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Effect of long-term intergenerational exposure to ocean acidification on ompa and ompb transcripts expression in European seabass (Dicentrarchus labrax)

Mazurais David [1,*], Neven Carolin J. [2], Servili Arianna [1], Vitre Thomas [1], Madec Lauriane [1], Collet Sophie [1], Zambonino Infante Jose-Luis [1], Mark Felix C. [2]

[1] IFREMER, Univ Brest, CNRS, IRD, LEMAR, F-29280, Plouzané, France
[2] Department of Integrative Ecophysiology, Alfred Wegener Institute Helmholtz Centre for Polar and Marine Research, 27570 Bremerhaven, Germany

* Corresponding author : David Mazurais, email address : david.mazurais@ifremer.fr

Abstract :

Since sensory system allows organisms to perceive and interact with their external environment, any disruption in their functioning may have detrimental consequences on their survival. Ocean acidification has been shown to potentially impair olfactory system in fish and it is therefore essential to develop biological tools contributing to better characterize such effects. The olfactory marker protein (omp) gene is involved in the maturation and the activity of olfactory sensory neurons in vertebrates. In teleosts, two omp genes (ompa and ompb) originating from whole genome duplication have been identified. In this study, bioinformatic analysis allowed characterization of the ompa and ompb genes from the European seabass (Dicentrarchus labrax) genome. The European seabass ompa and ompb genes differ in deduced amino acid sequences and in their expression pattern throughout the tissues. While both ompa and ompb mRNA are strongly expressed in the olfactory epithelium, ompb expression was further observable in different brain areas while ompa expression was also detected in the eyes and in other peripheral tissues. Expression levels of ompa and ompb mRNA were investigated in adult seabass (4 years-old, F0) and in their offspring (F1) exposed to pH of 8 (control) or 7.6 (ocean acidification, OA). Under OA ompb mRNA was down-regulated while ompa mRNA was up-regulated in the olfactory epithelium of F0 adults, suggesting a long-term intragenerational OA-induced regulation of the olfactory sensory system. A shift in the expression profiles of both ompa and ompb mRNA was observed at early larval stages in F1 under OA, suggesting a disruption in the developmental process. Contrary to the F0, the expression of ompa and ompb mRNA was not anymore significantly regulated under OA in the olfactory epithelium of juvenile F1 fish. This work provides evidence for long-term impact of OA on sensorial system of European seabass as well as potential intergenerational acclimation of omp genes expression to OA in European seabass.

Highlights

► We identified orthologous genes (*ompa* and *ompb*) in European sea bass. ► *Ompa* and *ompb* genes differ in amino acid sequences and in their expression pattern. ► Acidification induces intra- and intergenerational plasticity in *omps* expression. ► Both *ompa* and *ompb* mRNA could be used as novel molecular markers of OSN in sea bass.

Keywords : OMP, expression pattern, Dicentrarchus labrax, acidification, intergenerational

Introduction

Among the environmental constraints related to global change, ocean acidification (OA) due to increased concentrations of dissolved CO_2 in marine waters has been shown to disrupt olfactory system with consequences on behaviour in marine fish from both tropical and temperate environments (Ashur et al., 2017, Cripps et al., 2011, Dixson et al., 2015, Dixson et al., 2010, Doney et al., 2009, Esbaugh, 2018, Ferrari et al., 2011, Heuer and Grosell, 2014, Munday et al., 2009a, Rong et al., 2018, Williams et al., 2019, Velez et al., 2019, Chivers et al., 2014, Devine et al., 2012a, Porteus et al., 2018). Such effects can impact several traits of fish life including predator-prey relationships (prey detection and predator avoidance), navigation (e.g. migration, homing), and locating appropriate habitats, which may have severe consequences on the survival and dynamics of wild fish populations. Numerous studies demonstrated that fish sensitivities to OA are especially pronounced in early life stages (Munday et al., 2009b, Franke and Clemmesen, 2011a, Domenici et al., 2012, Devine et al., 2012b, Pimentel et al., 2016, Rong et al., 2018). Yet, OA effects on sensory system-mediated behaviour of fish have been recently questioned (Clark et al., 2020). To shed more light on this, additional investigation of both intra- and intergenerational impact of OA exposure in fish using proxies that provide information on the regulation affecting the maturation and activity of olfactory sensory neurons may be useful. While altered olfactory perception of chemical cues induced by OA have been demonstrated using electrophysiological analyses in fish at juvenile or adult stages, such an approach is difficult to achieve on small size individuals at larval stage (Porteus et al., 2018, Velez et al., 2019, Moore, 1994). Conversely, proxies based on the analysis of mRNA expression are very useful to investigate the physiological impact of environmental cues on small organisms since they do not require a lot of biological material. Moreover, numerous studies demonstrated that OA-induced physiological disturbances were associated with regulation of gene expression (Cline et al., 2020, Frommel et al., 2020, Hamilton et al., 2017, Huth and Place, 2016, Lai et al., 2017, Mazurais et al., 2020a, Mazurais et al., 2020b, Shrivastava et al., 2019, Preus-Olsen et al., 2014, Tseng et al., 2013, Mittermayer et al., 2019, Michael et al., 2016). In particular, changes in mRNA levels of proteins involved in neural signalling processes have been observed in olfactory systems of fish exposed to OA (Williams et al., 2019, Porteus et al., 2018), including early stages of development (Rong et al., 2018).

Olfactory marker protein (*omp*) genes encode for OMP proteins that are predominantly expressed in mature olfactory sensory neurons (OSN) of vertebrates in which they are expected to be involved in the maturation, the axon guidance and the physiological activity of olfactory

sensory neurons (Buiakova et al., 1996, Lee et al., 2011, St John and Key, 2005). While mammals possess a single-copy of the *omp* gene, teleost fish species have at least two *omp* gene paralogs resulting from the duplication of an ancestral *omp* gene (Suzuki et al., 2015). Sequences from paralog *ompa* and *ompb* genes have been identified from genomic resources in different teleost species including zebrafish (*Danio rerio*), stickleback (*Gasterosteus aculeatus*), fugu (*Takifugu rubripes*), tilapia (*Oreochromis niloticus*), medaka (*Oryzias latipes*), platyfish (*Xiphophorus maculatus*), goldfish (*Carassius auratus*) and gilthead sea bream (*Sparus aurata*) (Suzuki et al., 2015). In sockeye salmon (*Oncorhynchus nerka*), two *ompa* genes have been found which may have emerged with the additional whole genome duplication event in salmonids (Kudo et al., 2009, Suzuki et al., 2015). Very few information concerning the respective functions of the OMPa and OMPb proteins are available in teleost species. In zebrafish, *ompa* and *ompb* mRNA are mainly expressed in the superficial layer of the olfactory epithelium and in ciliated olfactory sensory neurons (OSNs) located in the deep layer, respectively (Suzuki et al., 2015). While both zebrafish *omp* genes are expressed in neurons expressing G-protein α-subunits (*Gαolf2*) genes, the almost completely non-overlapping expression pattern of *ompa* and *ompa* genes in neurons that project to different regions of the olfactory bulbs suggest that they have distinct roles. Suzuki et al (Suzuki et al., 2015) assumed that the distinct functions may result from the subfunctionalization of duplicated *omp* genes. Involvement of OMP proteins in OSN maturation and neuronal signal transduction makes *omp* mRNA expression a key molecular marker to study the regulation of olfactory function in different vertebrate species including fish (Kudo et al., 2009, Oboti et al., 2011, Sato et al., 2005, Suzuki et al., 2015). Particularly, quantitative analysis of *omp* mRNA expression levels can inform about deficiencies in the olfactory system in organisms (Kim et al., 2010, Tilton et al., 2008, Witt et al., 2009). This is of particular interest when it comes to revealing the impact of environmental stressors on the olfactory systems of fish (Tilton et al., 2008).

In this scientific context, investigation of *omp* transcript expression may provide advanced information about the intra- and intergenerational effects of acidification on the sensory system of fish at different life stages. The present study characterized the full length *ompa* and *ompb* mRNAs and protein sequences from European seabass (*Dicentrarchus labrax*), a commercially important species and their expression patterns during larval development and in different tissues at juvenile stage in normal condition by means of qPCR analysis. Based on these expression patterns, *omp* mRNA expression levels were then compared during early stages of larval development and in the olfactory rosette of adults from two successive generations (F0

and F1) of fish exposed to a pH of 8.0 for standard rearing conditions or to a pH of 7.6 for OA condition (Representative Concentration Pathway of the Intergovernmental Panel on Climate Change, RCP 8.5). This work contributes to better understand the impact of OA on the olfactory system of a marine fish species.

Material and methods

Identification and analysis of the omp sequences

Blast searches using the *ompa* (NM_001025185.1) and *ompb* (NM_173281.2) mRNA sequences from zebrafish as query against European seabass genome available on UCSC Genome Browser database (http://seabass.mpipz.mpg.de/index.html) allowed to identify two genome sequences, including the seabass *ompa* gene (Linking Group 13:27322839-27325677, DLAgn_00036760) and *ompb* gene (Linking Group 14:26037825-26038307, DLAgn_00046360). Linking group 13 and linking group 14 correspond to HG916830.1: 27,324,685-27,325,680 and HG916831.1: 26,036,773-26,038,307 in ensembl database, respectively. The full-length transcripts encoding the European seabass OMPs were then cloned by RT-PCR performed from olfactory epithelium cDNA using primers designed on the predicted mRNA sequences (table 1). After cloning, the cDNA sequences were obtained by GENEWIZ sequencing service (South Plainfield, USA).

