Feeding common sole (*Solea solea*) juveniles with increasing dietary lipid levels affects growth, feed utilization and gut health

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Abstract

Knowledge about the nutritional requirements of common sole (*Solea solea*) is limited and no information regarding the optimal dietary lipid level is available yet. Thus, this study was undertaken to assess the growth response and feed utilization of common sole juveniles fed diets with increasing lipid levels. Four isonitrogenous (59% protein) pelletized diets with different dietary lipid levels (8, 12, 16 and 20%; L8, L12, L16 and L20, respectively) were fed to triplicate fish groups of 80 individuals to apparent satiation over 150 days. A one-way ANOVA, Tukey's *post hoc* test and linear regression were used to analyse the data ($P \leq 0.05$). At the end of the trial, final body weight was significantly higher in fish fed L8 (40.7 ± 1.7 g), followed by those fed L12 (35.1 ± 1.2 g), L16 (27.9 ± 2.5 g) and L20 (22.1 ± 0.3 g). The specific growth rate was higher in fish fed L8 and L12, compared to the other treatments, and it was lowest in L20. Voluntary feed intake decreased with an increase in the dietary lipid level. The feed conversion rate, the protein efficiency ratio and the gross protein efficiency were lower in fish fed L20, while no significant differences were observed among L8, L12 and L16. Gross lipid efficiency was significantly higher in fish fed low lipid diets. Histological observations showed that 19 of 36 observed subjects had lipid droplets in the cytoplasm of enterocytes at the apex of the mucosal folds and, in some cases, also along the entire fold (intestinal steatosis). The number of fish with intestinal steatosis in groups L20 and L16 was significantly higher than the number in group L8. Ultrastructure showed large electrondense lipid droplets within the cytoplasm of enterocytes and warping of the cytoplasmic membrane (steatosis); in some cases, lipid droplets were also present within the Golgi apparatus.

In conclusion, the results of this trial suggest that the diet for *Solea solea* juveniles should include no more than 12% lipids. Higher lipid inclusions not only led to a substantial decline in performance but also affected gut health. This should be taken into consideration in formulating specific practical diets for common sole.

Keywords: Solea solea; lipid level; growth; nutrition; histology; ultrastructure

1. Introduction

Common sole (*Solea solea*) is a promising flatfish species for marine farming, especially due to its high market value, high flesh quality and increasing demand from consumers (Parma et al., 2013). Over the last few years, several aspects of the species' culture have been developed and optimized, in particular in terms of larviculture and larval physiology (Lund et al., 2008; Bonaldo et al., 2011; Ferraresso et al., 2013; Parma et al., 2013). However, variable and low growth remains one of the most important constraints for commercial sole farming activities (Mas-Muñoz et al., 2011). To reach large scale production of this species, some studies on juveniles and adults regarding optimal growth temperature (Schram et al., 2013), stocking density (Schram et al., 2006; Lund et al., 2013), feeding behaviour and attractants (Reig et al., 2003; Mas-Muñoz et al., 2011) have been carried out. Nevertheless, several information about the nutritional needs of juveniles of this species is still lacking. In fact, even though the protein requirement for maximum protein accretion in common sole has been estimated at 57% (Gatta et al., 2011), to our knowledge, no studies concerning the optimal dietary lipid level have been carried out for this species.

In other flatfish species, Borges et al. (2009) and Dias et al. (2004) have demonstrated a low lipid tolerance for Senegalese sole (*Solea senegalensis*), where diets should include no more than 8% lipids. In turbot (*Psetta maxima*), negative effects were reported at a dietary lipid level above 15% (Regost et al., 2001), while high levels of dietary lipids, up to 18%, enhanced growth and protein utilization in plaice (*Pleuronectes platessa*) (Cowey et al., 1975).

Although protein sparing by the increase of dietary lipid level up to an optimal level is well documented, the limits to its effectiveness have not been accurately defined for any fish species (Sargent et al., 2002). Furthermore, the use of increasingly high-lipid diets can have consequences for farmed fish by altering the lipid and fatty acid metabolism of the fish with implications for fish health and welfare (Sargent et al., 2002). Information on the nutritional status and lipid metabolism in fish species can be achieved thought the study of blood metabolites such as serum cholesterol and triglycerides (Borges et al 2013b; Peres et al 2014).

Thus, the effects of increasing the dietary lipid level (8 to 20 %) on growth, nutrient utilization, blood parameters (total cholesterol and triglycerides) and gut health (histology and ultrastructure) in common sole juveniles were studied over 150 days.

2. Materials and methods

2.1 Experimental diets

Four isonitrogenous (59% protein) diets were formulated to contain increasing lipid levels (8, 12, 16 and 20%; L8, L12, L16 and L20 respectively). The ingredients and

proximate composition of the experimental diets are presented in Table 1. Protein levels were defined on the basis of a previous trial carried out in our laboratory highlighting dietary protein requirements (Gatta et al., 2011). Increasing lipid levels were obtained by increasing fish oil inclusion and lowering the amounts of wheat meal.

For each diet, the ingredients were mixed and pelleted using a laboratory pelleting machine (La Monferrina, Asti, Italy) with a 1.0 mm die. The diets were dried at 45° C for 24 h. After drying, diet L20 was further greased apart by adding 5% oil to achieve the expected lipid level. The oil was sprayed manually, while diet was mixing using the same pelleting machine.

