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1 **Structural and functional characterization of an egg-laying hormone signaling system in**
2 **a lophotrochozoan – the Pacific oyster (*Crassostrea gigas*)**
3

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17

18 **Abstract.**

19 The egg-laying hormones (ELHs) of gastropod mollusks were characterized more than forty
20 years ago. Yet, they have remained little explored in other mollusks. To gain insights into the
21 functionality of the ELH signaling system in a bivalve mollusk – the oyster *Crassostrea gigas*,
22 this study investigates the processing of its ELH precursor (Cragi-ELH) by mass spectrometry.
23 Some of the ELH mature peptides identified in this study were subsequently investigated by
24 nuclear magnetic resonance and shown to adopt an extended alpha-helix structure in a micellar
25 medium mimicking the plasma membrane. To further characterize the ELH signaling system
26 in *C. gigas*, a G protein-coupled receptor phylogenetically related to ecdysozoan diuretic

27 hormone DH44 and corticotropin-releasing hormone (CRH) receptors named Cragi-ELHR was
28 also characterized functionally and shown to be specifically activated by the two predicted
29 mature ELH peptides and their N-terminal fragments. Both Cragi-ELH and Cragi-ELHR
30 encoding genes were mostly expressed in the visceral ganglia (VG). Cragi-ELH expression was
31 significantly increased in the VG of both fully mature male and female oysters at the spawning
32 stage. When the oysters were submitted to a nutritional or hyposaline stress, no change in the
33 expression of the ligand or receptor genes was recorded, except for Cragi-ELHR only during a
34 mild acclimation episode to brackish water. These results suggest a role of Cragi-ELH signaling
35 in the regulation of reproduction but not in mediating the stress response in our experimental
36 conditions.

37 **Keywords:** Oyster, GPCR, egg-laying hormone, reproduction, stress

38 **Highlights:**

- 39 - Cragi-ELHs adopt an extended alpha-helix structure in a micellar medium
- 40 - Cragi-ELHs activate a receptor phylogenetically related to DH44 and CRH receptors
- 41 - Cragi-ELH expression supports a role in the regulation of reproduction

42

43 1. Introduction

44 The egg-laying hormones (ELHs) of gastropods were among the first neuropeptides to be
45 functionally characterized in mollusks (Chiu et al., 1979; Ebberink et al., 1985). They are
46 synthesized as large precursor proteins that generate a set of neuropeptides in addition to ELHs
47 and whose release controls and coordinates egg-laying and associated behavioral events in this
48 class of mollusks [3-5]. With the development of transcriptomic and peptidomic resources for
49 a variety of mollusks and annelids, it has become clear that orthologs of ELH neuropeptides
50 exist in most lophotrochozoan species (Conzelmann et al., 2013; Jékely, 2013; Mirabeau and
51 Joly, 2013; Salzet et al., 1997; Stewart et al., 2014; Veenstra, 2010). Because lophotrochozoan

52 ELHs display structural homologies with ecdysozoan diuretic hormone 44 (DH44) and
53 deuterostome corticotropin-releasing hormone (CRH) family members (CRH, urocortins1-3:
54 Ucn1-3), the ancestor of these families of neuropeptides predated the split between the
55 protostome and deuterostome lineages.

56 Both vertebrate CRH family peptides and ecdysozoan DH44 are key players of the
57 physiological response or tolerance to stress (Cannell et al., 2016; Deussing and Chen, 2018)
58 and are involved in the regulation of a variety of physiological functions including feeding-
59 associated processes (Cannell et al., 2016; Czimmer and Tache, 2017; Dus et al., 2015; Stengel
60 and Taché, 2014; van Wielendaele et al., 2012), immunity (Lee et al., 2023), reproduction (van
61 Wielendaele et al., 2012), body fluid secretion in *Drosophila* (Cabrero et al., 2002) and the
62 renal function (Devetzis et al., 2013). Among Lophotrochozoa, besides gastropod ELHs, only
63 a first insight into the physiological role of ELH as an egg-laying (spawning) hormone had been
64 provided so far – in the Sydney rock oyster (*Saccostrea glomerata*) (In et al., 2016).
65 Interestingly, a recent phylogenetic analysis showed that gastropod ELH sequences segregate
66 apart from the clade including non-gastropod mollusk, annelid and nemertean counterparts (De
67 Oliveira et al., 2019). This singular clustering of gastropod ELHs raises the prospect that they
68 could have acquired specialized functions in relation with reproduction more recently
69 throughout the evolution of mollusks. To gain insights into the functional involvement and the
70 evolution of ELH signaling in mollusks, the present study characterizes functionally an ELH
71 receptor for the first time in a Lophotrochozoan, and investigates the specific tissue-dependent,
72 temporal and environment-induced expression of the genes encoding the components of the
73 ELH signaling system in a bivalve mollusk – the oyster *Crassostrea gigas*.

74

75 2. Material and methods.

76 2.1. Animals and tissue sampling

77 Two-year old adult oysters *Crassostrea gigas* (Thunberg 1793), purchased from a local farm
78 (Normandy, France), were used for peptide characterization and transcription analyses. Adult
79 tissues, mantle, mantle edges, gills, labial palps, digestive gland, gonad (mix of all stages),
80 heart, adductor muscle and the visceral ganglia (VG) were carefully dissected-out.

81 A one-year sampling of animals was undertaken to collect gonads and VG at different stages
82 of reproduction. Oysters were collected and their gonads and VG were immediately dissected.
83 VG and gonadal tissues were sampled for each individual, frozen in liquid nitrogen and stored
84 at -80°C. A part of each gonad sample was also fixed for histological analysis. Gonadal
85 development stage and sex were determined by histological methods according to the 4 stages
86 previously described (Rodet et al., 2005): stage 0 corresponding to the resting undifferentiated
87 stage with very few germinal stem cells; stage 1 corresponding to the gonial multiplication stage
88 with poorly developed tubules surrounded by a large matrix of vesicular connective tissue
89 (VTC); stage 2 corresponding to the maturation stage with tubule development and VCT
90 starting to regress, with vitellogenesis occurring in females and all the cells of the germline can
91 be observed in males (from spermatogonia to spermatozoa); stage 3 corresponding to sexual
92 maturity with tubules full of mature germinal cells.

93 The effect of trophic conditions was investigated on one-year-old adult oysters reared in water
94 tanks either in absence of food or in presence of *Isochrysis galbana* (clone T-Iso) maintained
95 at a concentration of 6 million of cells/mL during 4 weeks. To study the influence of osmotic
96 conditions, oysters were transferred from seawater (33‰) to brackish water (8‰) at 17°C by
97 addition of distilled water once in the sea water tank (Acute stress) or by diluting the sea water
98 with a continuous flow (0.5 Lh⁻¹) of distilled water during 3 days (Mild stress). To prevent the
99 closure of the shell, a wedge was inserted between the valves before applying the stress. VG
100 were sampled and frozen after 12 h of incubation of the oysters in brackish water at the final
101 salinity.

102 2.2. Mass spectrometry characterization of oyster endogenous ELH peptides.