A microsynteny analysis was performed using Genomicus web server (http://genomicus.biologie.ens.fr/genomicus). Location of *omp* and their neighbouring genes were compared among different fish species, using an ancestor species, the spotted gar (*Lepisosteus oculatus*), as query.

The OMPs amino acid sequences deduced from cDNA were obtained using the Expasy translate tool (https://web.expasy.org/translate/). cDNA and deduced protein sequences are available in Genbank nucleotide database (*ompa* sequence: MW536997; *ompb* sequence: MW536996). Identification of domains in OMPs amino acid sequences was performed using SMART (Simple Modular Architecture Research Tool) web resource (http://smart.embl-heidelberg.de/) (Letunic and Bork, 2017).

Table1: Primers used for European seabass ompa and ompb full length cDNA cloning and relative quantification by qPCR. Sequences used to design the primers are available in (*) Max Planck Institute (http://seabass.mpipz.mpg.de/index.html) and (**) genbank databases. Nd: not determined.

Amino acid sequences of OMPs from different vertebrate species were aligned by Mafft (Katoh et al., 2017) with default parameters [Auto strategy: L-INS-i). SnapGene software (version 5.2) was used to illustrate the alignment. The neighbor-joining method with the ITT model of amino acid substitution and 1000 bootstrap repetitions was used for the construction of a phylogenetic tree. Human (*Homo sapiens*), mouse (*Mus musculus*), tropical clawed frog (*Xenopus tropicalis*), African clawed frog (*Xenopus laevis*), spotted gar (*Lepisosteus oculatus*), zebrafish (*Danio rerio*), goldfish (*Carassius auratus*), common carp (*Cyprinus carpio*), gilthead sea bream (*Sparus aurata*), medaka (*Oryzias latipes*), Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*) OMP sequences were acquired from ensembl or genbank databases. Accession numbers: human ENSP00000436376; mouse ENSMUSP00000095882; tropical clawed frog ENSXETP00000098764; African clawed frog CAA09446.1 and CAA09447.1; spotted gar ENSLOCP00000022320; zebrafish ENSDARP00000139076.1 and ENSDARP00000108338.2; sea bream ENSSAUP00010011932.1 and ENSSAUP00010005277.1; goldfish ENSCARP00000109103 and ENSCARP00000136132; common carp ENSCCRP00000071916.1, ENSCCRP00000012058, ENSCCRP00000085754 and ENSCCRP00000092428; medaka ENSORLP00000038818 and ENSORLP00000018774; Atlantic salmon ENSSSAP00000032860, ENSSSAP00000002336, ENSSSAP00000121147 and ENSSSAP00000117049 and rainbow trout ENSOMYP00000010796, ENSOMYP00000092729 and ENSOMYP00000043590.

Animal and experimental conditions

F0 generation

Experiments were conducted under approved protocols in strict compliance with the EU Directive 2010/63/EU for animal experiments and according to the French legal requirements concerning welfare of experimental animals (APAFIS permit no. 17132-2018101614401562). The F0 population of European seabass used in the present experiment was the same as one used in previous works (Mazurais et al., 2020a). F0 larvae were obtained in October 2013 from a local commercial hatchery (Aquastream, Ploemeur, France). At two days post-hatch (dph), they were brought within the facilities of the laboratory 'Laboratoire Adaptation, Reproduction et Nutrition des poissons' which is part of Ifremer-Centre de Bretagne (Agreement number: B29-212-05). F0 European seabass were maintained from hatching in two PCO_2 conditions [Control group: pH 8, ~600 µatm, OA conditions group: pH 7.6, ~1600 µatm]. The ambient

169 PCO_2 was approximatively 600 µatm which corresponds to today's situation for coastal waters
170 of Brittany (Duteil et al., 2016). The experimental conditions were chosen based on the IPCC
171 Representative Concentration Pathway (RCP) 8.5 scenario (Stocker et al., 2013). The rearing
172 conditions of the F0 population throughout all life stages are detailed in the previous papers
173 (Mazurais et al., 2020a, Mazurais et al., 2020b) (see supplementary tables 1-3). Briefly, tanks
174 were supplied with sea water pumped from a depth of 20 m approximately 500 m from the
175 coastline in the Bay of Brest. Water was treated as follows: After the passage through a sand
176 filter (~500 µm) water was heated (tungsten, Plate Heat Exchanger, Vicarb, Sweden), degassed
177 using a column, filtered using a 2 µm membrane and finally UV sterilized (PZ50, 75W, Ocene,
178 France) assuring high water quality. Temperature and pH were checked daily with a WTW
179 3110 pH meter (Xylem Analytics Germany, Weilheim, Germany; with electrode: WTW Sentix
180 41, NBS scale) before feeding the fish. Each day the pH meter was calibrated with NBS certified
181 WTW technical buffers pH 4.01 and pH 7.00 (Xylem Analytics Germany, Weilheim,
182 Germany). Total alkalinity was measured once a week following the protocol of Strickland and
183 Parsons (Caspers, 1970): a 50 ml sample of filtered tank water was mixed with 15 ml HCl (0.01
184 M) and pH was measured immediately. Total alkalinity was then calculated with the following
185 formula:

$$TA = \frac{V_{HCl} \cdot c_{HCl}}{V_{sample}} - \frac{(V_{HCl} + V_{sample})}{V_{sample}} \cdot \frac{\{H^+\}}{\gamma_{H^+}} \left[\frac{mol}{l}\right]$$

186

187 With: TA—total alkalinity [mol * l-1], VHCl—volume HCl [l], cHCl—concentration HCl [mol
188 *l-1], Vsample—volume of sample [l], H+—hydrogen activity (10-pH), γH+—hydrogen
189 activity coefficient (here γH+= 0.758).

190 F0 larvae were maintained in triplicate tanks, with oxygen concentration around 95% air
191 saturation, salinity at 34‰ and the controlled photoperiod was set at 16L:8D (with
192 progressively increasing light intensity according to larval age from total darkness to 96 lux)
193 until 45 days post-hatching (dph). F0 larvae were fed from 6 dph (around mouth opening stage),
194 with live brine shrimp (*Artemia salina*) nauplii, hatched from High HUFA Premium cysts
195 (Catvis, AE's-Hertogenbosch, Netherlands). From 6 to 16 dph, a concentration of ~120 nauplii
196 per larva and day was continuously delivered from their storage tanks to the larval rearing tanks
197 for a duration of 6 hours, which was changed to a concentration of ~800 nauplii per larva per
198 day after 16 dph. From 28 dph until 45 dph larvae were fed with commercial feed diet (Néo-
199 start, Le Gouessant Aquaculture, France). From 2 years post-hatching, fish from triplicate tanks

were randomly distributed into duplicate tanks and reared under ambient temperature and natural photoperiod and fed a diet that meets the nutritional requirements of broodstocks (Vitalis Cal, Skretting, Stavanger, Norway). Apart from the pH conditions described above, F0 fish from the two groups experienced identical experimental conditions throughout their different life stages.

F1 generation

An artificial reproduction was performed from 4 years old F0 individuals. To produce a F1 generation, sperm and eggs were collected and pooled from 20 males and 6 females of each pH-group. To stimulate the synchronous oocytes final maturation, 3 females per tank (6 per pH treatment) were injected with LHRH (luteinizing hormone releasing hormone) hormone. 72 hours later LHRH-injected females and males (10 males per tank) were stripped and the eggs from each tank were fertilized. The eggs and sperm from each group were crossed separately to produce F1. The eggs were hatched and the resultant F1 fish were reared in the same pH as their parents. For each treatment (Control and OA) two replicates of tanks were used. Rearing condition was similar to those described for F1 population.

Sampling and RNA extraction

Before sampling, 24h-fasted fish were first lightly anesthetized (20 mg L^{-1}), and then euthanized with a lethal dose (200 mg L^{-1}) of tricaine methanesulfonate 222 (Pharmaq, Fordingbridge, Hampshire, UK).

F0 generation

Investigation of *ompa* and *ompb* mRNA expression patterns across different adult tissues (olfactory rosette, olfactory bulbs, diencephalon, optic tectum, cerebellum, spinal cord, gills, heart, muscle, liver, spleen, kidney and proximal intestine) were performed on tissues sampled from three adult males reared under control pH condition (4 years old).

Total RNA of olfactory rosettes was also extracted from 27 adults (4 years old) to analyse the potential long-term effect of pH on *ompa* and *ompb* mRNA expression within generation F0. For this purpose, 14 adults from the control group (10 females, 4 males) and 13 individuals from the OA group (6 females, 7 males) were sampled at the post-spawning period.