2.2 Experimental conditions

The experiment was carried out at the Laboratory of Aquaculture, Department of Veterinary Medical Sciences of the University of Bologna, Cesenatico, Italy. Common sole juveniles were obtained from a captive broodstock maintained at the above-mentioned facility. Before the experiment, the fish were kept in experimental tanks for three months and fed commercial diets (Aller Futura ex, Denmark; crude protein 64%, crude fat 12%). One week before the start of the trial they were fed with a mixture of the four experimental diets. At the beginning of the trial, 80 fish (initial average weight: 13.8 ± 0.4 g) per tank were randomly distributed into twelve 500-liter square flat bottom tanks (bottom surface: 0.64 m^2). Each diet was administered to triplicate groups, assigned in a completely random manner, over 150 days. Tanks were provided with natural seawater and connected to a closed recirculating system (overall water volume: 7,000 L). The rearing system consisted of a mechanical sand filter (0.4 m^3 of silica sand,

0.4–0.8 mm. PTK 1200, Astral Pool, Servaqua S.A. Barsareny, Spain), ultraviolet lights (PE 25mJ/cm^2: 16m^3 h^{-1}, Blaufish, Barcelona, Spain) and a biofilter (PTK 1200, Astral Pool, Servaqua S.A. Barsareny, Spain). The water exchange rate per tank was 100% every 2 h while the overall water renewal amount in the system was 5% daily. During the trial, the temperature was kept constant at 20.0 ± 1.0 °C and the photoperiod was maintained at 12 h light and 12 h dark through artificial light. Light intensity was regulated at 50 lx, measured on the water surface (Delta Ohm lightmeter HD2302.0; Probe LP 471 PHOT; Delta Ohm, Padua, Italy). The oxygen level was kept constant (7.5 ± 1.0 ppm) by a liquid oxygen system regulated by a software program (B&G Sinergia snc, Chioggia, Italy). A strong aeration (200 l min^{-1}) was applied in the stock tank to remove CO_2. Ammonia (total ammonia nitrogen ≤ 0.1 ppm), nitrite (≤ 0.2 ppm) and salinity (20 g L^{-1}) were spectrophotometrically (Spectroquant Nova 60, Merck, Lab business, Darmstadt, Germany) monitored daily. Sodium bicarbonate was added on a daily basis to keep pH constant at 7.8–8.0.

During the first 35 days, the fish were hand fed twice a day during the week and once on Sundays to determine the apparent satiation ration. After day 35, the fish were fed by automatic feeders for over 20 h a day^{-1} during the week and over 12 h on Sundays. Each day, all tanks were monitored to be certain that all feed was eaten. When some uneaten pellets remained at the bottom of the tank, the total amount of feed distributed each day was reduced by 10%, until no feed losses were recorded. When no feed losses were observed, the amount of food was maintained for four days and then augmented by 10% (Borges et al., 2009).

Uneaten feed, when present at the end of the meal, was removed and the amount estimated by counting each pellet and multiplying the number by the mean weight of a single pellet. This result was deducted from the daily feed intake of the tank.

2.3 Sampling

At the beginning and at the end of the experiment, all the fish in each tank were individually weighed. The total biomass was also determined at day 35, 70 and 105 by bulk weighing. Daily mortality was registered, and dead fish were weighed and registered.

The proximate composition of the carcasses was determined at the beginning of the trial on a pooled sample of 30 fish and on a pooled sample of ten fish per tank at the end of the trial. Furthermore, at the end of the trial, wet weight and viscera and liver weight were individually recorded for ten fish per tank to determine visceral somatic (VSI) and hepatosomatic indexes (HSI). At the end of the trial, three fish per tank (nine fish per dietary treatment) were sampled for histological examination of the intestines.

After the end of the trial, the fish left were fed for three more days to perform blood analyses of serum cholesterol and triglycerides. Blood from 14 fish per tank was collected six h postprandial from the caudal vein and then pooled into one sample (one pool per tank). Samples were then centrifuged (3,000 g for 10 min at 4 °C) and serum aliquots were stored at 4 °C and analysed during the same day.

All experimental procedures were evaluated and approved by the Ethical-scientific Committee for Animal Experimentation of the University of Bologna in accordance

with European directive 2010/63/UE on the protection of animals used for scientific purposes.

2.4 Chemical analyses

Diets and whole body samples were analysed for proximate composition. Moisture content was obtained by weight loss after drying samples in a stove at 105 °C until a constant weight was achieved. Crude protein was determined as total nitrogen (N) by using the Kjeldahl method and multiplying N by 6.25. Total lipids were determined according to Bligh and Dyer's (1959) extraction method. Ash content was estimated by incineration to a constant weight in a muffle oven at 450 °C. Gross energy was determined by a calorimetric bomb (Adiabatic Calorimetric Bomb Parr 1261; PARR Instrument, IL, USA). Total cholesterol (OSR6116) and triglycerides (OSR60118) of plasma were determined using an enzymatic colorimetric test (Beckman Coulter Olympus AU400 analyzers, Fullerton, CA, USA).