103 Peptide extraction: Fifteen VG, frozen and crushed in liquid nitrogen, were extracted in
104 acetonitrile/water (90/10 v/v) 0.1% TFA for 30 minutes at 4°C. After centrifugation for 20
105 minutes at 15 000 g at 4°C, supernatant was concentrated on C18 Sep-Pak cartridges. Half of
106 the recovered peptide sample was reduced with 100 mM DTT at 55 °C for 60 min and alkylated
107 with 50 mM iodoacetamide at 55 °C for 45 min. Both mature or reduced/alkylated peptides
108 were analyzed by mass spectrometry

109 For nano-LC fragmentation, peptide samples were first desalted and concentrated onto a µC18
110 Omix (Agilent) column before analysis. The chromatography step was performed on a
111 NanoElute (Bruker Daltonics) ultra-high-pressure nano flow chromatography system.
112 Approximately 200ng of each peptide sample were concentrated onto a C18 pepmap 100
113 (5mm x 300µm i.d.) precolumn (Thermo Scientific) and separated at 50°C onto a reversed
114 phase Reprosil column (25cm x 75µm i.d.) packed with 1.6µm C18 coated porous silica beads
115 (Ionopticks). Mobile phases consisted of 0.1% formic acid, 99.9% water (v/v) (A) and 0.1%
116 formic acid in 99.9% ACN (v/v) (B). The nanoflow rate was set at 300 nL/min, and the gradient
117 profile was as follows: from 2 to 15% B within 15 min, followed by an increase to 25% B
118 within 15 min and further to 35% within 12 min, followed by a washing step at 95% B and
119 reequilibration.

120 MS experiments were carried out on an TIMS-TOF pro mass spectrometer (Bruker Daltonics)
121 with a modified nano electrospray ion source (CaptiveSpray, Bruker Daltonics). A 1400 spray
122 voltage with a capillary temperature of 180°C was typically employed for ionizing. MS spectra
123 were acquired in the positive mode in the mass range from 100 to 1700 m/z. In the experiments
124 described herein, the mass spectrometer was operated in PASEF mode without exclusion of
125 single charged peptides. A number of 10 PASEF MS/MS scans was performed during 1.25
126 seconds from charge range 2-5 and the 1/K0 range 0.75-1.25.

127 Database searching was performed using the Peaks X+ software and a neuropeptide database
128 comprising all known neuropeptide precursors from *C. gigas*. The variable modifications
129 allowed were as follows: Carbamidomethylation (Cys residue), C-terminal amidation, Pyro-
130 glutamination (Gln residue) and oxidation (Met residue). “No Enzyme” was selected. Mass
131 accuracy was set to 20 ppm and 0.05 Da for MS and MS/MS mode, respectively. Data were
132 filtering according to an FDR of 1%.

133

134 2.3. Peptide synthesis

135 2.3.1. Chemical Reagents.

136 All Fmoc amino acid residues, O-benzotriazol-1-yl-*N,N,N',N'*-tetramethyluronium
137 hexafluorophosphate (HBTU), preloaded 4-hydroxymethyl-phenoxyethyl-copolystyrene-
138 1%-divinylbenzene resins (HMP) and Rink amide 4-methylbenzhydrylamine (MBHA) resin
139 were purchased from Novabiochem (Fontenay sous Bois, France) or IRIS Biotech
140 (Marktredwitz, Germany). *N,N*-Diisopropylethylamine (DIEA), piperidine, trifluoroacetic acid
141 (TFA), triisopropylsilane (TIS), tert-butylmethylether (TBME) were supplied from Sigma-
142 Aldrich (Saint-Quentin-Fallavier, France). *N*-methylpyrrolidone (NMP), dimethylformamide
143 (DMF), dichloromethane (DCM) and acetonitrile were from Fisher Scientific (Illkirch, France).

144 2.3.2. Peptide Synthesis.

145 Cragi-ELH1, Cragi-ELH1a, Cragi-ELH1b, Cragi-ELH1c, Cragi-ELH1₂₃₋₄₀, Cragi-ELH2,
146 Cragi-ELH2a and Cragi-ELH2b were synthesized by Fmoc solid phase methodology on a
147 Liberty microwave assisted automated peptide synthesizer (CEM, Saclay, France) using the
148 standard manufacturer’s procedures at 0.1 mmol scale as previously described (Touchard et al.,
149 2020). All Fmoc-amino acids (0.5 mmol, 5 eq.) were coupled on preloaded HMP resin (ELH1a
150 and ELH1c) or Rink amide resin (ELH1, ELH1b, ELH1₂₃₋₄₀, ELH2, ELH2a and ELH2b), by *in*
151 *situ* activation with HBTU (0.5 mmol, 5 eq.) and DIEA (1 mmol, 10 eq.) before Fmoc removal

152 with 20% piperidine in DMF. After completion of the chain assembly, peptides were
153 deprotected and cleaved from the resin by adding 10 mL of an ice-cold mixture of
154 TFA/TIS/H₂O (9.5:0.25:0.25, v/v/v) and agitating for 3 hours at room temperature. After
155 precipitation in TBME followed by centrifugation (4500 rpm, 15 min), the crude peptides were
156 purified by reversed-phase HPLC (Gilson, Villiers le Bel, France) on a 21.2 x 250 mm Jupiter
157 C18 (5 μm, 300 Å) column (Phenomenex, Le Pecq, France) using a linear gradient (10-60% or
158 20-50% over 45 min) of acetonitrile/TFA (99.9:0.1) at a flow rate of 10 mL/min. Peptides were
159 then characterized by MALDI-TOF mass spectrometry on a ultrafleXtreme (Bruker,
160 Strasbourg, France) in the reflectron mode using α-cyano-4-hydroxycinnamic acid as a matrix.
161 Analytical RP-HPLC, performed on a 4.6 x 250 mm Jupiter C18 (5 μm, 300 Å) column,
162 indicated that the purity of the peptides was > 99.9%.

163

164 2.4. NMR and structure determination

165 2.4.1. NMR measurements

166 The nuclear magnetic resonance (NMR) samples contained 1-3 mM of peptide Cragi-ELH1a
167 or Cragi-ELH2 dissolved in water or in presence of deuterated dodecylphosphocholine (DPC)
168 micelles (125 mM). All spectra were recorded on a Bruker Avance 500 spectrometer equipped
169 with a 5 mm TCI cryoprobe (1H, 13C, 15N). Homonuclear 2-D spectra -
170 DQF-COSY, TOCSY and ROESY or NOESY were typically recorded using standard Bruker sequences in
171 sensitive mode using the States-TPPI method. NOESY or ROESY spectra were acquired with
172 8 scans, 8 spectra were summed leading to less t1 noise as recently reported (Mo et al., 2017).
173 Typical spectra were acquired using matrices of 4096 x 320-600 zero filled in F1 to 2K x 1K
174 after apodization with shifted sine-square multiplication in both domains. Spectra were
175 processed with Topspin software (Bruker).

175 2.4.2 Structure calculations

176 ¹H chemical shifts were assigned according to classical sequential assignment procedure. NOE
177 cross-peaks were integrated and assigned within the CcpNmr (Vranken et al., 2005) and NMR
178 View software (Johnson, 2004). The volumes of NOE peaks between methylene pair protons
179 were used as reference of 1.8 Å. Structure calculations were performed with AMBER 17 (Case
180 et al., 2005) in two stages: cooking and simulated annealing in explicit solvent. The cooking
181 stage was performed at 1000 K to generate 100 initial random structures. Simulated annealing
182 calculations were carried during 20 ps (20000 steps, 1 fs long). First, the temperature was risen
183 quickly and was maintained at 1000 K for the first 5000 steps, then the system was cooled
184 gradually from 1000 K to 100 K from step 5001 to 18000 and finally the temperature was
185 brought to 0 K during the 2000 remaining steps. For the 3000 first steps, the force constant of
186 the distance restraints was increased gradually from 2.0 kcal.mol⁻¹.Å to 20 kcal.mol⁻¹.Å. For
187 the rest of the simulation (step 3001 to 20000), the force constant is kept at 20 kcal.mol⁻¹.Å.
188 The 20 lowest energy structures with no violations > 0.3 Å were considered as representative
189 of the compound structure. All the dihedral angles ϕ and ψ belonged to the allowed regions of
190 the Ramachandran plot. The representation and quantitative analysis were carried out using
191 MOLMOL (Koradi et al., 1996).

