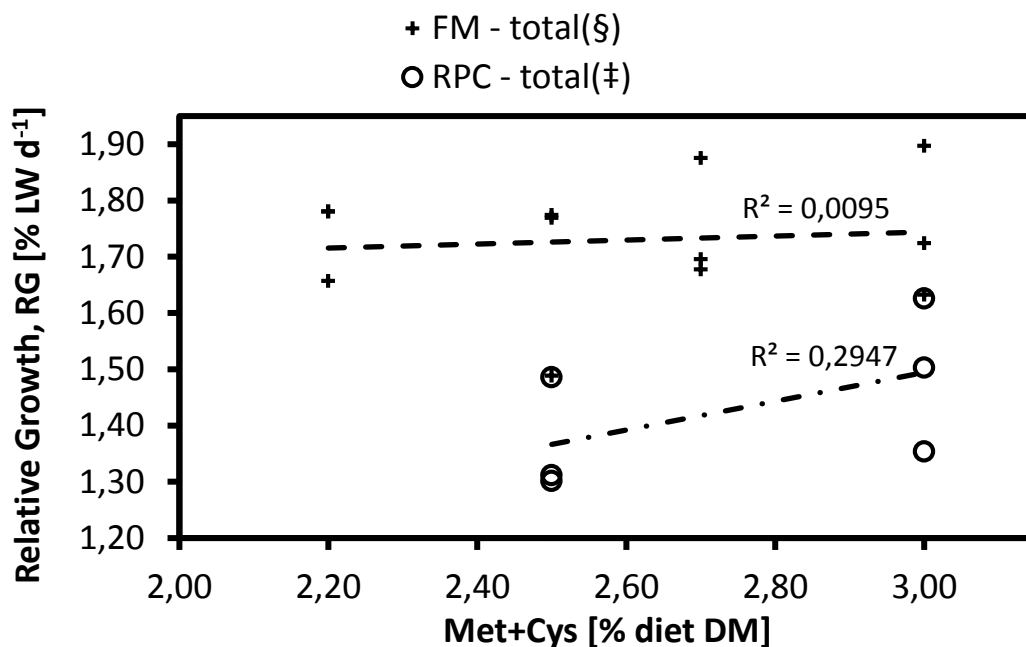


Figure 3.3.2 (Trial 2): Effect between Met+Cys concentration [% diet DM] from fish meal (FM) and rapeseed protein concentrate (RPC) and relative growth (RG) [% LW d⁻¹] on the total regression coefficients (FM-total, RPC-total). § FM-total: 2.2 - 3.0 % Met+Cys in diet DM; ‡ RPC - total: RPC1(2), RPC2(2), 2.5 - 3.0 % Met+Cys in diet DM; + FM – total = $y = 0.0 \pm 0.1x + 1.6 \pm 0.3$; ○ RPC – total = $y = 0.3 \pm 0.2x + 0.7 \pm 0.5$; slopes and intercepts are not significant ($P \geq 0.05$); ± standard error.

Table 3.6.1 (Trial 2): Arithmetic mean values (\pm standard deviation) of relative growth (RG), relative feed intake (RFI) and feed to gain ratio (FGR) in turbot, fed different Met+Cys concentrations provided by fish meal (FM) and rapeseed protein concentrate (RPC) (arithmetic mean \pm standard deviation).

| diet | C1(2) | FM1(2) | RPC-1(2) | C2(2) | FM2(2) | RPC2(2) |
|--|---------------|---------------|---------------|---------------|---------------|---------------|
| Met+Cys (g 100g ⁻¹ diet DM) | 2.2 | 2.5 | 2.5 | 2.7 | 2.7 | 3.0 |
| RG ¹ | 1.7 \pm 0.1 | 1.7 \pm 0.1 | 1.4 \pm 0.1 | 1.7 \pm 0.1 | 1.8 \pm 0.1 | 1.5 \pm 0.1 |
| RFI ² | 1.1 \pm 0.0 | 1.1 \pm 0.0 | 1.0 \pm 0.1 | 1.1 \pm 0.0 | 1.2 \pm 0.1 | 1.1 \pm 0.0 |
| FGR ³ | 0.6 \pm 0.0 | 0.7 \pm 0.0 | 0.7 \pm 0.0 | 0.6 \pm 0.0 | 0.7 \pm 0.0 | 0.7 \pm 0.0 |

¹RG = Relative growth (% LW d-1) = $(\ln(\text{final live weight (g)}) - \ln(\text{initial live weight (g)})) * 100 / \text{feeding days}$; ²RFI = Relative feed intake = feed intake in % DM of live weight d-1; ³FGR = Feed to Gain Ratio = g feed intake (DM) / g live weight gain.

Table 3.6.2 (Trial 2): Regression coefficients¹ for Met+Cys concentration in turbot at different ranges of oversupply, provided by rapeseed protein concentrate (RPC) and fish meal (FM) (\pm standard error).

| Parameter | range of oversupply | b (intercept) | | m (slope) | | | |
|------------------|--------------------------------------|---------------|-----------|-------------------|------|-----------|-------------------|
| RG ² | Oversupply-area A ⁵ – RPC | 4.5 | \pm 1.0 | ****a | -1.2 | \pm 0.4 | *A |
| | Oversupply-area B ⁶ – RPC | 4.1 | \pm 1.2 | ***a | -0.9 | \pm 0.4 | n.s. ^A |
| | Oversupply-area A ⁵ – FM | 2.2 | \pm 1.0 | *a | -0.2 | \pm 0.4 | n.s. ^A |
| | Oversupply-area B ⁶ – FM | 1.8 | \pm 1.2 | n.s. ^a | 0.0 | \pm 0.4 | n.s. ^A |
| | RPC - total ⁷ | 0.7 | \pm 0.5 | n.s. | 0.3 | \pm 0.2 | n.s. |
| | FM - total ⁸ | 1.6 | \pm 0.3 | *** | 0.0 | \pm 0.1 | n.s. |
| RFI ³ | Oversupply-area A ⁵ – RPC | 1.9 | \pm 0.5 | ***a | -0.4 | \pm 0.2 | n.s. ^A |
| | Oversupply-area B ⁶ – RPC | 1.7 | \pm 0.6 | *a | -0.2 | \pm 0.2 | n.s. ^A |
| | Oversupply-area A ⁵ – FM | 1.2 | \pm 0.5 | *a | -0.1 | \pm 0.2 | n.s. ^A |
| | Oversupply-area B ⁶ – FM | 0.9 | \pm 0.6 | n.s. ^a | 0.1 | \pm 0.2 | n.s. ^A |
| | RPC - total ⁷ | 0.7 | \pm 0.3 | * | 0.1 | \pm 0.1 | n.s. |
| | FM - total ⁸ | 1.0 | \pm 0.2 | *** | 0.1 | \pm 0.1 | n.s. |
| FGR ⁴ | Oversupply-area A ⁵ – RPC | -0.1 | \pm 0.2 | n.s. ^a | 0.3 | \pm 0.1 | **A |
| | Oversupply-area B ⁶ – RPC | 0.0 | \pm 0.2 | n.s. ^a | 0.2 | \pm 0.1 | *A |
| | Oversupply-area A ⁵ – FM | 0.5 | \pm 0.2 | *a | 0.1 | \pm 0.1 | n.s. ^A |
| | Oversupply-area B ⁶ – FM | 0.5 | \pm 0.2 | *a | 0.0 | \pm 0.1 | n.s. ^A |
| | RPC - total ⁷ | 0.8 | \pm 0.1 | *** | -0.4 | \pm 0.0 | n.s. |
| | FM - total ⁸ | 0.6 | \pm 0.1 | *** | 0.0 | \pm 0.0 | n.s. |

¹y = b + mx, describing the Met+Cys utilization efficiency as a function of concentration (% DM);

²RG = Relative growth (% LW d-1) = (ln (final live weight (g)) – ln (initial live weight (g))) * 100 / feeding days;

³RFI = Relative feed intake = feed intake in % DM of live weight d-1;

⁴FGR = Feed to Gain Ratio = g feed intake (DM) / g live weight gain;

⁵Oversupply-area A: C1(2), MC1(2), RPC1(2), 2.2 - 2.5 % Met+Cys in diet DM;

⁶Oversupply-area B: C2(2), MC2(2), RPC2(2), 2.7 - 3.0 % Met+Cys in diet DM;

⁷RPC - total: RPC1(2), RPC2(2), 2.5 - 3.0 % Met+Cys in diet DM;

⁸FM - total: C1(2), MC1(2), C2(2), MC2(2), 2.2 - 3.0 % Met+Cys in diet DM;

n.s. = not significant; *P<0.05; **P<0.01; *** P<0.001;

small superscript letters refer to a comparison of Oversupply-area A against B (within FM or RPC);

capital letters refer to a comparison of FM vs. RPC (within an Oversupply-area).