2.5 Calculations

The formulae employed were as follows:

Specific growth rate (SGR) (day^{-1}) = 100 * (ln FBW- ln IBW) / days (where FBW and IBW represent the final and the initial body weights). Feed intake (FI) (% day^{-1}) = 100 * (crude feed intake/ABW/days) (where ABW (g) = average body weight = (FBW + IBW) / 2). Feed conversion ratio (FCR) = feed intake/weight gain. Daily nutrient intake (NI) (g or KJ/kg/d) = nutrient intake/ABW/day. VSI (%) = 100 * (viscera weight/body

weight). HSI (%) = 100 * (liver weight/body weight). Protein efficiency ratio (PER) = (FBW – IBW) / protein intake. Gross protein efficiency (GPE) (%) = 100 * [(% final body protein * FBW) - (% initial body protein * IBW)] / total protein intake fish $^{-1}$. Gross lipid efficiency (GLE) (%) = 100 * [(% final body lipid * FBW) - (% initial body lipid * IBW)] / total lipid intake fish^{-1}. The survival rate (SR) was calculated as a percentage of the initial number of fish.

2.6 Histology and ultrastructure

Samples of proximal, middle and distal intestine were processed for routine histology and evaluated under a light microscope in a blinded fashion. Ultrastructure deepening was performed on a selection of subjects based on histological evidence of pathological changes. Transmission electron microscopy (TEM) was performed from formalin-fixed samples, which were dehydrated in a graded alcohols sequence and embedded in Durcupan AcM resin (Sigma–Aldrich, St Louis, MO, USA). Semithin sections (1 µm) were stained with toluidine blue and examined by light microscopy. Selected ultrathin sections (90 nm) were cut and stained with uranyl acetate and lead citrate. An ultrastructural observation was made with a Philips TEM 208 transmission electron microscope (Philips, Eindhoven, Holland) operating at 100 kV.

2.7 Statistical analysis

All data are presented as mean ± standard deviation (SD) of three replicate groups. All data except for histological data were analysed by a one-way ANOVA followed by a

Tukey's multiple comparison test. Histological data were analysed using Pearson's χ^2 test with Yates' continuity correction. All statistical analyses were performed using GraphPad Prism 6.0 for Windows (Graph Pad Software, San Diego, CA, USA). A linear regression analysis was conducted to analyse growth performance indices and feed utilization indices according to dietary lipid level and judged by the adjusted coefficient of determination (R^2). The differences among treatments were considered significant at $P \leq 0.05$.

3. Results

3.1 Growth

Survival rate, growth performance and daily nutrient intake are summarized in Table 2. No significant differences were recorded among groups (P = 0.0572). Final body weight, SGR and FI were inversely correlated to dietary lipid level (P < 0.0001), whereas the FCR was directly correlated (P < 0.001). The final body weight was significantly higher in fish fed diet L8 (40.7 ± 1.7), followed by those fed L12 (35.1 ± 1.2 g), L16 (27.9 ± 2.5 g) and L20 (22.1 ± 0.3 g). The lowest SGR was observed in fish fed L20, followed by fish fed L16, whereas fish fed L12 and L8 scored the highest. The FCR of fish fed L20 was higher than that of the other groups. FI was significantly lower when dietary lipid levels increased. From the group fed L8 to that fed L20, the protein intake significantly decreased whereas the lipid intake increased. Energy intake was significantly higher in fish fed L8 and L12 as compared to those fed L16 and L20.

Data on VSI, HSI, whole body composition and nutritional indices are shown in Table 3. VSI varied from 3.95 to 4.57%, a result significantly higher in fish fed high-lipid diets (L16 and L20) than in those fed low-lipid diets (L8 and L12). No significant differences were found in HSI. Whole body protein content was significantly lower in L20 in comparison with the other groups. Whole body lipid content ranged between 5.6% (L16) and 6.7% (L12), without significant differences among treatments. PER and GPE in fish fed L20 were significantly lower in comparison with that in the other groups. GLE was significantly higher in fish fed low-lipid diets (L8 and L12) than in those fed high-lipid diets (L16 and L20).

Serum total cholesterol and triglycerides concentrations are shown in Figure 1. No significant differences among treatments were found in total cholesterol (P = 0.1704) and triglycerides (P = 0.2232) concentrations even though L20 displayed lower values compared to the other groups.

3.2 Histology and ultrastructure

Histological observations showed lipid droplets in the cytoplasm of enterocytes at the apex of the mucosal folds and in some cases also along the entire fold (intestinal steatosis) (Fig. 2 A, D) in 19 of 36 subjects; the remaining 17 subjects did not show any pathological changes, and supranuclear vacuolation, mainly at the apex of mucosal folds, was present as a sign of normal absorptive activity.

The statistical correlation between histological observation and diet treatments is presented in Table 4. The number of fish with intestinal steatosis in groups L20 and L16 was significantly higher than that in group L8.

Ultrastructure showed large electrondense lipid droplets within the cytoplasm of enterocytes and warping of the cytoplasmic membrane (steatosis); in some cases, lipid droplets were also present within the Golgi apparatus (Fig. 2 E, F). In some cells, despite no evidence of cytoplasmic lipid droplets, several electrondense lamellar and whorled myelin bodies (myelinosomes), "zebra type," consistent with phospholipids were seen within lysosomes (Ghadially, 1988) (Fig. 2 B, C).

4. Discussion

Increasing the dietary lipid level from 8% to 20% had an overall impact on performance and gut health of the common sole juveniles. In particular, when comparing the different treatments, increasing the percentage of the dietary lipid level above 12% resulted in decreased growth. Also, in other flatfish, such as Senegalese sole (Dias et al., 2004; Borges et al., 2009) and turbot (Regost et al., 2001), increasing dietary lipid levels tends to depress growth performance. On the contrary, high-lipid diets improved growth in different pelagic marine species, as observed in parr (*Salmo salar*) (Nordgarden et al., 2002), Pacific bluefin tuna juvenile (*Thunnus orientalis*) (Biswas et al., 2009) and gilthead sea bream (*Sparus aurata*) (Mongile et al., 2014).

