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RESEARCH ARTICLE

Orally administered fatty acids enhance anorectic potential but do not activate central fatty acid sensing in Senegalese sole post-larvae

Cristina Velasco[1], Kruno Bonacic[2], José L. Soengas[1] and Sofia Morais[2,3,*]

ABSTRACT

Studies in fish have reported the presence and function of fatty acid (FA)-sensing systems comparable in many aspects to those known in mammals. Such studies were carried out in juvenile and adult fish, but the presence of FA-sensing systems and control of food intake have never been evaluated in early life stages, despite the importance of establishing when appetite regulation becomes functional in larval fish. In this study, we aimed to elucidate the possible effects of different specific FAs on neural FA-sensing systems and neuropeptides involved in the control of food intake in Senegalese sole post-larvae. To achieve this, we orally administered post-larvae with different solutions containing pure FA – oleate (OA), linoleate (LA), α-linolenate (ALA) or eicosapentaenoate (EPA) – and evaluated changes in mRNA abundance of neuropeptides involved in the control of food intake and of transcripts related to putative FA-sensing systems, 3 and 6 h post-administration. The changes in neuropeptide gene expression were relatively consistent with the activation of anorectic pathways (enhanced *cart4* and *pomcb*) and a decrease in orexigenic factors (*npy*) following intake of FA. Even though there were a few differences depending on the nature of the FA, the observed changes appear to suggest the existence of a putative anorectic response in post-larvae fish to the ingestion of all four tested FAs. However, changes in neuropeptides cannot be explained by the integration of metabolic information regarding FAs in circulation through FA-sensing mechanisms in the brain. Only the reduction in mRNA levels of the FA metabolism gene *acc* in OA-treated (6 h), ALA-treated (3 h) and EPA-treated (3 and 6 h) post-larvae could be indicative of the presence of a FA-sensing system, but most genes either were not significantly regulated (*fat/cd36-lmp2*, *acly*, *kir6.x*, *srebp1c*) or were affected in a way that was inconsistent with FA-sensing mechanisms (*fat/cd36-pg4l*, *fas*, *cpt1.1*, *cpt1.2*, *cpt1.3*, *sur*, *pparα* and *lxrα*).

KEY WORDS: Food intake, Larval fish, PUFA, Lipid sensing, Neuropeptides, Cocaine- and amphetamine-related transcript

INTRODUCTION

In previous studies, we characterized the presence and function of fatty acid (FA)-sensing systems in the hypothalamus of the teleost fish rainbow trout (*Oncorhynchus mykiss*) (Librán-Pérez et al., 2012, 2013, 2014a,b, 2015) and Senegalese sole (*Solea senegalensis* Kaup) (Conde-Sieira et al., 2015), which are comparable in many aspects to those in mammals (Blouet and Schwartz, 2010). These systems respond to increased levels of unsaturated long-chain FAs such as oleate (OA, C18:1 *n*-9) and correlate with the expression of neuropeptides involved in the control of food intake (Librán-Pérez et al., 2012, 2014a). FA-sensing mechanisms are based on: (i) FA metabolism via carnitine palmitoyltransferase 1 (CPT-1) inhibition to import FA-coenzyme-A (CoA) into the mitochondria for oxidation; (ii) binding to FA translocase (FAT/CD36) and modulation of the transcription factors peroxisome proliferator-activated receptor type α (PPARα) and sterol regulatory element-binding protein type 1c (SREBP1c); and (iii) production of reactive oxygen species in the mitochondria, resulting in inhibition of ATP-dependent inward rectifier potassium channel (K_{ATP}) activity (Soengas, 2014). The activation of these systems relates to the inhibition of the orexigenic factors agouti-related protein (AgRP) and neuropeptide Y (NPY), and the enhancement of the anorexigenic factors cocaine- and amphetamine-related transcript (CART) and pro-opiomelanocortin (POMC) (Librán-Perez et al., 2012, 2014a; Conde-Sieira et al., 2015), ultimately leading to decreased food intake.

In contrast to what is known in mammals, we also provided evidence in Senegalese sole juveniles for the activation of FA-sensing systems by polyunsaturated FAs (PUFAs). Furthermore, hypothalamic FA-sensing systems in Senegalese sole were activated by α-linolenate (ALA, C18:3 *n*-3) but not by eicosapentaenoate (EPA, C20:5 *n*-3) (Conde-Sieira et al., 2015), indicating that the response might be specific to certain PUFAs. Therefore, both the level of unsaturation and the chain length of the FA seem to be important factors for the hypothalamic sensing capacity, at least in Senegalese sole juveniles, but are probably not the only factors involved. The finding that FA-sensing systems in Senegalese sole respond to changes in PUFA levels may relate to the general importance of PUFAs (especially of the *n*-3, but also of the *n*-6 series) in marine fish (Tocher, 2003).

Studies in fish regarding FA-sensing systems and control of food intake have only been carried out in juveniles and adults so far (Libran-Pérez et al., 2012, 2014a,b; Conde-Sieira et al., 2015). However, it is of great importance to clearly establish when appetite regulation becomes functional in fish larvae, particularly in species of commercial interest to aquaculture. Very little is known concerning the neural regulation of feeding and appetite during early larval stages, with very few studies describing changes in the expression of brain neuropeptides (Kortner et al., 2011; Gomes et al., 2015). As far as we know, there are no available studies assessing the impact of specific nutrients on the regulation of food intake in fish larvae and post-larvae. In a preliminary study with Senegalese sole larvae (Bonacic et al., 2016), we observed differences in the intake of *Artemia* prey that had been enriched

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List of symbols and abbreviations

acc	acetyl-CoA carboxylase
acly	ATP-citrate lyase
agrp	agouti-related protein
ALA	α-linolenate
cart	cocaine- and amphetamine-related transcript
cpt1	carnitine palmitoyltransferase 1
dph	days post-hatching
ef1α	elongation factor 1α
EPA	eicosapentaenoate
FA	fatty acid
fas	fatty acid synthetase
fat/cd36	FA translocase
K_{ATP}	ATP-dependent inward rectifier potassium channel
kir6.x	inward rectifier K^+ channel pore type 6.x
LA	linoleate
lxrα	liver X receptor α
npy	neuropeptide Y
OA	oleate
pomc	pro-opiomelanocortin
pparα	peroxisome proliferator-activated receptor type α
PUFA	polyunsaturated fatty acid
qPCR	quantitative RT-PCR
srebp1c	sterol regulatory element-binding protein type 1c
sur	sulfonylurea receptor
ubq	ubiquitin

with different oils (olive, soybean, linseed and cod liver oil); specifically, there was a higher ingestion of prey enriched with cod liver oil. However, the expression of neuropeptides in whole larvae could not explain such differences, even though larvae on the cod liver oil-enriched *Artemia* diet had the most dissimilar gene expression pattern of the four dietary groups. Similar findings were obtained in the same experiment with post-larvae (although effects on food intake were less clear because of a higher variability). At this life stage, even though there was still no correlation between neuropeptide expression and diet intake, gene expression in the body and head of post-metamorphic larvae was generally more in accordance with the putative anorexigenic or orexigenic function of the analysed genes, which could suggest a slightly more developed regulatory system. The oils used were especially rich in specific FAs such as OA in olive oil, linoleate (LA, C18:2 *n*-6) in soybean oil, ALA in linseed oil and EPA in cod liver oil. However, other nutrients present in the diet could also have interacted to produce the effect, so the observed responses could not be unequivocally attributed to any of these specific FAs.