192

193 2.5 *In silico* analyses

194 Multiple sequence alignment was performed using Clustal Omega (Sievers et al., 2011).
195 Seaview (Gouy et al., 2010) was used for selecting the conserved protein regions and for manual
196 correction of the alignment. PhyML was used for generating the trees. The reliability of the
197 inferred trees was estimated by applying the bootstrap procedure with 1000 replications.
198 FigTree (<http://tree.bio.ed.ac.uk/software/figtree/>) was used to draw the tree.

199

200 2.6 Pharmacological characterization of Cragi-ELHR

201 2.6.1 Molecular cloning and transfection of mammalian cells

202 The CDS of Cragi-ELHR ([XP_034310400.1](#)) was amplified by PCR using the transcript
203 specific sense primer (5'-CACCATGTATAACCTGACGGACTATC-3') harboring a Kozak
204 consensus sequence and the antisense primer (5'-TTACAACATTTTCTCCGATTCTAGTCC-
205 3'). Ten nanograms of plasmid DNA (Pal 17.3 vector, Evrogen, Russia) from a *C. gigas* “all
206 developmental stages and adult central nervous system” normalized cDNA library (Fleury et
207 al., 2009) was used as template. PCR was carried-out in a 50 µl reaction volume containing 1.5
208 mM MgCl₂, 200 mM dNTPs, 1 mM each of the primer couples, 1.25 units of GoTaq®
209 polymerase and the appropriate buffer (Promega, Madison, WI, USA) in nuclease-free water.
210 Samples were subjected to the following cycling parameters (95 °C, 2 min; 30 cycles of: 95 °C-
211 45 s, 60°C-30 s, 72 °C-1 min, followed by 5 min at 72 °C).

212 The resulting PCR product was directionally cloned into the eukaryotic expression vector
213 pTARGET (Promega) and the correct insertion confirmed by Sanger sequencing.

214 Human Embryonic Kidney (HEK293T) cells were transiently co-transfected with the Cragi-
215 ELHR and pGloSensor™ - 22F cAMP (Promega) constructs using Fugene HD (Promega)
216 according to the manufacturer's instructions. Cells for negative control experiments were
217 transfected with an empty pcDNA3.1 and pGloSensor™ - 22F cAMP plasmids.

218 2.6.2 cAMP luminescence assay

219 Cragi-ELHR transfected HEK293 T cells were incubated with Glosensor cAMP reagent (4%
220 final concentration in the medium) (Promega) for 2 hours at room temperature prior to the
221 injection of the candidate ligands. The peptides to be tested were diluted at 5 times their final
222 concentration in 20mM HEPES in Hank's balanced salt solution pH 7.3 and distributed in a 96-
223 well plate. 25µL of the different peptide solutions were injected in the wells containing the
224 cells. cAMP luminescence response was measured for 30 min after injection using a FLEX
225 station 3 (Molecular Devices) at room temperature. Data were analyzed using SoftMax Pro

226 (Molecular Devices). Candidate peptide ligands were first tested in triplicate at a final
227 concentration of 10^{-5} M then at different peptide concentrations. Concentration–response
228 measurements of activating ligands were conducted in triplicate and for at least three
229 independent experiments.

230

231 2.7 Reverse transcription and quantitative PCR

232 RT-qPCR analysis was performed using the CFX96™ Real-Time PCR Detection System (Bio-
233 Rad, Hercules, CA, USA). Total RNA was isolated from adult tissues using Tri-Reagent
234 (Sigma-Aldrich) according to the manufacturer’s instructions. Recovered RNA was then further
235 purified on Nucleospin RNAII columns (Macherey-Nagel). After treatment for 20 min at 37 °C
236 with 1 U of DNase I (Sigma) to prevent genomic DNA contamination, 1 µg of total RNA was
237 reverse transcribed using 1 µg of random hexanucleotide primers (Promega), 0.5 mM dNTPs
238 and 200 U MMuLV Reverse Transcriptase (Promega) at 37 °C for 1 h in the appropriate buffer.
239 The reaction was stopped by incubation at 70 °C for 10 min. The GoTaq® qPCR Master Mix
240 (Promega) was used for real time monitoring of amplification (5 ng of cDNA template, 40
241 cycles: 95 °C/15s, 60 °C/15 s) with transcript specific primers for Cragi-ELH QF (5’-
242 GTTCCGATACGGCAAAGAA -3’) and QR (5’- AATCAAGGGCGTTTGATCTG -3’) and
243 for Cragi-ELHR QF2 (5’- GGTCCCAATCGTTGCAAATCAA- 3’) and QR2 (5’ -
244 CCCTCAACAAGCATCCAGAAGA-3’) as forward (QF) and reverse (QR) primers
245 respectively. Accurate amplification of the target amplicon was checked by performing a
246 melting curve. A parallel amplification of *C. gigas* Elongation Factor1α (EF1α) QS-Cg-EF1α
247 (5’-ACCACCCTGGTGAGATCAAG-3’) and QA-Cg-EF1α (5’-
248 ACGACGATCGCATTTCTCTT-3’) transcript (BAD15289) was carried out to normalize the
249 expression data of the analyzed transcripts. EF1α was used as a reliable normalization gene as
250 no significant difference ($p < 0.05$) of Ct values was observed between the different samples

251 compared. Coefficient of variation of EF1 α was less than 5% for all the tissue samples and
252 experimental conditions. Thus, the relative level of expression of the target gene was calculated
253 by using the following formula: $N = 2^{(Ct\ Cragi-EF1\alpha - Ct\ target\ cDNA)}$. The PCR amplification efficiency
254 ($E = 10^{(-1/slope)}$) for each primer pair was determined by linear regression analysis of a dilution
255 series to ensure that E ranged from 1.98 to 2.02.

256

257 2.8 Statistical analysis

258 Gene expression levels between different tissues and between samples at different reproduction
259 stages were compared using one-way ANOVA followed by a Tukey post hoc test. Expression
260 levels between animals in different feeding or salinity conditions were compared using an
261 unpaired Student's t test. Significance was set at $p < 0.05$.

262

263 3 Results.

264 3.1 Molecular processing of Cragi-ELH precursor.

265 The sequence of the precursor encoding *C. gigas* ELH (Cragi-ELH) described previously
266 (Stewart et al., 2014) harbors two predicted neurohormones Cragi-ELH1 and Cragi-ELH2.
267 Alignment of the predicted two oyster mature peptide sequences demonstrates sequence
268 similarity in terms of peptide length, C-terminal amidation and, especially in the N-terminal
269 and C-terminal regions, the occurrence of conserved amino acids at similar positions with
270 vertebrate corticotropin releasing hormone (CRH), insect diuretic hormone (DH 44) and
271 Lophotrochozoan egg-laying hormones (ELH) (Fig. 1). To determine the processing of Cragi-
272 ELH precursor, mass spectrometric methods were used. Analysis of VG extracts allowed the
273 detection of many mature peptides overlapping the entire precursor protein (Fig. 2A, Suppl
274 Fig.1, iProX database IPX0007327001). A peptide corresponding to the predicted full length
275 Cragi-ELH1 could not be detected whilst the corresponding peptides predicted to be generated

276 owing to the presence of prohormone convertase processing sites were all unambiguously
277 detected (Cragi-ELH1a: GRLSLTADLRSLARMLEAH, monoisotopic mass of m/z 528.2821
278 [4+], Cragi-ELH1b: FIASRFPYDSI m/z 658.34 [2+], Cragi-ELH1c: LFRYamide m/z 597.35
279 [1+]). For Cragi-ELH2, a single peptide of monoisotopic mass of m/z 845.4257 [5+],
280 corresponding to the full-length sequence could be detected. In contrast, despite the presence
281 of a monobasic putative processing site, no Cragi-ELH2 processed peptides could be detected.