In our trial, the decreased growth seemed due both to a decreased FI and an increased FCR in fish fed high lipid diets.

The decline in FI is in disagreement with a previous study on Senegalese sole that reported an increase in FI with increasing the level of lipids from 4 to 20% (Borges et al., 2009). The reduced FI observed in our study may be related to reduced feed palatability. Different authors have reported that the palatability of feed is one of the

major factors determining feed acceptance (de la Higuera, 2001; Glencross et al., 2007). Several research papers reported palatability problems in sole when fed fishmeal based feed. In fact, the trophic profile for common sole is mainly composed of polichaeta and molluscs, as well as crustaceans, while fish are not present in the natural diet of sole (Reig et al., 2003). Hence, the decreased feed intake observed in our study may be related to the increased levels of fish oil, which could have reduced feed palatability due to the organoleptic features of the oil (Reig et al., 2003).

Another explanation for the trend of FI may be due to the view that fish, like homeothermic animals, adapt feed intake to meet their energy requirements (Mongile et al., 2014). Feed intake depends upon the dietary digestible energy content (Lupatsch et al., 2001), and in the present study the high lipid diets had significantly higher energy contents. Therefore, sole may have adjusted feed intake to reduce energy intake. Furthermore, Borges et al. (2013b) suggested a low daily metabolic energy budget in Senegalese sole. Similarly, common sole in their natural habitat remain inactive, buried in the sand the whole day, with a passive behaviour and carry out all their activity only during the night (Reig et al., 2003), suggesting low energy requirements.

Similarly to FI, a worsening in FCR was observed in fish fed high lipid diets. Indeed, the FCR was significantly higher in fish fed L20 as compared to the other treatments, suggesting a negative influence of high lipid levels on nutrient utilization. This result is confirmed by a significantly reduced PER and GPE in L20 as compared to the other groups. In addition the results of GLE also confirm that lipids are not efficiently used for energy production and a dietary inclusion higher than 12% significantly depressed lipid utilization. It is well known that significant protein sparing can be achieved in many fish species by increasing dietary digestible energy levels in the diet through the

incorporation of fats and digestible carbohydrates (Rueda-Jasso et al., 2004). On the contrary, in our study, lipids in common sole not only were not efficiently used for energy production and protein sparing, but high levels also had a negative impact on gut health.

In fact, moderate to severe intestinal steatosis was found in fish fed the high lipids diets (L16 and L20). Moreover, the ultrastructure displayed a certain cellular engulfment due to lipid overload; large electrondense lipid droplets (triglycerides) were found in the cytoplasm of enterocytes and in the Golgi apparatus, while myelinosomes (stacked phospholipids) were found in lysosomes (Ghadially, 1988).

A study on *Solea senegalensis* fed two different lipid levels, which included histology and morphometry of the intestine, found a significant increase in goblet cell number in a higher lipid level diet (Borges et al., 2013b). In our study, there was no evidence of goblet cell hyperplasia in any of the treatments. To the best of our knowledge, there are few nutritional studies on lipid requirements that include the ultrastructure of fish intestine only studies regarding the substitution of fish oil with vegetable oils. Caballero et al. (2003) and Olsen et al. (2000) found lipid droplet accumulation in enterocytes and in intracellular spaces in sea bream (*Sparus aurata*) fed soybean oil and in Arctic char (*Salvelinus alpinus* L.) fed linseed oil. In our study, the accumulation of lipid droplets in intercellular spaces was not detected. This also applied to necrotic changes; on the other hand, a marked cell degeneration was seen during histological observation.

Regarding plasma total cholesterol and triglycerides levels, the range of values found in our study was similar to that obtained by Valente et al. (2011) and Borges et al. (2013b) for Senegalese sole when fed diets with two different lipid levels. Similar to our findings, total cholesterol did not show any variations at the same sampling time (six

hours after feeding) in both trials. On the other hand, triglycerides levels increased in fish fed the high lipid diet.

In our trial, high lipid diets not only affected the growth, but also the body composition. Indeed, whole body protein content was significantly lower in L20, providing additional evidence that dietary lipids do not favour protein accretion. Campos et al. (2010) observed a decrease in the expression of myogenic regulatory factors and myosins in the muscle of Senegalese sole fed increasing dietary lipid levels, supporting somehow the hypothesis that high lipid levels depress growth by reducing protein accretion. Nevertheless, whole body lipid content varied from 5.6 to 6.7 %, with no significant differences among treatments. The present work clearly showed that common sole is a lean fish with low body fat contents and with a scarce capacity to accumulate fat even when fed with high lipid diets, as already observed in Senegalese sole (Borges et al., 2009).

Regarding VSI, values were lower in the sole fed the low lipid diets than in the sole fed the high lipid diets. Borges et al. (2009) have also reported an increased VSI in Senegalese sole fed diets with lipid content above 16%. Moreover, the authors reported a two-fold increase in intestinal lipid content in Senegalese sole fed 16 and 20% dietary lipid levels as compared to those fed 4% lipid diets. In the present study no differences in HSI among treatments were found. These results are in agreement with a previous experiment on Senegalese sole where fish fed 4 and 17% lipid level diets did not have any changes in HSI levels (Borges et al., 2013a).