In the current study, we aimed to elucidate the possible effects of different specific FAs on neural FA-sensing systems and neuropeptides involved in the control of food intake in the early life stages of fish. To achieve these objectives, we orally administered different solutions containing OA, LA, ALA or EPA to Senegalese sole post-larvae. Because of the small size of the animals, which prevented dissection of specific brain regions, we evaluated changes in mRNA abundance of neuropeptides related to the control of food intake, such as *agrp2*, *npy*, *pomca*, *pomcb*, *cart1a*, *cart1b*, *cart2b* and *cart4* in head sections. Furthermore, we also evaluated changes in mRNA abundance of transcripts related to putative FA-sensing systems based on: (1) FA metabolism, such as acetyl-CoA carboxylase (*acc*), ATP-citrate lyase (*acly*), *cpt-1* and fatty acid synthetase (*fas*); (2) binding to FAT/CD36, such as *fat/cd36*, liver X receptor α (*lxrα*), *pparα* and *srebp1c*; and (3) mitochondrial activity, such as inward rectifier K^+ channel pore type 6.x (*kir6.x*) and sulfonylurea receptor (*sur*).

MATERIALS AND METHODS

Fish

Newly hatched Senegalese sole larvae were obtained from Stolt Sea Farm S.A. (Carnota, A Coruña, Spain) and distributed into two 100 l cylindroconical tanks at a density of 50 larvae l^{-1}. The tanks were connected to a recirculation system (IRTAMAR®) with 50% daily water renewal and regulated temperature (18–19°C), salinity (35 ppt) and dissolved oxygen (7.5 mg l^{-1}). The photoperiod was 16 h light:8 h dark, with a light intensity of <500 lx at the water surface. Feeding was performed twice a day (at 09:30 h and 17:30 h). Larvae were fed rotifers enriched with Algamac 3050 flake (Aquafauna Bio-Marine, Inc., Hawthorne, CA, USA), according to the manufacturer's instructions, at 10 rotifers ml^{-1} from 1 to 9 days post-hatching (dph). Algamac-enriched *Artemia* metanauplii were introduced at 7 dph and fed until 12 dph, in quantities gradually increasing from 0.5 to 3 metanauplii ml^{-1}. From 13 dph onwards, to avoid any influence from the high levels of lipids in the enrichment product, larvae were fed only non-enriched *Artemia* metanauplii (kept in clean seawater instead of the enrichment emulsion), at 4 metanauplii ml^{-1} during the planktonic stage (until 20 dph). After settling of the majority of the larvae, frozen non-enriched *Artemia* were also added in increasing amounts, at 6–12 metanauplii ml^{-1} in total, up to 40 dph. It is possible to feed Senegalese sole larvae on non-enriched *Artemia* without significantly affecting growth performance, given the relatively low requirements for lipids and essential fatty acids in this species compared with other marine species, particularly after settlement (Dâmaso-Rodrigues et al., 2010).

The experiments described here comply with the Guidelines of the European Union Council (2010/63/UE) and the Spanish Government (RD53/2013) for the use of animals in research, with protocols (including tube feeding, described below) approved by the ethics committee of IRTA.

Experimental design

Post-larvae were tube-fed one of four FA treatments on four consecutive days, from 36 to 39 dph. Test solutions consisted of the following pure free FAs: OA (≥99%), LA (≥99%), ALA (≥99%) or EPA (≥98.5%), all from Sigma-Aldrich (Madrid, Spain). On each preceding day, a group of post-larvae were taken from the rearing tanks and washed in a submerged 1 mm net (to remove any uneaten *Artemia*), transferred to a 4 l tray with clean water from the recirculation system and moved to the experimental room (maintained at 18°C), where they were kept with aeration and fasted for 18 h overnight. Post-larvae were gently anesthetized with 1 ml l^{-1} of tricaine methanesulfonate (MS-222, at 20 g l^{-1}). They were then individually tube-fed either a saline (control) or test FA solution under a dissecting microscope using a plastic capillary (0.19 mm inner diameter, Sigma-Aldrich) attached to a Nanoliter 2000 injector and micromanipulator (World Precision Instruments, Sarasota, FL, USA), to deposit ∼46 nl of the solution into the post-larvae foregut, as described in Rønnestad et al. (2001). Each post-larva was then transferred into an incubation system of plates containing 10 ml wells with clean seawater. Four post-larvae (N=4) were tube-fed per treatment and time point (3 and 6 h incubation, to allow for luminal absorption of FAs; Morais et al., 2005) and another four post-larvae (N=4) were tube-fed a saline solution and then immediately killed (control, reflecting the basal condition), every 4 consecutive days. The whole process took under 2 min per animal but, in order to avoid interference from the time of tube feeding, each day the order of administering the treatments was changed. Hence, each day a larva that was tube-fed a FA treatment in a different order was added to the sample pools, so that every sample (pool of four

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post-larvae) contained animals tube-fed at slightly different times, equally for each treatment. Larvae were killed by a lethal dose of MS-222, washed and transferred to an Eppendorf tube containing RNAlater stabilization buffer (Ambion, Life Technologies, Madrid, Spain), agitated at 4°C for 24 h, and then stored at −80°C. The post-larvae were later dissected on ice under a dissecting microscope, in order to separate and remove the body from the head section, which was then processed for molecular analysis of selected neuropeptide genes and genes involved in central FA-sensing pathways.

Analysis of relative expression by real-time quantitative RT-PCR

Total RNA was extracted from the head sections by homogenization in TRIzol (Ambion) with 50 mg of 1 mm diameter zirconium glass beads in a Mini-Beadbeater (Biospec Products Inc., Bartlesville, OK, USA). Solvent extraction was performed following the manufacturer's instructions and RNA quality and quantity were assessed by gel electrophoresis and spectrophotometry (NanoDrop2000, Thermo Fisher Scientific, Madrid, Spain). Total RNA (2 µg per sample) was reverse transcribed into cDNA using the High-Capacity cDNA RT kit (Applied Biosystems, Life Technologies, Carlsbad, CA, USA), following the manufacturer's instructions, but using a mixture of random primers (1.5 µl as supplied) and anchored oligo-dT (0.5 µl at 400 ng μl^{-1}, Eurogentec, Cultek, S.L., Madrid, Spain). Negative controls (containing no enzyme) were performed to check for genomic DNA contamination.

Gene expression levels were determined by real-time quantitative RT-PCR (qPCR) using the iCycler iQ (Bio-Rad, Hercules, CA, USA). Analyses were performed on 5 µl of diluted (1/50) cDNA using the SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad), in a total PCR reaction volume of 15 µl, containing 120–500 nmol l^{-1} of each primer. Sequences of the forward and reverse primers used for each gene expression assay are shown in Table 1. Relative quantification of the target genes was done using elongation factor 1 alpha (*ef1α*) and ubiquitin (*ubq*) as reference genes. Thermal cycling was initiated with incubation at 95°C for 2 min; 35 steps of qPCR were performed, each one consisting of heating at 95°C for 15 s, 30 s at each specific annealing temperature (Table 1) and 30 s at 72°C. Following the final PCR cycle, melting curves were systematically monitored (temperature gradient at 0.5°C s^{-1} from 55 to 94°C) to ensure that only one fragment was amplified. Samples without reverse transcriptase and samples without cDNA were run as negative controls, which were indeed negative, and confirmed no amplification of genomic DNA. Relative expression of the target genes was calculated using the delta-delta CT method ($2^{-\Delta\Delta CT}$), following Pfaffl (2001).

