Aquaculture 470 (2017) 169–181

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aquaculture

Effect of alternative oil sources at different dietary inclusion levels on food intake and appetite regulation via enteroendocrine and central factors in juvenile *Solea senegalensis* (Kaup, 1858)

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ARTICLE INFO

Article history:
Received 13 September 2016
Received in revised form 23 December 2016
Accepted 25 December 2016
Available online 28 December 2016

Keywords:
Fatty acid
Satiety
Gastrointestinal tract
Neuropeptides
Cocaine- and amphetamine-regulated transcript

ABSTRACT

Vegetable oils (VO) are increasingly used to substitute fish oil (FO) in aquafeeds in order to meet demands of the rapidly growing aquaculture industry. However, effects of dietary VO on appetite and food intake have hardly been investigated, despite the importance of these factors for determining growth and body composition of cultured fish. This study analyzes the effects of an alternative dietary oil source (75% FO replaced by VO, compared to 100% FO) included at different lipid levels (8% or 18%, at the expense of carbohydrates) on voluntary food intake (VFI) and on the expression of putative appetite–regulating genes in the intestine and brain of Senegalese sole (*Solea senegalensis*) juveniles before and after a meal. Decreased VFI was observed only in fish fed the 18FO diet, indicating a higher satiating effect of FO compared to VO at equally high dietary lipid levels. However, no obvious relationship was found between the VFI results and mRNA levels of the analyzed peripheral or central genes. Several putatively anorexigenic peripheral genes (*pyya*, *pyyb*, *glp1*) had higher basal expression in fish fed lower lipid diets whereas, 6 h after a meal, only *pyyb* and the orexigenic *gal* showed higher expression in the 8% lipid treatments. Conversely, basal mRNA levels of central neuropeptides were generally not regulated by diet, but most showed postprandial changes, with some slight differences in relation to lipid/carbohydrate level. Of all the studied genes, only the anorexigenic cocaine– and amphetamine–regulated transcript 1a and 1b were affected by dietary lipid source, with higher postprandial mRNA levels in fish fed FO, 1 h and 3 h after feeding the 18FO and 8FO diets, respectively, possibly relating to the decreased VFI of the 18FO treatment. This study provides new information on several key genes believed to be involved in the regulation of appetite and how they are affected by dietary lipid properties in Senegalese sole.

Statement of relevance: Insight into regulation of fish appetite by dietary lipids.

1. Introduction

In order to meet increasing demands of the growing aquaculture industry, and in the face of a substantial decline of FM and FO availability to use in compound feeds, considerable efforts to find suitable alternative ingredients are ongoing (Tacon and Metian, 2008). It is predicted that in the very near future FM and FO will be used as strategic ingredients at low dietary inclusion levels and differently for specific stages of production (FAO, 2014). Many studies have aimed at identifying suitable candidate vegetable oils (VOs) for the complete or partial replacement of FO in aquaculture feeds, focusing on their effects on growth, feed efficiency, fish health, reproduction and physiology, but results vary (Montero et al., 2003; Nasopoulou and Zabetakis, 2012; Sargent et al., 1999; Turchini et al., 2010, 2009; Zuo et al., 2015). Not all species perform well on these alternative oil sources, due to their lack of essential *n*-3 long-chain polyunsaturated fatty acids (LC-PUFA) and high levels of *n*-6 polyunsaturated fatty acids (PUFA), which leads to imbalances in the *n*-3/*n*-6 ratio (Turchini et al., 2010). While differences in

Abbreviations: FO, fish oil; VO, vegetable oil; FM, fish meal; PUFA, polyunsaturated fatty acid; LC-PUFA, long-chain polyunsaturated fatty acid; MUFA, monounsaturated fatty acids; SFA, saturated fatty acids; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FA, fatty acid; OEA, oleoylethanolamide; IBW, initial body weight; FBW, final body weight; avBW, average body weight; SGR, specific growth rate; FCR, food conversion ratio; VFI, voluntary food intake; CNS, central nervous system; ARC, arcuate nucleus; NTC, non-template control; RT-qPCR, reverse transcription quantitative polymerase chain reaction; CART, cocaine- and amphetamine-related transcript; POMC, proopiomelanocortin; CCKL, cholecystokinin (Leu) precursor; NPY, neuropeptide Y; AgRP2, agouti-related protein 2; PYY, peptide YY; GLP1, glucagon-like protein 1; GAL, galanin; UBQ, ubiquitin; RPS4, ribosomal protein s4; EF1A1, elongation factor 1 alpha; CD36/FAT, cluster of differentiation 36/fatty acid translocase; GRP, G-protein-coupled receptor; ACTH, adrenocorticotropic hormone; MSH, melanocyte-stimulating hormone.