F1 generation

One pool of F1 larvae was sampled per tank (two pools per pH group) and at seven larval stages 0, 1, 4, 10, 16, 20, 27 dph. One pool contained 30 mg biological material containing five individuals to several dozen individuals depending on the developmental stage. The number of pools (n=2) sampled per tank was limited by the quantity of larvae.

At juvenile stage (18 months old), total RNA of olfactory rosettes from 15 F1 fish from each group was sampled.

After sampling larvae and adults, tissues were stored in RNA later (Qiagen, Hilden, Germany) until total RNA extraction. The protocol of total RNA extraction is the same as previously described (Mazurais et al., 2020b). The RNA integrity number (RIN) of the extracted RNA were higher than nine allowing us to process to retro-transcription into cDNA and qPCR analysis.

Reverse transcription and qPCR analysis

The reverse transcription (RT) of cDNA for all larval and adult samples was carried out in duplicate using 500 ng of RNA with an iScript™ cDNA Synthesis kit (Bio-Rad Laboratories Inc., Hercules, CA, USA) following the protocol previously described in Mazurais et al. (2020b). Negative RT consisting in RT reaction without retro-transcriptase enzyme were also performed for all samples.

The relative quantification of mRNA of interest [*ompa, ompb, trypsin (prss1), amylase (amy1)*] and of the two housekeeping genes [elongation factor 1-alpha (*ef1α*) and Ribosomal Protein L13a (*rpl13a*)] was performed by qPCR using primers listed in table 1. *Prss1* and *amy1*genes were analysed as they are known to exhibit fluctuating expression with maturation of digestive function during early stages of sea bass larvae development (Zambonino-Infante and Cahu, 1994). The investigation of p*rss1* and *amy1*genes expression allowed the technical validation of the qPCR data and the evaluation of the physiological development of the larva. The primer pairs were designed using Primer 3 plus tool (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) and tested using a 2-fold serial dilution of pools of cDNA. The standard curves were performed for each primer pair to determine the efficiency of the qPCR reaction. In the present study, all qPCR efficiencies were around 100% with $R^2 > 0.999$.

Transcript expression was quantified using the CFX96 Touch Real-Time PCR Detection system (Bio-Rad Laboratories Inc.) and the protocol previously described (Mazurais et al., 2020b). The relative quantities of transcripts in juvenile and adult tissues were normalized with the ΔΔCt method using *ef1α* and/or *rpl13a* as reference genes. Only the *rpl13a* gene was used to normalize mRNA expression throughout larval development since the expression of *ef1α* gene was not stable. The choice of reference genes was performed based on their coefficient of variation (CV) and expression stability (M) values lower than 25% and 0.5, respectively.

Statistical analysis

All statistical analyses were performed with the free software R (R_Core_Team, 2018). A student's t-test was used to test significant differences in normalized *ompa* and *ompb* mRNA expression levels between control and OA groups at juvenile and adult stages. Two ways ANOVA was performed to analyse the potential effects of developmental stage and acidification factors on gene expression data at larval stage. The normality of residuals was checked graphically and homogeneity variance matrices were checked with the Box's Mtest. The level of significance was taken at 0.10 while being cautious for P value > 0.05.

Results

Sequence analysis of omp genes, cDNAs and proteins

Ompa and *ompb* genes are located in two different parts of the European seabass genome. The *ompa* gene, located in the Linking Group 13:27322839-27325677 on UCSC Genome Browser database (HG916830.1: 27,324,685-27,325,680 on Ensembl database), consists of two exons included in the non-coding sequence separating the exons 2 and 3 of the calpain 5a gene (*capn5a*) (figure 1A). Microsynteny analysis using an ancestor fish species as query was performed to compare the genomic structure around *omp* genes among fish species including European sea bass. The neighbouring genes of European sea bass *ompa* include *capna*, *cul5a* and *dcun1d5* that are retrieved within most of the flanking *ompa* regions in teleost species analysed (figure 1B). The *ompb* gene is included in the Linking Group 14:26037825-26038307 (HG916831.1: 26,036,773-26,038,307) and consists of a single exon incorporated between the exons 2 and 3 of the calpain 5b gene (*capn5b*) (figure 1A). The neighbouring genes of European sea bass *ompb* (i.e. *capnb, gdpd4b and myo7ab*) are well conserved among fish species. It is noteworthy that most of *ompa* neighbouring genes are the paralogs of the *ompb* neighbouring genes. Altogether, the present synteny analysis indicated that the genomic structures around *omp* genes are well conserved among species and resulted from duplications of an ancestral genome.

The *ompa* and *ompb* genes contain open reading frames (ORF) of 501 bp and 483 bp predicting primary translation products of 166 aa and 160 aa, respectively. The European seabass amino acids OMPa and OMPb sequences are 60.24% identical and exhibit 68.07% of homology. OMPa and OMPb sequences share high conservation with OMP from teleosts, gar (Holostei), and tetrapods, especially within the EphHB2-Receptor-like loop and in the protein area including the α1-helix, the α2-helix and the following β-7 strand (figure 2).

A phylogenetic analysis based on OMP amino acid sequences from tetrapods, gar and teleosts clearly separated teleost OMP sequences according to the class group (figure 3). A first cluster included tetrapod OMPs and was divided into mammals and amphibians. While spotted gar classified in a separate phylum, another monophyletic cluster (bootstrap value of 94%) included teleost OMPs divided in OMPa and OMPb subgroups. Within the groups of teleosts, zebrafish, goldfish and common carp clustered in Ostariophysi superorder, Atlantic salmon and Rainbow trout OMPs appeared included in a Protachanthopterygii cluster while European seabass OMPs

shared the closest relationship with species of Acanthopterygii superorder, the gilthead seabream and medaka.

Omp mRNA relative abundance in different tissues at juvenile stage

At juvenile stage, both *ompa* and *ompb* transcripts mRNA were mainly expressed in the olfactory rosette (figure 4). *Ompb* cDNA amplification was also observed to a lower level in cerebellum, spinal cord, olfactory bulbs, diencephalon and optic tectum. No significant *ompb* transcript expression was observed in eyes nor in non-central nervous system organs. Contrary to *ompb*, *ompa* transcript was expressed in the eyes. It was also expressed to a very low level in other tissues such as olfactory bulb, diencephalon, optic tectum, spinal cord, gills, heart, intestine, kidney, liver and spleen but not in cerebellum and skeletal muscle.

Omp mRNA expression in European seabass exposed to OA

The potential effects of OA on the relative abundance of the *ompa* and *ompb* mRNA were investigated in the olfactory epithelium of F0 adult fish exposed from hatch until four years-old to control (pH 8.0) or OA (pH 7.6) condition (figure 5). The *ompa* and *ompb* mRNA levels were shown to fluctuate differentially between control and OA condition. *Ompa* mRNA level was significantly higher (x1.31) in the olfactory epithelium of adults exposed to OA compared to the control group (t-test, p= 0.007). Inversely, the relative abundance of *ompb* mRNA level was significantly higher (x1.36) in the olfactory rosette of fish from control condition compared to the OA group (t-test, p=0.002).

Figure 6 (A, B) shows the levels of *ompa* and *ompb* transcripts during the first 27 days of development of larvae (F1) originating from F0 broodstock and reared under the same conditions as their parents. Both *ompa* and *ompb* transcript exhibited significant variation of expression level during larval development (p value < 10^{-4}). Under control pH condition, quantities of *ompa* and *ompb* transcript increased exponentially from 0 to 4 dph then decreased until day 16 post-hatching to remain almost stable afterwards. The OA factor tended to interact with stage of larval development (*P* value = 0.08 and 0.07 for *ompa* and *ompb*, respectively). Under OA condition, the expression profiles of both *ompa* and *ompb* transcripts are shifted compared to the control condition with a maximum of transcripts observed at day 10 post-hatching. The two genes, *amy1* and *prss1*, known to exhibit fluctuating expression patterns

during the early stages of digestive function development in fish were also analyzed (figure 6 C, D) (Zambonino-Infante and Cahu, 1994). The *amy1* and *prss1* mRNA expression levels exhibited significant variations during larval development being maximal at day 10 post-hatching in larvae under control pH condition (p value < 10^{-5}). Afterwards the *amy1* mRNA level dropped abruptly while the *prss1* mRNA level remained relatively stable before rising at day 27 post-hatching. OA did not change significantly the expression pattern of the *amy1* and *prss1* mRNA expression levels during larval development.

No significant difference (P value >0.1) in relative expression levels of both *ompa* and *ompb* mRNA levels was observed in the olfactory epithelium of F1 juveniles (18 months old) (figure 7).

Discussion

In the present study, we identified the genomic loci of the *ompa* and *ompb* genes in European seabass. Both *ompa* and *ompb* genes are separately included within intron 2 of European seabass *calpain 5a* and *calpain 5b*, respectively. This result is in agreement with previous data in the literature showing the location of the *ompa* and *ompb* genes between exon 2 and exon 3 of the duplicated *calpain 5* genes in different vertebrate species including teleosts (Suzuki et al., 2015, Nakashima et al., 2019). The microsynteny analysis showed that the genomic region surrounding the *ompb* gene is highly conserved between European seabass and zebrafish while the genomic area around the *ompa* gene is more heterogenous between these two species. Interestingly, the arrangement surrounding *SLC 35F2*, *cullin 5*, *omp* and *calpain 5* genes was found for both European seabass *ompa* and *ompb*genes suggesting that this region was probably entirely duplicated.