Statistics

Comparisons among groups were carried out using the statistical package SigmaStat (Systat Software, Inc.) with two-way ANOVA, with FA treatment and time as main factors. In cases where a significant effect was noted, *post hoc* comparisons were carried out by a Student–Newman–Keuls test. Differences were considered statistically significant at $P<0.05$.

RESULTS

The mRNA abundance of neuropeptides is shown in Fig. 1. The levels of *npy* (Fig. 1A) decreased after treatment with LA (6 h), ALA (3 and 6 h) and EPA (6 h) compared with the control; the levels in LA-treated fish after 6 h were also significantly reduced compared with the 3 h time point. The expression of *pomcb* (Fig. 1D) increased postprandially with all FA assessed, compared with the control, and then decreased in OA-treated fish from 3 h post-treatment to 6 h post-treatment. The mRNA levels of *cart1b* (Fig. 1F) were reduced in the ALA-treated group compared with the remaining groups, 6 h after FA administration. The expression of *cart4* (Fig. 1H) increased after treatment with OA (6 h), ALA (6 h), and EPA (3 and 6 h) compared with the control, and further increased in OA- and EPA-treated fish from 3 h post-treatment to 6 h post-treatment. No significant changes were observed for *agrp2* (Fig. 1B), *pomca* (Fig. 1C), *cart1a* (Fig. 1E) and *cart2b* (Fig. 1G) mRNA levels.

The expression of genes related to FA transport and metabolism is shown in Fig. 2. The mRNA levels of *fat/cd36-pg4l* (Fig. 2B) increased 6 h after treatment with OA, and this value was also higher than that for the same treatment at 3 h. The expression of *acc* (Fig. 2C) decreased after treatment with OA (6 h), ALA (3 h) and EPA (3 and 6 h); in OA-treated fish, *acc* mRNA levels were reduced from 3 h post-treatment to 6 h post-treatment. The levels of *fas* (Fig. 2E) increased after treatment with OA (3 and 6 h), LA (3 h), ALA (3 and 6 h) and EPA (3 h), but then decreased from 3 h post-treatment to 6 h post-treatment in all FA treatments. No significant changes were observed for *fat/cd36-lmp2* (Fig. 2A) and *acly* (Fig. 2D).

The mRNA level of genes involved in mitochondrial activity and the K_{ATP} channel is shown in Fig. 3. The level of *cpt1.1* (Fig. 3A) increased 6 h after treatment with OA compared with the control, and was also higher than that in the same treatment at 3 h. The expression of *cpt1.2* (Fig. 3B) increased after treatment with ALA (3 and 6 h) and decreased after treatment with EPA (6 h) compared with the control; in OA-treated fish there was an increase from 3 h post-treatment to 6 h post-treatment. The mRNA abundance of *cpt1.4* (Fig. 3C) increased in OA- (6 h), LA- (6 h), ALA- (6 h) and EPA-treated (3 and 6 h) fish compared with the control, and also in LA-treated fish from 3 h post-treatment to 6 h post-treatment. The expression of *sur* (Fig. 3E) increased after treatment with OA (3 h), LA (3 h), ALA (3 and 6 h) and EPA (3 h) compared with the control. No significant changes were observed for *kir6.x* (Fig. 3D).

The mRNA abundance of three transcription factors is shown in Fig. 4. The levels of *pparα* (Fig. 4A) increased after treatment with OA (3 h) and ALA (6 h) compared with the control, while its expression was reduced from 3 h post-treatment to 6 h post-treatment in OA- and EPA-treated fish. The expression of *lxrα* (Fig. 4C) was not different to the control for any of the treatments but was reduced from 3 h post-treatment to 6 h post-treatment in fish treated with OA and LA. In the case of *srebp1c* (Fig. 4B), there were no significant differences.

DISCUSSION

Orally administered FA enhances anorectic potential in Senegalese sole post-larvae

Oral treatment with different FAs induced changes in the mRNA abundance of some neuropeptides in sole post-larvae. Despite not all peptides displaying changes in their mRNA abundance, the changes observed generally suggest the existence of an anorectic response in fish post-larvae to the ingestion of different FAs. In particular, enhanced expression of anorexigenic neuropeptides (CART and POMC; at least one homologue – *cart4* and *pomcb*) was observed in parallel with decreased transcript levels of the orexigenic *npy* neuropeptide (although no effect was observed in *agrp2*). A similar effect was also suggested in juveniles of the same species after intraperitoneal (i.p.) injection with FA (Conde-Sieira et al., 2015), although there were important differences in the responses of the different neuropeptides and FAs assessed. For instance, in the present experiment there was no response of *cart2b*

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Table 1. Nucleotide sequences of the probes used to evaluate mRNA abundance by RT-PCR (qPCR)

Gene	Forward primer	AT (°C)	Database	Reference
acc	F: CAGCTGGGTGGAATTCAGAT R: ATGGGATCTTTGGCACTGAG	60	SoleaDB[1]	solea_v4.1_unigene15555
acly	F: CCACAGATTCACACCATTGC R: GCCAGGATGTTATCCAGCAT	60	SoleaDB[1]	solea_v4.1_unigene11536
agrp2	F: CAGGTCAGACTCCGTGAGCCC R: GTCGACACCGACAGGAGGCAC	64	SoleaDB[1]	solea_v4.1_unigene32957
cart1a	F: CGTCCACCACTGTCATTCTG R: CTTTCTCCTCCTGCGTCTTG	60	GenBank	KT189188
cart1b	F: TCGCTGAAAAGTCAACAAGAAA R: GCCAAGCTTTTTCTCCAGTG	60	GenBank	KT189189
cart2b	F: AGGACCATGCAGAGTTCCAG R: GGACTCGGTGTCCATCACTT	60	GenBank	KT189191
cart4	F: GTGAGCGAGAGCAGGAAACT R: TCGTGGTGAAATAAGGCAAA	60	GenBank	KT189194
cpt1.1	F: TAACAGCCACCGTCGACATA R: AGCGATTCCCTTGTGTCACT	63	GenBank	KR872890
cpt1.2	F: TCGCCAAGAACTAACCGAAC R: AGACCTGGCGTAGAGCTTCA	64	GenBank	KR872891
cpt1.3	F: CCTGACTGTTGACCCCAAGT R: TCACTCACAGTTACGCAGCA	60	GenBank	KR872892
ef1α	F: GATTGACCGTCGTTCTGGCAAGAAGC R: GGCAAAGCGACCAAGGGGAGCAT	70	GenBank	CAB326302
fas	F: CACAAGAACATCAGCCGAGA R: GAAACATTGCCGTCACACAC	60	GenBank	KP842777
fat/cd36-lmp2	F: TATGTGGCGGTAATGGATCA R: GCCGGTGTGGAATACAAACT	60	GenBank	KR872888
fat/cd36-pg4l	F: TGAATGAGACGGCTGAGTTG R: TGTTGTTTCTGCTCCTCACG	64	GenBank	KR872889
kir6.x	F: AGATGTTGGCGAGAAAGAGC R: GCTCGCGGATGTTCTTGT	60	SoleaDB[1]	solea_v4.1_unigene120423
lxrα	F: AAAGCAGGGCTTCAGTTTGA R: CAGCCTCTCCACCAGATCAT	60	SoleaDB[1]	solea_v4.1_unigene47872
npy	F: GAGGGATACCCGATGAAACC R: GCTGGACCTCTTCCCATACC	60	SoleaDB[1]	solea_v4.1_unigene466117
pomca	F: AAGGCAAAGAGGCGTTGTAT R: TTCTTGAACAGCGTGAGCAG	60	GenBank	FR851915
pomcb	F: GTCGAGCAACACAAGTTCCA R: GTCAGCTCGTCGTAGCGTTT	60	GenBank	FR851916
pparα	F: AAACCGCCTCTCATCATCC R: CACACCTGGAAACACATCTCC	60	GenBank	JX4240810
srebp1c	F: TCCAAGGCTTTCAGCAAGAT R: CTCCTCTGTCTTGGCTCCAG	60	SoleaDB[1]	solea_v4.1_unigene4060
sur	F: GCAGCACCTTCCGTTACCTA R: GCAGCAGCTTAGAGGACGAC	60	SoleaDB[1]	solea_v4.1_unigene446925
ubq	F: AGCTGGCCCAGAAATATAACTGCGACA R: ACTTCTTCTTGCGGCAGTTGACAGCAC	70	GenBank	CAB291588

