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

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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

38 Introduction

39 Among the environmental constraints related to global change, ocean acidification (OA) due to
40 increased concentrations of dissolved CO₂ in marine waters has been shown to disrupt olfactory
41 system with consequences on behaviour in marine fish from both tropical and temperate
42 environments (Ashur et al., 2017, Cripps et al., 2011, Dixson et al., 2015, Dixson et al., 2010,
43 Doney et al., 2009, Esbaugh, 2018, Ferrari et al., 2011, Heuer and Grosell, 2014, Munday et
44 al., 2009a, Rong et al., 2018, Williams et al., 2019, Velez et al., 2019, Chivers et al., 2014,
45 Devine et al., 2012a, Porteus et al., 2018). Such effects can impact several traits of fish life
46 including predator-prey relationships (prey detection and predator avoidance), navigation (e.g.
47 migration, homing), and locating appropriate habitats, which may have severe consequences on
48 the survival and dynamics of wild fish populations. Numerous studies demonstrated that fish
49 sensitivities to OA are especially pronounced in early life stages (Munday et al., 2009b, Franke
50 and Clemmesen, 2011a, Domenici et al., 2012, Devine et al., 2012b, Pimentel et al., 2016, Rong
51 et al., 2018). Yet, OA effects on sensory system-mediated behaviour of fish have been recently
52 questioned (Clark et al., 2020). To shed more light on this, additional investigation of both intra-
53 and intergenerational impact of OA exposure in fish using proxies that provide information on
54 the regulation affecting the maturation and activity of olfactory sensory neurons may be useful.
55 While altered olfactory perception of chemical cues induced by OA have been demonstrated
56 using electrophysiological analyses in fish at juvenile or adult stages, such an approach is
57 difficult to achieve on small size individuals at larval stage (Porteus et al., 2018, Velez et al.,
58 2019, Moore, 1994). Conversely, proxies based on the analysis of mRNA expression are very
59 useful to investigate the physiological impact of environmental cues on small organisms since
60 they do not require a lot of biological material. Moreover, numerous studies demonstrated that
61 OA-induced physiological disturbances were associated with regulation of gene expression
62 (Cline et al., 2020, Frommel et al., 2020, Hamilton et al., 2017, Huth and Place, 2016, Lai et
63 al., 2017, Mazurais et al., 2020a, Mazurais et al., 2020b, Shrivastava et al., 2019, Preus-Olsen
64 et al., 2014, Tseng et al., 2013, Mittermayer et al., 2019, Michael et al., 2016). In particular,
65 changes in mRNA levels of proteins involved in neural signalling processes have been observed
66 in olfactory systems of fish exposed to OA (Williams et al., 2019, Porteus et al., 2018),
67 including early stages of development (Rong et al., 2018).

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

71 sensory neurons (Buiakova et al., 1996, Lee et al., 2011, St John and Key, 2005). While
72 mammals possess a single-copy of the *omp* gene, teleost fish species have at least two *omp* gene
73 paralogs resulting from the duplication of an ancestral *omp* gene (Suzuki et al., 2015).
74 Sequences from paralog *ompa* and *ompb* genes have been identified from genomic resources in
75 different teleost species including zebrafish (*Danio rerio*), stickleback (*Gasterosteus*
76 *aculeatus*), fugu (*Takifugu rubripes*), tilapia (*Oreochromis niloticus*), medaka (*Oryzias latipes*),
77 platyfish (*Xiphophorus maculatus*), goldfish (*Carassius auratus*) and gilthead sea bream
78 (*Sparus aurata*) (Suzuki et al., 2015). In sockeye salmon (*Oncorhynchus nerka*), two *ompa*
79 genes have been found which may have emerged with the additional whole genome duplication
80 event in salmonids (Kudo et al., 2009, Suzuki et al., 2015). Very few information concerning
81 the respective functions of the OMPa and OMPb proteins are available in teleost species. In
82 zebrafish, *ompa* and *ompb* mRNA are mainly expressed in the superficial layer of the olfactory
83 epithelium and in ciliated olfactory sensory neurons (OSNs) located in the deep layer,
84 respectively (Suzuki et al., 2015). While both zebrafish *omp* genes are expressed in neurons
85 expressing G-protein α -subunits (*Gaolf2*) genes, the almost completely non-overlapping
86 expression pattern of *ompa* and *ompb* genes in neurons that project to different regions of the
87 olfactory bulbs suggest that they have distinct roles. Suzuki et al (Suzuki et al., 2015) assumed
88 that the distinct functions may result from the subfunctionalization of duplicated *omp* genes.
89 Involvement of OMP proteins in OSN maturation and neuronal signal transduction makes *omp*
90 mRNA expression a key molecular marker to study the regulation of olfactory function in
91 different vertebrate species including fish (Kudo et al., 2009, Oboti et al., 2011, Sato et al.,
92 2005, Suzuki et al., 2015). Particularly, quantitative analysis of *omp* mRNA expression levels
93 can inform about deficiencies in the olfactory system in organisms (Kim et al., 2010, Tilton et
94 al., 2008, Witt et al., 2009). This is of particular interest when it comes to revealing the impact
95 of environmental stressors on the olfactory systems of fish (Tilton et al., 2008).