282 3.2 Structure determination of Cragi-ELH peptides by NMR

283 The NMR analysis of ELH1a, as the longest processed peptide from predicted ELH1 sequence
284 and ELH2 in presence of deuterated DPC was performed using the sequential assignment
285 strategy (Wüthrich, 1986). As presented in supplementary tables 1 and 2, the complete chemical
286 shift assignment of ELH1a was successfully performed whereas severe overlapping prevented
287 the full assignment of ELH2. The three-dimensional structure of peptide ELH1a in DPC
288 micelles was solved. The superposition of the 18 lowest energy conformers, fitted on segment
289 4-17 backbone atoms is presented in Fig. 2B. Summary of the refinement statistics are given
290 (Suppl. table 3). An alpha helix is clearly defined between the residues 4 to 17. Incomplete
291 assignment of ELH2 prevents the structure determination, however the NOE connectivity
292 pattern is also characteristic of a helical structuration of the ELH2 peptide in DPC micelles with
293 numerous inter residue $i - i+3$ connectivities between the residues 4-19 (Suppl. Fig. 2).

294 3.3 Characterization of Cragi-ELHR

295 A BLAST search using the *Drosophila* DH44 Receptor 1 (DH44R1: AAF58250.1) sequence
296 in the Gigaton database (Riviere et al., 2015) allowed the identification of a full-length
297 transcript (CHOYP_CRHR.1.2) encoding a seven transmembrane domain protein typical of a
298 GPCR (Suppl. Fig. 3). Alignment of the oyster receptor named Egg-laying Hormone Receptor
299 (Cragi-ELHR) with vertebrate and protostome GPCRs highlights its high degree of homology

300 with functionally characterized insect DH44Rs, lophotrochozoan putative DH44/CRHRs and
301 vertebrate CRHRs (Suppl. Table 4 and Suppl. Fig. 4). A phylogenetic analysis showed that all
302 lophotrochozoan receptors form a single clade and were phylogenetically related to vertebrate
303 CRHRs and Ecdysozoan DH44Rs (Fig. 3). Transiently transfected HEK293T cells expressing
304 Cragi-ELHR were challenged with Cragi-ELH1 and Cragi-ELH2 as well as with all their
305 predicted or characterized processed peptides (Cragi-ELH1a, Cragi-ELH1b, Cragi-ELH1c,
306 Cragi-ELH123-40, Cragi-ELH2a and Cragi-ELH2b) at a single concentration of 10^{-5} M. Both
307 Cragi-ELH1 and Cragi-ELH2 activated Cragi-ELHR resulting in the activation of the adenylyl-
308 mediated cAMP transduction pathway. Only N-terminal peptides of both Cragi-ELH1 (Cragi-
309 ELH1a) and Cragi-ELH2 (Cragi-ELH2a) were efficient in activating Cragi-ELHR though C-
310 terminal and central peptides were not (Fig.4A). Thus, a dose-dependent activation of Cragi-
311 ELHR was investigated using the active peptides. Both Cragi-ELH1 and Cragi-ELH2 elicited
312 a gradual response with an EC50 of respectively 26.2 ± 3.2 nM and 81.3 ± 8.9 nM whilst ELH1a
313 and ELH2a showed an EC50 of 2.88 ± 0.76 μ M and of 3.11 ± 1.29 μ M respectively (Fig.4B).
314 Only micromolar concentrations of ELH1 and ELH2 triggered the phospholipase C β -mediated
315 calcium transduction pathway (data not shown)

316 3.4 Gene expression of Cragi-ELHR and Cragi-ELH.

317 The expression of Cragi-ELHR and Cragi-ELH precursor transcripts was investigated by RT-
318 qPCR in several adult tissues, in VG and gonads during reproduction and in VG of animals
319 exposed to different stresses (Supplementary table 5). Both Cragi-ELHR and Cragi-ELH
320 precursor genes were majorly expressed in the VG and at very much lower levels in most adult
321 tissues (Fig. 5A and B). During a cycle of reproduction, no significant change in expression of
322 Cragi-ELHR was observed both in male and female VG or gonads (Fig. 5C and D). In contrast,
323 a significantly higher expression of Cragi-ELH precursor gene was noticed only in VG at stage
324 3 in both males and females (Fig. 5C). Given the implication of vertebrate CRH in mediating

325 stress, two stress conditions were applied to oysters. The first stress was to starve animals for a
326 period of four weeks. In these conditions, no significant change of expression of Cragi-ELH or
327 Cragi-ELHR transcripts was observed in the VG of starved animals as compared to VG of fed
328 animals (Fig. 6A and B). The second stress was to expose oysters to brackish water either
329 suddenly (acute stress) or by progressively changing the salinity over a period of three days
330 (mild stress). Only Cragi-ELHR gene expression was found to be significantly stimulated in
331 mild stress conditions in the VG whilst no change was found for Cragi-ELH transcripts for both
332 stresses in the VG (Fig. 6C and D).

333 4 Discussion

334 The present work describes the functional characterization of a lophotrochozoan G protein-
335 coupled receptor (GPCR) phylogenetically related to insect DH44Rs and vertebrate CRH
336 family member receptors. Our phylogenetic analysis is congruent with previous studies
337 (Cardoso et al., 2014) and suggests that mollusk and annelid receptors are more similar to
338 chordate CRHRs than to insect DH44Rs. Interestingly, the receptors obviously cluster
339 according to the animal phylogeny, whereas among the ligands, gastropod ELHs segregate apart
340 and form an independent and specific clade beside the group gathering the remaining mollusk
341 and lophotrochozoan ELH/DH44 sequences (De Oliveira et al., 2019). This suggests a specific
342 evolution of gastropod ligands, potentially in association with their specific role in the control
343 of reproductive behavioral processes (Scheller et al., 1982; Vreugdenhil et al., 1988). Among
344 mollusks, cephalopods also display a complex pattern of reproductive behaviors (von Boletzky,
345 1983) involving a variety of neuropeptides (Henry et al., 1999; Zatylny-Gaudin et al., 2016;
346 Zatylny et al., 2000). However, no ELH-related peptide was identified from the neuro-
347 transcriptome and the neuropeptidome of spawning cuttlefish (*Sepia officinalis*) (Zatylny-
348 Gaudin et al., 2016). It has been suggested that the ELH-encoding gene may have been lost in
349 cephalopods (De Oliveira et al., 2019). The present study clearly shows the existence of an

350 ELHR in the cephalopod *Octopus bimaculoides*, noticeably phylogenetically distant from the
351 other lophotrochozoan receptors. This possibly indicates that it binds peptide ligands divergent
352 from their lophotrochozoan counterparts.

353 In the same way as other bivalve precursors (Stewart et al., 2014; Zhang et al., 2018), the Cragi-
354 ELH precursor potentially generates two homologous ELHs (Cragi-ELH1 and Cragi-ELH2) of
355 40 and 38 amino acids, respectively (Stewart et al., 2014). Both peptides specifically activate
356 Cragi-ELHR at nanomolar concentrations, as observed for insect DH44Rs and vertebrate
357 CRHRs and their respective ligands (Chen et al., 1993; Hector et al., 2009; Johnson et al., 2005;
358 Liaw et al., 1996). Cragi-ELHR increased cAMP levels upon activation, as most receptors of
359 this family do (Chen et al., 1993; Hector et al., 2009; Johnson et al., 2005; Liaw et al., 1996),
360 but which are otherwise promiscuous as to the G α type they activate (Grammatopoulos, 2012).

