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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 day lasting growth trial the lower and upper critical concentration 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 turbots (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 no. 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 lower and upper critical concentration 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 lower critical concentration 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 upper critical concentrations is larger (2.10–3.82% Met + Cys in diet DM; 3.80–6.91% in CP). The lower critical concentration 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 upper critical concentration 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 (no. 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.

Statement of relevance: High feed prices, especially for protein sources are a challenge for a sustainable aquaculture and fish feed industry. Therefore knowledge about amino acid requirement is necessary. This study determined the lower and upper critical concentrations of sulfur containing amino acids for turbot (*Psetta maxima*) at maximum performance. Based on these values optimum inclusion levels of protein sources can be derived.

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 turbots require high amounts of dietary crude protein (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

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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 sulfuric 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 of Met + Cys was determined.

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

2. Materials & 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). 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 and 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 and 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). 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). This helped to boost the Met ± Cys deficiency. The amino acid composition of all diets is given in Table 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 turbots 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 0.17 m^2, 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*, *200 mg O_3/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*) (Imsland 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*, *Birkerod*, *DK*). Average pH was 7.4 ± 0.2 (*GMH 3530*, *Digital pH-/mV-/Thermometer*, *Greisinger electronic*, *D*), total NH_4–N and NO_2–N levels were 0.2 ± 0.1 mg L^{-1} and 0.1 ± 0.04 mg L^{-1} (*Microquant test kits for NH_4^+ and NO_2^-*, *Merck KGaA*, *Darmstadt*, *Germany*), respectively.

Twenty-seven rectangular rearing tanks were stocked with 12 juvenile turbots each. The adaption period lasted 14 days and diet no. 6 (Tables 1 and 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 turbots 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 (TiO_2) was used as an inert marker to determine the apparent digestibility coefficients (ADC) (Table 1).

2.3. Sampling

At the beginning of the growth trial 2*6 turbots 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.