Table 3.7 (Trial 2): Growth performance, biometrical parameter and chemical body composition in turbot, fed different Met+Cys concentrations from fish meal (FM) and rapeseed protein concentrate (RPC) (arithmetic mean \pm standard deviation).

| diet | Initial | C1(2) | FM1(2) | RPC1(2) | C2(2) | FM2(2) | RPC2(2) |
|---|-----------------------------|------------------------------|-------------------------------|------------------------------|------------------------------|------------------------------|-------------------------------|
| Met+Cys [g 100g ⁻¹ in diet DM] | | 2.2 | 2.5 | 2.5 | 2.7 | 2.7 | 3.0 |
| initial ind. | | | | | | | |
| LW ¹ | 30.9 \pm 0.2 | 30.6 \pm 0.2 | 30.8 \pm 0.1 | 30.6 \pm 0.2 | 30.7 \pm 0.1 | 30.6 \pm 0.2 | 30.8 \pm 0.2 |
| final ind. LW ¹ | - | 79.4 \pm 25.2 ^b | 77.3 \pm 23.5 ^{ab} | 64.6 \pm 19.6 ^a | 80.4 \pm 23.7 ^b | 81.4 \pm 20.6 ^b | 70.1 \pm 16.3 ^{ab} |
| FCF ² | - | 2.1 \pm 0.2 | 2.0 \pm 0.2 | 2.0 \pm 0.2 | 2.1 \pm 0.2 | 2.1 \pm 0.2 | 2.0 \pm 0.2 |
| HSI ³ | - | 1.8 \pm 0.3 | 1.8 \pm 0.3 | 1.7 \pm 0.4 | 1.7 \pm 0.3 | 1.7 \pm 0.3 | 1.6 \pm 0.4 |
| SI ⁴ | - | 0.1 \pm 0.0 | 0.1 \pm 0.1 | 0.1 \pm 0.0 | 0.1 \pm 0.0 | 0.1 \pm 0.0 | 0.1 \pm 0.0 |
| moisture | 77.5 \pm 0.4 | 75.3 \pm 0.3 | 75.5 \pm 0.4 | 76.2 \pm 0.2 | 75.4 \pm 0.5 | 75.3 \pm 0.2 | 76.0 \pm 0.1 |
| Crude Protein | 13.9 \pm 0.1 ^a | 15.1 \pm 0.4 ^b | 15.0 \pm 0.3 ^b | 14.7 \pm 0.2 ^{ab} | 15.1 \pm 0.3 ^b | 15.3 \pm 0.2 ^b | 14.9 \pm 0.1 ^{ab} |
| Crude Lipid | 4.0 \pm 0.2 ^a | 5.1 \pm 0.2 ^b | 5.0 \pm 0.4 ^{ab} | 4.7 \pm 0.2 ^{ab} | 5.1 \pm 0.2 ^b | 5.0 \pm 0.3 ^{ab} | 4.8 \pm 0.3 ^{ab} |
| Crude Ash | 1.6 \pm 0.1 | 2.1 \pm 0.3 | 2.2 \pm 0.2 | 2.1 \pm 0.2 | 2.2 \pm 0.4 | 1.9 \pm 0.6 | 2.1 \pm 0.3 |
| Energy | 4.7 \pm 0.1 ^a | 5.3 \pm 0.0 ^b | 5.3 \pm 0.1 ^b | 5.2 \pm 0.1 ^b | 5.5 \pm 0.1 ^b | 5.5 \pm 0.1 ^b | 5.2 \pm 0.1 ^b |

Different superscript letters within the same row represent significant differences (Tukey test, $P < 0.05$); LW = live weight; ²FCF = Fulton's condition factor = (live weight in g / body length in cm³) * 100; ³HSI = hepatosomatic index = (g liver weight / g live weight) * 100; ⁴SI = splenic index = (g spleen weight / g live weight) * 100.

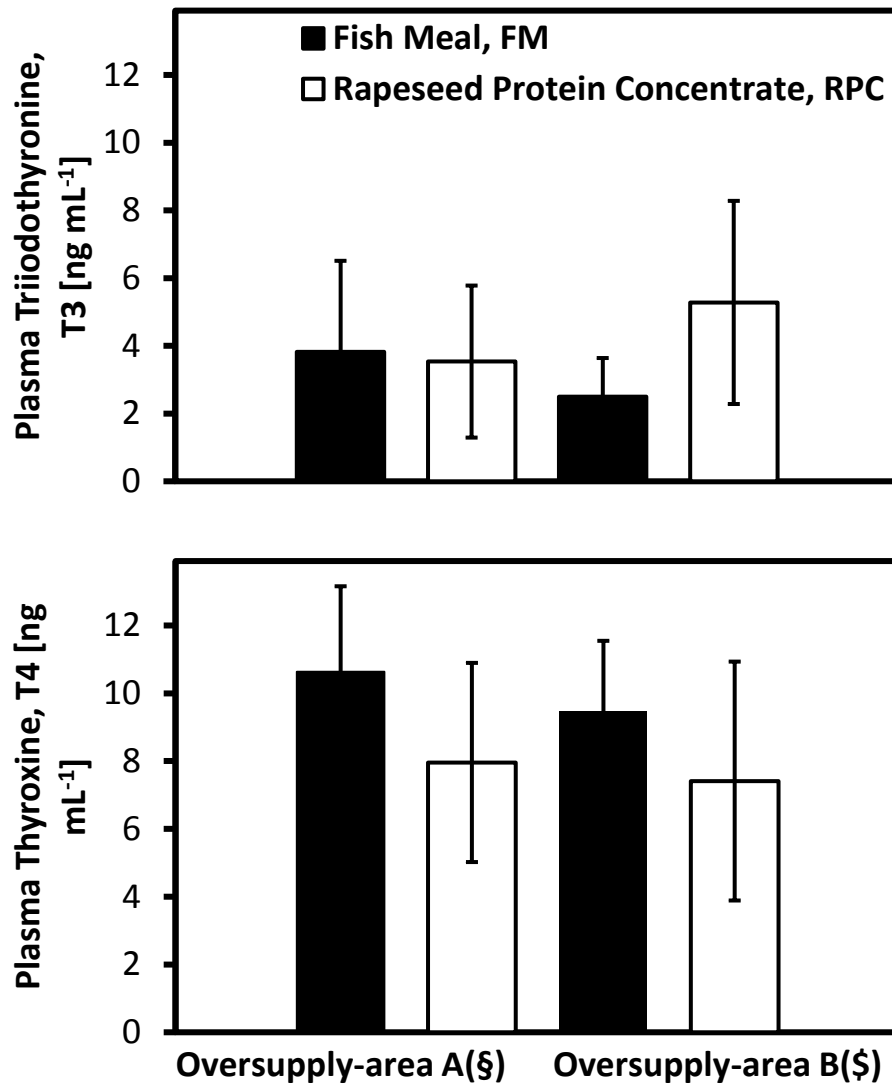


Figure 3.4 (Trial 2): Plasma thyroxine (T4) and triiodothyronine (T3) concentration [ng mL^{-1}] in turbot after 59 feeding days in fish meal (FM) and rapeseed protein concentrate (RPC) at different Met+Cys concentrations. § Oversupply-area A: MC1(2), RPC1(2), 2.5 % Met+Cys in diet DM; § Oversupply-area B: MC2(2), RPC2(2), 3.0 % Met+Cys in diet DM; no significant differences between Met+Cys concentration and RPC and FM ($P \geq 0.05$) ($n=3$) (\pm standard deviation).

4. Discussion

The two experiments investigated indirectly via growth response the intestinal availability of Met+Cys in RPC and FM (Trial 1), as well as the potential toxic effect of Met+Cys oversupply from RPC (Trial 2).

High inclusion levels of plant proteins sources can cause imbalances in the EAAs profile, resulting in less efficient protein accretion and growth of fish (Fournier et al. 2004; Klatt et al. 2016, chapter 1; Kaushik and Seiliez 2010; Tulli et al. 2010). As well plant proteins contain ANFs impairing fish performance (Francis et al. 2001). Especially rapeseed protein products, like RPC contain several ANFs (tannin, phytate, glucosinolates) (Table 3.1) (Nagel et al. 2014; Slawski et al. 2011). Likewise, RPC contains high concentrations of sulfur containing amino acids (AAs) (Met+Cys) (Table 3.1). Nevertheless, several ANFs can reduce the availability of protein and single (AAs), such as (Met+Cys). Likewise it was shown in previous studies with turbot that the oversupply with Met+Cys can cause depressions in feed intake and growth, when exceeding the upper critical concentration of 3.8 % (± 0.7 s.e.) in CP (Klatt et al. 2016, chapter 1; Klatt and Susenbeth 2016, chapter 2), like in studies using diets with high RPC concentrations (Nagel et al. 2014; von Danwitz et al. 2016).

Met is an EAA and Cys as a semi-EAA can replace Met to a rate of 40 – 60 % for several aquaculture species (hybrid striped bass (*Morone saxatilis* × *M. chrysops*); channel catfish (*Ictalurus punctatus*); rainbow trout (*Oncorhynchus mykiss*); red drum (*Sciaenops ocellatus*)) (Griffin et al. 1994; Harding et al. 1977; Kim et al. 1992; Moon and Gatlin 1991). Thus, it can be strongly assumed that the complete amount of Cys can replace Met (Lewis 2003) most probably in both presented experiments (trial 1 and 2) (Tables 3.1, 3.2 and 3.3).