361 In our experimental conditions, only micromolar concentrations of Cragi-ELH1 and Cragi-
362 ELH2 increased intracellular calcium, as found for DH44R1 in *Drosophila* (Johnson et al.,
363 2004). Structure-activity relationships were examined, and demonstrated that like vertebrate
364 CRHs (Deussing and Chen, 2018), synthetic N-terminal fragments of Cragi-ELH1 and Cragi-
365 ELH2 were efficient to activate Cragi-ELHR, though with less potency than their respective
366 full-length forms. Both the N- and C-terminal moieties of Cragi-ELHs display very high
367 conservation with other CRH/DH44/ELH peptides. This suggests that, as in vertebrate CRHs,
368 the N-terminal part of the ligand is crucial for receptor binding and activation, although the C-
369 terminal part would be responsible for the affinity and specificity of the ligand (Deussing and
370 Chen, 2018). Although the conformation of oyster ELHs had been predicted (Stewart et al.,
371 2014), their high-resolution structure was determined by NMR in this study. From a structural
372 point of view, there was no defined structure of the ELH1a and ELH2 peptides in water, as
373 often encountered with small peptides. However, an extended alpha helix was found in a
374 micellar medium after molecular modeling using NMR constraints. Also observed in human

375 and rat CRHs (Spyroulias et al., 2002) and more generally in Class B GPCR ligands (Neumann
376 et al., 2008), such a structuring of Cragi-ELH1a and Cragi-ELH2 in close proximity to the
377 membrane is consistent with the idea that peptides can adopt a helical structure in the vicinity
378 of their membrane receptors.

379 It was somewhat intriguing to observe that Cragi-ELH1 was much more potent than Cragi-
380 ELH2 to activate Cragi-ELHR. Yet, unlike Cragi-ELH2, Cragi-ELH1 does not seem to exist as
381 a molecular entity in oyster: it is processed into three unrelated peptides (ELH1a, ELH1b and
382 ELH1c). We cannot exclude the possibility that Cragi-ELH1 was not detected by MS due to an
383 inappropriate extraction procedure or poor ionization of the peptide. However, we think this is
384 unlikely because Cragi-ELH1 cleavage peptides have been easily identified by MS, which
385 means that Cragi-ELH1 occurs in rather small amounts if it does exist. As a result, Cragi-ELH2
386 appears as the main activator of the ELH signaling system in *C. gigas* given the poor potency
387 of ELH1a in activating Cragi-ELHR. Potency differences between Cragi-ELH2 and Cragi-
388 ELH1a may reflect respective roles as a neurohormone and a neurotransmitter. Nevertheless,
389 apart from Cragi-ELH1a, the other two neuropeptides generated by the processing of Cragi-
390 ELH1 constitute potential biologically active factors regulating target tissues *via* distinct
391 receptors. The processing of Cragi-ELH1 is somewhat reminiscent of that of *Aplysia californica*
392 ELH and *Lymnaea stagnalis* CDCH precursors: multiple short peptides (α/β BCPs, α/β
393 CDCPs) are generated that play a role as neurotransmitters (Scheller et al., 1983) and control
394 the activity of various target neurons implied in the reproductive behavior of *L. stagnalis*
395 (Koene, 2010). Out of the three neuropeptides generated from the processing of Cragi-ELH1,
396 the C-terminal tetrapeptide LFRYamide (Cragi-ELH1c) exhibited a sequence identical to the
397 C-terminal sequence of Cragi-LXRYamide (Réalís-Doyelle et al., 2021), and its structure
398 clearly resembled that of the LFRFamide family of neuropeptides identified in *C. gigas* (Bigot
399 et al., 2014). In agreement with a previous work (Réalís-Doyelle et al., 2021), the present study

400 shows that Cragi-ELH gene expression peaks in the VG of reproductively mature male and
401 female oysters. Interestingly, the expression of the Cragi-LXRYamide-encoding gene increased
402 in parallel to that of the Cragi-ELH-encoding gene in the VGs of reproductively mature oysters
403 (Réalis-Doyelle et al., 2021). In addition, LFRFamide signaling regulates reproduction and
404 feeding processes in mollusks (Bigot et al., 2014; Hoek et al., 2005). Thus, although encoded
405 by different genes, these closely structurally related neuropeptides may synergistically activate
406 the same receptor and contribute to regulate reproduction- and feeding-associated functions in
407 oysters. The significant increased expression of Cragi-ELH in the VGs of mature oysters
408 suggests a role in the regulation of reproduction. However, considering the low and stable
409 expression of Cragi-ELHR in oyster gonads during a reproductive cycle, it is not clear whether
410 Cragi-ELH signaling is directly involved in spawning. The highest levels of expression of
411 Cragi-ELHR and Cragi-ELH in the VGs compared to other adult tissues rather suggest a
412 reproduction-associated neuroregulatory role in the central nervous system. Cragi-ELH
413 signaling is likely not involved in the regulation of feeding, as no significant change was
414 recorded in the expression of ELH receptor- and ligand-encoding genes during a nutritional
415 stress. This is in contrast with the reported role of DH44 in inducing satiety in the desert locust
416 *Schistocerca gregaria* (van Wielendaele et al., 2012). Given the major role of CRH and DH44
417 in mediating the stress response and the regulation of osmolarity, it was rational to investigate
418 the possible role of Cragi-ELH signaling during a hypoosmotic stress – a common
419 environmental pressure experienced by oysters in estuaries (Guo et al., 2015). Cragi-ELH
420 tended to increase in the VG following a mild or acute hypoosmotic stress, but these changes
421 were not statistically significant, whereas Cragi-ELHR increased significantly in mild stress
422 conditions. Complementary experiments should be carried out on animals exposed to other
423 stress conditions or other stressors (i.e., temperature or air exposure), but our results suggest
424 that Cragi-ELH signaling does not represent a key neuroendocrine pathway of the stress

425 response or osmotic regulation in *C. gigas*. In contrast, decreased expression of the CCAP
426 signaling components has been evidenced in the gills of oyster (Realis-Doyelle et al., 2021) in
427 similar mild hyposaline conditions. Conversely, the two distinct calcitonin (CT) signaling
428 systems of *C. gigas* showed opposite responses in lower saline concentration conditions:
429 CT1b/CTR expression decreased, whereas CT2/CTR2 expression increased (Schwartz et al.,
430 2019).

431 5 Conclusion

432 An ELH signaling system was characterized for the first time in a lophotrochozoan species.
433 This signaling system is evolutionarily related to the vertebrate CRH and arthropod DH44
434 signaling systems. The involvement of ELH signaling in the regulation of reproduction-related
435 functions in oysters is very likely, while its involvement in mediating the stress response
436 remains doubtful.

437

438 6 References

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628 The authors declare no competing or financial interests

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633 Credit authorship contribution statement:

634 PF, JL and AB designed the study; MPD, JP, JS, BB, LM, GR, JL and AB performed the
635 experiments and analyzed, PF wrote the paper. All authors reviewed and edited the manuscript.