AT, annealing temperature; F, forward primer; R, reverse primer.

[1]SoleaDB: http://www.juntadeandalucia.es/agriculturaypesca/ifapa/soleadb_ifapa/.

acc, acetyl-CoA carboxylase; acly, ATP-citrate lyase; *agrp*, Agouti-related peptide 2; *cart*, cocaine- and amphetamine-related transcript; *cpt1.1*, carnitine palmitoyl transferase type 1, isoform 1; *cpt-1.2*, carnitine palmitoyl transferase type 1, isoform 2; *cpt1.3*, carnitine palmitoyl transferase type 1, isoform 3; *ef1α*, elongation factor 1α; *fas*, fatty acid synthetase; *fat/cd36-lmp2*, fatty acid translocase lysosome membrane protein 2-like; *fat/cd36-pg4l*, fatty acid translocase platelet glycoprotein 4-like; *kir6.x*, inward rectifier K^+ channel pore type 6.x; *lxrα*, liver X receptor α; *npy*, neuropeptide Y; *pomc*, pro-opiomelanocortin A1; *pparα*, peroxisome proliferator-activated receptor type α; *srebp1c*, sterol regulatory element-binding protein type 1c; sur, sulfonylurea receptor; *ubq*, ubiquitin.

or *agrp2* to any treatment, in contrast to juvenile sole where *cart2b* and *agrp2* were significantly regulated (increased and decreased, respectively) in the hypothalamus following i.p. treatment with OA or ALA (Conde-Sieira et al., 2015). Interestingly, *cart2b* was also significantly increased by the EPA treatment in juvenile sole, similar to the *cart4* homologue in the present study. Dissimilarities in the results could potentially be due to differences in the maturity of the fish or in the methodology employed, i.e. oral versus i.p. administration of free FAs. In the present study, the orally administered FAs will have been absorbed through the intestinal mucosa, where they can be at least partly metabolized, or reacylated into neutral or polar lipids and incorporated into chylomicrons for transport, reflecting the normal pathways in a fish feeding on a complete diet (only skipping the digestion step). However, an increased anorectic potential was also observed in the hypothalamus of rainbow trout fed a lipid-enriched diet (Librán-Pérez et al., 2015), which could suggest a conserved mechanism in teleosts in response to the ingestion of FAs.

All four assessed FAs appear to have the potential to enhance the anorectic response

In spite of a few differences, the present results do not seem to indicate major discrepancies between the different FAs tested with respect to their potential to affect the anorectic response of

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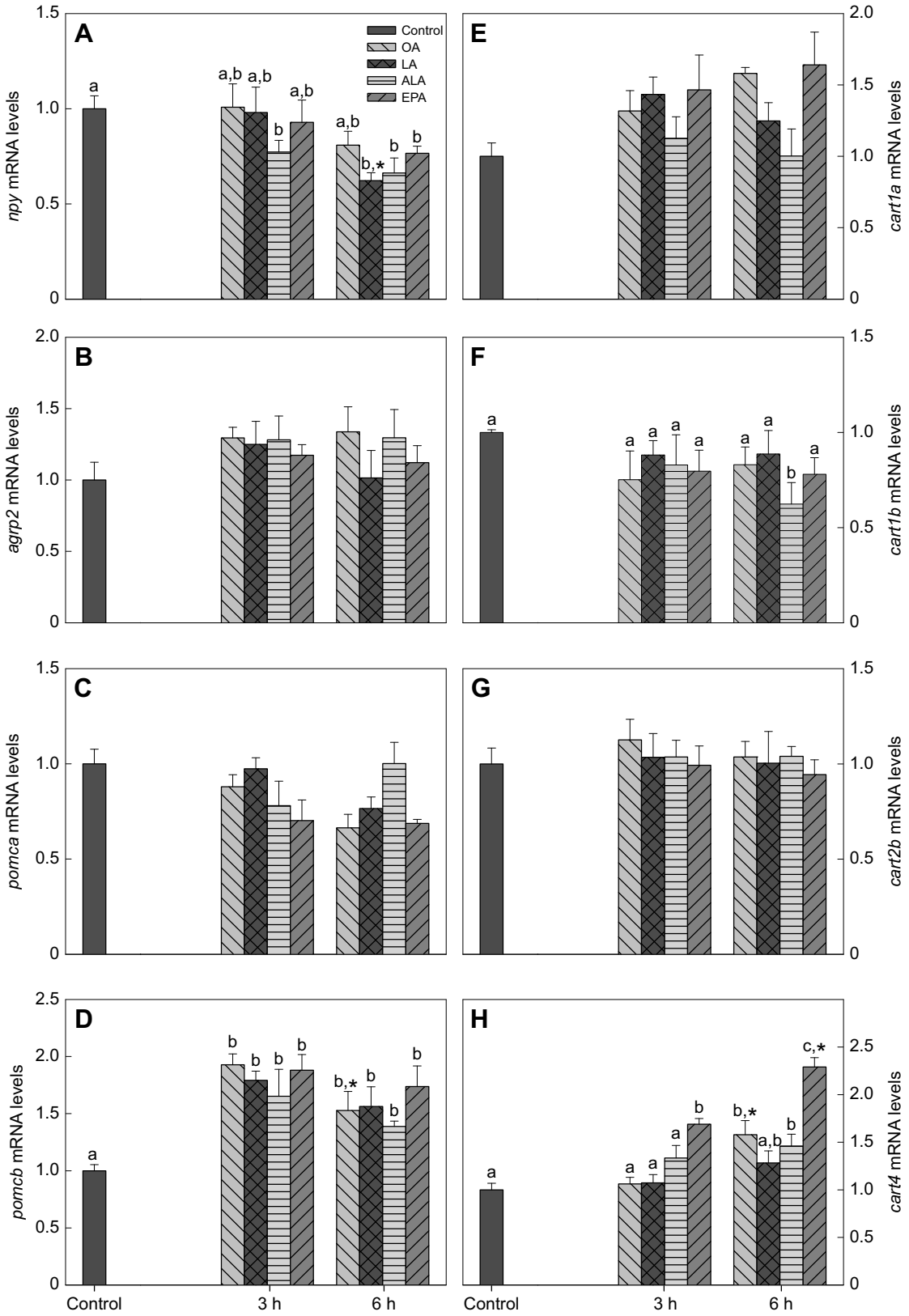