To better characterize European seabass OMP amino acid sequences, we conducted a phylogenetic analysis and analysed predicted functional domains of OMP proteins. Our phylogenetic analysis clustered on the one hand tetrapod OMP sequences and on the other hand the teleost OMP homologs that included OMPa and OMPb clades. The present phylogenetic analysis based on the full length OMP sequences confirmed a previous study indicating that teleost *ompa* and *ompb* genes were duplicated from an ancestor *omp* gene (Suzuki et al., 2015). The European seabass OMPa and OMPb sequences showed the closest relationship to the OMPa and OMPb from other members of the Acanthopterygii superorder, the gilthead sea bream and the medaka which validates the identity of European seabass *ompa* and *ompb* genes. Characterization of protein domains revealed that the predicted European seabass OMPa and OMPb proteins possess eight beta-strands, two long alpha-helices and an Eph2B-receptor-like loop domain. Our alignment analysis indicated that this latter domain is specially well conserved among OMP sequences of vertebrate species confirming that it should play a key role for protein function (Baldisseri et al., 2002, Smith et al., 2002).

To investigate basal *ompa* and *ompb* mRNA expression, we performed PCR assays in a variety of tissues from juvenile European seabass reared under basal environmental condition. As expected, *ompa* and *ompb* transcripts were highly expressed in the olfactory rosette. *Omp* transcripts are indeed known to be expressed mainly in the olfactory organ of vertebrates including fish (Rogers et al., 1987, Kang et al., 2015, Suzuki et al., 2015). Interestingly, to a lesser degree, *ompa* transcript is also highly expressed in the eye contrary to *ompb* transcript

379 which is more expressed in different parts of the central nervous system such as the cerebellum,
380 the olfactory bulb and the diencephalon. Differential expression of duplicated *omp* genes has
381 already been described in other teleost species. Indeed, the divergence of expression patterns
382 between *ompa* and *ompb* transcripts in brain and eye is in total agreement with expression data
383 obtained in zebrafish (Suzuki et al., 2015). This indicates that the distinct functions of the
384 duplicated *omp* genes suggested in zebrafish are likely conserved between the two species. In
385 zebrafish, *in situ* hybridization analyses indicated that *ompb* and *ompa* transcripts were mainly
386 expressed in non-overlapping ciliated OSN in the deep layer and the superficial layer of the
387 olfactory epithelium, respectively. Zebrafish *ompa* transcript expression was also shown to be
388 restricted in retinal horizontal cells in the outermost part of the inner nuclear layer (Suzuki et
389 al., 2015). Although additional studies are required to identify the cells expressing European
390 seabass *ompa* and *ompb* genes using *in situ* hybridization and/or immunohistochemical studies,
391 we assume that seabass *omp* transcripts have the same cellular distributions as their orthologs
392 in zebrafish in the olfactory and visual tissues. Further studies should also be performed to
393 determine the cell types expressing *ompa* and *ompb* transcripts in the different brain areas of
394 European seabass. To our knowledge, identification of *omp* gene expressing cells in non-
395 olfactory areas of the brain has only been performed in rodents (Baker et al., 1989). While OMP
396 protein has been localised in neurons of the pre-optic and hypothalamus region in three rodent
397 species, its expression patterns in other regions including cerebellum depends on the species
398 studied. Determining the nature of neurons expressing *ompa* and *ompb* transcripts in the
399 different areas of the teleost brain may offer novel opportunities to explore their functions in
400 non-olfactory brain regions. Especially since we found that *ompa* transcript was also
401 significantly expressed in many non-olfactory organs. This finding confirms previous data
402 obtained in mammals supporting the idea that OMP proteins may play a more general role in
403 chemosensing in addition to its role in the olfactory system (Kang et al., 2015).

404 The two paralogous of European seabass *omp* transcripts showed similar expression patterns
405 during larval development with maximum levels found around 4 dph. Interestingly, this
406 expression peak around 4 dph corresponds to the stage of mouth-opening in European seabass.
407 Data available in the expression atlas on the EMBL-EBI website confirm the high relative
408 expression level of *ompa* and *ompb* transcripts at larval protruding mouth stage in zebrafish.
409 The increasing expression of *omp* transcripts during the first days post-hatching is consistent
410 with the early differentiation of the olfactory organ during ontogenesis in European seabass
411 (Diaz et al., 2002). The peripheral olfactory organ is known to be the first chemosensory organ

to develop in fish (Hansen and Zielinski, 2005). The synchronization of the olfactory system development with mouth opening can be associated with the development of feeding behaviour during early life stages of larvae. The drop in *ompa* and *ompb* transcripts expression observed after 4 dph relies not necessarily to a decline in olfactory system formation, but could more probably arise from the decrease in olfactory organ/whole-body tissue mass ratio occurring during larval development. Other genes involved in the ontogenesis of sensory and nervous systems have been found to exhibit similar expression patterns during the early stage of European seabass development (Darias et al., 2008).

Another objective of the present study was to analyse *ompa* and *ompb* mRNA expression levels in two successive generations of fish reared under two pH conditions to evaluate the potential impact of OA on the olfactory system of European seabass. Surprisingly, our data revealed opposite effects of OA on *ompa* and *ompb* mRNA levels in the olfactory rosette of 4 years-old adult (F0) European seabass. Differential regulation of *omp* genes by OA indicates that the underlying molecular mechanisms differ between the two genes. Such differential regulation of paralogous genes have already been observed in teleost (Marandel et al., 2016). The absence of negative correlation between the expression of the two *omp* genes at the individual level seems to rule out the hypothesis that the regulation of one *omp* gene by OA could compensate for the opposite regulation of the paralog (data not shown). This data reinforces also the idea that *ompa* and *ompb* genes have distinct roles. Even if additional experiments would be necessary to confirm this regulation at the protein level, this result suggests that the opposite regulation of duplicated *omp* genes in the olfactory epithelium may significantly contribute to the long-term acclimation response (4 years exposure) of European seabass to OA. Such long-term impact of OA on transcript expression level in the olfactory rosette of adult (F0) European seabass was recently observed for the *cbln11* gene (Mazurais et al., 2020b). Altogether, these data suggest that the olfactory epithelium transcriptome may be durably impacted in F0 individuals exposed for long time to OA. Further determination of the roles of both *ompa* and *ompb* genes in teleost would be essential to better understand the physiological meaning of these opposite regulations and especially their potential impact on the olfactory system. However, these effects of OA on the expression of *ompa* and *ompb* transcripts expression were only observed in the olfactory epithelium of the first generation of fish. Indeed, no more significant effects of OA on the *ompa* and *ompb* mRNA expression levels were observed in the olfactory rosette of the juveniles from the F1 generation. While regulation of *omp* transcripts expression found in F0 adult relies on phenotypic plasticity associated to acclimation to environmental variation within a generation,

the absence of regulation observed in the olfactory epithelium of F1 juveniles may have different explanations. We cannot exclude the possibility that OA-induced regulation of genes involved in sensory system depends on the ontogenic development of the fish and particularly on its sexual maturation status. Interaction of the olfactory transcriptome with the progression of sexual maturation has been shown in Chum Salmon (*Oncorhynchus keta*)(Palstra et al., 2015). It may also be related to intergenerational acclimation and/or genetic adaptation (Munday, 2014). Intergenerational acclimation to OA has been mentioned in anemonefish (*Amphiprion melanopus*) in which the growth and survival is not impacted only in juveniles whose parents had been exposed to high CO_2 (Miller et al., 2012). It is uncertain whether intergenerational plasticity (including epigenetic regulation) and genetic adaptation interact for explaining the absence of regulation in the olfactory rosette of F1 juveniles in the present study. However, possible selection of individuals exhibiting an insensitivity to OA among the F1 is not supported by the apparent OA-induced regulation of *ompa* and *ompb* transcript expression found in larvae also being the offspring of long-term exposed F0 seabass. Indeed, the present expression data obtained at larval stage suggest a delay in the expression pattern of the *omp* transcripts during the early developmental stage of F1 larvae reared under OA. This delay in *omp* transcript expression suggests that the development of the external olfactory organ may be retarded under OA. Further histological and qPCR analyses with bigger sample size would confirm this hypothesis. This possible delay in the maturation of the peripheral olfactory tissue does not seem associated with a global developmental retardation in European seabass larvae as suggested by the OA-induced no significant effect on the expression of *prss1* and *amy1* transcripts encoding two enzymes involved in the digestive system. The increase in *amy1*and *prss1* expression observed between day 4 and day 10 post-hatching is in total agreement with the known peak of enzymatic activity observed around the mouth opening stage in European seabass larvae, which validates the gene expression data obtained in the present study (Zambonino-Infante and Cahu, 1994). The indicated OA-induced disturbance of the developmental process during the early larval stages of European seabass agrees with previous data obtained in other teleost species (Munday et al., 2009b, Pimentel et al., 2014, Baumann et al., 2012, Franke and Clemmesen, 2011b, Hurst et al., 2019). It would be interesting to investigate whether regulation of *ompa* and *ompb* genes expression is correlated to altered responses to sensory cues. From an ecological point of view, impairment of olfactory sensory system development during the early stages of larval development could have severe consequences in terms of predator avoidance, first feeding and survival in the natural environment.