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http://dx.doi.org/10.1016/j.aquaculture.2016.12.033
0044-8486/

performance mainly occur between species, numerous other factors such as trial duration, type of VO used, amount of fish meal (also a source of FO) in the diet, fish size, age or developmental stage, but also dietary lipid level, can lead to intra-species variation, and thus effects of dietary VO inclusions should be evaluated in each specific case (Sales and Glencross, 2011).

An important factor that has received little attention in the context of dietary FO substitution is appetite. This is quite surprising, since appetite directly governs food intake, which has a central role in determining growth and body composition of cultured animals (Forbes, 2007). Fish have generally been shown to regulate their food intake based on dietary energy density (Boujard et al., 2004; Gélineau et al., 2002; Sæther and Jobling, 2001). However, different macronutrients and even specific fatty acids (FAs) have been observed to elicit different responses from appetite-regulating mechanisms (Narnaware and Peter, 2002; Varricchio et al., 2012; Francis et al., 2014; Varricchio et al., 2015; Librán-Pérez et al., 2015). This has only begun to be investigated in fish, but ample evidence connecting dietary FA profile with appetite regulation exists in mammalian literature (Lawton et al., 2000; Reseland et al., 2001; Kratz et al., 2002; Feltrin et al., 2004; Bradford et al., 2008; Feltrin et al., 2008; Parra et al., 2008). A few studies in fish have analyzed the effects of dietary FO substitution at different lipid levels, most of which were related to growth performance, general indicators of health and condition and some aspects of lipid metabolism (Jobling et al., 2002; Kenari et al., 2011; Kim et al., 2012; Tocher et al., 2003). However, connections have been made between these dietary factors and appetite regulation through changes in lipid metabolism (Figueiredo-Silva et al., 2012; Morais et al., 2006).

Appetite regulation occurs in the central nervous system (CNS), mainly in the hypothalamus, by neuroendocrine secretion of several anorexigenic and orexigenic peptides that mutually interact to inhibit or stimulate appetite, respectively (Volkoff et al., 2005). It results from a combination of signals originating from sensory cues from the environment, the gastrointestinal tract (quality and quantity of ingested food), homeostatic regulatory mechanisms (lipostatic and glycostatic signals), and circadian control systems (Arora and Anubhuti, 2006; Rønnestad et al., 2013). However, the core of appetite regulation lies in the gut-brain axis and gastrointestinal endocrine signals will be mostly affected by changes in diet composition and feed entering the gut (Cummings and Overduin, 2007). These consist of anorexigenic and orexigenic enteroendocrine peptides that transmit signals to the CNS by activating vagal afferents or entering the circulatory system, both of which elicit responses in the hypothalamus to release relevant appetite-regulating neuropeptides (Breen et al., 2011). Ample studies with mammals suggest that changes in appetite caused by dietary FA composition are mediated through these central and peripheral peptides (French et al., 2000; Wang et al., 2002; Goncalves et al., 2005; Ramos et al., 2005; Relling and Reynolds, 2007; Dziedzic et al., 2007; Watanabe et al., 2009; Barson et al., 2011). The few studies examining these aspects in fish have mainly focused on peptides such as leptin and ghrelin, which have shown sensitivity to dietary FA composition (Francis et al., 2014; Ganga et al., 2005; Varricchio et al., 2012). Furthermore, circulating FA, differing in chain length and degree of saturation, were shown to elicit different responses from appetite-regulating neuropeptides in the hypothalamus (Conde-Sieira et al., 2015; Librán-Pérez et al., 2015).