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

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

Journal Pre-proof

108 **Material and methods**

109 *Identification and analysis of the omp sequences*

110 Blast searches using the *ompa* (NM_001025185.1) and *ompb* (NM_173281.2) mRNA
111 sequences from zebrafish as query against European seabass genome available on UCSC
112 Genome Browser database (<http://seabass.mpipz.mpg.de/index.html>) allowed to identify two
113 genome sequences, including the seabass *ompa* gene (Linking Group 13:27322839-27325677,
114 DLAgn_00036760) and *ompb* gene (Linking Group 14:26037825-26038307,
115 DLAgn_00046360). Linking group 13 and linking group 14 correspond to HG916830.1:
116 27,324,685-27,325,680 and HG916831.1: 26,036,773-26,038,307 in ensembl database,
117 respectively. The full-length transcripts encoding the European seabass OMPs were then cloned
118 by RT-PCR performed from olfactory epithelium cDNA using primers designed on the
119 predicted mRNA sequences (table 1). After cloning, the cDNA sequences were obtained by
120 GENEWIZ sequencing service (South Plainfield, USA).

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

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

131

132 *Table 1: Primers used for European seabass ompa and ompb full length cDNA cloning and*
133 *relative quantification by qPCR. Sequences used to design the primers are available in (*) Max*
134 *Planck Institute (<http://seabass.mpipz.mpg.de/index.html>) and (**) genbank databases. Nd: not*
135 *determined.*

136

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

156

157 **Animal and experimental conditions**

158 *F0 generation*

159 Experiments were conducted under approved protocols in strict compliance with the EU
160 Directive 2010/63/EU for animal experiments and according to the French legal requirements
161 concerning welfare of experimental animals (APAFIS permit no. 17132-2018101614401562).
162 The F0 population of European seabass used in the present experiment was the same as one
163 used in previous works (Mazurais et al., 2020a). F0 larvae were obtained in October 2013 from
164 a local commercial hatchery (Aquastream, Ploemeur, France). At two days post-hatch (dph),
165 they were brought within the facilities of the laboratory ‘Laboratoire Adaptation, Reproduction
166 et Nutrition des poissons’ which is part of Ifremer-Centre de Bretagne (Agreement number:
167 B29-212-05). F0 European seabass were maintained from hatching in two PCO₂ conditions
168 [Control group: pH 8, ~600 µatm, OA conditions group: pH 7.6, ~1600 µatm]. The ambient

169 PCO₂ 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], V_{HCl}—volume HCl [l], c_{HCl}—concentration HCl [mol
 188 *l⁻¹], V_{sample}—volume of sample [l], H⁺—hydrogen activity (10^{-pH}), γ_{H⁺}—hydrogen
 189 activity coefficient (here γ_{H⁺}= 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

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

205 *F1 generation*

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

215

216 **Sampling and RNA extraction**

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

220 *F0 generation*

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

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

229 *F1 generation*

230 One pool of F1 larvae was sampled per tank (two pools per pH group) and at seven larval stages
231 0, 1, 4, 10, 16, 20, 27 dph. One pool contained 30 mg biological material containing five
232 individuals to several dozen individuals depending on the developmental stage. The number of
233 pools (n=2) sampled per tank was limited by the quantity of larvae.

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

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

241

242 **Reverse transcription and qPCR analysis**

243 The reverse transcription (RT) of cDNA for all larval and adult samples was carried out in
244 duplicate using 500 ng of RNA with an iScript™ cDNA Synthesis kit (Bio-Rad Laboratories
245 Inc., Hercules, CA, USA) following the protocol previously described in Mazurais et al.
246 (2020b). Negative RT consisting in RT reaction without retro-transcriptase enzyme were also
247 performed for all samples.

248 The relative quantification of mRNA of interest [*ompa*, *ompb*, *trypsin (prss1)*, *amylase (amy1)*]
249 and of the two housekeeping genes [elongation factor 1-alpha (*ef1a*) and Ribosomal Protein
250 L13a (*rpl13a*)] was performed by qPCR using primers listed in table 1. *Prss1* and *amy1* genes
251 were analysed as they are known to exhibit fluctuating expression with maturation of digestive
252 function during early stages of sea bass larvae development (Zambonino-Infante and Cahu,
253 1994). The investigation of *prss1* and *amy1* genes expression allowed the technical validation
254 of the qPCR data and the evaluation of the physiological development of the larva. The primer
255 pairs were designed using Primer 3 plus tool ([http://www.bioinformatics.nl/cgi-
256 bin/primer3plus/primer3plus.cgi](http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi)) and tested using a 2-fold serial dilution of pools of cDNA.
257 The standard curves were performed for each primer pair to determine the efficiency of the
258 qPCR reaction. In the present study, all qPCR efficiencies were around 100% with $R^2 > 0.999$.