In conclusion, the results of this trial suggest that the diet for *Solea solea* juveniles should include no more than 12% lipids. Higher lipid inclusions not only led to a

substantial decline in performance but also affected gut health. This should be taken into consideration in formulating specific practical diets for common sole.

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References

Biswas, B.K., Ji, S.C., Biswas, A.K., Seoka, M., Kim, Y.S., Kawasaki, K.I., Takii, K., 2009. Dietary protein and lipid requirements for the Pacific bluefin tuna *Thunnus orientalis* juvenile. Aquaculture 288, 114–119.

Bligh, E.G., Dyer, W.J., 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Phys. 37(8), 911–917.

Bonaldo, A., Parma, L., Badiani, A., Serratore, P., Gatta, P.P., 2011.Very early weaning of common sole (*Solea solea* L.) larvae by means of different feeding regimes and three commercial microdiets: influences on performances, metamorphosis development and tank hygiene. Aquaculture 321, 237-244.

Borges, P., Oliveira, B., Casal, S., Dias, J., Conceição, L., Valente, L.M.P., 2009. Dietary lipid level affects growth performance and nutrient utilisation of Senegalese sole (*Solea senegalensis*) juveniles. British Journal of Nutrition 102, 1007-1014.

Borges, P., Medale, F., Dias, J., Valente, L.M.P., 2013a. Protein utilisation and intermediary metabolism of Senegalese sole (*Solea senegalensis*) as a function of protein:lipid ratio. British Journal of Nutrition 109, 1373–1381.

Borges, P., Medale, F., Veron, V., Pires, M.A., Dias, J., Valente, L.M.P., 2013b. Lipid digestion, absorption and uptake in *Solea senegalensis*. Comparative Biochemistry and Physiology, Part A 166, 26–35.

Caballero, M.J., Izquierdo, M.S., Kjørsvik, E., Montero, D., Socorro, J., Fernández, A.J., Rosenlund, G., 2003. Morphological aspects of intestinal cells from

gilthead seabream (*Sparus aurata*) fed diets containing different lipid sources. Aquaculture 225, 325–340.

Campos, C., Valente, L.M.P., Borges, P., Bizuayehu, T., Fernandes, J.M.O., 2010. Dietary lipid levels have a remarkable impact on the expression of growth-related genes in Senegalese sole (*Solea senegalensis* Kaup). Journal of Experimental Biology 213, 200-209.

Cowey, C.B., Adron, J.W., Brown, D.A., Shanks, A.M., 1975. Studies on nutrition of marine flatfish – metabolism of glucose by plaice (*Pleuronectes platessa*) and effect of dietary energy-source on protein utilization in plaice. British Journal of Nutrition 33, 219–231.

Dias, J., Rueda-Jasso, R., Panserat, S., Conceição, L., Gomes, E.F., Dinis, M.T., 2004. Effect of dietary carbohydrate-to-lipid ratios on growth, lipid deposition and metabolic hepatic enzymes in juvenile Senegalese sole (*Solea senegalensis*, Kaup). Aquaculture Research 35, 1122–1130.

Ferraresso, S., Bonaldo, A., Parma, L., Cinotti, S., Paola Massi, P., Bargelloni, L., Gatta, P.P., 2013. Exploring the larval transcriptome of the common sole (*Solea solea* L.). BMC Genomics 14, 315-337.

Gatta, P.P., Parma, L., Guarniero, I., Mandrioli, L., Sirri, R., Fontanillas, R., Bonaldo, A., 2011. Growth, feed utilization and liver histology of juvenile common sole (*Solea solea* L.) fed isoenergetic diets with increasing protein levels. Aquaculture Research 42, 313-321.

Ghadially, F.N., 1988. Lysosomes, in: Ghadially, F.N. (Ed.), Ultrastructural Pathology of the Cell and Matrix, third edition. Butterworth-Heinemann, London, pp. 589-765.

Glencross, B.D., Booth, M., Allan, G.L., 2007. A feed is only as good as its ingredients – a review of ingredient evaluation strategies for aquaculture feeds. Aquaculture Nutrition 13, 17–34.

de la Higuera, M., 2001. Effects of nutritional factors and feed characteristics on feed intake, in: Houlinan, D., Boujard, T., Jobling, M. (Eds), Food Intake in Fish. Wiley-Blackwell Science Ltd, Oxford, UK, pp. 250–268.

Lund, I., Steenfeldt, S.J., Banta, G., Hansen, B.W., 2008. The influence of dietary concentrations of arachidonic acid and eicosapentaenoic acid at various stages of larval ontogeny on eye migration, pigmentation and prostaglandin content of common sole larvae (*Solea solea* L.). Acquaculture 276, 143-153.

Lund, I., Steenfeldt, S.J., Herrmann, B., Pedersen, P.B., 2013. Feed intake as explanation for density related growth differences of common sole *Solea solea*. Aquaculture Research 44, 367–377.

Lupatsch, I., Kissil, G.W., Sklan, D., Pfeffer, E., 2001. Effects of varying protein and energy supply on growth, body composition and protein utilization in gilthead seabream (*Sparus aurata* L.). Aquaculture Nutrition 7, 71-80.