Several studies investigated the available Met for the metabolism (direct bioavailability) by determining the concentration in the blood for species like European seabass (*Dicentrarchus labrax*) (Thebault 1985) or rainbow trout (*Oncorhynchus mykiss*) (Schuhmacher et al. 1997). However, no studies investigated availabilities of Met or Cys for turbot, until now. The approach used in trial 1 indirectly evaluated the intestinal availability of the sum of sulfur containing amino acids (Met+Cys) in FM and RPC by the response of weight gain (WG) in % of LW and retained crude protein (RCP) in $\text{mg kg}^{-0.8} \text{d}^{-1}$ (Table 3.4.2; Figure 3.2).

To determine the availability of Met+Cys in a slope ratio assay, it is necessary that these AAs are first-limiting (Klatt et al. 2016, chapter 1; Levesque et al. 2010; Littel et al. 1997), to ensure a linear response on the intake (Figures 3.1 and 3.2). At a higher concentration of Met+Cys ≥ 2.26 % in CP this relationship (Figures 3.1 and 3.2) would change to a diminishing response and thereafter to non-response as an asymptote (plateau) (Klatt et al. 2016, chapter 1), where further performance improvements cannot be recorded (Susenbeth et al. 1999). Therefore, the efficiency of the utilization of Met+Cys will decrease at the point of transition or at a point where other nutrients or energy become the first-limiting factor. The

feed intake in the basal treatment (Basal(1)) was low (Table 3.2), and the energy intake ($\text{kJ kg}^{-0.8} \text{d}^{-1}$) was not the first limiting factor compared to Met+Cys intake ($\text{mg kg}^{-0.8} \text{d}^{-1}$) (Figures 3.1 and 3.2). It is illustrated by the slopes of Figure 3.1 that the increment of energy onto the diet Basal(1) without extra Met+Cys supplementation (0-Met(1), excl. Met+Cys suppl.) does not increase RCP, whereas the addition of free Met+Cys to the diet (1-Met(1), incl. Met+Cys suppl.) increased RCP. Therefore, the different slopes illustrated in Figure 3.2 for WG and given in Table 3.4.2 for WG and RCP are solely due to the Met+Cys intestinal availabilities of the different components. Hence, the preconditions to use a slope ratio assay were fulfilled in the present trial 1 (Levesque et al. 2010; Littell et al. 1997).

To determine the availability of AA via a slope ratio assay it is further necessary that the maintenance requirement is fulfilled, and furthermore a sufficient response (WG, RCP) is required. For fish, Hua (2013) determined in a meta-study the maintenance requirement of Met to be $18.4 (\pm 1.9 \text{ s.e.})$ in $\text{mg kg}^{-0.75} \text{d}^{-1}$ or 11.9 % as a proportion of maintenance for protein accretion. Even though, data for Cys maintenance is not included, the lowest Met+Cys intake (Basal(1)) exceed the recommended maintenance amount for requirement of Hua (2013) (Tables 3.2 and 3.4.1). Hence, all diets which were used in trial 1 (Table 3.2) supplied sulfur containing AA (Met+Cys) that exceeded the maintenance requirement of turbot and provided additional Met+Cys for growth (Tables 3.4.2 and 3.5; Figure 3.3.2).

Restrictions in trial 1, were of a short duration (15 feeding days) and concentrations of free AAs in the diets 0-Met(1) and 1-Met(1) were high (up to 26.5 % in diet; Table 3.2). Other studies detected lower growth and feed intake already at concentrations ≥ 22.9 % of free AAs in diet for turbot (Peres and Oliva-Teles 2005) or ≥ 20.9 % for rainbow trout (*Oncorhynchus mykiss*) (Bodin et al. 2012). However, these thresholds were determined for diets containing much higher inclusion levels of FM and have therefore a stronger control to determine significant effects than in the present study where all diets had a low growth performance.

Compared to trial 2 (Table 3.7) and other studies with well-nourished turbot (Bonaldo et al. 2015; Nagel et al. 2014; von Danwitz et al. 2016) CA concentration is higher and CL and energy concentrations in the fish body are lower in trial 1 (Table 3.5). Corresponding observations were made for the low HSI and FCF in Table 3.5, compared to trial 2 (Table 3.7) and other studies with turbot (Bonaldo et al. 2015; Klatt et al. 2016, chapter 1; von Danwitz et al. 2016). These effects are mainly due to restricted Met+Cys and feed intake (Table 3.2).

RCP is a precondition for growth (e.g.: WG) as shown in manifold reviews and research studies for aquaculture species (e.g.: Fournier et al. 2002; Kaushik and Seiliez 2010; Kroeckel et al. 2013, 2015), as well as for terrestrial livestock as pigs (e.g.: Susenbeth 1995, 2006). Nevertheless, in the present study this connection cannot be made, due to the low availability of FM for RCP (71.4 %) but high availability for WG (95.7 %) (Table 3.4.2). Physiological reasons for this gap cannot be identified, however may be associated with nutrient and energy restrictions in trial 1, as mentioned above. Furthermore, it has to be critically appraised that

for the reference Met+Cys source (free Met+Cys) which was assumed to have an availability of 100% does not show a significant slope for RCP. Therefore, all availabilities calculated for RCP in trial 1 are less valid than for WG (Table 3.4.2).

Compared to FM, the Met+Cys availabilities in RPC are on a low level for both investigated parameters, RCP (58.0 %) and WG (65.3 %) (Table 3.4.2). Previous studies which investigated RPC in diets of turbot already assumed (Slawski et al. 2011) or proved (von Danwitz et al. 2016), that specific ANFs in RPC (Table 3.1) reduce the availability of CP compared to FM.

One reason might be the high tannin concentration for the RPC used in this study, which was approximately three times higher (Table 3.1) than in the RPC used by Nagel et al. (2014). High tannin concentrations in RPC deteriorate the availability of protein due to complexes, as shown for several non-aquaculture species, such as ruminants (Ahnert et al. 2015; Komolong et al. 2001) or chicken (Mitaru et al. 1985), but as well addressed in a review of Francis et al. (2001) as an ANF for fish.

Another ANF deteriorating CP availability is phytate. In a recent study using turbot and high RPC concentrations of 70 % FM substitution in CP, von Danwitz et al. (2016) indicate that the inclusion of the enzyme phytase could improve phosphorus utilization, as well as apparent digestibility and retention of CP, due to the break-down of phytate. Due to the lower retention and apparent digestibility of CP (von Danwitz et al. 2016), it can be assumed that phytate in RPC reduces the performance in the RPC diets in both trials. Even though, the phytate concentration is lower (Table 3.1) compared to RPC used in other studies (Nagel et al. 2014; von Danwitz et al. 2016). Thus, tannins and phytate are seen as the main factors reducing protein availability and therefore for the low Met+Cys availability in RPC.

As mentioned above, high concentration of Met+Cys in rapeseed proteins (Table 3.1; Nagel et al. 2012, 2014; Slawski et al. 2011) can lead to toxic oversupplies in turbot (Klatt and Susenbeth 2016, chapter 2; Klatt et al. 2016, chapter 1). Therefore, trial 2 separates a potential negative Met+Cys-effect from the ANF-effect of rapeseed protein (RPC) inclusion (e.g.: tannin, phytate, glucosinolates) (Burel et al. 2000a, 2000b; Cheng et al. 2010; Nagel et al. 2014; Slawski et al. 2011; von Danwitz et al. 2016).

For RG it can be seen that the slope for RPC in Oversupply-area B decreases less than in Oversupply-area A (Figure 3.3.1). Likewise there are no significant differences between all Oversupply-areas for FM as well as for RPC. This strengthens the independency of Met+Cys concentration and performance decrease of RPC towards FM for RG, RFI and FGR (Figure 3.3.1; Table 3.6.2).

No breakpoint for all investigated parameter (RG, RFI, FGR) could be identified, neither within both Oversupply-areas (A, B), nor for the overall slope (FM-total, RPC-total) (Figures 3.3.1 and 3.3.2; Table 3.6.2).

Therefore, it has to be considered that the upper critical concentrations of Met+Cys detected in previous studies (Klatt and Susenbeth 2016, chapter 2; Klatt et al. 2016, chapter 1) cannot be confirmed by the data-set of the current trial 2 for all investigated parameter (RG, RFI, FGR). The inclusion of RPC shows partly negative effects.

The decrease of RG (commonly expressed as specific growth rate – SGR) in diets with high RPC inclusions (Nagel et al. 2014; Slawski et al. 2011; von Danwitz et al. 2016) is also demonstrated in the current trial 2 (Figure 3.3.1; Tables 3.6.1 and 3.6.2).

Whereas other studies with similar high inclusion levels of RPC observed a decrease in feed intake compared to FM diets (Nagel et al. 2014; Slawski et al. 2011; von Danwitz et al. 2016), the RFI in this study did not decrease significantly (Tables 3.6.1 and 3.6.2). The increase in the slopes of FGR, due to high RPC replacement (66 % of FM CP) (Table 3.6.2) can also be confirmed by other studies where the feed conversion was impaired (Slawski et al. 2011; von Danwitz et al. 2016).

Similar relations can be observed for the final LW in Table 3.7, where only RPC₁₍₂₎ is significantly different from all FM diets, except for diet FM₁₍₂₎. The FCF is not significantly influenced by RPC, like in the study of von Danwitz et al. (2016), whereas Nagel et al. (2014) found significantly reduced FCF, due to high RPC inclusion. The same can be observed for HSI, Nagel et al. (2014) detected partly significantly lower HSI, whereas von Danwitz et al. (2016) did not detect differences (Table 7). Concordant with the study of Klatt and Susenbeth (2016) (chapter 2), the SI was on a similar level and not significantly influenced by Met+Cys concentration, as well as by RPC inclusion (Table 3.7).