Fig. 1. Neuropeptide mRNA abundance in Senegalese sole head. mRNA abundance of *npy* (A), *agrp2* (B), *pomca* (C), *pomcb* (D), *cart1a* (E), *cart1b* (F), *cart2b* (G) and *cart4* (H) in the head of Senegalese sole post-larvae after oral administration of saline (control), or 3 and 6 h after administration of oleate (OA), linoleate (LA), α-linolenate (ALA) or eicosapentaenoate (EPA). Gene expression results are given relative to the control group and are normalized by *ef1α* and *ubq* expression. Each value is the mean±s.e.m. of *N*=4 pools of four fish each per treatment. Differences were assessed by two-way ANOVA, with fatty acid (FA) treatment and time as main factors. Different letters indicate significant differences ($P<0.05$) between treatment groups; asterisks indicate a significant difference ($P<0.05$) from the same treatment at 3 h.

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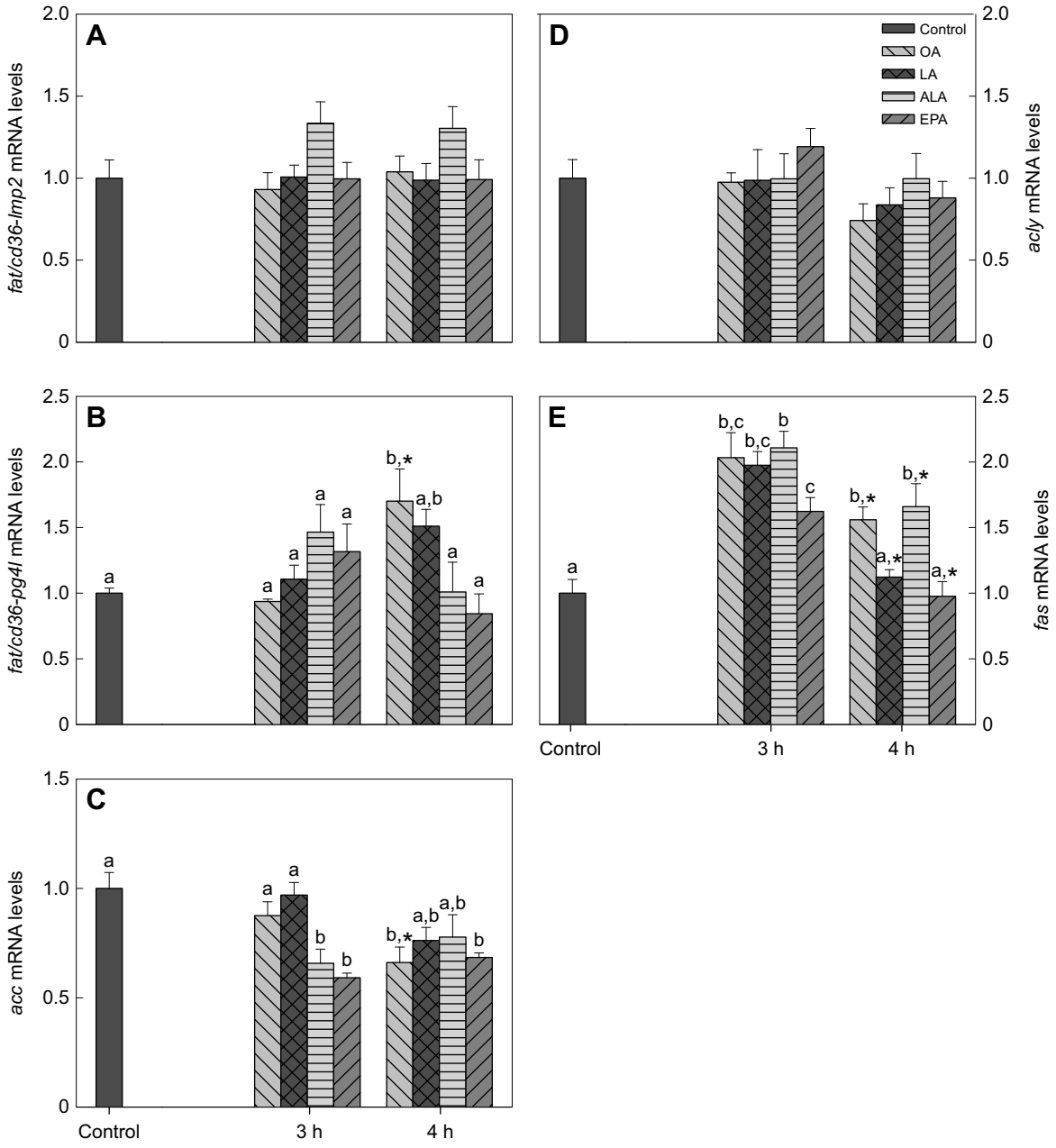

Fig. 2. Parameters related to FA transport and metabolism in Senegalese sole head. mRNA abundance of *fat/cd36-lmp2* (A), *fat/cd36-pg4l* (B), *acc* (C), *acly* (D) and *fas* (E) in the head of Senegalese sole post-larvae after oral administration of saline (control), or 3 and 6 h after administration of OA, LA, ALA or EPA. Gene expression results are given relative to the control group and are normalized by *ef1α* and *ubq* expression. Each value is the mean±s.e.m. of *N*=4 pools of four fish each per treatment. Differences were assessed by two-way ANOVA, with FA treatment and time as main factors. Different letters indicate significant differences ($P<0.05$) between treatment groups; asterisks indicate a significant difference ($P<0.05$) from the same treatment at 3 h.

Senegalese sole post-larvae. The treatment with OA induced an increased anorectic potential based on the increased expression of the anorectic peptides *pomcb* and *cart4* postprandially. This increased anorectic potential after OA treatment is similar to that already observed in juvenile fish of the same species (Conde-Sieira et al., 2015), as well as in rainbow trout (Librán-Pérez et al., 2012, 2014a). Treatment with LA also stimulated the anorectic potential based on the increased mRNA abundance of *pomcb* and the decreased expression of the orexigenic neuropeptide *npy*. As far as we are aware, this is the first time that the effects of this FA have been assessed in any species. ALA intake similarly induced clear responses in the mRNA abundance of neuropeptides including increased expression of *pomcb* and *cart4* and decreased expression of *npy*. The resulting anorectic potential is comparable to that observed in the hypothalamus of juvenile fish of the same species when fish were i.p. treated with the same FA (Conde-Sieira et al., 2015). EPA treatment induced basically the same response as ALA, which is very interesting as in juvenile sole i.p. treatment with EPA did not result in relevant changes in the expression of these peptides (only of *cart2b*; Conde-Sieira et al., 2015).