Taking into consideration the importance of food intake in farmed fish and its potential regulation by dietary lipid composition, for which there is ample evidence in mammals and some emerging in fish (see above), the main goal of the current study was to analyze the effects of diets differing in lipid level and oil source (FA composition) on food intake and appetite regulation via neuro- and gastrointestinal peptides. The underlying hypothesis was that the FO treatments (high in LC-PUFA) may have a higher satiating effect than the VO-based diets, as has been observed in mammals (French et al., 2000; Lawton et al., 2000; Goncalves et al., 2005; Ramos et al., 2005; Relling and Reynolds, 2007; Dziedzic et al., 2007; Parra et al. 2008; Barson et al., 2011), which could possibly be also affected by the inclusion level of the lipids in the diet. To approach this subject, we chose the marine flatfish Senegalese sole (*Solea senegalensis*) as the model organism. It is a commercially important aquaculture species in southern Europe (Morais et al., 2015a), which brings additional value to this research, as it may aid the development of sustainable feeds for this species. Furthermore, contrary to many cultured marine finfish species, Senegalese sole is sensitive to high dietary lipid inclusions (≥12%; Borges et al., 2009; Valente et al., 2011), but tolerant to even total FO substitution (at 9% dietary lipids) (Borges et al., 2014), with unique capabilities of synthesizing docosahexaenoic (DHA) from eicosapentaenoic acid (EPA) via the Δ4 desaturation pathway (Morais et al., 2015b). With this in mind, Senegalese sole is a very interesting candidate to investigate how appetite-regulating mechanisms, potentially sensitive to lipid levels, might function in varying dietary conditions. The study was set up to analyze the effects of diets differing in lipid level and oil source on the VFI of Senegalese sole juveniles during a 13 week culture period, after which mRNA levels of key gastrointestinal and central appetite-regulating genes were evaluated after fasting (24 h) and re-feeding.

2. Materials and methods

2.1. Experimental diets and fish culture

Four isoproteic extruded diets were formulated and manufactured by Sparos Lda. (Olhão, Portugal). They differed in total lipid level (~8% or ~18%, at the expense of carbohydrates) and FA composition, using FO or VO as the main lipid source. The 8FO and 18FO diets had 100% of the lipid supplied by FO, while 75% of the FO in diets 8VO and 18VO was replaced by a VO blend (rapeseed, soybean, and linseed oil in a ratio of 1:1:1; Table 1). However, all diets contained an estimated 5.3% of FO originating from the marine meals, which implies that diets 8VO and 18VO contained a theoretical amount of 5.9% and 8.4% of marine derived fats, respectively. Formulation and proximate analysis ($n = 3$) of the experimental diets are presented in Table 1 and FA composition ($n = 3$) in Table 2. The analyses were performed using standard methods, as described in Bonacic et al. (2016).

Senegalese sole juveniles with an average initial body weight (IBW) of 5.0 ± 0.1 g were distributed into twelve rectangular flat bottom 20 l tanks (50 fish per tank) connected to a recirculation system at CCMAR, University of Algarve, Faro (Portugal), and maintained at a temperature of 19.3 ± 1.2 °C, a salinity of 32, and a 12 h light/12 h dark photoperiod for 13 weeks. They were fed 2 mm extruded diets using automatic feeders in several meals spread throughout 22 h of the day (Navarro et al., 2009) in order to avoid possible interference of feed expectation, as has been observed in some central neuropeptides (Kehoe and Volkoff, 2007). Administered feed was recorded and adjusted daily to ensure the fish were fed to satiety. In the case of an excess of uneaten feed, rations were reduced by 10% and in the absence of uneaten feed increased by 10%. Fish were weighed (FBW) at the end of the experiment ($n = 3$ pools of 30 individuals) and specific growth rate, SGR = [(ln FBW (g) − ln IBW(g)) / t] × 100, where t is experimental period (91 days); food conversion ratio, FCR = feed intake (g) / [FBW (g) − IBW (g)]; and voluntary food intake, VFI = {(feed intake (g) × 100) / [(IBW (g) + FBW(g)) / 2]} / t (days), were calculated.

The study was conducted according to the guidelines on the protection of animals used for scientific purposes from the European directive 2010/63/UE.

2.2. Sampling for molecular analysis

During the last week of the trial, fish were sampled for molecular analysis. The expression of putative gastrointestinal satiety-related genes was analyzed in pre- and postprandial conditions. For this, sections of the middle intestine (100–150 mg) were taken from two fish per tank ($n = 6$ per treatment) after a fasting period of 24 h, after