The chemical body composition of trial 2 and shown in Table 3.7 is similar to the composition presented in studies with turbot of a similar weight and growth (Dietz et al. 2012; Klatt and Susenbeth 2016, chapter 2; von Danwitz et al. 2016). All parameter are not significantly affected by Met+Cys concentration or RPC inclusion, just partly significantly different from the initial body composition and comparable to the findings in other studies (Nagel et al. 2014; von Danwitz et al. 2016). In the study of von Danwitz et al. (2016) energy and CL decreased in RPC diets without phytase treatment. Furthermore, Nagel et al. (2014) presented partly decreasing energy and DM concentration at high RPC inclusions for the fish bodies.

Beside the impairments of the ANFs tannin and phytate, as discussed above for trial 1 (Table 3.4.2), as well other factors in RPC could be responsible for a lower performance of turbot (*P. maxima*) at RPC inclusions (Table 3.6.2).

A further factor of lower performance of rapeseed protein products in fish diets is assumed to inferior palatability, resulted in lower feed intake and assumed to be due to ANFs like glucosinolates and sinapic acid (Burel et al. 2000a, 2000b; Nagel et al. 2014). As shown by Nagel et al. (2014) the feed intake of turbot (*P. maxima*) could be improved by the inclusion of blue mussel meal as feed attractant in RPC diets. Nevertheless, reduced feed intakes are also related to imbalances of nutrient supply and are not automatically related to a worse palatabil-

ity (Fournier et al. 2002; Kroeckel et al. 2015; Klatt et al. 2016, chapter 1; Tulli et al. 2010; Tusche et al. 2011).

ANFs like glucosinolates in rapeseed products can impair the thyroid hormonal (thyroxine (T₄), triiodothyronine (T₃)) status of fish. Due to the impact of T₄ and T₃ on the growth performance and energy metabolism, growth depressions of fishes fed diets with high glucosinolates level can be explained. Even though, glucosinolates could not be measured in the current RPC product, due to the feed matrix. Nevertheless, it can be strongly assumed that the product used in the current study (Table 3.1) contains glucosinolates, as in other studies with similar RPC products ranging between 0.1 – 1.32 $\mu\text{mol g}^{-1}$ (Nagel et al. 2014; Slawski et al. 2011).

Influences on thyroid hormones (T₄, T₃) were investigated for turbot (Burel et al. 2000a; Pradet-Balade et al. 1999), where a partly significant decrease of T₄ and negatively correlated to glucosinolates concentrations could be observed, but similar to own results a reduction of T₃ did not occur (Burel et al. 2000a; Pradet-Balade et al. 1999) (Figure 3.4). However, no significant differences occur for T₄ and T₃ concentration in trial 2, neither influenced by Met+Cys concentration nor by RPC inclusion. Although it can be seen that T₄ concentration tended to decrease in RPC diets (Figure 3.4). Nevertheless, glucosinolates level in RPC are much lower than in rapeseed meals used by Burel et al. (2000a) and Pradet-Balade et al. (1999). Therefore the impact of glucosinolates on growth depressions, due to T₄ and T₃ status, observed for high RPC inclusions in diets for turbot can be assumed to be low.

5. Conclusion

The intestinal availability, indirectly determined by the slope ratio assay, of Met+Cys in RPC is relatively low. This might probably be the reason why a toxic effect due to an oversupply of Met+Cys from high proportions of RPC in the diet could not be detected. It can be concluded that growth depressions by RPC in diets for turbot are mainly due to ANFs rather than by high Met+Cys concentrations. Furthermore, the low intestinal availability of Met+Cys in RPC decreases the risk of Met+Cys toxicity.

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GENERAL DISCUSSION

The aim of the present thesis was to evaluate the supply of sulfur containing amino acids (AA), methionine (Met) and cyst(e)ine (Cys), in diets of juvenile turbot (*Psetta maxima*). Accordingly, three different approaches were used: (i) determination of the lower and the upper critical concentration (LCC, UCC) of Met+Cys (chapter 1), (ii) the reevaluation of the UCC (chapter 2) and (iii) the evaluation of the intestinal availability and toxicity of Met+Cys in rapeseed protein concentrate (RPC) as fish meal (FM) replacement (chapter 3).

Methionine and Cystine ratio towards Lysine requirement - comparison of the results of chapter 1 with literature data for turbot and other aquaculture, as well as terrestrial species

The increasing replacement rates of alternative protein sources for FM (FAO 2014) are especially relevant for fish species with a high quantitative crude protein (CP) requirement like for juvenile turbot of up to 55 % (Cho et al. 2005). However, a focus merely on the quantity of CP does not take into account the specific requirements for essential amino acids (EAA), such as Met and the semi-EAA Cys.

The first study determined the EAA requirements for turbot via the determination of whole body protein concentration (Kaushik 1998). By using this approach it has to be considered that maintenance costs, e.g. potential functions of Met+Cys in the metabolism, as discussed in chapter 1, are neglected. The disregard of maintenance costs can lead to an underestimation of Met+Cys requirement and accordingly to potential deficiencies in diets for turbot. In a meta-study the maintenance costs in fish were determined on 11.9 % Met (Hua 2013) and for sea bass (*Dicentrarchus labrax*) on 23 % Met+Cys of total requirement (Tulli et al. 2010), respectively, which illustrates its relevance.

As an alternative, dose-response trials for single AA are taking into account the total requirement (maintenance + accretion) as presented for Met+Cys or Lysine (Lys) of several fish species (Tables 1.7 and 4.1).

Beside the EAA Lys, Met+Cys are frequently first-limiting AAs in fish nutrition (Figueiredo-Silva et al. 2015; El-Saidy and Gaber 2002; Mambrini et al. 1999). Especially high inclusion levels of plant protein sources to replace FM increase the risk of deficiencies (Kaushik and Seiliez 2010). Therefore, in dose-response trials the total requirement for turbot of Met+Cys were determined by Ma et al. (2013), as well as by the current study entitled as LCC (chapter 1), and Lys by Peres and Oliva-Teles (2008). Except for the maintenance requirement of Arginine (Fournier et al. 2002), the requirements of other EAAs for turbot are not determined until now.

Table 4-1: Literature overview about the requirements of Lysine [g 16 g⁻¹ N] and Methionine + Cystine [g 16 g⁻¹ N; in % of Lysine] for growth of aquaculture finfish species.

| Aquaculture finfish species | Lysine | | | Methionine + Cystine | | | |
|--|--------------------------------------|--|---------------------------------|--------------------------------------|--|--------------------------------|-------------------------|
| | Requirement (g 16 g ⁻¹ N) | Growth parameter (statistical regression analysis) | References | Requirement (g 16 g ⁻¹ N) | Growth parameter (statistical regression analysis) | References | % of Lysine requirement |
| Turbot (<i>Psetta maxima</i>) | 5.0 | WG ¹ (kinetic saturation model) | Peres and Olivares Teles (2008) | 2.3 | RG ² (broken-line) | Klatt et al. (2016), chapter 1 | 46 |
| Atlantic salmon fry (<i>Salmo salar</i>) | 5.0 | WG ¹ (kinetic saturation model) | Peres and Olivares Teles (2008) | 4.2 * | RG ² (polynomial) | Ma et al. (2013) | 83 |
| Rainbow trout (<i>rainbow trout</i>) | 5.3 | N Gain (linear) ³ | Rollin et al. (2003) | 3.4 | N Gain (linear) ³ | Rollin et al. (2003) | 64 |
| Black sea bream (<i>Sparus macrocephalus</i>) | 3.7 | WG ¹ (broken-line) | Kim et al. (1992b) | 2.3 | WG ¹ (broken-line) | Kim et al. (1992a) | 62 |
| European seabass (<i>Dicentrarchus labrax</i>) | 8.6 | RG ² (polynomial) | Zhou et al. (2010) | 5.3 | RG ² (polynomial) | Zhou et al. (2011) | 62 |
| Groupers (<i>Epinephelus coioides</i>) | 4.8 | WG ¹ (broken-line) | Tibaldi and Lanari (1991) | 3.0 | WG ¹ (broken-line) | Tulli et al. (2010) | 63 |
| Large yellow croaker (<i>Pseudosciaena crocea</i>) | 5.6 | WG ¹ (broken-line) | Luo et al. (2006) | 3.3 * | WG ¹ (broken-line) | Luo et al. (2005) | 59 |
| Yellowtail (<i>Seriola quinqueradiata</i>) | 5.8 | RG ² (broken-line) | Zhang et al. (2008) | 4.0 | RG ² (polynomial) | Mai et al. (2006) | 69 |
| Yellowtail (<i>Seriola quinqueradiata</i>) | 4.1 | WG ¹ (broken-line) | Ruchimat et al. (1997a) | 3.3 * | WG ¹ (broken-line) | Ruchimat et al. (1997b) | 79 |