In a previous experiment, we analysed changes in gene expression of peripheral peptides and neuropeptides in Senegalese sole larvae and post-larvae after feeding with *Artemia* enriched with different oils, especially rich in the same FAs that were administered in the present study (Bonacic et al., 2016). However, it is difficult to correlate the results of the two experiments given that we previously

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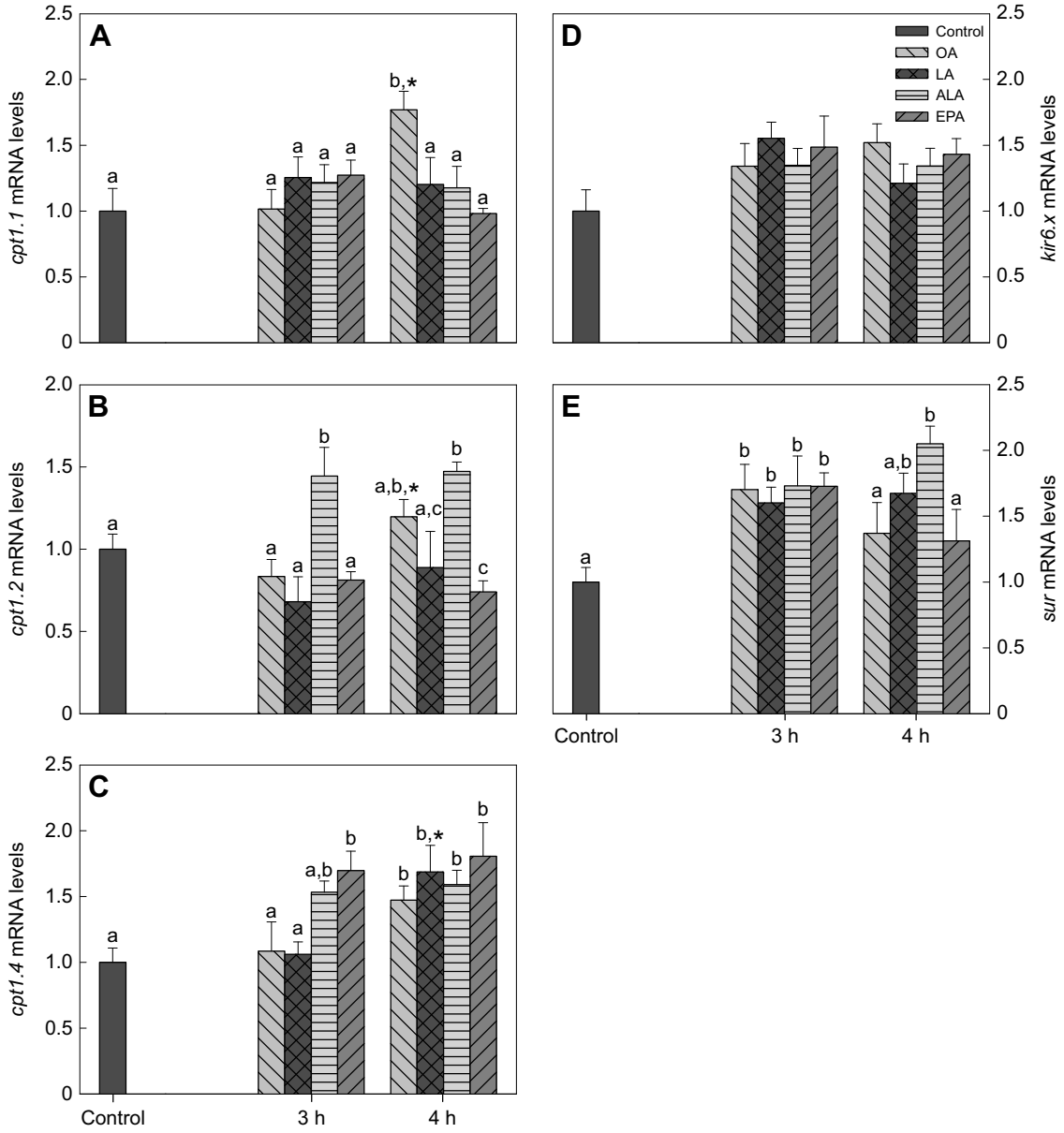

Fig. 3. Parameters related to mitochondrial activity in Senegalese sole head. mRNA abundance of *cpt1.1* (A), *cpt1.2* (B), *cpt1.4* (C), *kir6.x* (D) and *sur* (E) in the head of Senegalese sole post-larvae after oral administration of saline (control), or 3 and 6 h after administration of OA, LA, ALA or EPA. Gene expression results are given relative to the control group and are normalized by *ef1α* and *ubq* expression. Each value is the mean±s.e.m. of *N*=4 pools of four fish each per treatment. Differences were assessed by two-way ANOVA, with FA treatment and time as main factors. Different letters indicate significant differences ($P<0.05$) between treatment groups; asterisks indicate a significant difference ($P<0.05$) from the same treatment at 3 h.

evaluated more complex (complete) diets, probably with lower total amounts of the tested FA, and fed during the whole larval and post-larval stage, rather than a single meal as in the present study. The previous experiment showed that larvae and post-larvae fed a diet containing higher EPA levels (enriched with cod liver oil) tended to have a higher food intake than those in the remaining treatments, and hence we expected to see differences in neuropeptide expression between treatments. Nevertheless, this did not correlate with a higher orexigenic and/or lower anorexigenic potential in either of the two experiments. In fact, EPA-treated fish showed higher levels of the putatively anorexigenic *cart4* than those in the remaining treatments in the present experiment. However, different classes of FA can have diverse effects on metabolism and energy homeostasis, and on the release of peripheral appetite-regulating peptides, which can potentially affect food intake through different routes (French et al., 2000; Lawton et al., 2000; Wang et al., 2002; Tocher, 2003; Relling and Reynolds, 2007; Parra et al., 2008; Soengas, 2014). Furthermore, a peptide such as CART, for instance, is known to have multiple physiological functions besides the regulation of feeding (e.g. regulation of energy metabolism) in mammals (Lau and Herzog, 2014). In teleosts, its roles have not been well documented but the retention of several duplicated genes (in sole, up to seven homologues have been reported) suggests the conservation of multiple functions, or even the development of new ones (Bonacic et al., 2015). In this respect, Bonacic et al. (2016) reported the transcriptional regulation of *cart1b*, which did not show a response to the ingestion of food in the brain of sole juveniles (Bonacic et al., 2015), but was affected by the FA composition of the diet in a manner consistent with an anorexigenic role (expression reduced in larvae fed the cod liver oil, EPA-rich diet). Similarly,