In conclusion, we found that the European seabass *ompa* and *ompb* gene products exhibit similar structural and expression characteristics with zebrafish orthologs suggesting that the function of ortholog genes are conserved between these species. In addition, the present data revealed that under acidification conditions which could occur in the ocean by the end of this century, OA induces intra- and intergenerational plasticity in *ompa* and *ompb* mRNA expression. While further research is needed to better understand the role of *ompa* and *ompb* genes in European seabass, our data suggest potential long-term impact of OA on sensorial system of European seabass.

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Legends

Figure 1: Microsynteny analysis of OMP loci. The microsynteny was performed using Genomicus web server at http://genomicus.biologie.ens.fr/genomicus. A: *ompa* and *ompb* genes from European sea bass (*Dicentrarchus labrax*) are included in the non-coding sequence separating the exons 2 and 3 of the calpain 5a (*capn5a*) and calpain 5b (*capn5b*) genes, respectively. B: Overview of microsynteny analysis of *omp* genes and the neighbouring genes in their flanking regions among different fish species, using an ancestor species, the spotted gar (*Lepisosteus oculatus*), as query. Syntenic genes are represented by arrow colour. All orthologs are drawn with the same color and the lettering or number inside refer to subtype. Shaded genes correspond to genes that are not orthologous to any genes from the spotted gar species. The map is centralized in *omp* gene. Genes are aligned in columms and kept in the order in which they appear in chromosomes (Chr) without consideration for distance, while the transcriptional sense is represented by the pentagon tip. Red square nodes represent duplication events of an ancestral version of the gene used as query. Blue square nodes represent ancestral species

leading from the same "root" ancestral species to orthologs and/or paralogs of the gene used as query. Open blue square nodes represent extant species. European sea bass species is indicated by a black arrow. The black horizontal line separates *omp*, *gdpd4*, *myo7 and capn*5 subgroups.

Figure 2: Alignment of OMP amino acid sequences of teleosts, gar and tetrapods including *Dicentrarchus labrax* OMPa and OMPb. SnapGene software (version 5.2) was used to illustrate the alignment. Homologies among the sequences are illustrated by grey blocks above the alignment. Amino acids are marked with color highlighting based on properties and conservation. Secondary structure prediction based on Smith et al. (2002), Suzuki et al. (2015) is indicated as followed: the eight beta strands (beta-1 to beta-8) are boxed, the two α-helical regions (α1-Helix and α2-Helix) and the EphHB2-Receptor Like Loop (RLL) domain are indicated by solid and open arrows, respectively.

Figure 3: Phylogenetic analysis constructed from OMP amino acid sequences of gar, tetrapods and teleosts. Phylogenetic tree was performed using neighbour-joining method with the ITT model of amino acid substitution after Mafft alignment. OMP sequences were acquired from ensembl and genbank databases. Numbers next to the branching points indicate the relative support from 1000 bootstrap replicates. Arrows indicate European seabass OMP sequences.

Figure 4: Boxplot showing *ompa* (A) and *ompb* (B) relative mRNA abundance (arbitrary units, a.u.) throughout different European seabass tissues. Three individuals were analysed by sampling tissue. *Omp* mRNA abundances were normalized using *ef1α* and *rpl13a* as housekeeping genes. The cross in each column of the plot represents the mean mRNA relative abundance value. Mean non-normalized Ct values for each tissue are indicated in brackets. Upper and lower whiskers indicate maximum and minimum values, respectively.

Figure 5: Relative mRNA abundance (arbitrary units, a.u.) of *ompa* and *ompb* in the olfactory epithelium of 4 years-old adult European seabass (F0) exposed (gray boxes) or not exposed (black boxes, control condition) to ocean acidification (OA) from hatching

stage. *Omp* mRNA abundances were normalized using *ef1α* and *rpl13a* as housekeeping genes. The relative level of *omp* mRNA was fixed to 1 for each control group. The cross and the line in each column of the plot represents the mean and the median mRNA relative abundance value, respectively. Asterisks indicate statistically significant effects of ocean acidification related to the respective control group (t-test, *** <0.01).

Figure 6: *ompa* (A), *ompb* (B), *amy1* (C) and *prss1* (D) relative mRNA abundance (arbitrary units, a.u.) by days post-hatching (dph) under control (solid black line) and ocean acidification (OA, dashed gray line) conditions determined using qPCR analysis. Two pools of F1 larvae were analysed per condition and by sampling date. mRNA abundance was normalized using *rpl13a* as housekeeping gene. Each point represents the relative mRNA level from one pool of larvae. Each panel integrates results of two-way ANOVA test. F and P-values of significant effects of dph and/or OA and interaction between them are highlighted using the following signification codes: ****<0.001<***<0.01<**<0.05<*<0.1

Figure 7: Relative mRNA abundance (arbitrary units, a.u.) of *ompa* and *ompb* in the olfactory epithelium of F1 juvenile European seabass (18 months old) exposed (gray boxes) or not exposed (black boxes, control condition) to ocean acidification (OA). *Omp* mRNA abundances were normalized using *ef1α* and *rpl13a* as housekeeping genes. The relative level of *omp* mRNA was fixed to 1 for each control group. The cross and the line in each column of the plot represents the mean and the median mRNA relative abundance value, respectively.