Continuation(1) of Table 4.1

| Aquaculture finfish species | Lysine | | | Methionine + Cystine | | | |
|--|--------------------------------------|--|---------------------------------|--------------------------------------|--|---------------------------------|-------------------------|
| | Requirement (g 16 g ⁻¹ N) | Growth parameter (statistical regression analysis) | References | Requirement (g 16 g ⁻¹ N) | Growth parameter (statistical regression analysis) | References | % of Lysine requirement |
| Red drum (<i>Sciaenops ocellatus</i>) | 4.4 | WG ¹ (broken-line) | Craig and Gatlin (1992) | 3.0 | WG ¹ (broken-line) | Moon and Gatlin (1991) | 68 |
| Indian major carp (<i>Cirrhinus mrigata</i> , <i>Labeo rohita</i>) | 5.8 | WG ¹ (polynomial) | Ahmed and Khan (2004) | 3.2 | WG ¹ (broken-line) | Murthy and Varghese (1998) | 56 |
| Hybrid striped bass (<i>Morone chrysops</i> x <i>M. saxatilis</i>) | 4.0 | WG ¹ (broken-line) | Keembiyehetty and Gatlin (1992) | 2.9 | WG ¹ (broken-line) | Keembiyehetty and Gatlin (1993) | 72 |
| | | | | | | arithm. mean | 65 ± 9.6s.d. |
| | | | | | | median | 63 |

¹WG = weight gain in % or g of initial live weight

²RG = relative growth (% LW gain d⁻¹) = (ln(final live weight (g)) - ln(initial live weight (g))) * 100 / feeding days

³amino acid requirements determined by dilution (Wang and Fuller 1989)

*Conversion from g 100 g⁻¹ in diet DM into g 16 g⁻¹ N and / or addition of Cys to Met requirement, conducted by the author.

To compare the requirements of Lys and Met+Cys with other fishes, Table 4.1 presents findings of 22 studies for eleven aquaculture finfish species, including turbot. The values were derived from growth parameter (weight gain – WG; relative growth – RG; N gain), and analyzed by broken-line, polynomial or kinetic saturation regression models. The ratio of Met+Cys to Lys was calculated because Lys is used as the reference amino acid in an ideal protein (Firman and Boling 1998; Wang and Fuller 1989) (Table 4.1).

The ratio of Met+Cys to Lys broadly ranges from 46 – 83 %. However, when excluding the values for yellowtail (*Seriola quinqueradiata*) and turbot the variation narrow to values between 56 – 72 % (Table 4.1). The median lies at 63 % and the arithmetic mean value is 65 %. The Met+Cys requirement determined by Ma et al. (2013) is 83 %, but 46 % Met+Cys (% of Lys) in the present study in chapter 1 (Table 1.3.2; Figure 1.1), based on the Lys requirement value determined by Peres and Oliva-Teles (2008) (Table. 4.1). Surprisingly, turbot represents the lower and the upper extreme value of the ratio of Met+Cys to Lys.

The great discrepancies in the Met+Cys requirement for turbot can be due to methodological differences as discussed in chapter 1 and presented in Table 1.7. Responsible factors might be differences in amino acid availability (chapter 3), the limitation of fishes in reaching their maximum performance, and Met-sparing ingredients (inter alia Taurine) in the diets (Kaushik and Seiliez 2010; Kampman-Van de Hoek 2013; Qi et al. 2012). The main reason for these discrepancies is evidently the different type of the regression model applied. Broken-line regressions tend to result in lower values, whereas polynomial regressions estimate higher values as shown for the Gibel carp (*Carassius auratus gibelio*) by Wang et al. (2015). These facts can also be observed in the literature overviews for requirements of Met+Cys and Lys (Tables 1.7 and 4.1).

The main failure of polynomial regressions is that they neglect the plateau-phase of the dose response relationship, rather they misleadingly show a further positive response before and a decrease after the maximum value, which is taken as the optimum supply, however, the values are just above the minimum supply necessary to reach the plateau phase. Polynomial regressions were used by Ma et al. (2013), which leads to an overestimation of the determined Met+Cys requirement. In the present study broken-line regression analyses were applied (chapter 1 and 2), where the plateau-phase is adequately described and evaluated by Akaike Information Criteria (AIC) (Figures 1.1 and 2.1; Tables 1.3.2 and 2.2.2).

To verify this effect we recalculated the published data using a broken-line regression (slope – plateau) instead of the kinetic saturation model used by Peres and Oliva-Teles (2008), or the polynomial regression used by Ma et al. (2013). Using the same type of regression analysis (broken-line) to determine Met+Cys as well as Lys requirement, the range of the Met+Cys to Lys ratio is distinctly narrow, ranging from 60 – 75 %, as compared to the previous range (46 – 83 %) (Table 4.2).

Table 4.2: Impact of the type of regression analysis on the estimated requirements for Lysine [g 16 g⁻¹ N] and Methionine + Cystine [g 16 g⁻¹ N; in % Lysine] of turbot (Psetta maxima).

| Lysine | | Methionine and Cystine | | | | | | |
|--------------------------------------|--|--------------------------------|--------------------------------------|--------------------------------------|--|--------------------------------|--------------------------------------|-------------------------|
| Requirement [g 16 g ⁻¹ N] | Growth parameter (statistical regression analysis) | Reference of data [§] | Regression analyses performed by ... | Requirement [g 16 g ⁻¹ N] | Growth parameter (statistical regression analysis) | Reference of data [§] | Regression analyses performed by ... | % of Lysine requirement |
| 5.0 | WG ¹ (kinetic saturation model) | Peres and Oliva-Teles (2008) | Peres and Oliva-Teles (2008) | 2.3 | RG² (broken-line) | chapter 1 | the author / chapter 1 | 46 |
| 3.9 | WG¹ (broken-line[§]) | Peres and Oliva-Teles (2008) | the author | 2.3 | RG² (broken-line) | chapter 1 | the author / chapter 1 | 60 |
| 5.0 | WG ¹ (kinetic saturation model) | Peres and Oliva-Teles (2008) | Peres and Oliva-Teles (2008) | 4.2 * | RG ² (polynomial) | Ma et al. (2013) | Ma et al. (2013) | 83 |
| 3.9 | WG¹ (broken-line[§]) | Peres and Oliva-Teles (2008) | the author | 4.2 * | RG ² (polynomial) | Ma et al. (2013) | Ma et al. (2013) | 107 |
| 5.0 | WG ¹ (kinetic saturation model) | Peres and Oliva-Teles (2008) | Peres and Oliva-Teles (2008) | 2.9 * | RG² (broken-line[§]) | Ma et al. (2013) | the author | 58 |
| 3.9 | WG¹ (broken-line[§]) | Peres and Oliva-Teles (2008) | the author | 2.9 * | RG² (broken-line[§]) | Ma et al. (2013) | the author | 75 |

¹WG = weight gain in % or g of initial live weight

²RG = relative growth (% LW gain d⁻¹) = (ln(final live weight (g)) - ln(initial live weight (g))) * 100 / feeding days

[§]regression analysis using broken-line (slope - plateau) (cp. chapter 2)

[§]arithmetic mean values, used for regression analyses

*Conversion from g 100 g⁻¹ in diet DM into g 16 g⁻¹ N and / or addition of Cys to Met requirement, conducted by the author.

It has to be noted that the ratios of total Met+Cys to total Lys in the Tables 4.1 and 4.2 are not necessarily identical with the ideal protein (IP) concept. The IP describes, theoretically, the ideal balance of what an animal truly needs (Firman and Boling 1998), therefore, requirements should base on truly available AAs (chapter 3; Kaushik and Seiliez 2010). Nevertheless, the study of Rollin et al. (2003) presents the available AA requirements (Table 4.1) and the ideal ratio of Met+Cys to Lys for Atlantic salmon fry (*Salmo salar*), determined by dilution (Wang and Fuller 1989). This ratio is located at 64 %, close to the median (63 %) of all values presented in Table 4.1.

The literature overview in Table 4.3 presenting the ratio Met+Cys to Lys for growing pigs (*Sus scrofa domestica*) and chicken (*Gallus gallus domesticus*), indicates that, even though differences within a species occur, it can clearly be seen that the requirement of sulfur containing amino acids related to Lys can differ among species. For instance broiler chicken show higher ratios of Met+Cys to Lys than pigs (Table 4.3), which is caused by the additional requirement for feather growth (Khan et al. 2015).

Table. 4.3: Range of Methionine + Cystine requirement [in % of Lysine] for growing terrestrial species (broiler chicken, pig).

| terrestrial species | Range of Methionine + Cystine requirement in % of 100 % Lysine | References |
|---|--|---|
| Broiler chicken (<i>gallus gallus domesticus</i>) | 69 – 75 | Mack et al. (1999); Vieira et al. (2004); Emmert and Baker (1997) |
| Pig (<i>Sus scrofa domestica</i>) | 51 – 63 | Baker (2000); Boisen (1997); Wang and Fuller (1989) |

In fish nutrition the IP concept is less precisely investigated (Rollin et al. 2003) than for terrestrial animals (Baker 2000; Boisen 1997; Firman and Boling 1998; Mack et al. 1999; Wang and Fuller 1989), and much more controversially discussed (Bureau and Encarnaç o 2006). One reason could be that protein and AAs are used for energy metabolism to a larger extent in fish than in mammals like pigs, due to a higher protein energy utilization efficiency of fish (Bureau and Encarnaç o 2006) increasing the relevance of an accurate definition of the IP. Another, perhaps more relevant reason might be that other macro-nutrients are not sufficiently available to replace protein to a higher extent as energy source, mainly due to physiological restrictions (Catacutan and Coloso 1995; Wilson 1994). Likewise, differences in the IP between fish species might occur, similar like for terrestrial species (Table 4.3). Due to the high number of farmed finfish species, the question arises whether for each species their respective IP should be determined.