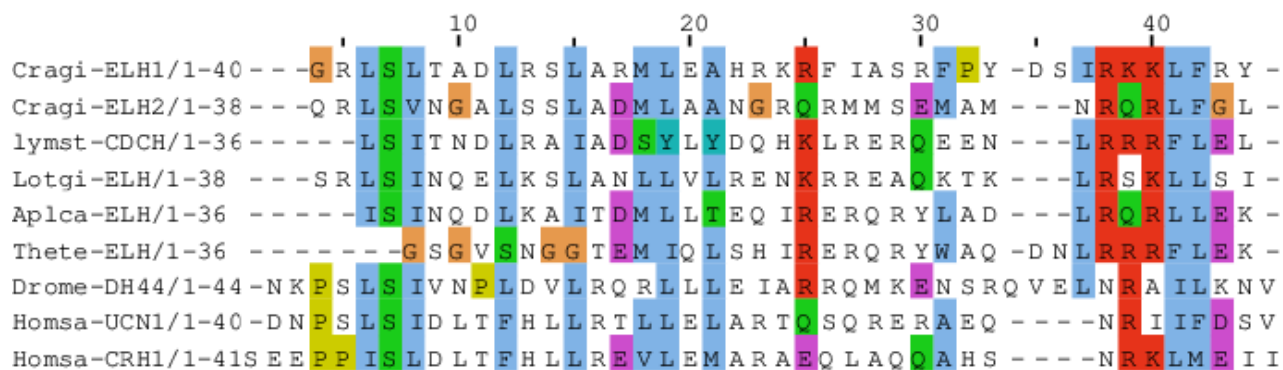


Figure 1: Multiple sequence alignment of Cragi-ELH and ELH-related family

members. *Crassostrea gigas* **predicted** Cragi-ELH1 and Cragi-ELH2 (FQ667900.1) were aligned

with sequences from mollusks: *Lymnaea stagnalis*: Lymst-CDCH (P06308); *Lottia gigantea*: Lotgi-

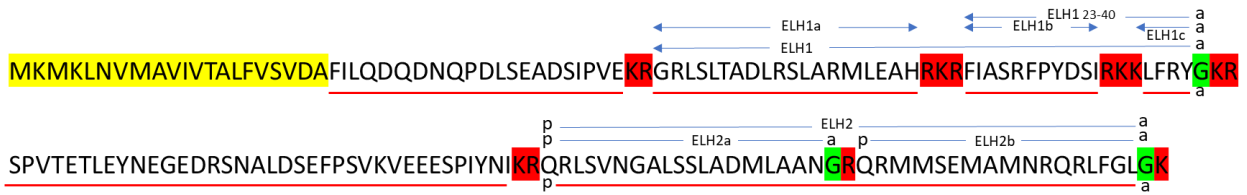
ELH (V3YYG7); *Aplysia californica*: Aplca-ELH (P01362); annelids: *Theromyzon tessalum*:

Thete-ELH (P80594.1); arthropods: *Drosophila melanogaster*: Drome-DH44 (Diuretic Hormone

44) (AAF54421.4) and vertebrates: *Homo sapiens*: Homsa-UCN (urocortin 1) (NP_003344.1) and

Homsa-CRH1 (corticoliberin) (NP_000747.1). Conserved amino acids are highlighted.

A



B

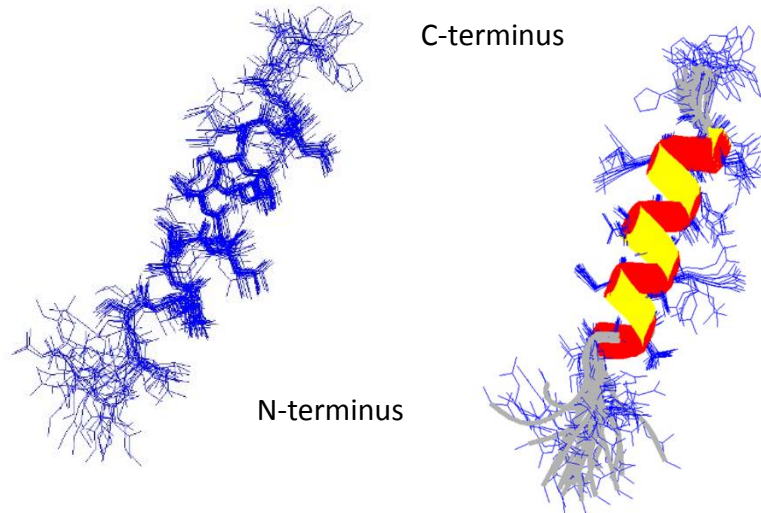


Figure 2: Structure of Cragi-ELH precursor and of some mature peptides. A: Amino acid sequence of *Crassostrea gigas* ELH precursor (Cragi-ELH). The predicted signal peptide is highlighted in yellow; the likely convertase processing sites are highlighted in red, the glycine residues likely to be converted into a C-terminal amide are highlighted in green, the mature neuropeptides detected by MS are underlined in red. The post-translational modifications: pyroglutamate (p) and C-terminal amide (a) are indicated. The various peptides synthesized are indicated in blue. B: Superimposition of the 18 lowest energy conformers of peptide Cragi-ELH1a in presence of deuterated DPC micelles, fitted on segment 4-17 backbone atoms (rmsd: 0.28) and ribbon representation of the structure.