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

266 **Statistical analysis**

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

274 **Results**275 ***Sequence analysis of omp genes, cDNAs and proteins***

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

292 The *ompa* and *ompb* genes contain open reading frames (ORF) of 501 bp and 483 bp predicting
293 primary translation products of 166 aa and 160 aa, respectively. The European seabass amino
294 acids OMPa and OMPb sequences are 60.24% identical and exhibit 68.07% of homology.
295 OMPa and OMPb sequences share high conservation with OMP from teleosts, gar (Holostei),
296 and tetrapods, especially within the EphHB2-Receptor-like loop and in the protein area
297 including the α 1-helix, the α 2-helix and the following β -7 strand (figure 2).

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

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

307

308 ***Omp mRNA relative abundance in different tissues at juvenile stage***

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

316

317 ***Omp mRNA expression in European seabass exposed to OA***

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

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

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

343 No significant difference (P value >0.1) in relative expression levels of both *ompa* and *ompb*
344 mRNA levels was observed in the olfactory epithelium of F1 juveniles (18 months old) (figure
345 7).

346

347 **Discussion**

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

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

373 To investigate basal *ompa* and *ompb* mRNA expression, we performed PCR assays in a variety
374 of tissues from juvenile European seabass reared under basal environmental condition. As
375 expected, *ompa* and *ompb* transcripts were highly expressed in the olfactory rosette. *Omp*
376 transcripts are indeed known to be expressed mainly in the olfactory organ of vertebrates
377 including fish (Rogers et al., 1987, Kang et al., 2015, Suzuki et al., 2015). Interestingly, to a
378 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

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

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

445 the absence of regulation observed in the olfactory epithelium of F1 juveniles may have
446 different explanations. We cannot exclude the possibility that OA-induced regulation of genes
447 involved in sensory system depends on the ontogenic development of the fish and particularly
448 on its sexual maturation status. Interaction of the olfactory transcriptome with the progression
449 of sexual maturation has been shown in Chum Salmon (*Oncorhynchus keta*)(Palstra et al.,
450 2015). It may also be related to intergenerational acclimation and/or genetic adaptation
451 (Munday, 2014). Intergenerational acclimation to OA has been mentioned in anemonefish
452 (*Amphiprion melanopus*) in which the growth and survival is not impacted only in juveniles
453 whose parents had been exposed to high CO₂ (Miller et al., 2012). It is uncertain whether
454 intergenerational plasticity (including epigenetic regulation) and genetic adaptation interact for
455 explaining the absence of regulation in the olfactory rosette of F1 juveniles in the present study.
456 However, possible selection of individuals exhibiting an insensitivity to OA among the F1 is
457 not supported by the apparent OA-induced regulation of *ompa* and *ompb* transcript expression
458 found in larvae also being the offspring of long-term exposed F0 seabass. Indeed, the present
459 expression data obtained at larval stage suggest a delay in the expression pattern of the *omp*
460 transcripts during the early developmental stage of F1 larvae reared under OA. This delay in
461 *omp* transcript expression suggests that the development of the external olfactory organ may be
462 retarded under OA. Further histological and qPCR analyses with bigger sample size would
463 confirm this hypothesis. This possible delay in the maturation of the peripheral olfactory tissue
464 does not seem associated with a global developmental retardation in European seabass larvae
465 as suggested by the OA-induced no significant effect on the expression of *prss1* and *amyl*
466 transcripts encoding two enzymes involved in the digestive system. The increase in *amyl* and
467 *prss1* expression observed between day 4 and day 10 post-hatching is in total agreement with
468 the known peak of enzymatic activity observed around the mouth opening stage in European
469 seabass larvae, which validates the gene expression data obtained in the present study
470 (Zambonino-Infante and Cahu, 1994). The indicated OA-induced disturbance of the
471 developmental process during the early larval stages of European seabass agrees with previous
472 data obtained in other teleost species (Munday et al., 2009b, Pimentel et al., 2014, Baumann et
473 al., 2012, Franke and Clemmesen, 2011b, Hurst et al., 2019). It would be interesting to
474 investigate whether regulation of *ompa* and *ompb* genes expression is correlated to altered
475 responses to sensory cues. From an ecological point of view, impairment of olfactory sensory
476 system development during the early stages of larval development could have severe
477 consequences in terms of predator avoidance, first feeding and survival in the natural
478 environment.

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

487

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494

495 **Legends**

496

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

510 leading from the same "root" ancestral species to orthologs and/or paralogs of the gene used as
511 query. Open blue square nodes represent extant species. European sea bass species is indicated
512 by a black arrow. The black horizontal line separates *omp*, *gdpd4*, *myo7* and *capn5* subgroups.

513

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

522

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

529

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

536

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

540 **stage.** *Omp* mRNA abundances were normalized using *ef1 α* and *rpl13a* as housekeeping genes.
541 The relative level of *omp* mRNA was fixed to 1 for each control group. The cross and the line
542 in each column of the plot represents the mean and the median mRNA relative abundance value,
543 respectively. Asterisks indicate statistically significant effects of ocean acidification related to
544 the respective control group (t-test, *** <0.01).