On the other hand, protein sources are rising in price and became one of the most relevant cost factors in fish production (FAO 2014; Martínez-Llorens et al. 2011; World Bank 2013). Furthermore, nitrogen excretions of fish should be limited especially in recirculating systems to reduce costs for water treatment and backlashes on fish health (van Bussel et al. 2012). Therefore, it is highly relevant to reduce the amount of protein in fish nutrition, especially for species like turbot with a high CP consumption, ultimately reducing their environmental impacts (Aubin et al. 2006; Cho et al. 2005; Samuel-Fitwi et al. 2013).

Upper critical concentration of Methionine and Cystine – Relevance of oversupply on the performance of juvenile turbot (chapter 1, 2 and 3)

In addition to investigate deficiencies and determining the LCC of sulfur containing amino acids (Met+Cys) (chapter 1), emphases of the current thesis were put on evaluating the effects of oversupplies on health and growth performance of farmed turbot (*P. maxima*) (chapters 1, 2 and 3).

Similarly other AAs, like branched-chain Leucine, were found to be toxic when fed in excess to pig (Edmonds and Baker 1987; Wiltafsky et al. 2010) or trout (*Oncorhynchus mykiss*) (Choo et al. 1991). Nevertheless, Met+Cys are the most toxic AAs when given in excess, as shown in a study with rats (Sauberlich 1961). Met+Cys toxicities were investigated for several other species, like humans (Rees et al. 2006), pigs (Edmonds and Baker 1987) and dogs (Merino et al. 1975). Another study with pigs detected increased odor emission and intensity, due to a surplus of sulfur containing amino acids above the requirement, whereas other AAs (Tryptophan, Phenylalanin, Tyrosin) did not show these effects (Le et al. 2007). However, Le et al. (2007) did not detect a depression in growth performance, similar to several dose-response studies in aquaculture, where a decrease of performance due to Met+Cys oversupply could not be observed (Alam et al. 2000; Niu et al. 2013; Ruchimat et al. 1997a; Simmons et al. 1999; Tulli et al. 2010; Wang et al. 2015). This shows that moderate oversupplies for some finfish species is trivial, as discussed in chapter 1 and 2.

Whereas, a high number of studies estimated the minimum requirement of Met+Cys (Tables 1.7 and 4.1), referred to as LCC in chapter 1, studies which investigated Met+Cys toxicity for fish nutrition in detail are rare. Even though, some studies in fish nutrition realized toxic effects of Met+Cys, when feeding in excess after reaching a plateau (Mai et al. 2006; Murthy and Varghese 1998; Zhou et al. 2006, 2011). However, studies who investigated the upper critical concentration (UCC) of Met+Cys for fish or terrestrial species are rare (Hu et al. 2015; Xie et al. 2007). Therefore the objective of the present thesis was to evaluate the UCC of sulfur containing amino acids for turbot.

In chapter 1 the UCC for several growth performance parameters were determined, admittedly with a high standard error (s.e.) (Table 1.3.2). In almost the same manner the study described in chapter 2, was focused only on the UCC, with smaller increments in Met+Cys con-

centration for a more precise determination. Nevertheless, the s.e. of UCCs was not reduced, when comparing the studies described in chapter 1 and 2 (Tables 1.3.2 and 2.2.2). No UCCs for Met+Cys could be detected in the oversupply experiment (trial 2) presented in chapter 3, neither for the FM control, nor for the RPC diets (Figure 3.3.2). However reasons for these discrepancies could not be identified. Even though, the ranges of Met+Cys oversupply monitored for toxicity are very similar with 2.2 – 3.0 (chapter 3) and 1.8 – 3.0 (chapter 2). Exemplarily for all growth performance parameter determined in the current thesis, Table 4.4 presents the UCC of Met+Cys for RG of all studies performed in this thesis. It has to be mentioned that the UCC in chapter 3 could not be identified within the observed range and is therefore higher than 3.0 % Met+Cys in diet DM (>5.1 % Met+Cys in CP). The UCC of the first experiment (chapter 1) is around 1 % Met+Cys in CP higher compared to the data presented in chapter 2 (Table 4.4). The high range for the UCCs for RG determined in all experiments showed the difficulty to determine precisely an UCC for sulfur containing amino acids, even though the methodology, e.g. the statistical analyses were similar and a strong bias, thereof, can be excluded.

Table 4.4: Overview about the upper critical concentrations (UCC) estimated for Met+Cys [in diet DM and protein] of turbot (*Psetta maxima*) for the parameter relative growth (RG)¹ (\pm standard error).

| upper critical concentration of Met+Cys in diet DM [%] | upper critical concentration of Met+Cys in CP [%] | References |
|--|---|--------------------------------------|
| 2.7 \pm 0.2*** | 4.9 \pm 0.4*** | chapter 1; Table 1.3.2; Figure 1.1 |
| 2.3 \pm 0.4*** | 3.8 \pm 0.7*** | chapter 2; Table 2.2.2; Figure 2.1 |
| >3.0 [§] | >5.1 [§] | chapter 3; Table 3.6.2; Figure 3.3.2 |

¹ RG = Relative growth (% LW d⁻¹) = (ln (final live weight (g)) – ln (initial live weight (g))) * 100 / feeding days;

*** P<10⁻⁴;

[§] maximum value used, since upper critical concentration (UCC) could not be detected.

For fish, as far as my knowledge, solely one study estimated for the Met-analogue 2-hydroxy-4-(methylthio) butanoic acid (HMTBa) a safety level for turbot of below 5 % in diet (Hu et al. 2015). For terrestrial species merely for duck (*Anas platyrhynchos domesticus*) an UCC of Met was determined to be lower than 1.38 % in diet (Xie et al. 2007). The UCC for ducks given as percentage in the diet is much lower than the UCCs presented in Table 4.4, considering a much higher CP concentration in diets for turbot than in poultry as ducks (Cho et al. 2005; Xie et al. 2007). The UCC determined by Hu et al. (2015) and Xie et al. (2007) via comparisons of means, leading to less precise values than the far more precise regression analyses

(broken-line) used in the current thesis. Therefore, the reported UCC are hardly comparable with the UCC defined in the current thesis (Table 4.4). However, no experiments for turbot or other fishes were conducted to determine an UCC for Met+Cys to define the upper limit of supply higher than the LCC (chapter 1) is safe.

The relevance of a sound derived UCC increases by the need of the inclusion of protein sources high in Met+Cys to replace FM in diets for turbot, inter alia in studies where high inclusion levels of Met+Cys rich rapeseed proteins are used (Nagel et al. 2012, 2014; von Danwitz et al. 2016). However, it should be noted that the low intestinal availability of Met+Cys for RPC of 65.3 % (Table 3.4.2) is reducing the risk of Met+Cys toxicity in diets rich in rapeseed proteins, as shown in trial 1 of chapter 3. Whereas protein sources high in Met+Cys like wheat gluten and free Met+Cys with higher intestinal availability are more critical in reaching the risk of toxicity, e.g. in the study of Fournier et al. (2004) or Kroeckel et al. (2015).

The reasons for Met+Cys toxicity are multifactorial and complex and were discussed in the respective chapters. One factor was identified in chapter 1, where an increased S-Adenosylhomocysteine (SAH) was found (Figure 1.2), resulting into adverse effects due to increasing Homocysteine in the blood (Regina et al. 1993; Tulli et al. 2010). Whereas the splenic index (SI) measured in chapter 2 and 3 was not related to Met+Cys toxicity or concentration within the investigated ranges (Tables 2.3 and 3.7). However, van Bussel et al. (2012) identified a correlation between nitrate toxicity and SI for turbot. The hepatosomatic index (HSI) is significantly lower only at very strong Met+Cys oversupplies of 6 % in diet as shown in chapter 1 (Table 1.4).

The relation of Met which can be replaced by Cys is investigated for several finfish species (Griffin et al. 1994; Harding et al. 1977), but not yet determined for turbot. Nevertheless, if high inclusions of dietary protein sources are used which have a high ratio of Cys to Met+Cys, like feather meals (Li et al. 2011), this could lead into a specific oversupply solely of Cys. Thus, Cys will not be utilized for protein accretion or maintenance and will be therefore catabolized, which increases the risk of toxic effects inter alia due to sulfur oversupply. From this case it would follow that only Cys would become toxic, regardless the total UCCs for Met+Cys determined in this thesis would not be exceeded. Therefore, it might be a relevant goal to investigate the ratio of the semi-EAA Cys which is able to replace the EAA Met for turbot.

Further perspectives

To contribute knowledge for the qualitative protein requirement of juvenile turbot, the current thesis defined the range of sulfur containing amino acids (Met+Cys) concentration from where the requirement was met (LCC), until where the oversupply did not lead to negative effects (UCC). Thus, recommendations for an optimum inclusion of protein sources regarding an adequate Met+Cys supply in diets of juvenile turbot can be derived more precisely.