Mas-Muñoz, J., Komen, H., Schneider, O., Visch, S.W., Schrama, J.W., 2011. Feeding behaviour, swimming activity and boldness explain variation in feed intake and growth of sole (*Solea solea*) reared in captivity. PloS One 6(6):1.

Mongile, F., Bonaldo, A., Fontanillas, R., Mariani, L., Badiani, A., Bonvini, E., Parma, L., 2014. Effects of dietary lipid level on growth and feed utilisation of gilthead seabream (*Sparus aurata* L.) reared at Mediterranean summer temperature. Italian Journal of Animal Science 13, 30-35.

Nordgarden, U., Hemre, G.I., Hansen, T., 2002. Growth and body composition of Atlantic salmon (*Salmo salar* L.) parr and smolt fed diets varying in protein and lipid contents. Aquaculture 207, 65– 78.

Olsen, R.E., Myklebust, R., Ringø, E., Mayhew, T.M., 2000. The influences of dietary linseed oil and saturated fatty acids on caecal enterocytes in Arctic char (*Salvelinus alpinus* L.): a quantitative ultrastructural study. Fish Physiology and Biochemistry 22, 207–216.

Parma, L., Bonaldo, A., Massi, P., Yufera, M., Martínez-Rodríguez, G., Gatta, P.P., 2013. Different early weaning protocols in common sole (*Solea solea* L.) larvae: Implications on the performances and molecular ontogeny of digestive enzyme precursors. Aquaculture 414-415, 26-35.

Peres, H., Guerreiro, I., Pérez-Jiménez, A., Oliva-Teles, A., 2013. A non-lethal faeces collection method for Senegalese sole (*Solea senegalensis*) juveniles. Aquaculture 414-415, 100-102.

Peres, H., Santos, S., Oliva-Teles, A., 2014. Blood chemistry profile as indicator of nutritional status in European seabass (*Dicentrarchus labrax*). Fish Physiology and Biochemistry 40, 1339–1347. DOI 10.1007/s10695-014-9928-5

Regost, C., Arzel, J., Cardinal, M., Robin, J., Laroche, M., Kaushik, S.J., 2001. Dietary lipid level, hepatic lipogenesis and flesh quality in turbot (*Psetta maxima*). Aquaculture 193, 291–309.

Reig, L., Ginovart, M., Flos, R., 2003. Modification of the feeding behaviour of sole (*Solea solea*) through the addition of a commercial flavour as an alternative to betaine. Aquatic Living Resources 16, 370–379.

Rueda-Jasso, R., Conceição, L.E.C., Dias, J., De Coen, W., Gomes, E., Rees, J.F., Soares, F., Dinis, M.T., Sorgeloos, P., 2004. Effect of dietary non-protein energy levels on condition and oxidative status of Senegalese sole (*Solea senegalensis*) juveniles. Aquaculture 231, 417-433.

Sargent, J.R., Tocher, D.R., Bell, J.G., 2002. The lipids, in: Halver, J.E. and Hardy R.W. (Eds.), Fish Nutrition, third edition. Elsevier Science, USA, pp. 181-257.

Schram, E., Van der Heul, J.W., Kamstra, A., Verdegem, M.C.J., 2006. Stocking density-dependent growth of Dover sole (*Solea solea*). Aquaculture 252, 330–347.

Schram, E., Bierman, S., Teal, L.R., Haenen, O., van de Vis, H., Rijnsdorp, A.D., 2013. Thermal Preference of Juvenile Dover Sole (*Solea solea*) in Relation to Thermal Acclimation and Optimal Growth Temperature. PloS One 8(4):1.

Valente, L.M.P., Linares, F., Villanueva, J.L.R., Silva, J.M.G., Espe, M., Escórcio, C., Pires, M.A, Saavedra, M.J., Borges, P., Medale, F., Alvárez-Blázquez, B., Peleteiro, J.B., 2011. Dietary protein source or energy levels have no major impact on growth performance, nutrient utilisation or flesh fatty acids composition of market-sized Senegalese sole. Aquaculture 318, 128–137.

Figure captions

Figure 1. Serum triglycerides and cholesterol levels 5 h postprandial. Data are given as the mean ± SD from triplicate groups.

Figure 2. A, B, C moderate steatosis. **A)** histological section shows some lipid droplets in the cytoplasm of apical enterocytes (H&E, 50 µm); **B)** ultrastructure shows several electrondense lamellar and whorled myelin bodies (myelinosomes) (arrow) in lysosomes consistent with phospholipids (bar=10 µm); **C)** higher magnification displays myelinosomes, "zebra type" (arrow), characterized by stacked lamellar structures (bar=1 µm). D, E, F severe steatosis. **D)** histological section shows lipid droplets in the cytoplasm of enterocytes along the entire intestinal fold (H&E, 50 µm); **E)** ultrastructure shows large electrondense lipid droplets occupying the cytoplasm (arrow) and warping of the cytoplasmic membrane (bar=5 µm); **F)** higher magnification displays lipid droplets within the Golgi apparatus (arrow) (bar=2 µm).