545

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

554

555 **Figure 7: Relative mRNA abundance (arbitrary units, a.u.) of *ompa* and *omb* in the**
556 **olfactory epithelium of F1 juvenile European seabass (18 months old) exposed (gray**
557 **boxes) or not exposed (black boxes, control condition) to ocean acidification (OA).** *Omp*
558 mRNA abundances were normalized using *ef1 α* and *rpl13a* as housekeeping genes. The relative
559 level of *omp* mRNA was fixed to 1 for each control group. The cross and the line in each column
560 of the plot represents the mean and the median mRNA relative abundance value, respectively.

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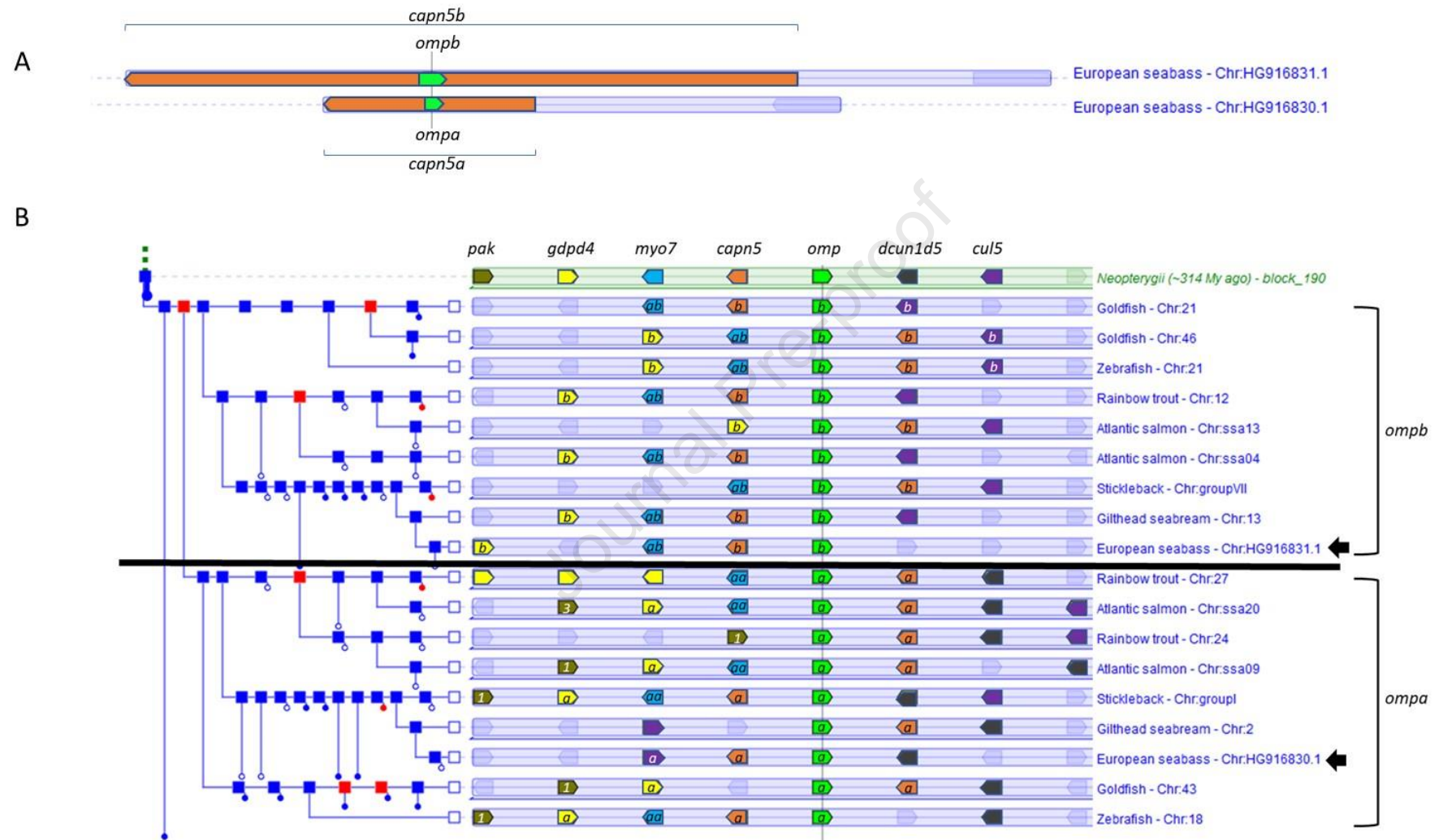
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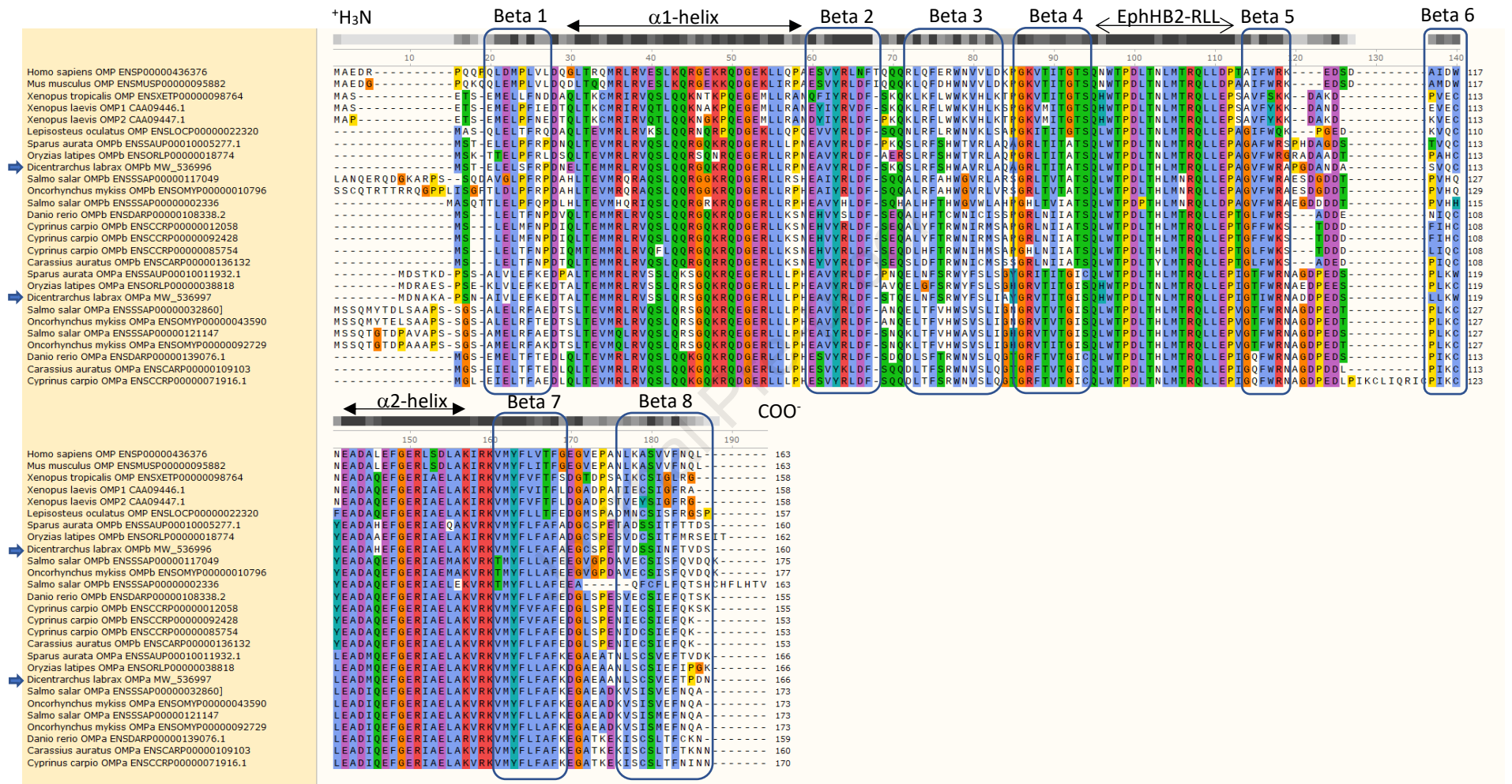
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Figure 1