Whereas the single LCCs were defined precisely (low s.e.), the determination of the UCCs were less precisely (high s.e.) and high variations within the different UCCs were observed. The recommended LCCs and UCCs could be improved by providing the truly intestinal available LCC and UCC for Met+Cys.

Due to an enhanced knowledge for the supply of sulfur containing amino acids combined with more investigations on other EAA requirements of turbot in future, protein utilization efficiency might increase further. Thus, ultimately economical and environmental traits of turbot farming will improve.

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SUMMARY

A key factor in feeding carnivorous fish species like turbot (*Psetta maxima*) is crude protein (CP) supply, and the knowledge about the requirement of essential amino acids. Especially for sulfur containing amino acids (methionine – Met; cyst(e)ine – Cys) it has to be considered to be likely the first limiting amino acid. Likewise, Met+Cys are well-known to be toxic if provided at levels of strong oversupply. Therefore, the objectives of the present thesis were to generate knowledge for an adequate supply of Met+Cys to improve dietary formulations for fish feeds used for juvenile turbot farming.

In chapter 1, the experiment determined the lower and the upper critical concentration (LCC, UCC) of Met+Cys via a dose-reponse trial. For 56 feeding days, nine different iso-nitrogenous (CP = 55.3 % on dry matter (DM) basis) and iso-energetic diets (gross energy (GE) = 21.8 MJ/kg DM) were fed once daily until apparent satiation to three tanks per diet. Each tank was stocked with 12 juvenile turbot (arithm. mean live weight (LW) 25.8 g ± 3.0 s.d.). The first seven diets ranged between 0.8 and 2.0 % Met+Cys in DM, realized by 0.2 % increments of L-Met. For the last two diets, increments of 2.0% L-Met were used, respectively, to cover a range of strong Met+Cys oversupply. Cys concentration remained constant at 0.3 % in diet DM. By applying a segmental linear regression model (increase / decrease – Plateau – increase / decrease) the LCC and UCC of Met+Cys was determined for relative growth (% LW d⁻¹) (RG), relative feed intake (% LW d⁻¹ in DM) (RFI), feed to gain ratio (FGR), protein efficiency ratio, retained CP (mg kg^{-0.8} d⁻¹) (RCP) and retained energy (kJ kg^{-0.8} d⁻¹) (RE).

The LCC of all parameter, except FGR, varied within a small range of 1.23 – 1.28 % in diet DM (2.22 – 2.31 % in CP), whereas the UCC for Met+Cys within 2.10 – 3.82 % in diet DM and 3.80 – 6.91 % in CP. The derived LCC for RG and RFI are 1.25 % (± 0.02 s.e.) in diet DM (2.26 % in CP) and 1.24 % (± 0.03 s.e.) Met+Cys in diet DM (2.24 % in CP), respectively. For RG the UCC is 2.68 % (± 0.21 s.e.) (4.85 % in CP) and for RFI 2.10 % (± 0.41 s.e.) Met+Cys in diet DM (3.80 % in CP). Chemical body composition is influenced by Met+Cys concentration. The lower concentrations of CP in fat free reflect deficiencies of Met+Cys are leading to lower values, different to the ratios with an adequate until strong oversupply. Hepatic S-Adenosylhomocysteine concentrations showed a negative correlation towards growth performance and might be a useful tool to detect Met+Cys toxicity. For the coefficients of apparent digestibilities, investigated for three diets with 1.2, 1.6 and 2.0 % Met+Cys in diet DM no significant differences within any investigated parameter were found, as well neither between the Met+Cys concentrations, nor among the feces collection methods (dissection vs. stripping).

The dose-response trial in chapter 2, reevaluated solely the UCC of Met+Cys. For 56 feeding days five iso-nitrogenous diets (CP = 59.1 % DM) and iso-energetic (GE = 22.7 MJ/kg DM) diets were fed until apparent satiation once a day. Altogether, the diets covered a range of Met+Cys oversupply between 1.8 and 3.0 %, realized by addition of 0.2 % L-Met and 0.1 % L-Cys per increment. Diets were fed to three tanks stocked with 20 juvenile turbot (arithm. mean LW 30.9 g ± 3.2 s.d.), respectively. For the parameter individual weight gain (iWG), RG, RFI, FGR and RCP the UCCs were determined by a segmental linear regression analysis (broken-line; Plateau – increase / decrease).

The values for the UCCs ranged between 2.23 % (± 0.44 s.e.) for iWG and 2.56 % (± 0.45 s.e.) Met+Cys for RFI in diet DM (3.77 % and 4.33 % in CP). The UCC for RG was 2.27 % (± 0.44 s.e.) (3.84 % in CP). This range is smaller than the range of the UCCs determined in the experiment in chapter 1, nevertheless the s.e. remained high. Final individual live weights are not significantly different between the Met+Cys concentrations, as well as the biometrical parameter. For chemical body compositions solely CP concentration is influenced by Met+Cys concentration.

In chapter 3, two experiments (trial 1 and 2) evaluated the potential of rapeseed protein concentrate (RPC) with regard to an adequate supply of sulfur containing amino acids (Met+Cys) in diets of juvenile turbot. Trial 1 investigated in 15 feeding days the intestinal availability of Met+Cys in RPC and fish meal (FM), where free Met+Cys was used as a 100 % control. Likewise, the effect of additional energy incl. Met+Cys vs. additional energy excl. Met+Cys supplementation was evaluated to confirm Met+Cys as first limiting factor vs. energy intake. To ensure a linear response, all diets were deficient in Met+Cys and the feed was provided restrictively twice per day. The tanks were stocked with 15 turbot of an arithm. mean LW of 42.12 g (± 4.2 s.d.). Diets in trial 1 were formulated to be iso-nitrogenous (CP = 56.1 % DM) and iso-energetic (GE = 22.0 MJ/kg DM). The intestinal availabilities were determined via slope ratio assay.

For RCP, the intestinal availabilities of Met+Cys are 58.0 % for RPC and 71.4 % for FM. For WG (in %) the intestinal availabilities are 65.3 % and 95.7 % for RPC and FM. The additional energy excl. Met+Cys supplementation tend to have lower RCP compared to additional energy incl. Met+Cys supplementation. For trial 1, biometrical parameter and the parameter of chemical body composition did not indicate any significant differences.

In trial 2 (chapter 3), the effect of a potential Met+Cys toxicity compared to other anti-nutritional factors (ANF) in RPC diets (66 % in CP) was investigated. Diets were fed for 56 days once a day until apparent satiation. The tanks were stocked with 20 turbot with an arithm. mean LW of 30.7 g (± 3.1 s.d.), respectively. A range from 2.2 of up to 3.0 % Met+Cys in diet DM, including four FM (2.2 %, 2.5 %, 2.7 %, 3.0 % Met+Cys in diet DM) and two RPC diets (2.5 %, 3.0 %) were formulated by L-Met and L-Cys supplementation. As well, diets

were designed to be iso-nitrogenous (CP = 59.1 % DM) and iso-energetic (GE = 22.6 MJ/kg DM). The influence of Met+Cys oversupply or ANFs from RPC on performance (RG, RFI and FGR) was analyzed via linear regression analyses.

In trial 2 (Chapter 3), no differences due to Met+Cys oversupply occurred, not for FM and not for RPC diets. Whereas it can be assumed that ANFs in RPC diets have a negative impact on the performance (RG, RFI, FGR). Neither biometric parameter, nor any parameter of chemical body composition was influenced by any treatment in trial 2.

In the present thesis the LCC and UCC of Met+Cys were derived, describing the range of maximum performance of Met+Cys supply in diets for turbot. While the LCC could be determined precisely, the UCC seems to be more variable. The evaluation of indicates that growth depression by RPC in diets for turbot is mainly due to ANFs rather than by high Met+Cys concentrations. Due to an enhanced knowledge of Met+Cys supply for turbot, the dietary inclusion of other protein sources can be improved, resulting inter alia into a higher protein utilization efficiency.

ZUSAMMENFASSUNG

Bei der Fütterung karnivorer Fischarten wie dem Steinbutt (*Psetta maxima*) kommt der Versorgung mit Rohprotein (XP) eine Schlüsselrolle zu. Folglich ist hinreichend Fachwissen über den Bedarf an essentiellen Aminosäuren notwendig. Insbesondere bei schwefelhaltigen Aminosäuren (Methionin - Met; Cyst(e)ine - Cys) ist die Wahrscheinlichkeit erstlimitierend zu sein, vergleichsweise hoch. Bei starker Überversorgung sind Met+Cys allerdings auch dafür bekannt toxisch zu sein. Daher waren die Ziele der vorliegenden Arbeit neue Erkenntnisse für eine adäquate Versorgung mit Met+Cys zu liefern, umso die Formulierung von Rationen für die Fütterung juveniler Steinbutte zu verbessern.