Table 1 Ingredients and proximate composition of the experimental diets

	Experimental diet			
	L8	L12	L16	L20
Dietary ingredients (%)				
Fishmeal	35.0	35.0	35.0	35.0
Pea protein concentrate	20.0	20.0	20.0	20.0
Wheat gluten	20.0	20.0	20.0	20.0
Mussel meal	5.0	5.0	5.0	5.0
Fish oil	0	4.0	8.0	12.0
Wheat meal	19.0	15.0	11.0	7.0
Mix vit-min[1]	1.0	1.0	1.0	1.0
Proximate composition (% or specified)				
Crude protein	60.2	59.6	59.8	57.8
Total lipids	8.0	12.6	15.5	19.4
Ash	7.5	7.4	7.4	7.0
Moisture	3.8	4.5	4.5	3.9
Gross energy (KJ/g)	19.9	21.1	22.2	23.6

[1]Vitamins (mg kg^{-1} diet or specified): retinol acetate, 18,000 (IU kg^{-1} diet); cholecalciferol, 2,000 (IU kg^{-1} diet); alpha tocopherol acetate, 35; sodium menadione bisulphate, 10; thiamin-HCl, 15; riboflavin, 25; calcium pantothenate, 50; nicotinic acid, 200; pyridoxine HCl, 5; folic acid 10; cyanocobalamin, 0.02; biotin, 1.5; ascorbic acid, 400; inositol, 400, choline chloride (50%), 2000. Minerals (mg kg^{-1} diet or specified): cobalt sulphate, 1.91; copper sulphate, 19.6; iron sulphate, 200; sodium fluoride, 2.21; potassium iodide, 0.78; magnesium oxide, 830; manganese oxide, 26; sodium selenite, 0.66; zinc oxide, 37.5; dibasic calcium phosphate, 5.93 (g kg^{-1} diet); potassium chloride, 1.15 (g kg^{-1} diet); sodium chloride, 0.40 (g kg^{-1} diet).

Table 2 Growth performance, survival rate and daily nutrient intake of sole fed the experimental diets

	Experimental diet				Linear regression line	r^2	*P*
	L8	L12	L16	L20			
Growth							
IBW (g)	13.8 ± 0.4	13.8 ± 0.4	13.8 ± 0.4	13.8 ± 0.4			
FBW (g)	40.7 ± 1.7^{d}	35.1 ± 1.2^{c}	27.9 ± 2.5^{b}	22.1 ± 0.3^{a}	Y = 53.58 – 1.582x	0.96	< 0.0001
SGR (day^{-1})	0.71 ± 0.03^{c}	0.63 ± 0.03^{c}	0.48 ± 0.05^{b}	0.30 ± 0.01^{a}	Y = 1.015 – 0.035x	0.94	< 0.0001
FI (% day^{-1})	0.88 ± 0.01^{d}	0.78 ± 0.02^{c}	0.69 ± 0.02^{b}	0.63 ± 0.01^{a}	Y = 1.037 – 0.021x	0.97	< 0.0001
FCR	1.32 ± 0.05^{a}	1.35 ± 0.04^{a}	1.63 ± 0.11^{a}	2.66 ± 0.29^{b}	Y = 0.233 + 0.108x	0.72	< 0.001
Survival	100 ± 0.0	100 ± 0.0	98 ± 3.5	92 ± 9.2	Y = 106.6 – 0.650x	0.25	0.0572
Nutrient daily intake (g or kJ/kg ABW / day)							
Protein	5.27 ± 0.07^{d}	4.68 ± 0.15^{c}	4.15 ± 0.13^{b}	3.62 ± 0.07^{a}	Y = 6.346 – 0.137x	0.98	< 0.0001
Lipid	0.70 ± 0.01^{a}	0.99 ± 0.03^{b}	1.07 ± 0.03^{c}	1.22 ± 0.02^{d}	Y = 0.425 + 0.041x	0.92	< 0.0001
Energy	174.3 ± 2.19^{b}	165.8 ± 5.21^{b}	153.8 ± 4.74^{a}	147.5 ± 2.98^{a}	Y = 192.6 – 2.306x	0.89	< 0.0001

Data are given as the mean (n=3; n=80 for IBW and FBW) ± SD. In each line, different superscript letters indicate significant differences among treatments ($P \leq 0.05$).

IBW, Initial body weight.

FBW, Final body weight.

SGR, Specific growth rate.

FI, Feed intake.

FCR, Feed conversion rate.

Table 3 Viscerosomatic index (VSI), hepatosomatic index (HSI), body composition and nutritional indices of common sole fed the experimental diets

	Experimental diet						
	L8	L12	L16	L20	Linear regression line	r^2	*P*
Somatic indices							
VSI	4.04 ± 0.36^{a}	3.95 ± 0.32^{a}	4.57 ± 0.19^{b}	4.55 ± 0.23^{b}	Y = 3.525 + 0.054x	0.37	< 0.05
HSI	1.68 ± 0.15	1.46 ± 0.16	1.63 ± 0.17	1.54 ± 0.26	Y = 1.660 – 0.006x	– 0.08	0.6446
Whole body composition							
Moisture	73.5 ± 0.22^{b}	72.6 ± 0.37^{c}	72.7 ± 0.64^{c}	74.5 ± 0.41^{a}	Y = 72.23 + 0.078x	0.08	0.1931
Crude Protein	17.4 ± 0.19^{a}	17.2 ± 0.41^{a}	17.0 ± 0.38^{a}	16.3 ± 0.57^{b}	Y = 18.17 – 0.086x	0.46	< 0.001
Total Lipids	5.9 ± 0.55	6.7 ± 0.76	5.6 ± 0.37	6.3 ± 1.17	Y = 6.035 + 0.007x	– 0.09	0.8512
Ash	2.3 ± 0.09^{b}	2.7 ± 0.13^{a}	2.1 ± 0.16^{b}	2.9 ± 0.31^{a}	Y = 2.00 – 0.035x	0.12	0.1436
Nutritional indices							
PER	1.2 ± 0.05^{b}	1.3 ± 0.05^{b}	1.1 ± 0.10^{b}	0.8 ± 0.03^{a}	Y = 1.601 – 0.035x	0.71	< 0.001
GPE	22.0 ± 0.9^{b}	22.2 ± 1.1^{b}	19.2 ± 2.0^{b}	12.9 ± 1.9^{a}	Y = 29.59 – 0.752x	0.70	< 0.001
GLE	58.5 ± 3.5^{b}	46.6 ± 7.3^{b}	26.5 ± 3.5^{a}	21.1 ± 3.8^{a}	Y = 84.49 – 3.306x	0.89	< 0.0001