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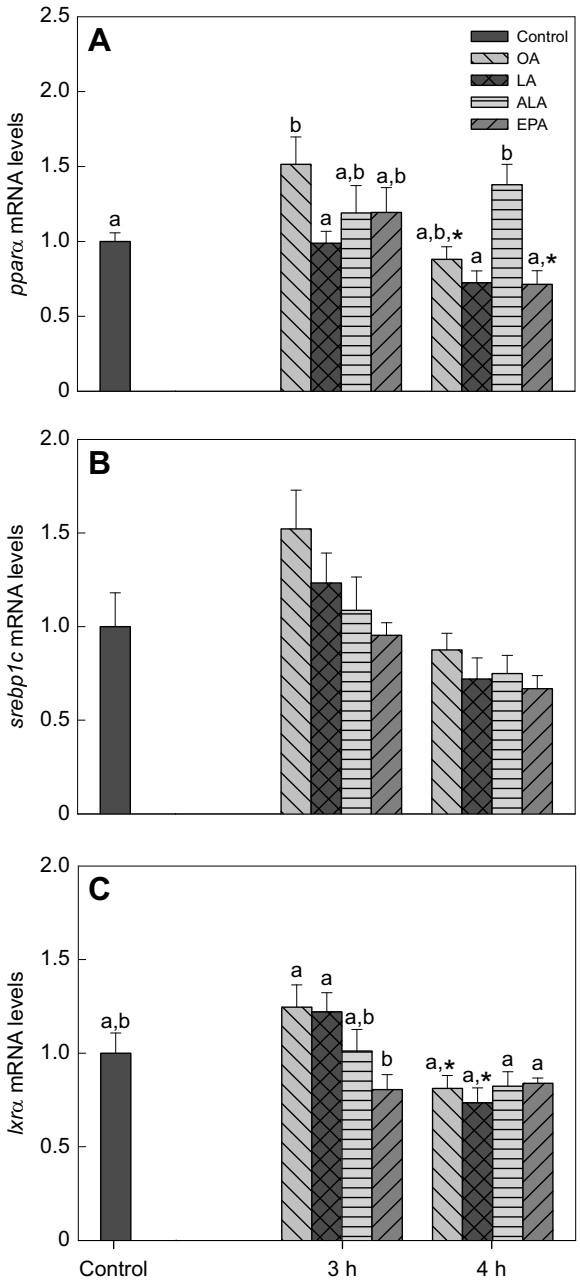

Fig. 4. Transcription factors in Senegalese sole head. mRNA abundance of *pparα* (A), *srebp1c* (B) and *lxrα* (C) in the head of Senegalese sole post-larvae after oral administration of saline (control), or 3 and 6 h after administration of OA, LA, ALA or EPA. Gene expression results are given relative to the control group and are normalized by *ef1α* and *ubq* expression. Each value is the mean ±s.e.m. of *N*=4 pools of four fish each per treatment. Differences were assessed by two-way ANOVA, with FA treatment and time as main factors. Different letters indicate significant differences ($P<0.05$) between treatment groups; asterisks indicate a significant difference ($P<0.05$) from the same treatment at 3 h.

cart1a and *cart1b* seemed to respond to the FA composition (lipid source) of the diet, correlating with food intake in juvenile sole (Bonacic et al., 2017). In the present study, the transcription of *cart1a* was not significantly regulated postprandially or by the FA treatment, which suggests that dietary effects may be exerted in these genes via long-term changes in metabolism and energy homeostatic status rather than FA sensing following a meal. In fact, FA-sensing systems did not appear to be activated in the present study (see below), supporting this hypothesis. In the case of *cart1b*, its expression was not affected 3 h after feeding with any FA treatment but was significantly reduced 6 h after administration of ALA, but not in any of the remaining treatments, including EPA (as might have been expected from Bonacic et al., 2016). The reasons for this response are elusive at the moment, but do not seem to be related to FA sensing. Therefore, although preliminary and still speculative, data are starting to emerge in fish suggesting different roles of CART homologues in modulating the feeding response to not only dietary lipid levels but also FA composition, and this should be further evaluated in future studies.

Post-larval FA-sensing systems are not activated by oral treatment with FAs

The presence of several mechanisms involved in FA sensing in the mammalian brain was investigated in Senegalese sole in response to the orally administered FA treatments, for the first time in a teleost at such an early stage of development. Highly variable responses were observed in this study, which might suggest that FA-sensing systems are not active in the post-larval stage, contrary to what was reported previously after i.p. administration of FA in sole juveniles (Conde-Sieira et al., 2015), as well as in rainbow trout (Librán-Pérez et al., 2012, 2013, 2014a). In the hypothalamus of juvenile sole, several FA systems were activated by OA and ALA treatment: (1) those based on FA metabolism (decrease in mRNA levels of *acly*, *acc* and *fas*); (2) mechanisms based on binding of FAs to FAT/CD36 and subsequent modulation of transcription factors (no effect on *fat/cd36* but reduction of *pparα*, *sbrepb1c* and *lxrα* expression, although only in OA-treated fish in the case of *lxrα*); and (3) based on mitochondrial activity (decreased expression of *kir6.x* and *sur*). Furthermore, a differential effect of FAs depending on chain length and degree of saturation was described, with a saturated FA such as stearate lacking a response and EPA inducing only a few changes in the transcription of these genes, probably unrelated to a role in FA sensing (Conde-Sieira et al., 2015). In the present study, the only result that is in line with previous observations is the reduction in mRNA levels of *acc* in OA- (only at 6 h) ALA- (3 h) and EPA-treated (3 h and 6 h) post-larvae, while the remaining genes were either not significantly regulated (*fat/cd36-lmp2*, *acly*, *kir6.x*, *srebp1c*) or affected in a way inconsistent with FA-sensing mechanisms (*fat/cd36-pg4l*, *fas*, *cpt1.1*, *cpt1.2*, *cpt1.3*, *sur*, *pparα* and *lxrα*) (Soengas, 2014). However, we cannot disregard the fact that previous results demonstrating the presence of FA-sensing systems in sole juveniles were obtained after i.p. injection of free FAs, obviating the steps of intestinal absorption, which is likely to result in different timing and bioavailability of circulating FAs.

Interestingly, important changes were measured in the postprandial expression of *cpt1* genes after treatment with different FAs but, just as observed in juvenile sole, the response was quite variable depending on the isoform that was measured. However, we would need more information, particularly on the functional characterization of these genes, in order to be able to interpret these results.

Therefore, it appears that oral administration of FAs to sole post-larvae in this study did not activate central FA-sensing systems, even when fish were administered with OA and ALA, which were previously demonstrated to induce the activation of these systems in juvenile sole (Conde-Sieira et al., 2015). The lack of response to any of the tested FAs in Senegalese sole post-larvae could suggest that the metabolic control of food intake involving FA sensing is not yet functional at this early stage of development. The finding that the anorectic potential was enhanced irrespective of the FA treatment also supports this possibility. A gradual development of FA-sensing

capacities is likely as the operability of other mechanisms involved in the control of food intake, such as gastrointestinal remodelling, is also dependent on developmental stage (Kortner et al., 2011; Gomes et al., 2015). However, these results raise an interesting question: if FA-sensing systems are not responding to the intake of FAs, how do neuropeptides respond to FA administration? This differential behaviour may rely on the fact that neurons expressing neuropeptides not only integrate metabolic information on specific nutrients in circulation but also integrate information from other sources, including nervous inputs from the gastrointestinal tract and/or levels of peripheral hormones (leptin, insulin and ghrelin, among others) (Wang et al., 2002; Blouet and Schwartz, 2010). Hence, we might speculate that the capacity to integrate this kind of information in brain centres involved in metabolic regulation of food intake might develop earlier than that of FA-sensing mechanisms.