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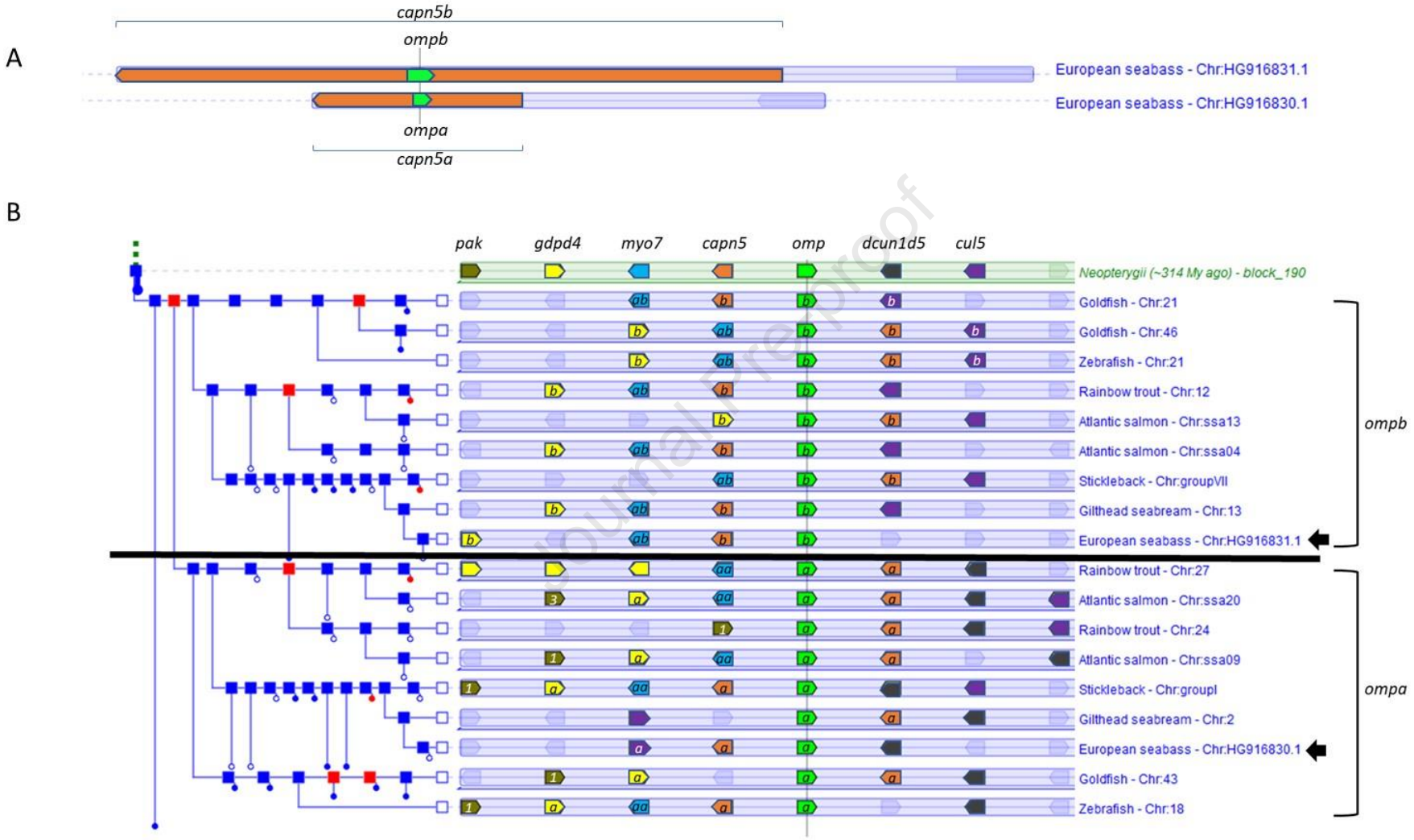
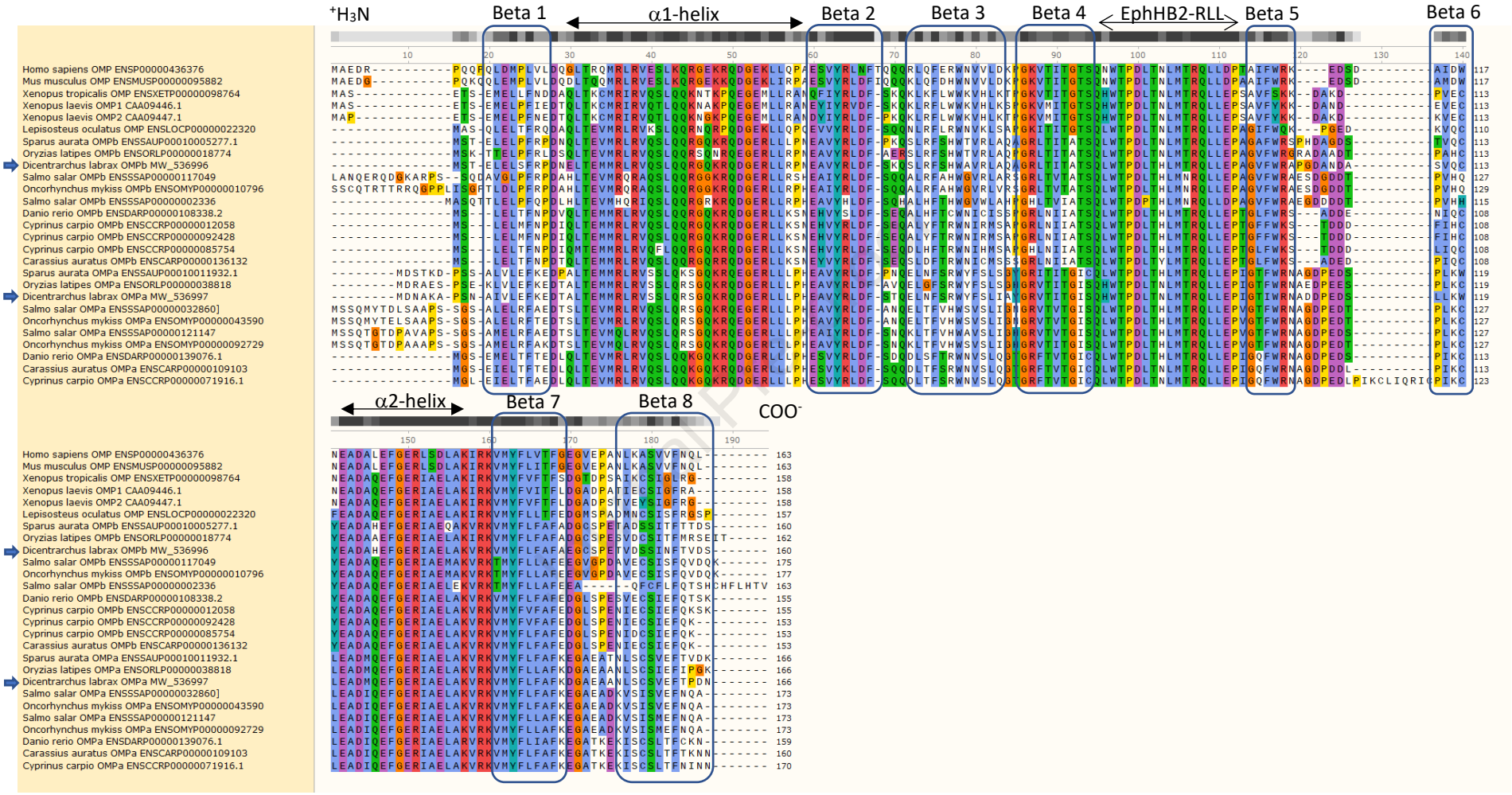
Figure 1


A
capn5b
ompb
ompa
capn5a
European seabass - Chr:HG916831.1
European seabass - Chr:HG916830.1
B
pak
gdpd4
myo7
capn5
omp
dcun1d5
cul5
Neopterygii (~314 My ago) - block_190
Goldfish - Chr:21
Goldfish - Chr:46
Zebrafish - Chr:21
Rainbow trout - Chr:12
Atlantic salmon - Chr:ssa13
Atlantic salmon - Chr:ssa04
Stickleback - Chr:groupVII
Gilthead seabream - Chr:13
European seabass - Chr:HG916831.1
Rainbow trout - Chr:27
Atlantic salmon - Chr:ssa20
Rainbow trout - Chr:24
Atlantic salmon - Chr:ssa09
Stickleback - Chr:groupI
Gilthead seabream - Chr:2
European seabass - Chr:HG916830.1
Goldfish - Chr:43
Zebrafish - Chr:18
ompb
ompa


+H3N
Beta 1
α1-helix
Beta 2
Beta 3
Beta 4
EphHB2-RLL
Beta 5
Beta 6
α2-helix
Beta 7
Beta 8
COO-
Homo sapiens OMP ENSP00000436376
Mus musculus OMP ENSMUSP00000095882
Xenopus tropicalis OMP ENSXETP00000098764
Xenopus laevis OMP1 CAA09446.1
Xenopus laevis OMP2 CAA09447.1
Lepisosteus oculatus OMP ENSLOCP00000022320
Sparus aurata OMPb ENSSAUP00010005277.1
Oryzias latipes OMPb ENSORLP00000018774
Dicentrarchus labrax OMPb MW_536996
Salmo salar OMPb ENSSSAP00000117049
Oncorhynchus mykiss OMPb ENSOMYP00000010796
Salmo salar OMPb ENSSSAP00000002336
Danio rerio OMPb ENSDARP00000108338.2
Cyprinus carpio OMPb ENSCCRP00000012058
Cyprinus carpio OMPb ENSCCRP00000092428
Cyprinus carpio OMPb ENSCCRP00000085754
Carassius auratus OMPb ENSCARP00000136132
Sparus aurata OMPa ENSSAUP00010011932.1
Oryzias latipes OMPa ENSORLP00000038818
Dicentrarchus labrax OMPa MW_536997
Salmo salar OMPa ENSSSAP00000032860]
Oncorhynchus mykiss OMPa ENSOMYP00000043590
Salmo salar OMPa ENSSSAP00000121147
Oncorhynchus mykiss OMPa ENSOMYP00000092729
Danio rerio OMPa ENSDARP00000139076.1
Carassius auratus OMPa ENSCARP00000109103
Cyprinus carpio OMPa ENSCCRP00000071916.1