Data are given as the mean (n=3;n=30 for VSI and HSI)± SD. In each line, different superscript letters indicate significant differences among treatments ($P \leq 0.05$).

VSI, Visceral somatic index.

HIS, Hepato somatic index.

PER, Protein efficiency ratio ((FBW-IBW)/protein intake).

GPE, Gross protein efficiency (100*[(%final body protein * FBW)-(%initial body protein * IBW)]/total protein intake $fish^{-1}$).

GLE, Gross lipid efficiency (100*[(%final body lipid * FBW)-(%initial body lipid * IBW)]/total lipid intake $fish^{-1}$).

Table 4 Number of common sole per dietary treatment that displayed intestinal steatosis (n=9)

	Morphological changes of enterocytes				
	Normal	Steatosis		χ^2	*P*
Treatments					
L8	8	1	a	14.60	0.0022
L12	6	3	ab		
L16	1	8	b		
L20	2	7	b		

Different superscript letters indicate significant differences among treatments ($P \leq 0.05$).

Figure 1

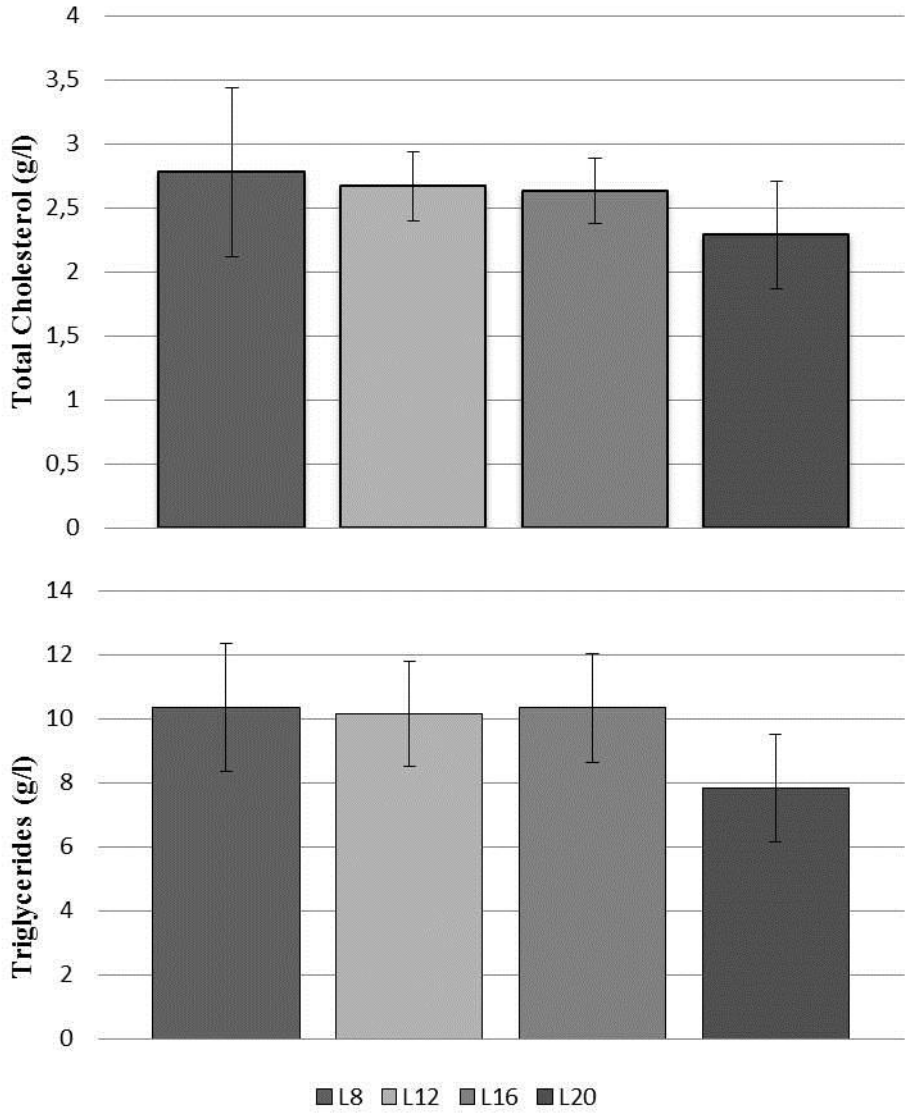
Total Cholesterol (g/l)
4
3,5
3
2,5
2
1,5
1
0,5
0
Triglycerides (g/l)
14
12
10
8
6
4
2
0
L8
L12
L16
L20

Figure 2