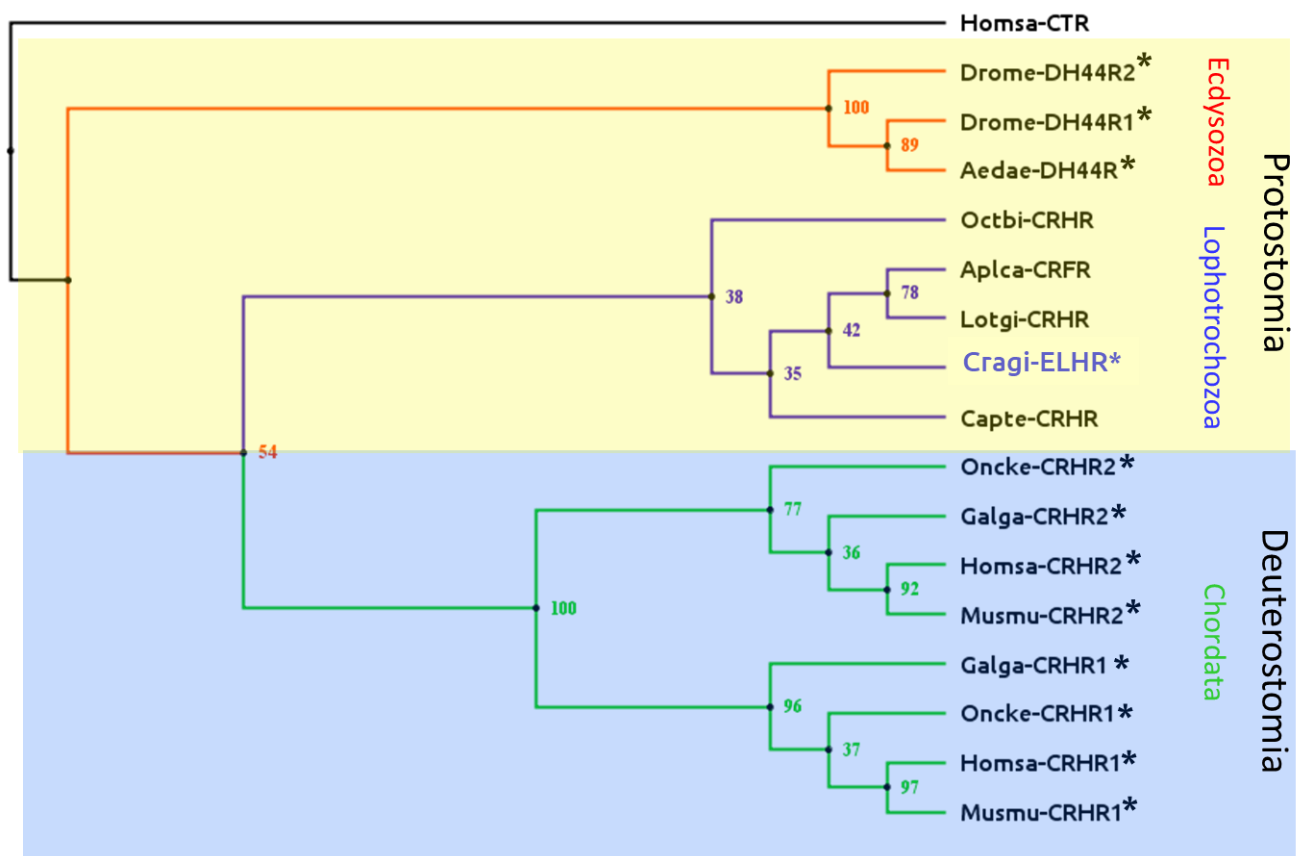


Figure 3: Phylogenetic representation of the relationship between Cragi-ELHRs and other ELHR family members. Phylogenetic and molecular evolutionary analyses were conducted using the maximum likelihood methods using the edited sequence alignment of the deduced receptor protein sequences obtained from Seaview. The accession numbers and the sequences used to construct the tree are: *Aedes aegypti*: Aedae-DH44R (QBC65449.1); *Aplysia californica*: Aplca-CRHR (XP_012938918.2); *Crassostrea gigas*: Cragi-ELHR* (CHOYP_CRHR.1.2, Gigaton); *Capitella telleta*: Capte-CAPTEDRAFT_93223 (ELU10073.1); *Drosophila melanogaster*: Drome-DH44R1* (AAF58250.1), Drome-DH44R2* (AAM68690.3); *Gallus gallus*: Galga-CRHR1* (NP_989652.1), Galga-CRHR2* (XP_015136531.2); *Homo sapiens*: Homsa-CRHR1* (NP_004373.2), Homsa-CRHR2* (NP_001874.2), Homsa-CTR* (calcitonin receptor) (NP_001733.1); *Lottia gigantea*: Lotgi-LOTGIDRAFT_141716 (XP_009049617.1); *Mus musculus*: Musmu-CRHR1* (NP_031788.1), Musmu-CRHR2* (NP_001275547.1); *Octopus*

bimaculoides: Octbi-CRHR-like (XP_014771477.1); *Oncorhynchus keta*: Oncke-CRHR1 (CAC81753.1), Oncke-CRHR2 (CAC81754.1). (*): functionally characterized receptors. Green branches correspond to vertebrate receptors, blue branches to lophotrochozoan (molluscs and annelids) receptors, red branches to ecdysozoan receptors. Branch node labels correspond to likelihood ratio test values.

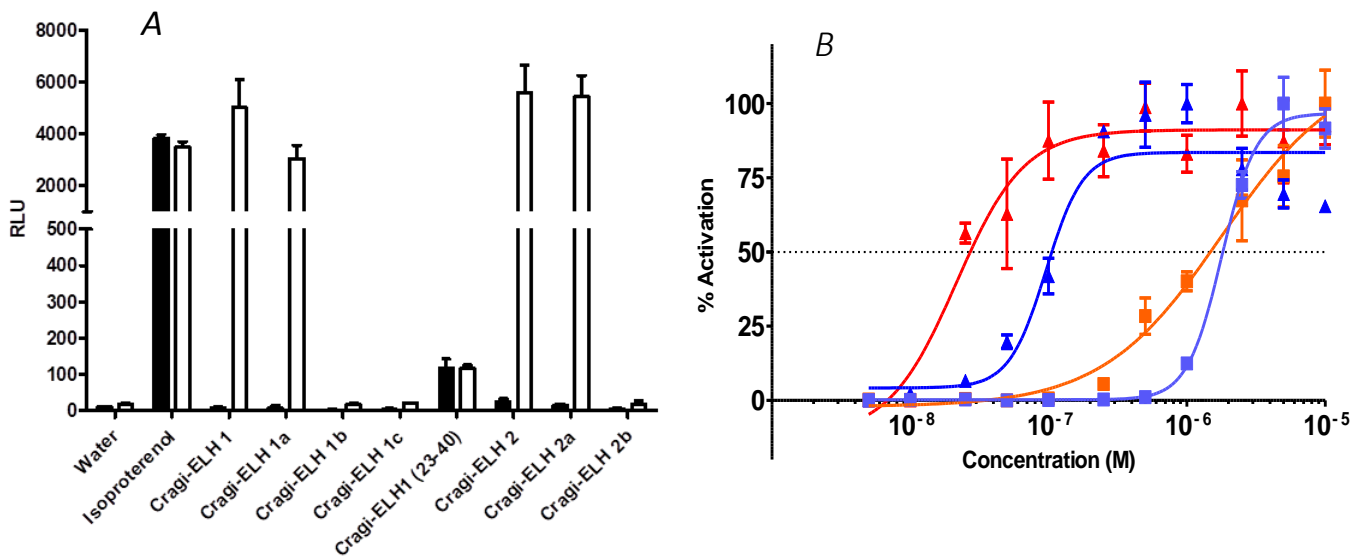


Figure 4: Activation modalities of Cragi-ELHR by Cragi-ELH peptides. A: Activation of *Crassostrea gigas* ELH receptor (Cragi-ELHR) by Cragi-ELH1 and CragiELH2 synthetic peptides at a concentration of 10^{-5} M (n=3). B: Dose-dependent effect of Cragi-ELH1 (▲) Cragi-ELH1a (■), Cragi-ELH2 (▲) and Cragi-ELH2a (■) on Cragi-ELHR expressed in HEK293T cells. Data are shown as relative (%) to the highest value (100% activation) and represent the mean of an experiment (n=3) performed in triplicate. Vertical bars represent the standard error of the mean (SEM). Black bar: empty plasmid, open bar: Cragi-ELHR plasmid.