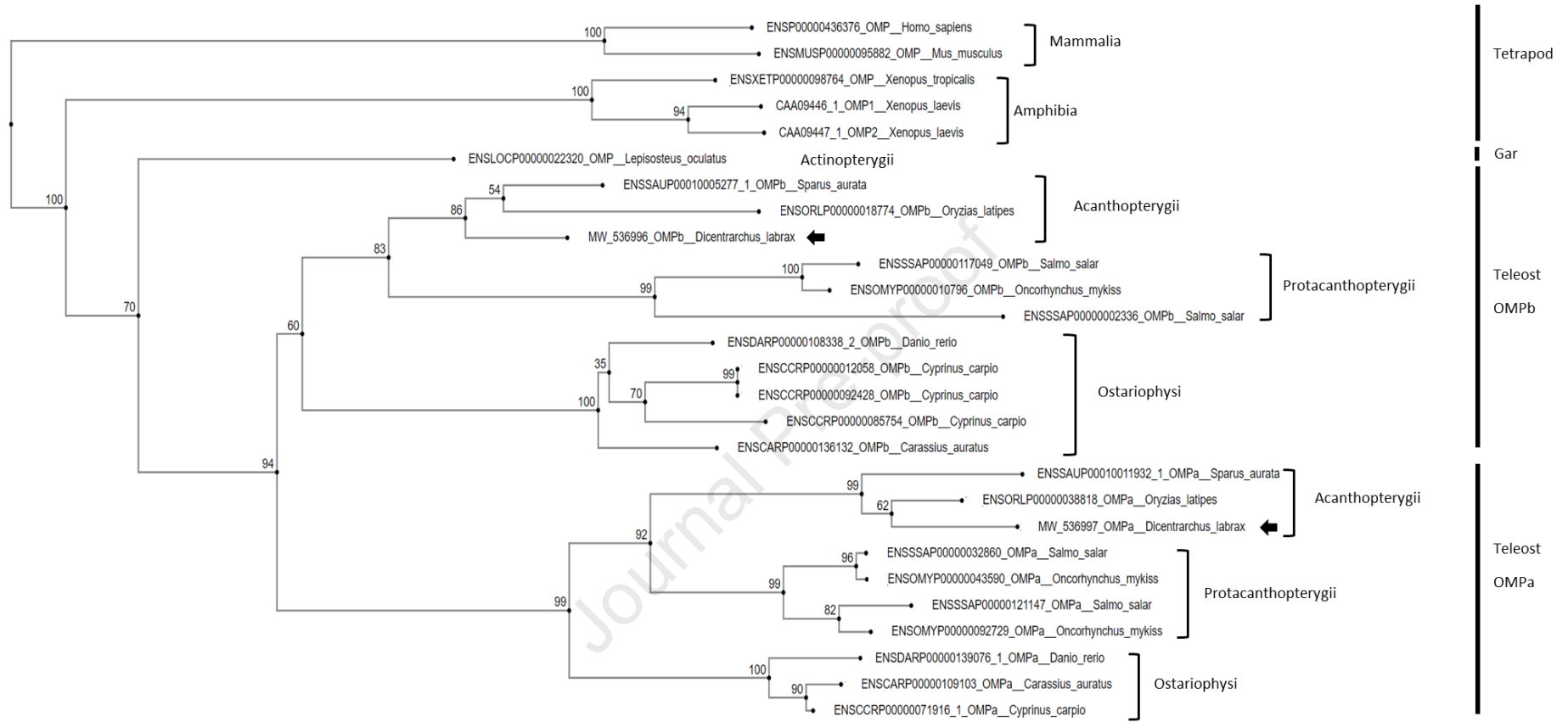


Figure 4

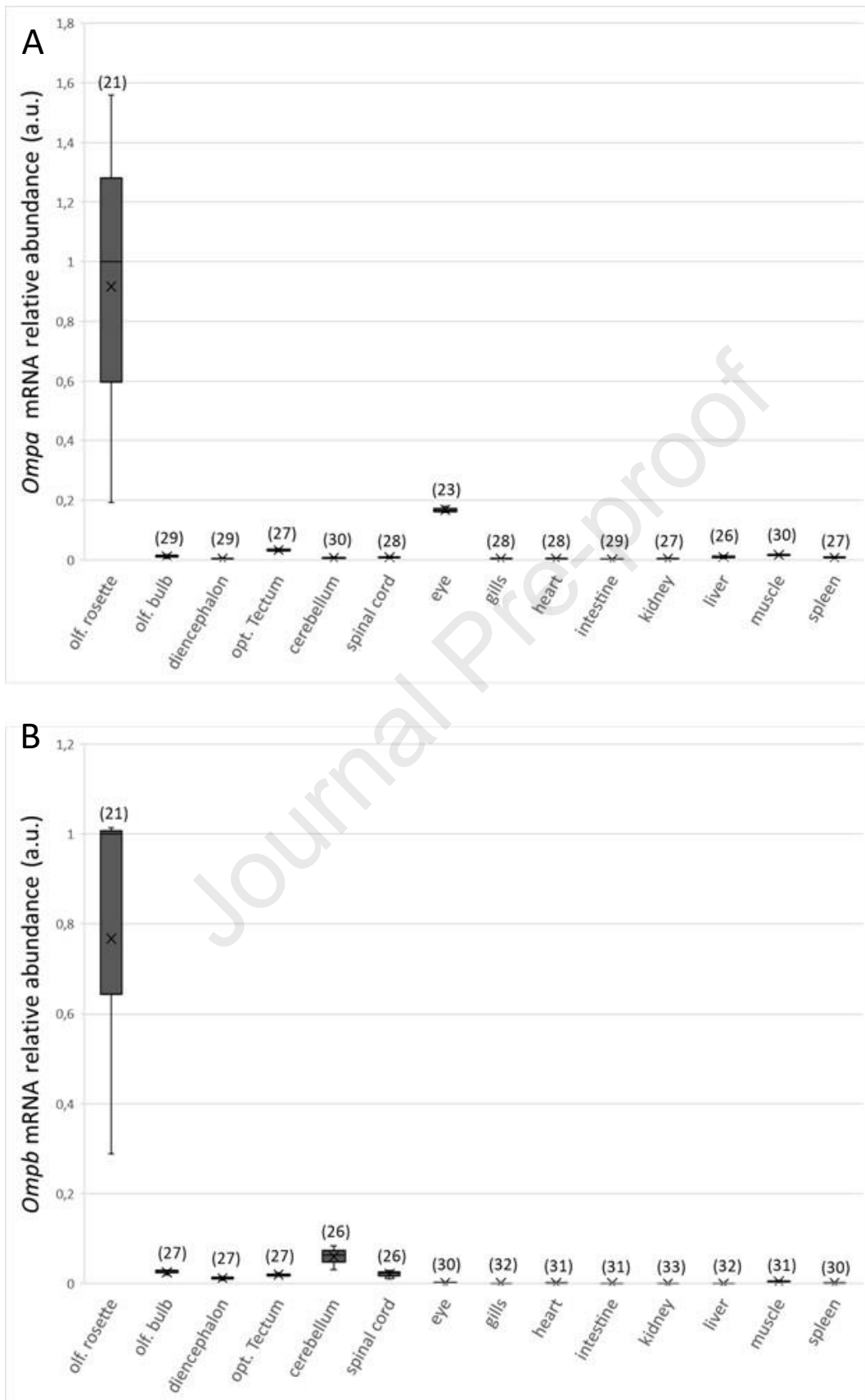


Figure 5

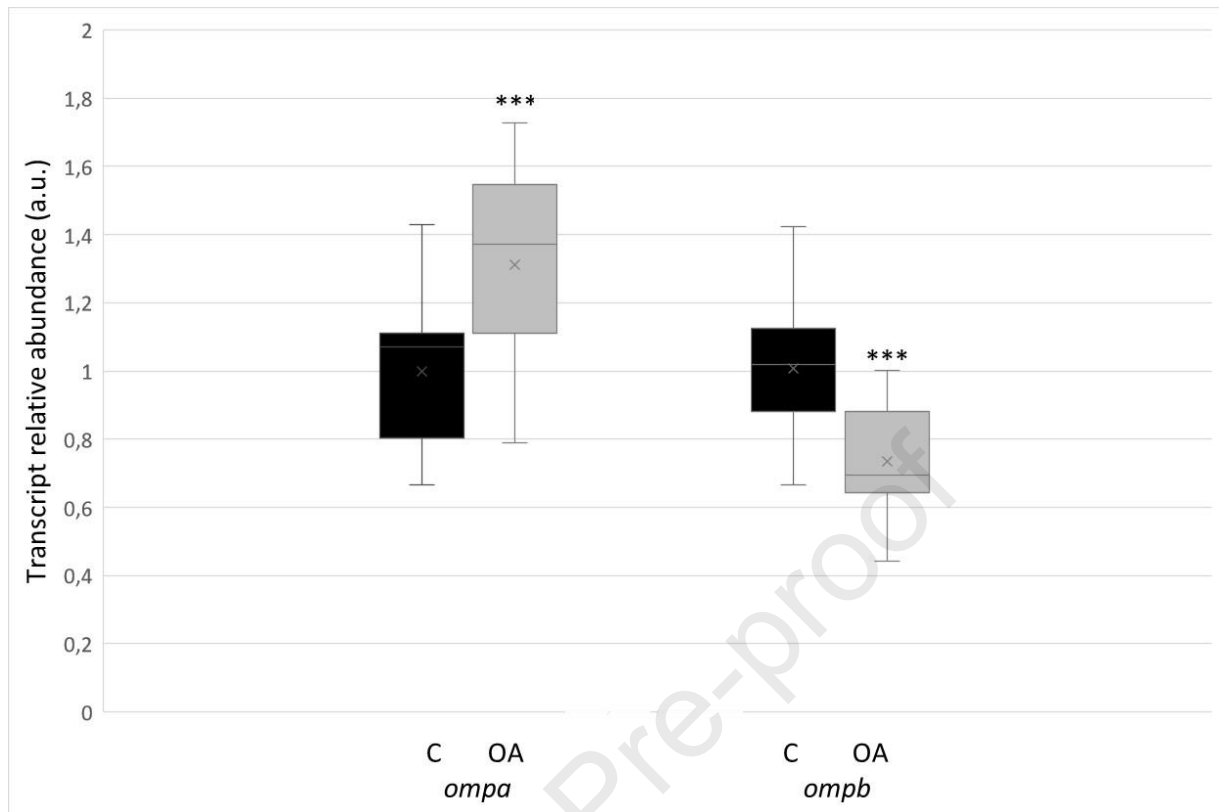


Figure 6