In Kapitel 1 wurde in einem Experiment die untere und die obere kritische Konzentration (UKK, OKK) von Met+Cys über einen Dosis-Wirkungsversuch ermittelt. Für 56 Fütterungstage wurden neun verschiedene iso-nitrogene (XP = 55,3 % auf Trockensubstanz (TS) - Basis) und iso-energetische (Gesamtenergie (GE) = 21,8 MJ/kg TS) Rationen einmal täglich bis zur scheinbaren Sättigung an jeweils drei Tanks je Ration verfüttert. Jeder Tank wurde mit 12 juvenilen Steinbutten besetzt (arithm. mittleres Lebendgewicht (LG) 25,8 g ± 3,0 s.d.). Die ersten sieben Rationen lagen im Bereich zwischen 0,8 bis 2,0 % Met+Cys in TS, dies wurde über Zulagenstufen von jeweils 0,2 % L-Met realisiert. Für die letzten beiden Rationen lagen die jeweiligen Zulagenstufen bei 2,0 % L-Met, um einen Bereich mit starker Met+Cys Überversorgung zu erreichen. Die Konzentration von Cys wurde bei 0,3 % in der Ration TS konstant gehalten. Mittels einer segmentalen linearen Regression (Anstieg/Abfall – Plateau – Abfall/Anstieg) wurde die UKK, sowie die OKK für Met+Cys bezogen auf relatives Wachstum (% LG Tag⁻¹) (RW), relative Futteraufnahme (% LG Tag⁻¹ in TS) (RF), Futterquotient (FQ), Proteinwirkungsverhältnis, retiniertes XP (mg kg^{-0.8} Tag⁻¹) (RXP), sowie retinierte Energie (kJ kg^{-0.8} Tag⁻¹) (RE) berechnet.

Für die UKK lagen sämtliche Parameter, mit Ausnahme des FQ, in einem vergleichsweise kleinen Bereich von 1,23 – 1,28 % Met+Cys in der Ration TS (2,22-2,31% im XP), im Vergleich zu dem Bereich für die OKK der zwischen 2,10 – 3,82 % Met+Cys in der Ration TS und 3,80 – 6,91 % im XP lag. Die UKK Werte für RW und RF sind jeweils 1,25 % (± 0,02 s.e.) und 1,24 % (± 0,03 s.e.) Met+Cys in der Ration TS (2,26 % und 2,24 % im XP). Für RW ist die OKK 2,68 % (± 0,21 s.e.) (4,85 % im XP) und entsprechend für RF 2,10 % (± 0,41 s.e.) Met+Cys in der Ration in TS (3,80 % im XP). Die chemische Körperzusammensetzung ist durch die Met+Cys Konzentration beeinflusst. So kann etwa für das Verhältnis von XP zu fettfreier Substanz beobachtet werden, dass ein Mangel an Met+Cys zu niedrigeren Werten führt, wohingegen dies bei einer adäquaten bis starken Überversorgung nicht beobachtet werden kann. Die Analyse des hepatischen S-Adenosylhomocysteins zeigte eine negative Korrelation zur Wachstumsleistung und könnte eine mögliche Methode zur Erkennung von

Met+Cys Toxizität sein. Die scheinbaren Verdaulichkeiten die für drei Rationen mit 1,2 %, 1,6% und 2,0 % Met+Cys in der Ration TS untersucht wurden, zeigten keine signifikanten Unterschiede für alle untersuchten Parameter, weder innerhalb der Met+Cys Konzentrationen, noch zwischen zwei unterschiedlichen Kotsammelmethoden (Dissektion vs. Abstreifen).

Der Dosis-Wirkungsversuch in Kapitel 2 führte eine erneute Bestimmung der OKK für Met+Cys durch. In einem 56 Tage dauernden Fütterungsversuch wurden fünf iso-nitrogene (XP = 59,1 % TS) und iso-energetische (GE = 22,7 MJ/kg TS) Rationen bis zur scheinbaren Sättigung einmal täglich verfüttert. Die Rationen haben einen Bereich zwischen 1,8 und 3,0 % Met+Cys in der Ration TS, durch die Zulage von 0,2% L-Met und 0,1% L-Cys je Stufe, abgedeckt. Verfüttert wurden die Rationen an jeweils drei Tanks, jeder Tank war mit 20 juvenilen Steinbutten (arithm. mittleres LG 30,9 g ± 3,2 s.d.) besetzt. Die OKK für die einzelnen Leistungsparameter wie individueller Gewichtszuwachs (iGZ), RW, RF, FQ und RXP wurde durch eine segmentale lineare Regressionsanalyse (Plateau – Anstieg/Abfall) bestimmt.

Der Bereich für die OKK lag zwischen 2,23 % (± 0,44 s.e.) für iGZ und bei 2,56 % (± 0,45 s.e.) in der Ration in TS für RF (3,77 % und 4,33 % im XP). Die OKK für RW lag bei 2,27 % (± 0,44 s.e.) (3,84 % im XP). Dieser Bereich der OKK ist kleiner als der Bereich der im Experiment in Kapitel 1 bestimmt worden ist, allerdings blieb der s.e. hoch. Die Met+Cys Konzentration zeigt keinen signifikanten Einfluss auf die finalen LG, ebenso wenig wie auf die biometrischen Parameter. Für die chemischen Zusammensetzungen der Ganzkörper ist ausschließlich das XP zum Teil von der Met+Cys Konzentration beeinflusst.

In Kapitel 3 wurde in zwei Versuchen (Versuch 1 und 2) die Rolle von Rapsproteinkonzentrat (RPK) für eine bedarfsgerechte Versorgung mit schwefelhaltigen Aminosäuren (Met+Cys) in der Ernährung von juvenilen Steinbutten untersucht. In Versuch 1 wurde für 15 Fütterungstage die intestinale Verfügbarkeit von Met+Cys in RPK und Fischmehl (FM) untersucht, hierbei diente freies Met+Cys als 100 % Kontrolle. Ebenso ist die Wirkung von zusätzlicher Energie inkl. Met+Cys gegenüber zusätzlicher Energie exkl. Met+Cys Supplementierung bewertet worden, um Met+Cys als erstlimitierenden Faktor vs. Der Energieaufnahme zu bestätigen. Um eine lineare Wirkung zu erzielen, wurden alle Rationen Met+Cys defizitär gestaltet und das Futter wurde zweimal pro Tag restriktiv verfüttert. Sämtliche Tanks wurden mit je 15 juvenilen Steinbutten und einem arithm. mittleren LG von 42,12 g (± 4,2 s.d.) besetzt. Die Rationen in Versuch 1 wurden iso-nitrogen (XP = 56,1 % TS) und iso-energetisch (GE = 22,0 MJ/kg TS) formuliert. Die intestinale Verfügbarkeit wurde via Slope-Ratio-Assay bestimmt.

Für RXP liegt die intestinale Verfügbarkeit bei 58,0 % für RPK und 71,4 % für FM. Für den Parameter iGZ (in %) ist die intestinale Verfügbarkeit 65,3% und 95,7% für RPK und FM. Ebenso lässt sich feststellen, dass die zusätzliche Energie exkl. Met+Cys Supplementierung

zu niedrigeren RXP Werten tendiert, im Gegensatz zu zusätzlicher Energie inkl. Met+Cys Ergänzung. Die biometrischer Parameter, sowie die Parameter für die chemische Ganzkörperzusammensetzung zeigen keine signifikanten Unterschiede in Versuch 1.

In Kapitel 3 wurde in Versuch 2 die Wirkung einer möglichen Toxizität von Met+Cys im Vergleich zu anderen antinutritiven Faktoren (ANF) in RPK-Rationen (66% im CP) untersucht. Die Rationen wurden für 56 Tage einmal täglich bis zur scheinbaren Sättigung verfüttert. Die Tanks wurden mit je 20 Steinbutten mit jeweils einem arithm. mittleren LG von 30,7 g (\pm 3,1 s.d.) besetzt. Ein Bereich von 2,2 bis 3,0 % Met+Cys in der Ration in TS, darunter vier FM (2,2 %, 2,5 %, 2,7 %, 3,0 % Met+Cys in der Ration in TS) und zwei RPK Rationen (2,5%, 3,0%) wurden mit L-Met und L-Cys Zulagen formuliert. Die Rationen wurden ebenfalls iso-nitrogen (XP = 59,1 % TS) und iso-energetisch (GE = 22,6 MJ/kg TS) konzipiert. Der Einfluss von Met+Cys als Überangebot, oder von ANF aus RPK wurde für RW, RF und FQ mittels linearer Regressionsanalyse bestimmt.

Es konnten keine Unterschiede aufgrund von Met+Cys Überversorgungen ermittelt werden, nicht für FM und nicht für die RPK Rationen. Während davon ausgegangen werden kann, dass ANF in RPK Rationen die Leistung (RW, RF, FQ) negativ beeinflussen. Weder biometrische Parameter, noch irgendein Parameter der chemischen Körperzusammensetzung unterscheidet sich in Versuch 2 signifikant.

In der vorliegenden Arbeit wurde die UKK und OKK von Met+Cys hergeleitet, dadurch kann der Bereich der Met+Cys Versorgung mit maximaler Leistung für die Futterformulierung von Steinbutten bestimmt werden. Während die UKK präzise ermittelt werden konnte, scheint die OKK weniger präzise bestimmbar zu sein. Die Bewertung von RPK für eine adäquate Versorgung mit Met+Cys zeigt, dass Wachstumsdepressionen in RPK Rationen für den Steinbutt vor allem auf ANF und nicht durch hohe Met+Cys Konzentrationen erklärt werden können. Darüber hinaus kann aufgrund von erweiterten Kenntnissen über die Met+Cys Versorgung des Steinbutts die Nutzung von anderen Proteinquellen in den Rationen verbessert werden, was unter anderem zu einer höheren Proteinnutzungseffizienz führt.

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