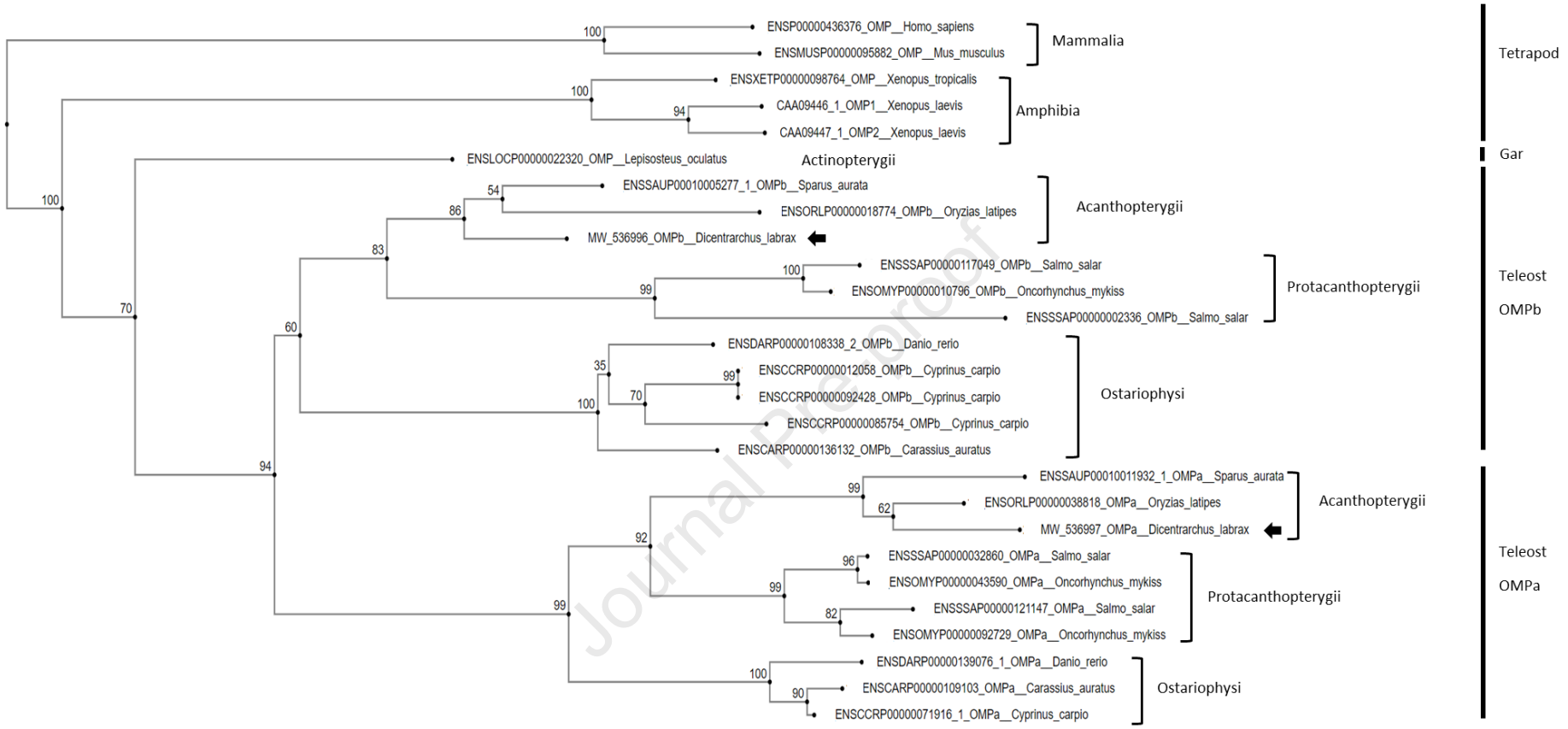
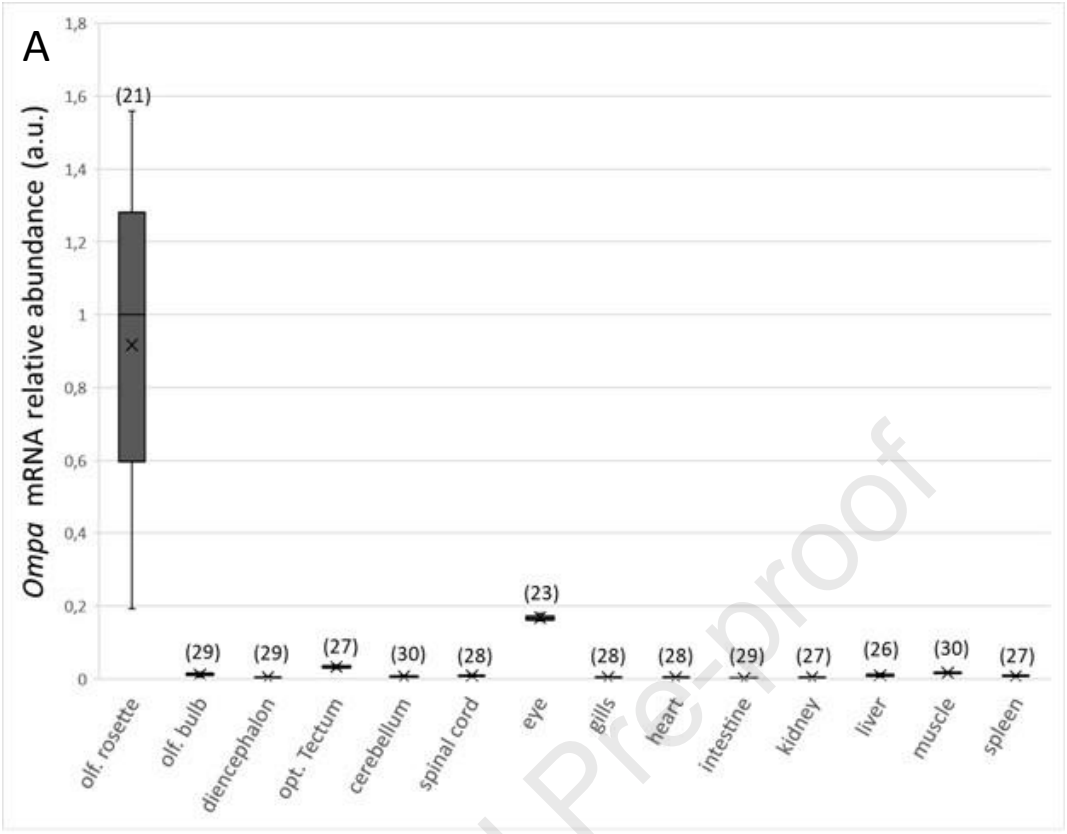