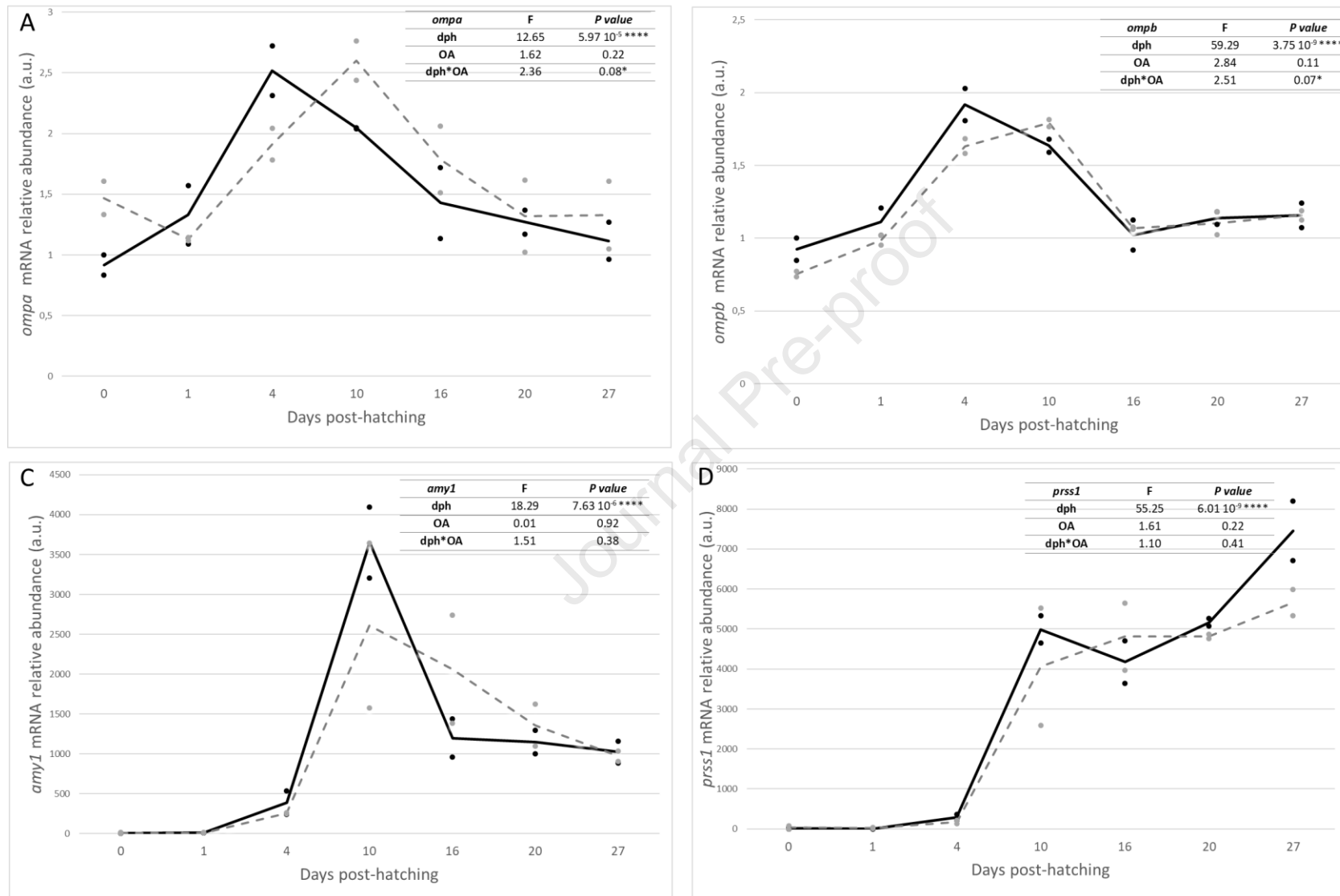


Figure 7

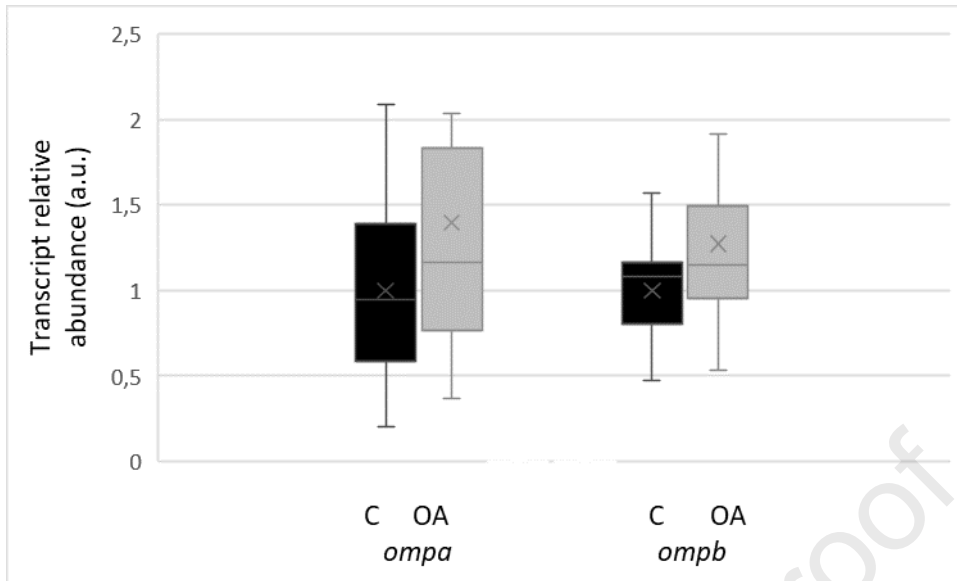


Table1

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

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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:

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