In summary, we have described for the first time in fish post-larvae the effect of feeding long-chain FAs of different nature (including PUFAs), such as OA, LA, ALA and EPA, on the metabolic regulation of food intake in Senegalese sole. All FAs induced changes in the expression of neuropeptides involved in the control of food intake. Although not all the assessed neuropeptides changed their mRNA abundance, the changes observed generally suggest an enhanced anorectic potential irrespective of the FA administered. However, none of the treatments activated central FA-sensing systems. These results are different from those observed in the hypothalamus of juveniles of the same species, where anorectic responses, in parallel with activation of FA-sensing systems, were observed in response to specific FAs injected i.p. (Conde-Sieira et al., 2015). This different behaviour between post-larvae and juveniles allows us to suggest that the metabolic control of food intake involving the function of FA-sensing systems and integration of this metabolic information through changes in neuropeptide expression might not be functional at this early stage of development.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
S.M. and J.L.S. conceived and designed the study; K.B. and S.M. performed the experiments; C.V. and K.B. performed the molecular analysis; all authors interpreted the results of the experiments; C.V. and J.L.S. prepared the figures; S.M. and J.L.S. had the main responsibility in drafting the manuscript and all authors revised the manuscript.

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References
Blouet, C. and Schwartz, G. J. (2010). Hypothalamic nutrient sensing in the control of energy homeostasis. *Behav. Brain Res.* **209**, 1-12.

Bonacic, K., Martínez, A., Martín-Robles, J., Muñoz-Cueto, J. A. and Morais, S. (2015). Characterization of seven cocaine- and amphetamine-regulated transcripts (CARTs) differentially expressed in the brain and peripheral tissues of Solea senegalensis (Kaup). *Gen. Comp. Endocrinol.* **224**, 260-272.

Bonacic, K., Campoverde, C., Gómez-Arbonés, J., Gisbert, E., Estevez, A. and Morais, S. (2016). Dietary fatty acid composition affects food intake and gut-brain satiety signaling in Senegalese sole (*Solea senegalensis*, Kaup 1858) larvae and post-larvae. *Gen. Comp. Endocrinol.* **228**, 79-94.

Bonacic, K., Martínez, A., Gisbert, E., Estevez, A. and Morais, S. (2017). 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). *Aquaculture* **470**, 169-181.

Conde-Sieira, M., Bonacic, K., Velasco, C., Valente, L. M. P., Morais, S. and Soengas, J. L. (2015). Hypothalamic fatty acid sensing in Senegalese sole (*Solea senegalensis*): response to long-chain saturated, monounsaturated, and polyunsaturated (n-3) fatty acids. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **309**, R1521-R1531.

Dâmaso-Rodrigues, M. L., Pousão-Ferreira, P., Ribeiro, L., Coutinho, J., Bandarra, N. M., Gavaia, P. J., Narciso, L. and Morais, S. (2010). Lack of essential fatty acids in live feed during larval and post-larval rearing: effect on the performance of juvenile *Solea senegalensis*. *Aquacult. Int.* **18**, 741-757.

French, S. J., Conlon, C. A., Mutuma, S. T., Arnold, M., Read, N. W., Meijer, G. and Francis, J. (2000). The effects of intestinal infusion of long-chain fatty acids on food intake in humans. *Gastroenterology* **119**, 943-948.

Gomes, A. S., Jordal, A.-E. O., Olsen, K., Harboe, T., Power, D. M. and Rønnestad, I. (2015). Neuroendocrine control of appetite in Atlantic halibut (*Hippoglossus hippoglossus*): changes during metamorphosis and effects of feeding. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **183**, 116-125.

Kortner, T. M., Overrein, I., Øie, G., Kjørsvik, E. and Arukwe, A. (2011). The influence of dietary constituents on the molecular ontogeny of digestive capability and effects on growth and appetite in Atlantic cod larvae (*Gadus morhua*). *Aquaculture* **315**, 114-120.

Lau, J. and Herzog, H. (2014). CART in the regulation of appetite and energy homeostasis. *Front. Neurosci.* **8**, 313.

Lawton, C. L., Delargy, H. J., Brockman, J., Smith, F. C. and Blundell, J. E. (2000). The degree of saturation of fatty acids influences post-ingestive satiety. *Br. J. Nutr.* **83**, 473-482.

Librán-Pérez, M., Polakof, S., López-Patiño, M. A., Míguez, J. M. and Soengas, J. L. (2012). Evidence of a metabolic fatty acid-sensing system in the hypothalamus and Brockmann bodies of rainbow trout: implications in food intake regulation. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **302**, R1340-R1350.

Librán-Pérez, M., López-Patiño, M. A., Míguez, J. M. and Soengas, J. L. (2013). Oleic acid and octanoic acid sensing capacity in rainbow trout *Oncorhynchus mykiss* is direct in hypothalamus and Brockmann bodies. *PLoS ONE* **8**, e59507.

Librán-Pérez, M., Otero-Rodiño, C., López-Patiño, M. A., Míguez, J. M. and Soengas, J. L. (2014a). Central administration of oleate or octanoate activates hypothalamic fatty acid sensing and inhibits food intake in rainbow trout. *Physiol. Behav.* **129**, 272-279.

Librán-Pérez, M., Velasco, C., López-Patiño, M. A., Míguez, J. M. and Soengas, J. L. (2014b). Counter-regulatory response to a fall in circulating fatty acid levels in rainbow trout. Possible involvement of the hypothalamus-pituitary-interrenal axis. *PLoS ONE* **9**, e113291.

Librán-Pérez, M., Geurden, I., Dias, K., Corraze, G., Panserat, S. and Soengas, J. L. (2015). Feeding rainbow trout with a lipid-enriched diet: effects on fatty acid sensing, regulation of food intake and cellular signaling pathways. *J. Exp. Biol.* **218**, 2610-2619.

Morais, S., Rojas-Garcia, C. R., Conceição, L. E. C. and Rønnestad, I. (2005). Digestion and absorption of a pure triacylglycerol and a free fatty acid by *Clupea harengus* L. larvae. *J. Fish. Biol.* **67**, 223-238.

Parra, D., Ramel, A., Bandarra, N., Kiely, M., Martínez, J. A. and Thorsdottir, I. (2008). A diet rich in long chain omega-3 fatty acids modulates satiety in overweight and obese volunteers during weight loss. *Appetite* **51**, 676-680.

Pfaffl, M. W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* **29**, e45.

Relling, A. E. and Reynolds, C. K. (2007). Feeding rumen-inert fats differing in their degree of saturation decreases intake and increases plasma concentrations of gut peptides in lactating dairy cows. *J. Dairy. Sci.* **90**, 1506-1515.

Rønnestad, I., Rojas-García, C. R., Tonheim, S. K. and Conceição, L. E. C. (2001). *In vivo* studies of digestion and nutrient assimilation in marine fish larvae. *Aquaculture* **201**, 161-175.

Soengas, J. L. (2014). Contribution of glucose- and fatty acid sensing systems to the regulation of food intake in fish. A review. *Gen. Comp. Endocrinol.* **205**, 36-48.

Tocher, D. R. (2003). Metabolism and functions of lipids and fatty acids in teleost fish. *Rev. Fish. Sci.* **11**, 107-184.

Wang, H., Storlien, L. H. and Huang, X.-F. (2002). Effects of dietary fat types on body fatness, leptin, and ARC leptin receptor, NPY, and AgRP mRNA expression. *Am. J. Physiol. Endocrinol. Metab.* **282**, E1352-E1359.