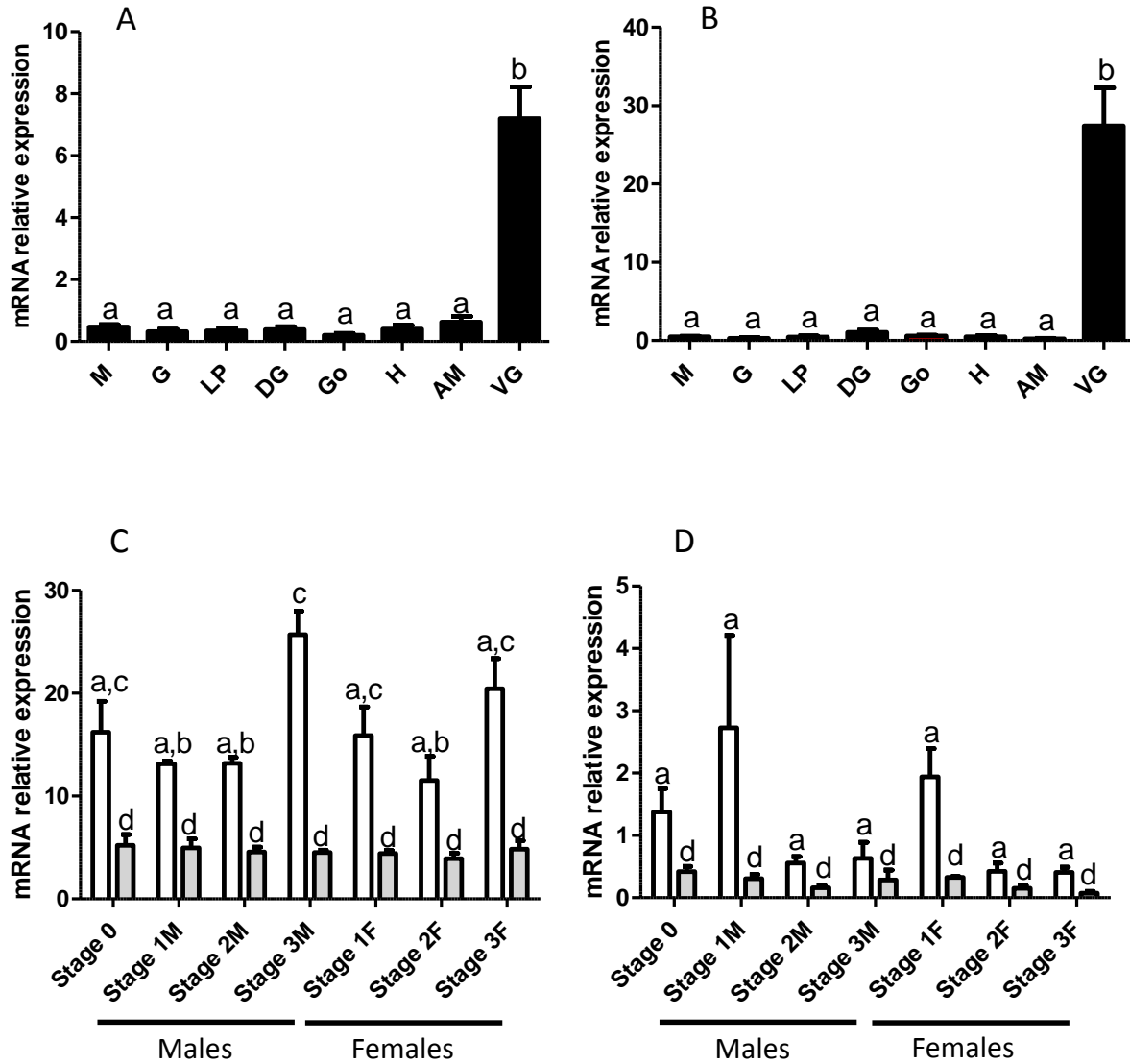


Figure 5: Expression of mRNAs encoding Cragi-ELHR and Cragi-ELH precursor in adult tissues. Distribution of mRNAs encoding Cragi-ELHR (A) and Cragi-ELH precursor (B) in adult tissues: M: Mantle (n=10); G: Gills (n=5); LP: Labial palps (n=5); DG: Digestive gland (n=5); Go: Gonad (mix of all stages) (n=5); H: Heart (n=10); AM: Adductor muscle (n=5); VG: Visceral ganglia (n=5). Distribution of mRNAs encoding Cragi-ELH (open bars) and Cragi-ELHR (grey bars) in the VG (C) and in the gonad (D) during gametogenesis: Stage 0: sexual resting stage; stage 1: gonial multiplication stage; stage 2: tubule development and maturation stage and stage 3: sexual maturity stage. Each value is the mean + SEM of at least 4 pools of 5 animals. Expression levels were normalized to Elongation Factor 1 α (EF1 α) mRNA. Results were statistically tested with a

one-way ANOVA $p < 0.05$. Vertical bars represent the standard error of the mean (SEM). Bars with different labels are statistically significantly different.

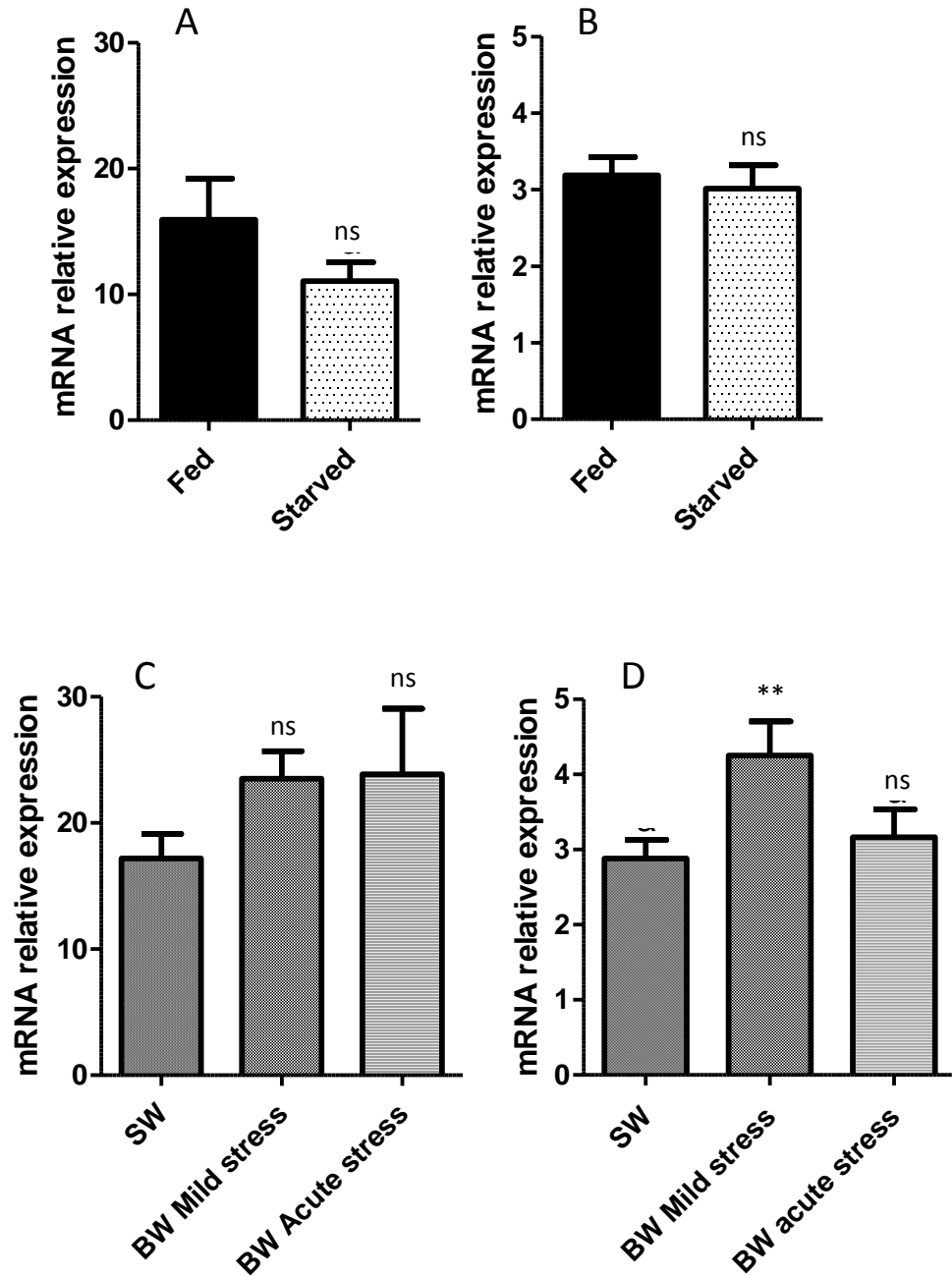


Figure 6: Expression of mRNAs Cragi-ELH precursor and Cragi-ELHR in VG of oysters after starvation and after a hyposaline stress. Expression of Cragi-ELH precursor (A) and Cragi-ELHR (B) in the VG of fed (n=13 and n=14) and starved oysters (n=14 and n=15). Expression of Cragi-ELH precursor (C) and Cragi-ELHR (D) in the VG of oysters acclimatized to brackish water. Each value is the mean + SEM of n=18 VG (SW), n=6 VG (BW Mild stress) and n=16 VG (BW acute stress). Expression levels were normalized to Elongation Factor 1 α (EF1 α) mRNA. Results were statistically tested with a Student's t test *p<0.05, **p<0.01, ns: non-

significant. Vertical bars represent the standard error of the mean (SEM). SW: Sea water, BW: Brackish water.



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Supplementary Material

Revision Suppl data Cragi ELH signaling.pdf