Figure 4



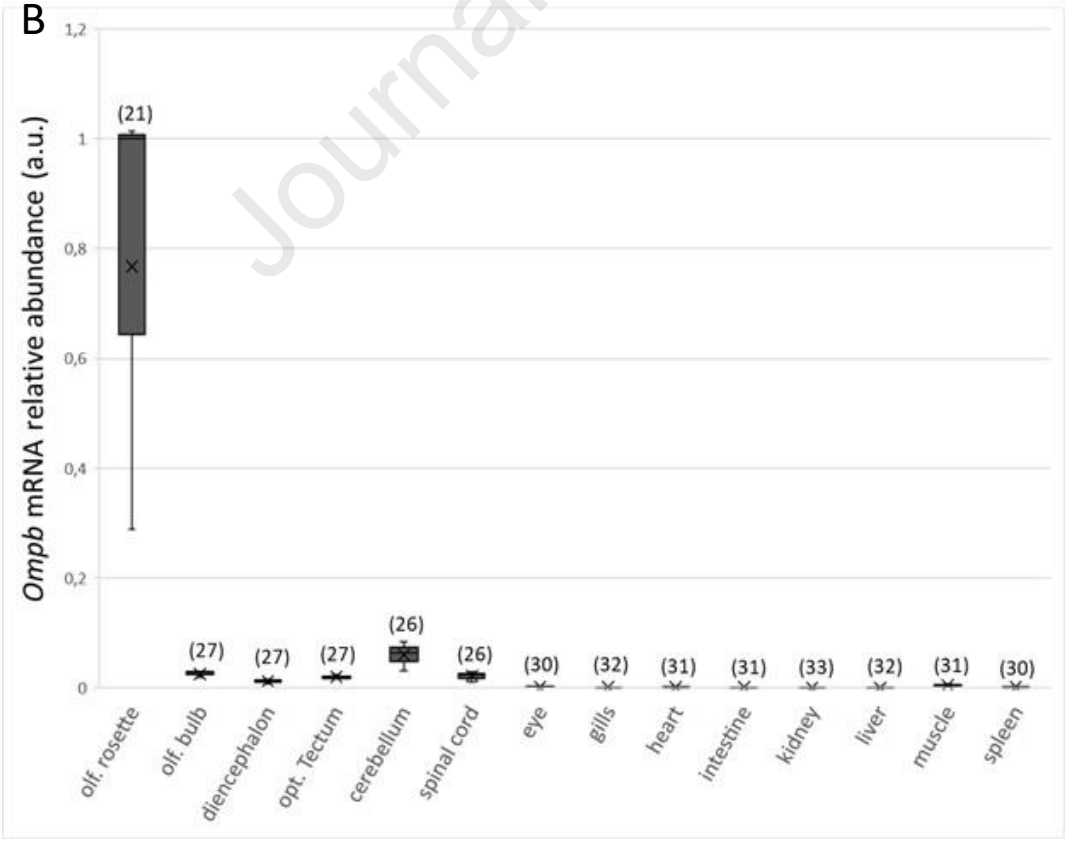


Figure 5

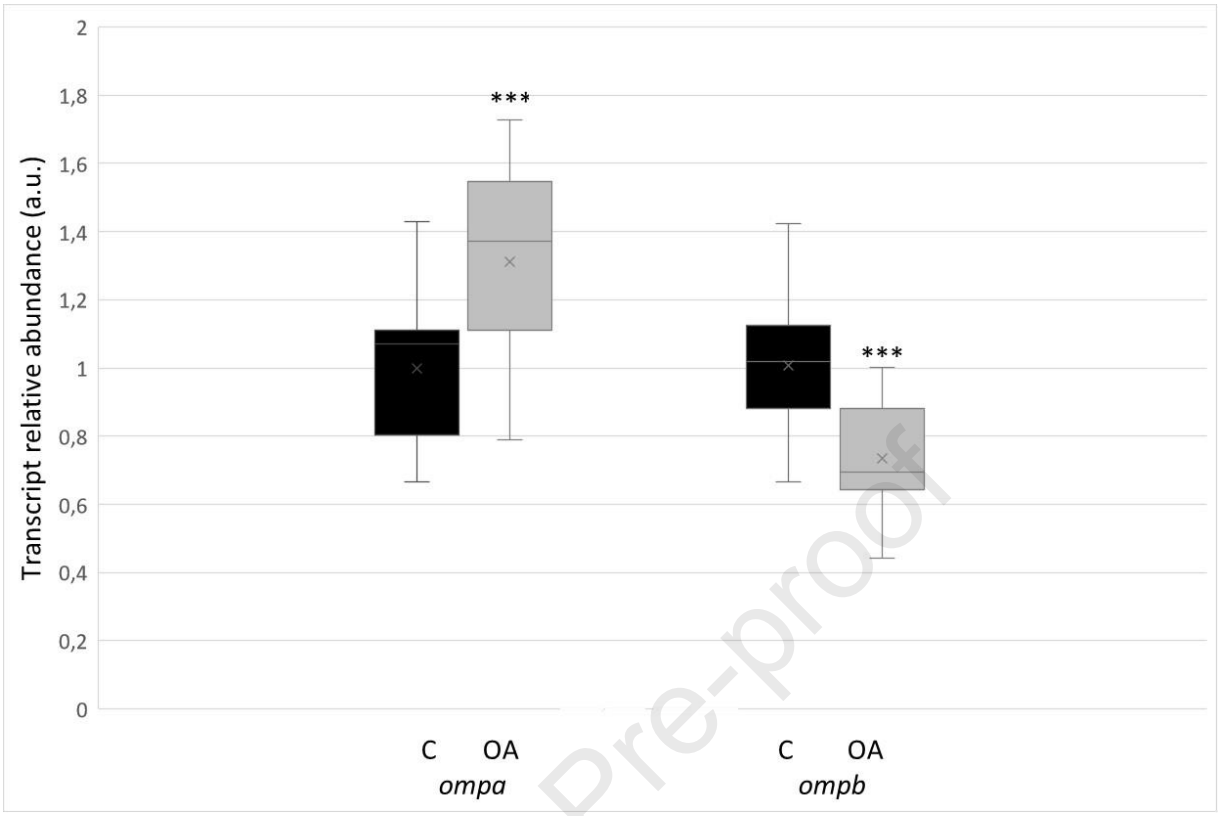


Figure 6

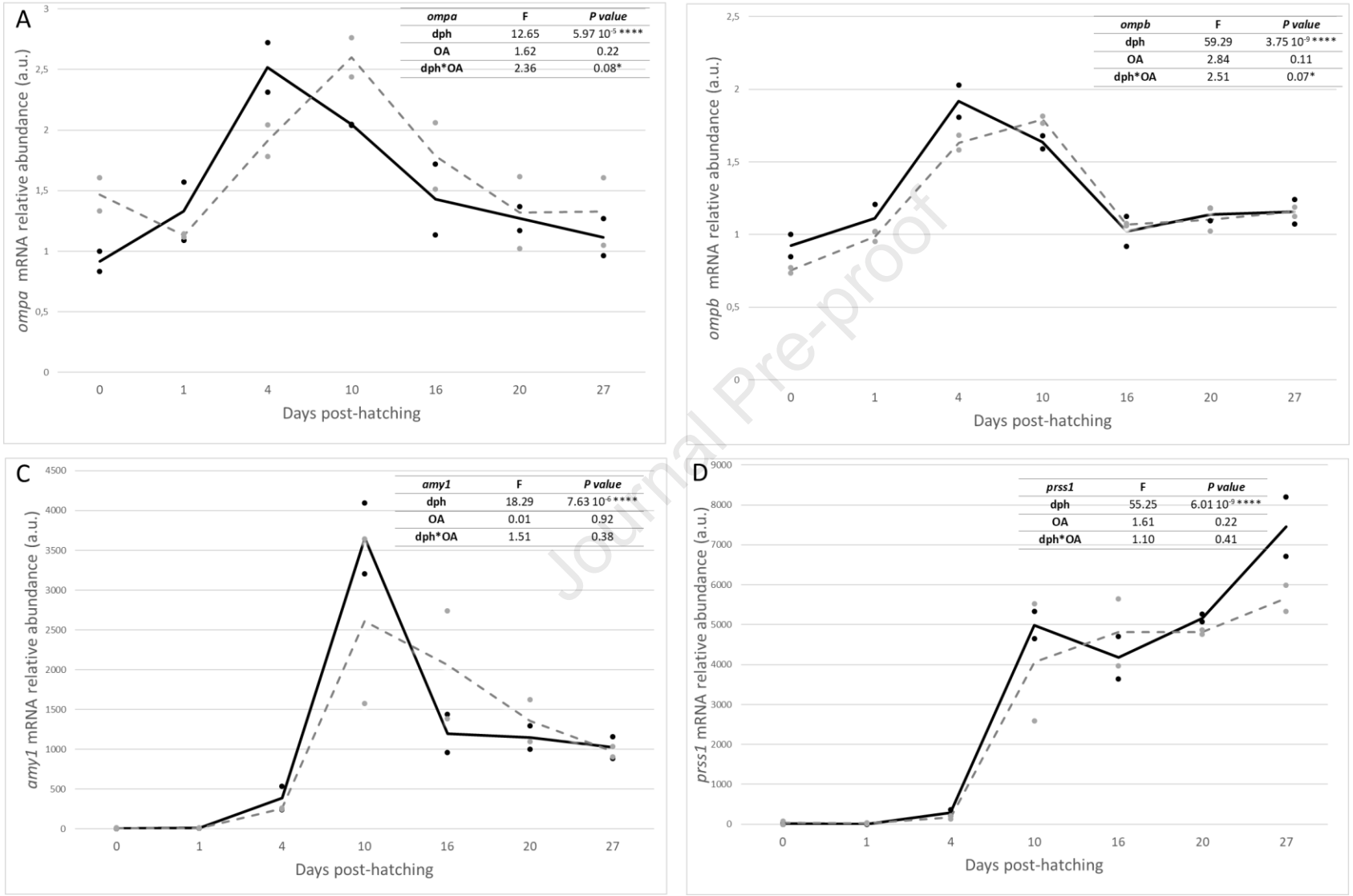


Figure 7

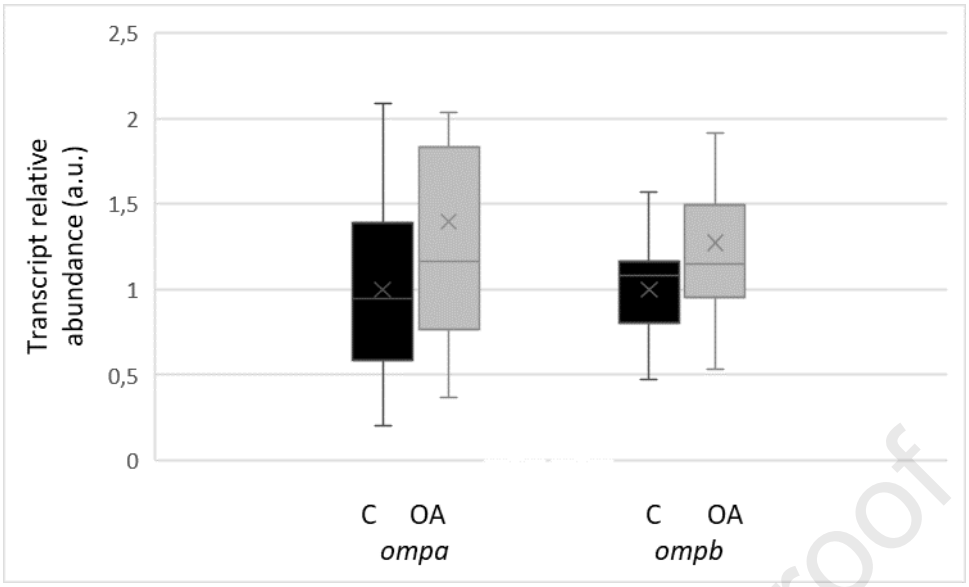


Table1

Gene name	Ref seq	Application	5'/3' Forward primer	5'/3' Reverse primer	Efficiency (%)	Melting temp. (°C)	Amplicon size (bp)
ompa	Linking Group 13:27322839-27325677 *	full length cDNA cloning	AACCTTGAAGTCGGACATGG	GAGAAGAGTCAATTATCTGGTGTGAA	nd	nd	525
ompa	DLAgn_00036760*	qPCR	ATTTCCCAACACTGGACCCC	AGCGTTTCGCCAAATCGTTC	95	84	84
ompb	Linking Group 14:26037825-26038307*	full length cDNA cloning	TTTCGACATAGCTGCCAATC	ACAGCCAGGCCTCAGCTATC	nd	nd	570
ompb	DLAgn_00046360*	qPCR	CTCACCCACCTGATGACACG	CCTCGTAGCACTGAACGGAC	99	88	97
amylase	DLAgn_00008180*	qPCR	GATCACCAGATGCAACAACG	CTGAACCAGCTTCCACATGA	97	85	114
trypsin	AJ006882.1 **	qPCR	CTCCACTGCTGACAGGAACA	CATGCCAGGGTAGGAGTTGT	95	82	85
ef1α	AJ866727.1 **	qPCR	CTGGAGGGCAGTGAAAAGAT	CATCAAGAGCCTCCAGCAGT	98	84.5	97
rpl13A	DLA_LG12_004180*	qPCR	TCTGGAGGACTGTCAGGGGCATGC	AGACGCACAATCTTGAGAGCAG	96	86	148

Highlights:

- We identified orthologous genes (*ompa* and *ompb*) in European sea bass
- *Ompa* and *ompb* genes differ in amino acid sequences and in their expression pattern
- Acidification induces intra- and intergenerational plasticity in *omps* expression
- Both *ompa* and *ompb* mRNA could be used as novel molecular markers of OSN in sea bass

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: