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1 **Vitellogenin gene expression in marine mussels exposed to ethinylestradiol:**
2 **no induction at the transcriptional level**

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15

16 **Abstract**

17 Vitellogenin (Vtg), a large multidomain protein precursor of egg-yolk proteins, was first
18 used as an endocrine disruption biomarker in fish and more recently also in invertebrates
19 like mollusks. However, it remains unclear whether invertebrate endocrine system
20 produces Vtg in response to estrogens, like it occurs in oviparous vertebrates. In a
21 previous study, no evidence of induction of Vtg expression at protein level was found in
22 gonads of the marine mussel *Mytilus galloprovincialis* after exposure to the estrogenic
23 chemical 17α-ethinylestradiol (EE2). In the present study, it was investigated whether
24 there is any effect of EE2 on Vtg abundance at transcriptional level in *M.*
25 *galloprovincialis* gonads. To this aim, RT-qPCR analysis targeting three different
26 domains of Vtg transcript was performed on mussels that were exposed either 4 or 24
27 days to 100 ng/L EE2. In addition, several reference genes were analysed and a selection
28 of these for potential use in further RT-qPCR analyses on mussel male and female gonads
29 is provided. Results showed higher expression in females than in males for the three
30 analysed Vtg domains, and no evidence of Vtg mRNA induction due to EE2 either in
31 females or males. The present results support that Vtg is not an adequate biomarker of
32 endocrine disruption in marine mussels. Additionally, nucleotide sequences of Vtg
33 transcripts of three closely-related species from *Mytilus edulis* complex (*M.*
34 *galloprovincialis*, *M. edulis* and *M. trossulus*) are provided and compared with Vtg
35 sequences from other mollusk species to assess the level of conservation and evolutionary
36 relationships among species.

37 **Key words**

38 Endocrine disruption; Biomarker; RT-qPCR; Mollusks; *Mytilus galloprovincialis*

39 **1. Introduction**

40 Mussels from the genus *Mytilus* have long been used as sentinel species in marine
41 pollution biomonitoring programmes, including the assessment of the effects of
42 contaminants through the use of biomarkers (Beiras, 2018; Kimbrough et al., 2008).
43 Aquatic environments can be polluted with a wide range of chemicals including
44 Endocrine Disrupting Chemicals (EDCs). These are a structurally diverse group of
45 compounds that can interact with the endocrine system of vertebrates and invertebrates
46 causing adverse consequences like decrease on fertility, demasculinization and
47 defeminization among others (Colborn et al. 1993). EDCs include a broad and
48 heterogeneous group of substances that have the capacity to mimic female hormones
49 known as xenoestrogens (Matozzo et al., 2008), which are responsible of detrimental
50 effects in fishes and other organisms such as male feminization and low fertility rates
51 (Sumpter and Jobling, 1995). In order to detect the presence and effects of these
52 substances in the coastal environments, it would be desirable to find a biomarker of
53 xenoestrogenicity and/or other signs of endocrine disruption that can be used in marine
54 mussels and incorporated in marine monitoring programs.

55 For the study of the effects of xenoestrogens, one of the most used biomarkers is the
56 induction of the large multidomain protein precursor of egg yolk proteins - vitellogenin
57 (Vtg) - in oviparous females (Matozzo et al., 2008; Porte et al., 2006). Vitellogenin is
58 synthetized in most oviparous and ovoviviparous animals during the process of yolk
59 formation (vitellogenesis). In natural conditions, Vtg is only present in females and it is
60 undetectable in males or in immature females, but in the presence of xenoestrogens, it is
61 synthesised also in males and juveniles, being an early warning signal for feminization
62 (Hara et al., 2016). This biomarker has proven useful in vertebrate species, such as fish
63 (Hiramatsu et al., 2006), but its use was later extended to invertebrates, including

64 mollusks, based on the assumption that mollusk endocrine system is homologous to
65 vertebrate endocrine system (Cuvillier-Hot and Lenoir, 2020; Matozzo et al., 2008; Porte
66 et al., 2006). However there are reasonable arguments to think that Vtg could not be an
67 adequate biomarker of endocrine disruption in mollusks (Scott, 2013). For instance, there
68 is a paucity of studies testing for the positive expectation that vertebrate sex steroids have
69 endocrine effects on mollusks, and most studies were based on the generalized use of an
70 inadequate technique (alkali labile phosphates, ALP) for mollusk Vtg measurement
71 (Morthorst et al., 2014; Sánchez-Marín et al., 2017).

72 In a previous study, using a direct technique to measure Vtg abundance at protein level,
73 no evidence was found of Vtg induction in *Mytilus galloprovincialis* gonads after
74 exposure to 17α-ethinylestradiol (EE2), a synthetic estrogen (Fernández-González et al.
75 2020). However, due to conflicting evidence in the literature, it was hypothesised that Vtg
76 gene expression might be induced by estrogens at the transcript level but not at the protein
77 level (Fernández-González et al. 2020). Due to post-transcriptional gene regulation events
78 some transcripts could be either not translated or do it at different rates, hence
79 contributing to the well-known lack of correlation found between the abundance of some
80 gene products at transcript and protein level (Diz and Calvete, 2016; Vogel and Marcotte,
81 2012). Thus, Vtg gene expression could be still a valid biomarker of estrogenicity as far
82 as induction at transcript level could be demonstrated.

83 Protein isoforms can be produced from the same gene due to alternative splicing
84 mechanisms or due to the expression of different paralogue genes (Stastna and Van Eyk,
85 2012). In the case of Vtg, different isoforms have been described for many organisms, in
86 varying numbers depending on the species. In the case of vertebrates, it has been
87 described that the canonical (complete) form of Vtg presents a multi-domain structure,
88 and the diverse isoforms may present different combinations of these protein domains in

89 their structure (Hiramatsu et al., 2006; Schubiger and Wahli, 1986; Wang et al., 1983;
90 Yilmaz et al., 2018). In the case of invertebrates, several Vtg isoforms have been
91 described also for some species. Some of them contain similar domains as those described
92 for vertebrates, while other species present Vtgs with a different structure (Barnett et al.
93 1980; Riddell et al. 1981; Spieth et al. 1985; Wahli 1988). In mollusks, there is not yet
94 solid evidence of the existence of different Vtg isoforms, despite it has been suggested in
95 a few studies (Llera-Herrera et al. 2014; Qin et al. 2012). Only one Vtg sequence has been
96 described in *M. galloprovincialis* (Sánchez-Marín et al., 2017), based on published RNA-
97 seq project data from different mussel tissues including gills, muscle, mantle and
98 haemocytes (Moreira et al. 2015). This sequence contains three conserved domains also
99 described for other mollusks: N-terminal LDP-N domain, a central DUF1943 domain
100 with unknown function and the C-terminal von Willebrand Factor type D domain (Tran
101 et al., 2016; Wu et al., 2015). However, it might be possible that different isoforms (partial
102 versions of the canonical sequence) are expressed in *M. galloprovincialis* as occurs in
103 other invertebrate and vertebrate species.

104 Real-time quantitative polymerase chain reaction (RT-qPCR) has been a widely used
105 technique for accurate quantification of expression of target genes, including studies
106 involving ecotoxicological effects in mollusks (Volland et al., 2017). Despite its wide use
107 and the advantages offered by this technique (high sensitivity, flexibility and efficiency),
108 RT-qPCR has a few drawbacks, since the results can be affected by some procedures
109 which can lead to bias and errors (Bustin et al., 2009; Dheda et al., 2005; Taylor et al.,
110 2010). One of these sources of bias is the selection of reference genes used for data
111 normalization. Due to the difficulty in finding one gene that is stable across different
112 samples and conditions, many authors have recommended the use of several reference
113 genes to accurately measure the expression of target genes (Huggett et al., 2005; Pfaffl et

114 al., 2004; Tricarico et al., 2002; Vandesompele et al., 2002). Despite this, it is far from
115 rare to find studies in bivalve ecotoxicology in which reference genes were not quality-
116 checked and/or a single gene for normalization was used and even applied across different
117 tissues, despite not being a recommended procedure (Volland et al., 2017).

118 The first objective of the present study was to find an adequate set of reference genes that
119 could be used for data normalization in RT-qPCR studies performed in either female or
120 male mussel gonads under EE2 exposure. The second objective was to analyse by RT-
121 qPCR whether exposure to the synthetic hormone EE2 induces Vtg expression in female
122 or male gonads of *M. galloprovincialis* after different exposure times (4 and 24 days). For
123 this, three different Vtg domains were used in order to account for potential expression
124 changes due to the formation of different Vtg isoforms. Results were compared with
125 other studies of Vtg mRNA expression performed in other bivalve species to discuss its
126 validity as a reliable biomarker of xenoestrogenicity in bivalves. Finally, the level of Vtg
127 conservation between three closely-related species of the *Mytilus edulis* complex (*M.*
128 *galloprovincialis*, *M. edulis* and *M. trossulus*) and evolutionary relationships among Vtg
129 sequences from mollusk species were studied.

## 130 2. Materials and methods

### 131 2.1. Exposure experiment to EE2

132 Mussels (*Mytilus galloprovincialis)* sized in a range between 40 and 50 mm of shell
133 length were obtained from mussel rafts located in an unpolluted area in the Ría de Vigo
134 (Galicia, NW Iberian Peninsula) in October 2017. Mussels were acclimated to the
135 laboratory conditions during three weeks before the experiment in an open system with
136 running seawater at 13ºC, and fed three times per week with a mixture of three microalgae
137 species: *Rhodomonas lens*, *Phaeodactylum tricornutum* and *Chaetoceros neogracilis*, in
138 a flagellates-diatoms proportion 50:50 in volume. During acclimation, mussel feeding

139 regime was a ration of a 6% of mussel tissue dry weight (MTDW) and during the exposure
140 experiment it was 12.95% of MTDW. These regimes were designed to maintain mussels
141 in neutral energy balance during acclimation and in positive energy balance during the
142 experiment (Fernández-González et al., 2020). The exposure experiment was performed
143 in 30 L glass aquaria with continuous aeration (0.22 µm filtered air) and with a winter
144 photoperiod (12h:12h light:dark). Seawater used during the experiment was filtered (1
145 µm pore-size) and UV-treated, with a salinity and a temperature of 35 ppt and 15 ºC.
146 During the exposure experiment, mussels were exposed to one of these three treatments
147 for 24 days: Control (C) (seawater), Solvent Control (SC) (seawater + acetone) and 100
148 ng/L of the synthetic hormone 17α-ethinylestradiol (EE2) (seawater + EE2 dissolved in
149 acetone). Additions of EE2 (98% HPLC, PHR1480 Sigma-Aldrich) were made from a
150 concentrated stock of 0.03 mg/ml in acetone (HPLC grade, Sigma-Aldrich). Total amount
151 of acetone added in SC and EE2 treatments was 100 µl per each 30 L aquaria (0.00033%).
152 Number of mussels per aquarium were between 23-24 during all exposure experiment for
153 the purpose of maintaining water quality, and to minimize EE2 depletion in solution.
154 Exposure experiment was performed in closed systems in semi-static exposure, with
155 complete water changes made three times per week, after mussel feeding. Water samples
156 were taken before and after water changes in glass bottles to evaluate EE2 concentration
157 in aquaria.

158 After 4 and 24 days of exposure, 36 mussels were homogeneously sampled among all
159 aquaria of each treatment (C, CS and EE2) and gonads were divided in three pieces for
160 histological, proteomic and RT-qPCR analysis. Gonadal pieces for RT-qPCR analysis
161 were quick-frozen in liquid nitrogen and kept at -80 ºC until analysis. For RT-qPCR
162 analysis, three male and three female samples were selected per treatment (C, CS and
163 EE2) and sampling time (t4 and t24).

164 Further specific details of exposure experiment can be found in Fernández-González et al
165 (2020), corresponding to "Experiment 2017 - high regime" there described. Samples
166 analyzed in the present study correspond to three of the five individuals of each sex and
167 treatment used for proteomic analysis. Results of histological, proteomic, biometric and
168 EE2 concentrations in water were presented in the cited previous study.

169 **2.2. RT-qPCR analyses**

170 The procedures followed in the different stages of RT-qPCR meet the best practises for
171 this type of analysis described in the MIQE guidelines (Taylor et al. 2010).

172 **2.2.1. RNA isolation and cDNA synthesis**

173 Total RNA was extracted from 20-30 mg of gonad tissue in 1.2 ml of TRI® Reagent
174 (Molecular Research Center, Inc (MCR)) under micro-pestle potter homogenization at
175 room temperature. Samples were incubated (4 ºC, 15 min), centrifuged (12.000 g, 4 ºC,
176 10 min), 1 ml of supernatant transferred to a new microtube avoiding the superficial layer
177 followed by the addition of 200 µl of pre chilled chloroform (-20 ºC) and 100 µl of
178 nuclease-free water. Total volume was transferred to a preconditioned Phasemaker$^{TM}$ tube
179 (Invitrogen) and centrifuged (13,000 g, 4 ºC, 15 min). This step was performed two times
180 to ensure an accurate separation between phenol-chloroform phase and aqueous phase
181 with RNA. Finally, the aqueous phase was transferred to a new tube and one volume of
182 pre chilled ethanol (-20 ºC) was added. RNA was cleaned and concentrated with RNA
183 Clean and Concentrator TM5 kit (Zymo Research), and treated with RNAse-free DNAse
184 (Zymo Research). RNA concentration, purity and integrity of all RNA samples were
185 assessed by measuring the absorbance ratio at 260/280 nm and at 230/260 nm in a
186 NanoDrop 2000 spectrophotometer (Termo Scienctific) and automated electrophoresis
187 (Agilent 2100 Bioanalyzer, Agilent Technologies). Total RNA (0.055 µg) from each
188 sample was reverse transcribed using a Maxima First strand cDNA synthesis kit (Thermo

189 Fisher Scientific) in a final volume of 20 µl. cDNA samples were diluted 1:10 in nuclease-
190 free water and stored at -20 °C until subsequent RT-qPCR analysis.

191 **2.2.2. Selection of candidate reference genes for RT-qPCR**

192 Due to generalized and well-known difficulties, leading to a lack of consensus among
193 different researchers to select an appropriate set of reference genes for RT-qPCR analysis
194 in bivalve mollusk gonads (see Martínez-Escauriaza et al. 2018), we searched for any
195 previous RT-qPCR studies that was focused on validation of different reference genes in
196 bivalve gonads; we found eleven studies that met this criteria. Based on the results of
197 gene stability obtained in these studies, an initial selection was made considering the three
198 genes in each study with the best ranking according to their stability. Among them, those
199 best ranked in studies using *M. galloprovincialis,* and those showing good stability in
200 several studies including other bivalves, were finally chosen resulting in seven candidate
201 reference genes (Table 1). Note that the 18S (rRNA) and actine genes, the two most
202 commonly used reference genes for RT-qPCR in ecotoxicological studies (Volland et al.,
203 2017), were not finally included in our selection because despite they were within the
204 three most stable genes in some algorithm rankings in some of the studies, this was not
205 consistent across algorithms rankings among revised studies.

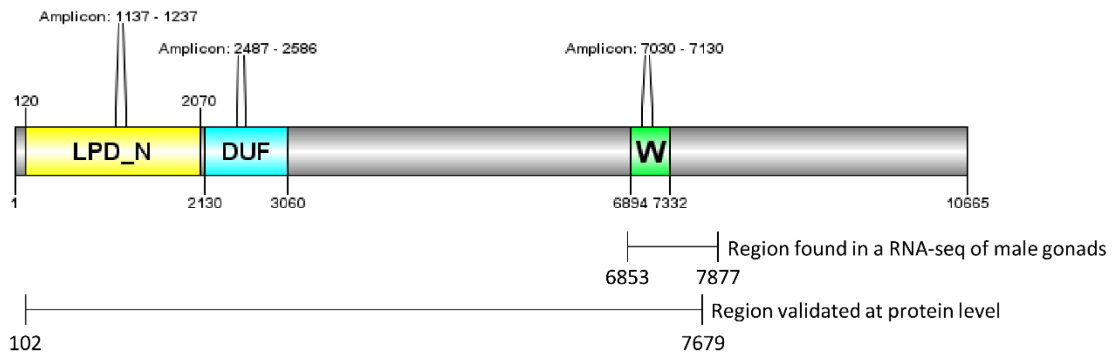
206

| Gene name | Amplicon name | Primer sequences (5'-3') | Sequence code | Amplicon size (bp) | E (%) | r2 | References |
|---|---|---|---|---|---|---|---|
| Vitellogenin | Vtg_LPD_N | **F:** GATGAAGTCGAAGCAGAGG / **R:** GCATACTCCTCCCAAACAG | Unigene21_All | 101 | 102.37 | 0.999 | |
| | Vtg_DUF1943 | **F:** CAGCAATCCGAGGAAAGTAG / **R:** GGTTTGGTGATCGTCTCTG | | 100 | 100.42 | 0.996 | |
| | Vtg_VWD | **F:** AGCAGCCATTGTCACAG / **R:** CACATCTTCTCCTTCTTGGG | | 101 | 94 | 0.992 | |
| Glyceraldehyde-3-phosphate dehydrogenase | gapdh | **F:** GACGCTGGAGCAGAATATG / **R:** GTCACTTGATGGTGCTGAG | CL5131.Contig1_All | 117 | 100.64 | 0.994 | 1, 2, 3, 4, 5, 6 |
| Eukariotic translation elongation factor 1 alpha | ef1 | **F:** CAGTGCCAGTAGGTAGAGTAG / **R:** GAGACTCGTGGTGCATTTC | CL8632.Contig1_All | 114 | 99.59 | 0.992 | 1, 2, 6, 7, 8, 9, 10 |
| 40S ribosomal protein S3 | rps3 | **F:** GGCACAGTGGAGTTGTATG / **R:** CAGCAAGACCACCAATCAG | Unigene35875_All | 100 | 96.98 | 0.998 | 11 |
| 40S ribosomal protein S4 | rps4 | **F:** TGACGGCAAAGTCAGAAC / **R:** GGTGGACAGCAAATCTACC | CL12349.Contig1_All | 122 | 101.60 | 0.994 | 4, 5 |
| 40S ribosomal protein S27 | rps27 | **F:** GGGCAAGACCATCACAC / **R:** CCATCCTCCAACTGCTTAC | CL7546.Contig1_All | 132 | 100.92 | 0.990 | 4, 5 |
| 60S ribosomal protein L13A | L13A | **F:** CTGGAAGCCAGACGTAAAG / **R:** GGTAGCCATAGCCTTCAATC | CL6519.Contig5_All | 139 | 98.07 | 0.997 | 11 |
| 60S ribosomal protein L19 | L19 | **F:** AGAGGAAGGCAGAGAAGAG / **R:** TACGAGCCTCCTTGACTC | CL8858.Contig1_All | 81 | 94.69 | 0.997 | 11 |

207 **Table 1.** Gene name, given amplicon name, primer sequences, sequence code in Moreira et al., (2015) database, amplicon size, primer efficiencies E(%) and
208 coefficient of determination ($r^2$) of Vtg_LPD_N, Vtg_DUF1943, Vtg_VWD and candidate reference genes gapdh, ef1, rps3, rps4, rps27, L13A and L19. In the
209 last column there are listed the references of the revised studies for reference gene selection,1: Dheilly et al. (2011); 2: Mauriz et al. (2012); 3: Feng et al. (2013);
210 4: Lozano et al. (2015); 5: Martínez-Escauriaza et al. (2018); 6: Xin et al. (2018); 7: Fleury et al. (2010); 8: Llera-Herrera et al. (2012); 9: Cubero-León et al.
211 (2012); 10: Yue et al. (2018); 11: Jarque et al. (2014). F, forward primer. R, reverse primer

212 **2.2.3. Primers design**

213 Primer sequences were designed for three different domains of the complete Vtg sequence
214 and for the seven selected candidate reference genes indicated above (see Table 1) using
215 Primer Quest Tool and Oligo Analyzer (IDT Integrated DNA Technologies). The three
216 conserved domains of Vtg sequence (Figure 1): lipoprotein N-terminal domain (LPD_N)
217 (hereafter Vtg_LPD_N), domain of Unknown Function 1943 (DUF1943) (hereafter
218 Vtg_DUF1943) and von Willebrand factor type D domain (VWD) (hereafter Vtg_VWD)
219 were chosen to ensure gene expression in case that different isoforms in mussel gonads
220 could be generated. The Vtg_VWD domain was specifically selected in this study due to
221 a reported short sequence matching this domain in a RNA-seq of mature male gonad of
222 *Mytilus* spp (Figure 1) (Diz et al., 2018; Romero et al., 2019).



223

224 **Figure 1.** Vitellogenin (Vtg) nucleotide sequence of *Mytilus galloprovincialis*. This sequence
225 (CL1077.Contig3_All_5) was extracted from an RNA-seq database published by Moreira et al.
226 (2015) and validated at protein level in Sánchez-Marín et al., (2017) and Fernández-González et
227 al., (2020). Fragment of nucleotide sequence validated at protein level is indicated below the
228 sequence (covering from nucleotide 102 to 7679). Domains identified in CL1077.Contig3_All_5
229 at protein level were: lipoprotein N-terminal domain (LPD_N), Domain of Unknown Function
230 1943 (DUF) and von Willebrand factor type D domain (W). It is also represented the sequence
231 found in a RNA-seq of mature male gonad of *Mytilus* spp which includes von Willebrand domain

232 (Diz et al., 2018; Romero et al., 2019). In the sequence there are represented the three amplicons
233 analyzed by RT-qPCR in this study.
234
235 Nucleotide sequences for Vtg and reference genes available in a transcriptomic database
236 obtained from a RNA-seq analysis of different tissues of *M. galloprovincialis* (Moreira
237 et al., 2015) were used for primers design. This transcriptome database includes two
238 sequences annotated as Vtg, CL1077.Contig3_All and Unigene21_All. Both sequences
239 present the three conserved Vtg domains and both only differ in 3'-URT region (See
240 Alignment 4 in supplementary material file S3). Designed primer sequences are shown
241 in Table 1. Primers were synthesized by Eurofins Genomics (Germany).

242 **2.2.4. Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR)**

243 RT-qPCR reactions were performed in triplicate in 96-well reaction plates including
244 negative controls without cDNA. Each sample well contained a final volume of 12.5 µl,
245 including 6.25 µl of Maxima SYBR Green/ROX RT-qPCR master mix (Fisher
246 Scientific), 1.25 µl of each forward and reverse primer solution (3 µM), 2.75 µl of
247 nuclease-free water and 1 µl of 1:10 diluted cDNA sample. RT-qPCR analyses were
248 conducted in a 7900HT Fast Real-Time PCR System (Thermo Fisher Scientific) under
249 the following conditions: 10 min at 95 °C for initial denaturation followed by 40 cycles
250 of 15 sec for denaturation at 95 °C, 1 min for annealing/elongation at 60 °C. Specificity
251 of amplification reaction was checked by melting curve analysis and agarose gel
252 electrophoresis (1% agarose) to verify that a single PCR product was amplified.
253 Polymerase chain reaction efficiency (E) for each primer pair was calculated based on the
254 slopes of standard curves from seven serial five-fold dilutions of template cDNA,
255 following the equation described in Kubista et al. (2006), $E=(10^{(-1/slope)}-1)$ multiplied by

256 100 if expressed in percentage (E%). Both E% and the coefficient of determination ($r^2$)
257 of the standard curves (Cq vs $Log_{10}$ (dilution)), where Cq was the fractional PCR cycle
258 used for quantification as described in Bustin et al. (2009), were used for the evaluation
259 of RT-qPCR assay.

260 **2.2.5. Expression stability of reference genes**

261 Reference gene expression stability was evaluated in 12 females and 12 males (2 samples
262 for treatment and sampling time) using geNorm (Vandesompele et al. 2002), BestKeeper
263 (Pfaffl et al., 2004) and NormFinder (Andersen et al. 2004) algorithms using RStudio
264 v3.6.1 packages "ctrlGene" and "generefer". In geNorm, expression stability of reference
265 genes is defined by pairwise comparison of variation of expression ratios, therefore, the
266 suitabilty of a reference gene depends on the number and the set of reference genes
267 included in the analysis, and this algorithm tends to select the gene with the highest
268 degree of similitude to the expression pattern of other reference genes included in the
269 whole data set (Hibbeler et al., 2008). BestKeeper determines the quality of each
270 reference gene tested only by the standard deviation (SD) of its expression in different
271 samples (Hibbeler et al., 2008). NormFinder determines the stability of reference genes
272 based on an estimate of the inter- and intragroup variation, thus combining the advantages
273 of geNorm and BestKeeper (Andersen et al., 2004). Due to these differences, results may
274 vary depending on the algorithm used. Analysis were performed in female and male
275 gonads and both sexes together to observe the possible influence of sex on the stability of
276 the tested reference genes. For BestKeeper analysis raw Cq obtained from RT-qPCR
277 analysis were used (Pfaffl et al., 2004), whereas for geNorm and NormFinder analyses,
278 raw Cq values were transformed to relative quantities (RQ) following the equation
279 $RQ=(1+E)^{\Delta Cq}$ (Hellemans et al., 2007), where E = primer pair efficiency and $\Delta Cq=$
280 (lowest Cq value of all samples of the selected gene – Cq value of the sample). Reference

281 genes were ranked assigning a number to each gene (being 1 the most stable one and 7
282 the least stable one) according to each individual algorithm. A final consensus ranking
283 was obtained from the Geometric mean (GE) of the three individual algorithm rankings
284 (Table 2).

| Rank | **geNorm** | Mean M value | **NormFinder** | Stability Value | **BestKeeper** | SD | **Consensus ranking** | GE |
|---|---|---|---|---|---|---|---|---|
| ♀ | | | | | | | | |
| 1 | ef1 | 0.068 | L19 | 0.110 | ef1 | 0.322 | ef1 | 1.4 |
| 2 | L19 | 0.068 | rps4 | 0.148 | gapdh | 0.382 | L19 | 1.6 |
| 3 | rps27 | 0.092 | ef1 | 0.201 | rps27 | 0.471 | rps27 | 3.8 |
| 4 | gapdh | 0.110 | L13A | 0.206 | L19 | 0.483 | gapdh | 3.8 |
| 5 | rps4 | 0.137 | rps3 | 0.284 | rps3 | 0.573 | rps4 | 3.9 |
| 6 | rps3 | 0.151 | rps27 | 0.334 | rps4 | 0.682 | rps3 | 5.3 |
| 7 | L13A | 0.189 | gapdh | 0.336 | L13A | 2.375 | L13A | 5.8 |
| ♂ | | | | | | | | |
| 1 | rps27 | 0.154 | L13 | 0.072 | rps27 | 0.335 | rps27 | 1.0 |
| 2 | rps3 | 0.154 | L19 | 0.126 | ef1 | 0.353 | ef1 | 2.5 |
| 3 | gapdh | 0.171 | rps4 | 0.171 | L19 | 0.371 | L19 | 3.6 |
| 4 | ef1 | 0.209 | ef1 | 0.257 | gapdh | 0.455 | gapdh | 3.6 |
| 5 | L19 | 0.227 | rps3 | 0.306 | rps3 | 0.512 | rps3 | 3.7 |
| 6 | rps4 | 0.250 | rps27 | 0.348 | rps4 | 0.579 | rps4 | 5.6 |
| 7 | L13A | 0.274 | gapdh | 0.362 | L13A | 2.992 | L13A | 7.0 |
| ♀+♂ | | | | | | | | |
| 1 | rps27 | 0.114 | L19 | 0.071 | rps27 | 0.759 | rps27 | 1.8 |
| 2 | rps3 | 0.114 | rps4 | 0.114 | ef1 | 0.799 | L19 | 2.5 |
| 3 | gapdh | 0.132 | L13A | 0.120 | L19 | 0.851 | ef1 | 3.2 |
| 4 | ef1 | 0.159 | ef1 | 0.139 | rps3 | 0.861 | rps3 | 3.4 |
| 5 | L19 | 0.171 | rps3 | 0.159 | rps4 | 1.031 | rps4 | 3.9 |
| 6 | rps4 | 0.185 | rps27 | 0.172 | gapdh | 1.313 | gapdh | 5.0 |
| 7 | L13A | 0.210 | gapdh | 0.190 | L13A | 2.659 | L13A | 5.3 |

285 **Table 2.** Individual algorithm (geNorm, NormFinder and BestKeeper) and consensus ranking
286 among the 3 algorithms (based on calculated geometric mean (GE)) of the seven candidate
287 reference genes tested for normalising qPCR results on *M. galloprovincialis* gonads of either
288 females, males or both sexes together. Gene stability is evaluated by the M value in geNorm
289 algorithm, stability value in NormFinder and by the standard deviation (SD) in BestKeeper. In all
290 cases, lower values indicate higher gene stabilities.

291

### 2.2.6. RT-qPCR data analysis

293 Pfaff method for multiple reference genes (Pfaffl, 2001) was used in order to normalize
294 Cq values of the three Vtg gene domains (Vtg_LPD_N, Vtg_DUF1943 and Vtg_VWD).
295 This method was selected based on the slopes obtained in primer efficiency tests
296 performed for all primer pairs. For this method slopes must be between -4 and -2.7
297 following recommendations provided in Pfaffl (2001). Results from our analysis were
298 within this range (see Table S1 in supplementary material File S1 for slopes of all primer
299 standard curves). In order to compare mRNA expression levels in the two different tissues
300 (female gonads vs male gonads), Cq values were used to estimate the relative difference
301 in gene expression using the formula: Fold change = $2^{(Cq_male\ gonads - Cq_female\ gonads)}$.

### 2.3. Statistical analyses

303 Shapiro-Wilk and Levene tests were used to check for data normality and
304 homoscedasticity of normalized Vtg gene expression (i.e. mRNA expression levels). For
305 statistical analysis, normalized Vtg gene expression were transformed to logarithms to
306 improve the fit to the assumptions of the parametric tests. Two-way ANOVA was used
307 to check for differences between Vtg mRNA normalized expression for the three different
308 Vtg domains (Vtg_LPD_N, Vtg_DUF1943 and Vtg_VWD) in female and male gonads
309 after different exposure time (t4 and t24) and different treatments (C, SC and EE2).
310 Statistical significance was accepted at $p < 0.05$. Statistical analysis was performed with
311 IBM SPSS Statistics 23. In order to compare the expression levels among the three
312 different Vtg domains for males and females independently, expression ratios of different
313 domains were calculated for all individual samples, and the mean expression ratio for

314 each sex and for each pairwise comparison was tested for its significant deviation from
315 1, using a standard t-test in Microsoft Excel.

316 **2.4.Assembly of Vtg sequences from *M. edulis* and *M. trossulus* using RNA-seq data**

317 *M. galloprovincialis* Vtg protein sequence, resulting from the translation of the longest
318 CL1077.Contig3_All sequence (RNA-seq data) annotated as Vtg (Moreira et al. 2015)
319 (see Sequence S2 in supplementary material File S2) and validated by proteomic analysis
320 (Sánchez-Marín et al., 2017; Fernández-González et al., 2020) was chosen as query
321 sequence in order to find Vtg sequences form closely related to *Mytilus* species. This
322 sequence was searched (query) using tblastn against Transcriptome Shotgun Assembly
323 (TSA) database restricted to *M. edulis* and *M. trossulus* taxa. Matched TSA sequences
324 were input in GeneStudio ™ Professional sequence analysis software version 2.2.0.0
325 (GeneStudio, Inc) (using default parameters) in order to produce larger contig sequences.
326 Resulting contigs were aligned against the complete *M. galloprovincialis* Vtg sequence
327 in Jalview software (Waterhouse et al., 2009) using MUSCLE algorithm to produce a
328 unique larger *M. edulis* and also *M. trossulus* Vtg sequence that were manually edited
329 where it was necessary. See supplementary material File S2 for TSA sequences and
330 contigs used for the assembly. ExPASy translation tool (https://web.expasy.org/translate)
331 was used (using default parameters) to get Vtg protein sequences from the new assembled
332 contigs and ascertain the correct protein reading frame. Additionally, to assess the query
333 cover and the percentage of identity between Vtg sequences of the three *Mytilus* species,
334 blastn (nucleotidic Vtg sequences) and blastp (aminoacidic Vtg sequences) were used.
335 Vtg sequences from the three *Mytilus* species were aligned and compared using Jalview
336 (MUSCLE algorithm) in order to complement this preliminary overview about the
337 conservation degree of Vtg sequence (both at nucleotide and amino acid level) among
338 species.

339 A phylogenetic analysis was carried out to study the evolutionary relationships between
340 Vtg sequences from several mollusk species, and also to check the reliability of the *M.*
341 *edulis* and *M. trossulus* Vtg sequences assembled in this study. To this aim we used
342 complete nucleotide Vtg sequences from the three *Mytilus* species (see Sequences S2, S3
343 and S4 in supplementary material File S2) and other available mollusk species in nrNCBI
344 with the following accessions: *Crassostrea virginica* (XM_022461020.1), *Pecten*
345 *maximus* (AM943022.2), *Haliotis discus hannai* (AB360714.1), *Saccostrea glomerata*
346 (KU194474.1), *Mimachlamys nobilis* (JN638064.1), *Chlamys farreri* (GQ227743.1),
347 *Mizuhopecten yessoensis* (KC138552.1) and *Scapharca broughtonii* (MG580782.1). Vtg
348 sequence from *Danio rerio* (AF406784.1) was used as outgroup. We checked that all
349 selected Vtg sequences contained the three conserved domains: Vtg_LPD_N,
350 Vtg_DUF1943 and Vtg_VWD. Highly variable 3'-UTR (untranslated regions) detected
351 in *M. edulis*, *M. galloprovincialis*, *M. trossulus*, *S. broughtonii* and *S. glomerata*
352 sequences and Poly A tails detected in *P. maximus*, *M. yessoensis*, *M. nobilis*, *H. discus*
353 *hannai*, *C. farreri* and *D. rerio* were removed before sequence alignment. All nucleotide
354 sequences were aligned using MUSCLE algorithm in MEGA software version 10.1.8
355 (Kumar et al., 2018) using default parameters, and ignoring gaps. The evolutionary
356 history of Vtgs was inferred using the Neighbor-Joining method (Saitou et al. 1987). Next
357 to the branches there are represented the percentage of replicate trees in which the
358 associated taxa clustered together in the boostrap test (10,000 replicates) (Felsenstein
359 1985). The tree is represented to scale, presenting branch lengths in the same units as
360 those of the evolutionary distances employed to infer the phylogenetic tree. The
361 evolutionary distances were calculated using the p-distance method (Masatoshi and
362 Sudhir 2000) and are in the units of the number of base variations per site. All positions
363 presenting gaps and missing data were eliminated (complete deletion option). The final

364 dataset contains a total of 3603 positions. Evolutionary analyses were conducted in
365 MEGA X (Kumar et al. 2018).

366

367 # 3. Results

368 ## 3.1. Assessment of RNA quality

369 Average total RNA concentration (ng/μl) obtained in female and male samples were
370 531.82 ± 242 and 110.46 ± 31.56 respectively. Purity of extracted RNA was assessed by
371 A260/280 and A260/230 ratios. In the case of females, values were on average 2.06 ±
372 0.03 and 1.81 ± 0.22 respectively, whereas for males were 1.89 ± 0.05 and 0.93 ± 0.13.
373 An initial analysis of RNA integrity in a selected group of female and male samples using
374 an Agilent 2100 Bioanalyzer (Agilent Technologies) confirmed good RNA integrities
375 since a single peak was observed (18S rRNA) in each sample, an expected result for
376 mollusks and other invertebrates (Gayral et al. 2011) (see Figure S4 in supplementary
377 material File S1). Values lower than 2.0 for A260/230 ratio indicates the remain of
378 solvents like phenol/chloroform or chaotropic salts like guanidine isothiocyanate that
379 usually remains in RNA samples extracted by phenol-based methods (Cirera 2013). The
380 presence of these compounds is due to the problems in chloroform phase separation that
381 was not produced correctly due to the high viscosity of the gonad samples, which was
382 noted especially in male samples. This problem was partially solved by slightly
383 modifying the standard RNA extraction protocol (i.e. repeating twice the chloroform
384 washing step) (see section 2.2.1). However it is important to note that sub-optimum
385 A260/230 ratio values do not necessarily compromise the reliability of RT-qPCR reaction
386 (Cicinnati et al. 2008), especially in situations where A260/280 ratio (between 1.8 and 2)
387 and RNA integrity analysis results are good.

### 3.2. Specificity and efficiency of RT-qPCR analysis

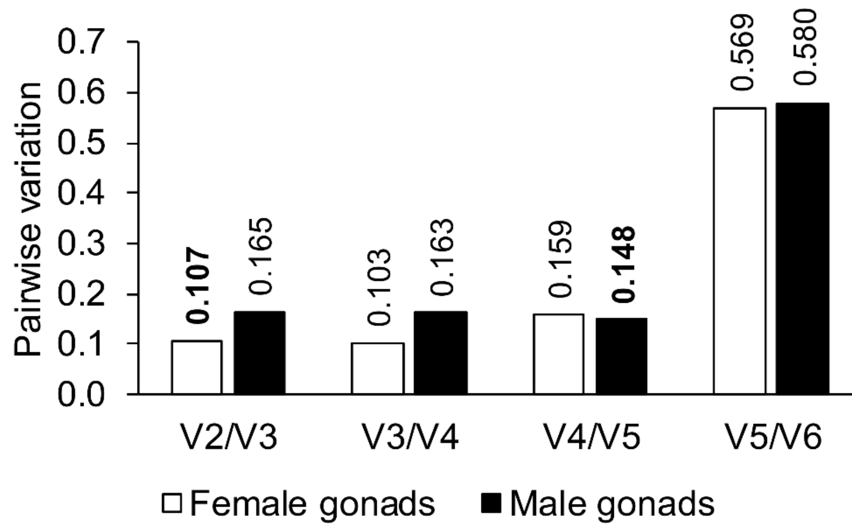
Primer efficiency (E%) values ranged from 94 to 101.60% and coefficient of determination of the standard curves varied from 0.990 to 0.998 (Table 1), which are all above accepted threshold values recommended for reliable RT-qPCR analysis (Taylor et al., 2010). Melting curve analysis of all evaluated primer pairs showed a single peak indicating the presence of a unique PCR product (see Figure S5 in supplementary material File S1). Agarose gel electrophoresis confirmed these results together with expected amplicon size for each selected primer pair, with the only exception of rps3 primer pair where an extra nonspecific amplification faint band was observed (see Figure S3 d) in supplementary material File S1).

### 3.3.Validation of reference genes

Results obtained for expression stability of seven potential reference genes evaluated by geNorm, NormFinder and BestKeeper are provided for females, males, and both sexes combined to assess whether there are any differences in stability gene rankings between females and males (Table 2). geNorm algorithm results showed that all reference genes tested were adequate since M values were all below the 1.5 cut-off value established in Vandesompele et al. (2002). Bestkeeper algorithm results showed that some reference genes are not adequate since SD values were above the cut-off (SD < 1) in the case of females and males separately (L13A) and in the case of both sexes together (rps4, gapdh and L13A). Finally, for NormFinder a cut-off value has not been established, and reference genes are only ranked from best to worst stability (Vandesompele et al., 2002). The final consensus rank (among results of geNorm, NormFinder and BestKeeper) was (in order to the most stable to the least stable) ef1>L19>rps27>gapdh>rps4>rps3>L13A for females, rps27>ef1>L19>gapdh>rps3>rps4>L13A for males, and rps27>L19>ef1>rps3>rps4>gapdh>L13A after combining samples from both sexes

413 (Table 2). The four most stable reference genes (ef1, L19, rps27 and gapdh) were
414 coincident in males and female individual rankings, although in different order, being ef1
415 and L19 the best two in females and rps27 and ef1 in males. For both sexes combined,
416 rps27, L19 and ef1 were also between the best ranked genes, but gadph ranked on $6^{th}$
417 position (compared to the $4^{th}$ position when evaluated separately for either males or
418 females respectively) while rps3 ranked $4^{th}$. Given the slightly different ranking obtained
419 for males and females, and based on the observation that SD values obtained from
420 BestKeeper were higher for samples that include both rather than separated sexes, we
421 decided to choose a different set of reference genes for males and females.

422 In order to choose the number of reference genes required for an optimal normalization,
423 we used the pairwise variation ($V_{n/n+1}$) tool in geNorm R package (Figure 2). This was
424 done excluding the rps3 gene, because this gene, despite being well ranked in geNorm
425 for males, was not well ranked in the other two algorithms (see Table 2), and also because
426 agarose gel electrophoresis showed possible primer non-specific amplification (see
427 section 3.2). An arbitrary cut-off value of 0.15 was proposed for the pairwise variation,
428 indicating that if $V_n/V_{(n+1)}$ is below this value, n reference genes are enough, and an
429 additional reference gene (n + 1) is not necessary for normalization process
430 (Vandesompele et al. 2002). According to this, two reference genes, ef1 and L19, were
431 used for normalization of female mRNA expression data (V2/V3 = 0.107; Figure 2) and
432 four reference genes, rps27, ef1, L19 and gapdh, were used for normalization of male
433 mRNA expression data (V4/V5 = 0.148; Figure 2). Unless otherwise noted, Vtg mRNA
434 expression in subsequent results was normalized using these reference genes. However,
435 in order to assess the impact that using different number of reference genes can have on
436 results, normalization of Vtg mNRA expression values was also performed with different
437 number of reference genes (see next section).



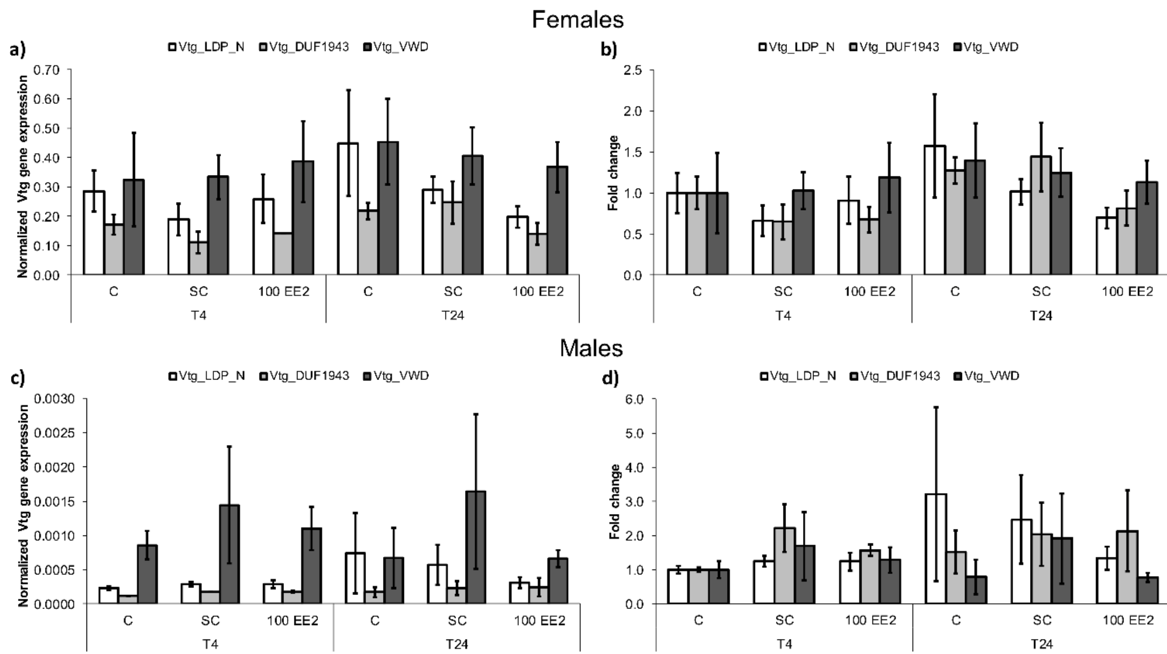

438

439 **Figure 2.** Pairwise variation ($V_{n/n+1}$) made using geNorm for the determination of the number of
440 reference genes needed for an optimal normalization of qPCR results. Pair-wise variation was
441 performed in female and male gonads including six reference genes gapdh, ef1, rps4, rps27, L13A
442 and L19 (rps3 gen was excluded, see section 3.3). In bold the first value below the cut off (0.150),
443 indicating the optimal number of reference genes for normalization.

444

445 **3.4. Vtg expression analysis**

446 Results of Vtg normalized gene expression in female and male gonads of *M.*
447 *galloprovincialis* in the different experimental treatments and for the three Vtg domains
448 are presented in Figure 3.




449

450 **Figure 3.** Vtg mRNA expression measured in three domains of the Vtg sequence (Vtg_LPD_N,
451 Vtg_DUF1943 and Vtg_VWD) in female and male gonads of *M. galloprovincialis* exposed for 4
452 (T4) or 24 days (T24) to control seawater (C), a solvent control (SC) or 100 ng/L EE2 (100 EE2).
453 Values are represented as normalized expression levels after normalization with the two most
454 stable reference genes (ef1 and L19) for females (a) and with the four most stable reference genes
455 (rps27, ef1, L19 and gapdh) for males (c), or as fold change compared to the control at T4 (b, d).
456 Mean ± SE 95% CI (n = 3) is represented. Note different Y-scale between females and males in
457 a) and c). No significant differences were found among treatments (two-way ANOVA, $p > 0.05$)
458 (see Table 3).

459

460 After statistical analysis, results did not show evidence of any effect of the factors
461 chemical exposure (C, SC, EE2) or exposure time (t4, t24) in Vtg mRNA normalized
462 expression levels in any of the three Vtg domains in females or males, neither was the
463 interaction between the two factors significant (Table 3). To rule out the possibility that
464 using a different number of reference genes for data normalization could produce
465 different results and conclusions, the same analyses were repeated using Vtg expression
466 data normalized with a different number of reference genes following the reference gene
467 ranking obtained after reference gene validation (See Figure S1 and S2 in supplementary
468 material File S1). Similar results and the same conclusion was obtained, *i.e.*, there was no
469 evidence of any EE2 effect on Vtg mRNA expression in either females or males (see
470 Table S2 in supplementary material File S1 for Two-way ANOVA results).

| Sex | Vtg domain | Factors | F. | P value Two-Way ANOVA |
|---|---|---|---|---|
| ♀ | Vtg_LPD_N | Time (t4, t24) | 0.247 | 0.629 |
| | | Chemical (C, SC, EE2) | 1.295 | 0.313 |
| | | Time × chemical | 0.654 | 0.539 |
| | Vtg_DUF1943 | Time (t4, t24) | 3.554 | 0.086 |
| | | Chemical (C, SC, EE2) | 1.542 | 0.257 |
| | | Time × chemical | 0.753 | 0.494 |
| | Vtg_VWD | Time (t4, t24) | 0.446 | 0.518 |
| | | Chemical (C, SC, EE2) | 0.050 | 0.952 |
| | | Time × chemical | 0.195 | 0.826 |
| ♂ | Vtg_LPD_N | Time (t4, t24) | 0.166 | 0.692 |
| | | Chemical (C, SC, EE2) | 0.047 | 0.954 |
| | | Time × chemical | 0.063 | 0.939 |
| | Vtg_DUF1943 | Time (t4, t24) | 0.099 | 0.759 |
| | | Chemical (C, SC, EE2) | 0.423 | 0.665 |
| | | Time × chemical | 0.272 | 0.765 |
| | Vtg_VWD | Time (t4, t24) | 0.968 | 0.346 |
| | | Chemical (C, SC, EE2) | 0.721 | 0.508 |
| | | Time × chemical | 0.104 | 0.902 |

471 **Table 3.** Results of two-way ANOVA testing for differences in log-transformed normalized
472 mRNA expression data in gonads for the 3 different Vtg domains among sample groups. Sample
473 groups: C, Control. SC, Solvent Control. 100 EE2, 100 ng/L EE2. t, exposure time in days. Data
474 from female and male samples were analyzed independently. Expression levels were normalized

475 with two reference genes in females (ef1 and L19) and with four reference genes in males (rps27,
476 ef1, L19 and gapdh).

477 Vtg mRNA expression was detected in both female and male gonads, but its level was
478 much higher in females (Figure 3a, c). For the different domains Vtg_LPD_N,
479 Vtg_DUF1943 and Vtg_VWD, it was estimated that RNA expression levels were 447,
480 1184 and 107 times higher for females than for males, respectively. Normalized Vtg gene
481 expression levels of the three domains were also different within sex (Figure 3a, c). In
482 females, Vtg_VWD was more expressed than Vtg_LPD_N (expression ratio
483 Vtg_VWD/Vtg_LPD_N: $1.57 \pm 0.88$, $p = 0.013$) and Vtg_DUF1943 (expression ratio
484 Vtg_VWD/Vtg_DUF1943: $2.43 \pm 0.98$, $p < 0.001$). In addition, Vtg_LPD_N was more
485 expressed than DUF1943 (expression ratio Vtg_LPD_N/Vtg_DUF1943: $1.82 \pm 0.88$, $p =$
486 $0.001$). In males, Vtg_VWD was more expressed than Vtg_LPD_N (expression ratio
487 Vtg_VWD/Vtg_LPD_N: $3.93 \pm 3.59$, $p= 0.003$) and Vtg_DUF1943 (expression ratio
488 Vtg_VWD/Vtg_DUF1943: $6.55 \pm 5.72$, $p= 0.0007$), while no differences were found
489 between Vtg_LPD_N and Vtg_DUF1943 expression (expression ratio
490 Vtg_LPD_N/Vtg_DUF1943: $3.12 \pm 5.63$, $p= 0.12$).

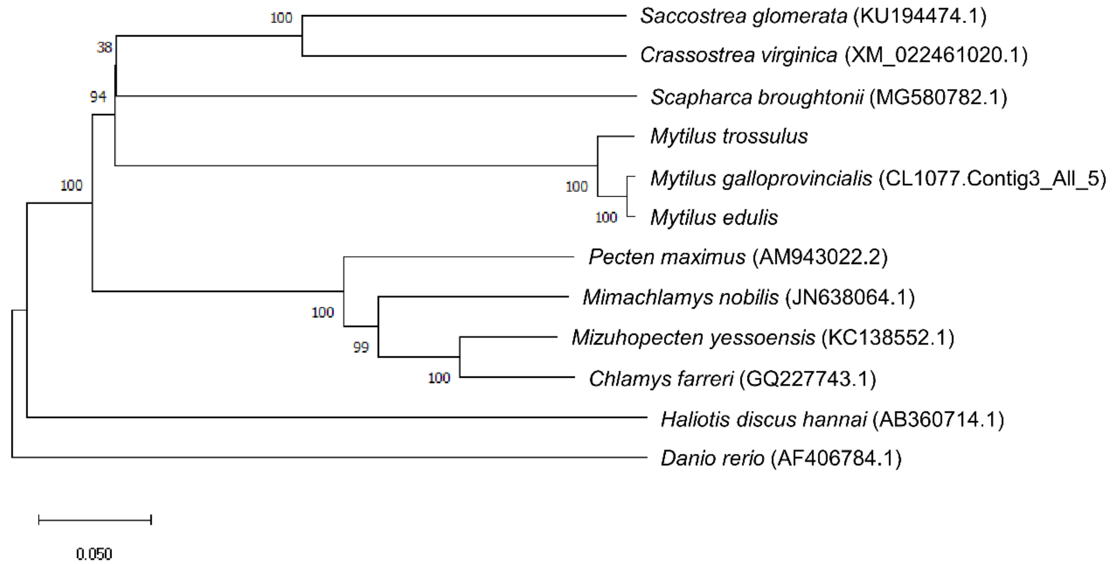
491 **3.5. *M. edulis* and *M. trossulus* Vtg sequences**

492 Using contigs obtained from transcripts from TSA databases, nucleotide sequences of *M.*
493 *edulis* and *M. trossulus* Vtgs were assembled, avoiding overlaps, using contigs 1, 2, 5 and
494 6 for *M. edulis* and 2, 1, 7 and 4 for *M. trossulus* (see Contig S1 and S2 in supplementary
495 material file S2). In the case of *M. edulis*, transcripts matching *M. galloprovincialis* Vtg
496 sequence were obtained from *M. edulis* larvae (Ramesh et al., 2019) and mantle tissue
497 (Knöbel et al., 2020) RNA-seq projects, whereas for *M. trossulus* transcripts were from a
498 RNA-seq project on *M. trossulus* mantle tissue (Knöbel et al. 2020). Assembled

499 nucleotide and subsequent aminoacidic sequences for *M. edulis* and *M. trossulus* are
500 provided in supplementary material File S2, Sequences S3 and S4.

501 Results from either nucleotide or aminoacidic sequence pairwise alignments using Blast
502 tools with default parameters showed that the degree of conservation of Vtgs were higher
503 in the first 2500/2600 aa, depending on the species (See Alignment 10 in supplementary
504 material file S3). This region corresponds with the region validated at protein level for *M.*
505 *galloprovincialis* Vtg in Sánchez-Marín et al. (2017) and Fernández-González et al.
506 (2020) with a 71.08% of peptide coverage. On the contrary, the biggest differences were
507 detected in the final part of the sequence, which probably correspond to a 3'-UTR.

508 The results obtained after comparison of Vtg sequences of *M. edulis, M. galloprovincialis*
509 and *M. trossulus* are in agreement with current evidence about evolutionary history of
510 these taxa pointing to a first allopatric speciation which gave rise to *M. trossulus* and the
511 ancestor form of *M. edulis* and *M. galloprovincialis* 3.5 million years ago (mya) and a
512 later allopatric isolation that gave rise to *M. edulis* and *M. galloprovincialis* 2.5 mya
513 (Mathiesen et al., 2017). Results shown in the phylogenetic tree (Figure 4) are in
514 agreement with the described above as well as with the phylogenetic (evolutive)
515 relationships described for mollusks (Wanninger and Wollesen, 2019), and therefore
516 reinforce the reliably of the *M. edulis* and *M. trossulus* Vtg sequences assembled and
517 provided in the present study.




518

519 **Figure 4.** Phylogenetic tree reconstruction based on calculated distance matrix from Vtg
520 nucleotide sequences of 11 mollusk and one fish (outgroup) species using the Neighbor-Joining
521 method. Note this is the consensus tree summarizing all those obtained from boostraped data
522 (10,000 replicates), indicating branch support in their nodes, i.e. the percentage of reconstructed
523 trees following boostrapping recovering the same result. Scale represents the branch length in
524 terms of distance units employed to infer the phylogenetic tree. Accessions for the different Vtg
525 sequences, obtained from nrNCBI database, and from Moreira et al. (2015) transcript database in
526 the case of *Mytilus galloprovincialis*, are provided in parenthesis beside species name, while the
527 three *Mytilus* sequences are those provided after the analysis of previous RNA-seq data in this
528 study (see M&M).

529

530 **Discussion**

531 **4.1. Expression patterns of conserved Vtg domains**

532 RT-qPCR has been a widely used technique to measure Vtg expression in bivalve
533 mollusks like freshwater and marine mussels (*Elliptio complanata*, *M. edulis, M.*
534 *galloprovincialis*), oysters (*S. glomerata, Cassostrea angulata*) and scallops (*C. farreri,*

535 *Agropecten purpuratus*) exposed to different estrogenic EDCs in field and laboratory
536 experiments (Gagne et al. 2005; Boutet et al. 2008; Qin et al. 2012; de los Ríos et al. 2013,
537 2016 2018; Llera-Herrera et al. 2014; Ni et al. 2014; Tran et al. 2019 and cites therein).
538 In most cases, primers were designed in the LPD-N domain (Matsumoto et al. 2003;
539 Puinean and Rotchell 2006; Puinean et al. 2006; Ciocan et al. 2010; Andrew-Priestley et
540 al. 2012; Qin et al. 2012; de los Ríos et al. 2013, 2016, 2018; Tran et al. 2016) and in less
541 cases in other central regions (Boutet et al., 2008; Llera-Herrera et al., 2014) or in C-
542 terminal region (Llera-Herrera et al., 2014).

543 Despite certain limitations described in gene expression studies using RT-qPCR
544 technique (see Zhang et al. 2019), this technique has been widely used successfully in
545 numerous studies with very diverse objectives in a wide variety of organisms. Perhaps,
546 one of the greatest limitations is related to the analysis of the expression level of certain
547 genes from the amplification of a single specific region of the target gene. This common
548 and simple approach could provide an incomplete vision of the expression of the gene by
549 not considering the possibility of differential expression at the isoform level. Therefore,
550 considering this, for the present study primers were designed for three conserved domains
551 of *M. galloprovincialis* Vtg sequence (Vtg_LPD_N, Vtg_DUF1943 and Vtg_VWD).
552 This approach was also motivated by the fact that a short sequence (1025 nucleotides)
553 containing Vtg_VWD domain was obtained from a RNA-Seq database of *M.*
554 *galloprovincialis* male gonads described in Diz et al. (2018) and Romero et al. (2019)
555 (Locus_22135_Transcript_1/2) (Figure 1). We hypothesised that this short sequence
556 could correspond to a Vtg isoform present in male gonads of *M. galloprovincialis*. The
557 results obtained in the present study show that Vtg_VWD is the most expressed domain
558 in males (except for T24 C, Figure 3c), which is in concordance with our hypothesis.
559 However, the expression of Vtg_LPD and Vtg_DUF1943 at lower levels indicate that, in

case this isoform would exist, it would not be the only one present in males. These results also indicate that the study of different domains of the Vtg sequence by RT-qPCR analysis is a good strategy since it can be observed that the different domains show different expression levels. One of the most likely explanations about the observed differences in the expression levels of the three domains (Vtg_VWD > Vtg_LPD_N > Vtg_DUF1946) could be the potential existence of different Vtg isoforms.

### 4.2. Vtg gene expression in male and female gonads of *M. galloprovincialis*

The much lower male Vtg mRNA expression levels compared to females in the present study are in agreement with the results obtained for males of *M. galloprovincialis* by de los Ríos et al. (2013, 2016, 2018) that used primers designed from a partial Vtg sequence of *M. galloprovincialis* (GenBank No. KC793981.1) that corresponds with LPD_N domain. There is also agreement with other studies in which Vtg was detected in female and male gonads, but Vtg levels were sex-biased, being much higher in females than in males; for example, several studies performed in Vtg_LPD_N of other bivalve species like *C. angulata*, *C. gigas* and *C. farreri* (Matsumoto et al., 2003; Ni et al., 2014; Qin et al., 2012) as well as others performed in different Vtg domains such as the region between Vtg_DUF1943 and Vtg_VWD in *A. purpuratus* (Boutet et al., 2008) and *C. gigas* (Llera-Herrera et al., 2014) and the C-terminal region after Vtg_VWD of *C. gigas* (Llera-Herrera et al., 2014). Also one study using massive sequencing technique (Illumina RNA-seq) in gonads of *Pactinopecten yessoensis* showed high Vtg expression in females but a very low level of Vtg expression in males (Hou et al., 2011). In addition, Vtg expression in gonads of *S. glomerata* using semi-quantitative real time PCR technique (sqRT-PCR) was detected in female gonads but in males it was not detected or was detected at very low levels, depending on the sample origin (Anderson et al., 2010).

584 However our results are in disagreement with those from other RT-qPCR transcriptomic
585 studies performed in Vtg_LPD_N in gonads of *M. edulis* (Ciocan et al., 2010; Puinean
586 and Rotchell, 2006; Puinean et al., 2006) where Vtg mRNA expression was detected in
587 the same range of expression in both sexes. In those studies, Vtg primers were designed
588 using a partial sequence of *M. edulis* Vtg described by Puinean and Rotchell (2006)
589 (GenBank No. AY679116), nevertheless, as already noted in Fernández-González et al.
590 (2020), sequence AY679116 showed higher similarity with Vtg sequences described for
591 different species of the *Crassostrea* genus than with Vtg sequence of the more related
592 species *M. galloprovincialis*. When the sequence AAT72932.1 (subsequent aminoacidic
593 sequence of AY679116) was searched (query) using tblastn against TSA database
594 restricted to *M. edulis* only one match was detected (GHII01269945.1) from *M. edulis*
595 mantle tissue (Knöbel et al., 2020) with a percentage of identity of only 47.03%. All
596 described above strongly suggest that AY679116 sequence does not belong to *M. edulis*
597 Vtg, which could explain the discrepancies between present results and those from
598 referenced studies.

599 The fact that Vtg mRNA abundance for the three analysed domains was much higher in
600 females than in males is expected given that the main function of Vtg is to produce egg-
601 yolk in developing oocytes during embryogenesis. Its presence in males, despite at low
602 amount, can be explained by the fact that Vtg (or Vtg isoforms) could have other
603 functions. It has been described that in vertebrates and chordates Vtg can be implicated
604 in defense responses like antibacterial activity, phagocytosis of microbes or antioxidant
605 activity (Zhang et al. 2015; Carducci et al. 2019). Also in invertebrates, alternative
606 functions of Vtg have been described beyond its role in reproduction. These functions
607 include reduction of oxidative stress by scavenging free radicals (Seehuus et al., 2006),
608 involvement in social organization (Guidugli et al., 2005), and immune-relevant activity

609 (including bacterial growth inhibition) (Zhang et al. 2011; Wu et al. 2015) among others.
610 These potential additional functions of Vtg can explain the presence of Vtg mRNA
611 expression in males. However, no evidence of Vtg protein expression in *M.*
612 *galloprovincialis* male gonads was found in previous proteomic studies (Sánchez-Marín
613 et al 2017, Fernández-González et al 2020). A possible explanation for these results could
614 be due to the level of Vtg in males which was below the detection limit of the high-
615 resolution mass spectrometry device used for protein identification and quantification.
616 An alternative explanation could be that despite there might be some basal Vtg
617 transcription, Vtg mRNA is not finally translated to protein in male gonads. This
618 hypothetical scenario is not rare because there are several reports informing about the
619 lack of correlation found for some proteins and their respective transcripts, as well as the
620 non-translation of some effectively transcribed genes within specific tissues (Diz and
621 Calvete, 2016; Vogel and Marcotte, 2012).

622 **4.3. Effect of EE2 on Vtg mRNA expression levels**

623 Comparison of results from Vtg_LPD_N, Vtg_DUF1943 and Vtg_VWD mRNA
624 expression levels between mussels exposed to EE2 and controls (C and SC) do not
625 provide any evidence of an effect of EE2 on Vtg expression either in female or male
626 gonads (Figure 3). Despite the limited sample size used, a visual observation of the results
627 does not show any tendency towards higher values in EE2 exposed individuals. On the
628 contrary, Vtg expression was even higher in some C and SC individuals compared to EE2
629 exposed ones (see Figure S3 in supplementary material File S1). These results are in
630 disagreement with other RT-qPCR studies that showed induction of Vtg mRNA
631 expression after estrogen exposure in different mollusk species like mussels, scallops and
632 oysters. Studies performed in *M. edulis* showed Vtg mRNA induction in female and male
633 gonads exposed to different estrogenic compounds (including EE2 at a similar

634 concentration as in the present study) during 10 days of exposure only when mussels were
635 in early stage of gametogenesis (Ciocan et al., 2010; Puinean et al., 2006). However, the
636 cited studies could have important limitations in terms of primer design specificity as
637 previously described (see section 4.2) that could compromise reported results.

638 Another study performed in scallop *C. farrery* also showed Vtg induction in ovaries after
639 30 days being injected with 60 μg of 17β-estradiol every 10 days (Qin et al., 2012).
640 Several studies performed in oysters (*S. glomerata* and *C. angulata*) showed induction of
641 Vtg mRNA expression by 17β-estradiol (E2) (Ni et al., 2014; Tran et al., 2016). Tran et
642 al (2016) showed that E2 induced Vtg mRNA expression after 14 days of exposure (but
643 not after 28 days of exposure) to 100 ng/L E2, while lower concentrations did not cause
644 any induction in relation to controls. In addition, Vtg mRNA expression increased in
645 ovary explants after *in vitro* exposure to E2 at nominal concentrations ranging from 2.7
646 μg/L to 2.7 mg/L (Tran et al., 2016). In the study with *C. angulata*, temperature and diet
647 regimes were designed in order to facilitate gonad development, as confirmed by the
648 increase in Vtg mRNA expression in females during the course of the experiment (Ni et
649 al., 2014). Furthermore, Vtg mRNA expression increased earlier in females exposed to
650 E2 (at concentrations of 5 and 50 ng/L) as compared to controls. Also for males, induction
651 of Vtg mRNA expression was detected after 21 and 48 days of exposure to different E2
652 concentrations (5 ng/L, 50 ng/L and 200 ng/L) (Ni et al., 2014). In addition, HPLC
653 analysis showed that Vtg protein synthesis was induced in oyster *S. glomerata* female and
654 male gonads after EE2 exposure (Andrew et al., 2010, 2008). It is important to highlight
655 that *C. farrery*, *S. glomerata* and *C. angulata* are protandrous hermaphrodite species that
656 can change from males to females during the experiment if the conditions are favorable,
657 or depending on the age of individuals (Andrew et al., 2008; Mori et al., 1969; Silina,
658 2018; Zapata-Restrepo et al., 2019), thus complicating the interpretation of results from

659 these studies. Also, the functioning of the endocrine system of protandric species,
660 including its possible regulation by estrogens, could work in a different way compared to
661 other bivalves.

662 Despite the “publication bias” whereby there is a trend among researchers (and journals)
663 to preferentially publish research with positive outcome, few studies reporting also a
664 negative outcome have been published concerning Vtg regulation by estrogens in bivalve
665 mollusks (*Unio tumidos* and *M. galloprovincialis*). No evidence of Vtg protein induction
666 by estrogens was found in *U. tumidos* after exposure to 17β-estradiol (100, 320 and 100
667 ng/L) after 55-57 days (Morthorst et al., 2014) and *M. galloprovincialis* after 4 and 24
668 days of exposure to EE2 (100 ng/L) (Fernández-González et al., 2020).

669 Similar negative results were obtained in other invertebrates, such as *Crangon crangon*
670 and *Carcinus maenas* exposed to diethylstilbestrol (DES) and 4-nonilphenol (4-NP) (both
671 at $1\times10^8$ ng/L) after 7, 4 and 21 days of exposure (Allen et al., 2002) and in males of *C.*
672 *maenas* after 12 weeks of exposure to NP (10,000-100,000 ng/L) (Lye et al., 2008). At
673 transcritomic level, Vtg mRNA induction has not been detected in *Eurytemora affinis*
674 after exposure to 4-NP (1000 and 5000 ng/L) for 24 h (Boulangé-Lecomte et al., 2017).

675 **4.4. Controversy about the use of mollusks in endocrine disruption studies**

676 The fact that, up to date, no consensus has yet been reached about whether EDCs can
677 have any effect on Vtg production in bivalve mollusks reveals that current knowledge
678 about the functioning of the endocrine system of invertebrates is scarce if compared with
679 vertebrates (Katsiadaki, 2019; Porte et al., 2006). Since the discovering of vertebrate-like
680 steroids in marine invertebrates in the 1950s (Hagerman et al., 1957), it has been long
681 assumed that the endocrine system in invertebrates is analogous to that described for
682 vertebrates, i.e. steroids act at the genomic level, binding to nuclear receptors and

683 stimulating expression of inducible genes (Lafont, 2000), and thus canonical EDCs that
684 disrupt vertebrate endocrine system are relevant to invertebrates as well. In the last
685 decade, some authors have questioned this assumption (Fodor et al., 2020; Katsiadaki,
686 2019; Scott, 2018, 2013, 2012). Aspects of this questioning have been recently discussed
687 in Fodor et al. (2020) and can be summarized as follows: some essential key enzymes of
688 vertebrate steroid synthesis are absent or there is no firm evidence of their presence in
689 invertebrates, there is no firm evidence that invertebrate estrogen receptors have the
690 capacity to bind vertebrate steroids and finally, the presence of vertebrate steroids in
691 aquatic invertebrates can be explained by their ability for the rapid uptake,
692 biotransformation and esterification of vertebrate sex steroids present in the environment
693 (Schwarz et al., 2017a, 2017b). Other reproductive regulation mechanisms have been
694 proposed that might be present in invertebrates. As reviewed in Katsiadaki (2019) the
695 absence of endocrine glands in mollusks (except in gastropods and cephalopods) should
696 be considered as an indication that these are first order systems that work in a simple way
697 responding to neurotransmitters/neurohormones acting locally (Lafont, 2000). An
698 alternative possibility could be the presence in invertebrates of other type of steroids, such
699 as the "non-cleaved steroids" or "paraestrols" that occur in corals and sponges and that
700 could be potential ancestral ligands for estrogen receptors (Markov et al., 2017).

701 Other authors have proposed that reproductive regulation in bivalves, including Vtg
702 induction, are regulated by steroid hormones (such as estrogens) but through a different
703 pathway compared to vertebrates, i.e. through non-genomic regulation of kinase cascades
704 (Tran et al., 2019). However, the present results do not provide evidence for this pathway
705 neither, since Vtg was not induced after EE2 exposure, disregarding if the pathway
706 involved is either a genomic or non-genomic one, at least concerning *M.*
707 *galloprovincialis*. Might non-genomic regulation events occur in *M. galloprovincialis* in

708 response to estrogens (Canesi et al., 2004a,b; Blalock et al., 2018), these may produce
709 other effects, but not Vtg production, at least under the conditions of the present
710 experiment.

711 Results obtained in the present study and in Fernández-González et al. (2020) indicated
712 that Vtg expression both at transcript and protein level in female and male gonads of *M.*
713 *galloprovincialis* were not affected after laboratory exposure to EE2. In addition, field
714 studies evaluating Vtg mRNA expression in *M. galloprovincialis* exposed to WWTP
715 effluents, the main source of EDCs contribution to the marine environment, do not
716 provide evidence of disruption of Vtg mRNA expression in male mussels (de los Ríos et
717 al. 2016, 2018). These results strongly suggests that canonical EDCs such as EE2 do not
718 have any effect in mussels Vtg expression, at least for this species.

719 **4. Conclusion and perspectives**

720 Results from this study do not provide evidence of Vtg mRNA expression induction in
721 *M. galloprovincialis* gonads for the three Vtg domains analysed by RT-qPCR after
722 exposure to the synthetic hormone EE2 at a concentration of 100 ng/L neither at short (4
723 days) or longer (24 days) exposure period. This together with other results from previous
724 studies at protein level strongly suggests that Vtg should not be used as biomarker of
725 endocrine disruption in marine mussels. Differences reported here in the Vtg mRNA
726 expression levels from the three analysed Vtg domains suggest the presence of different
727 Vtg isoforms in *M. galloprovincialis* female and male gonads, though this hypothesis
728 would need to be confirmed in following up studies. Finally, in this work the complete
729 Vtg sequences of *M. edulis* and *M. trossulus* are provided, which will allow to design
730 primers for different Vtg domains and perform similar studies in these two closely related
731 species to *M. galloprovincialis* that should contribute to strengthen the evidence and
732 conclusions presented here.

733 **Apendix A. Supplementary data**

734 The following is supplementary data to this article:

735 File S1. Microsoft word file "Supplementary material File S1 – Tables and figures"
736 including additional Tables S1 and S2 and additional figures S1 to S6.

737 File S2. Compressed file "Supplementary material File S2 – Sequences" including
738 nucleotic and aminoacidic Vtg sequences of *M. galloprovincialis*, *M. edulis* and *M.*
739 *trossulus* (Sequences S1 to S4), the accession number of *M. edulis* and *M. trossulus* TSA
740 sequences used to generate contigs (Matched sequences S1 and S2) and contigs generated
741 from TSA transcripts of *M. edulis* and *M. trossulus* (Contigs S1 and S2).

742 File S3. Compressed file "Supplementary material File S3 – Alignments" including
743 vitellogenin alignments in nucleotidic and aminoacidic format of M. *galloprovincialis*,
744 *M. edulis* and *M. trossulus*.

745

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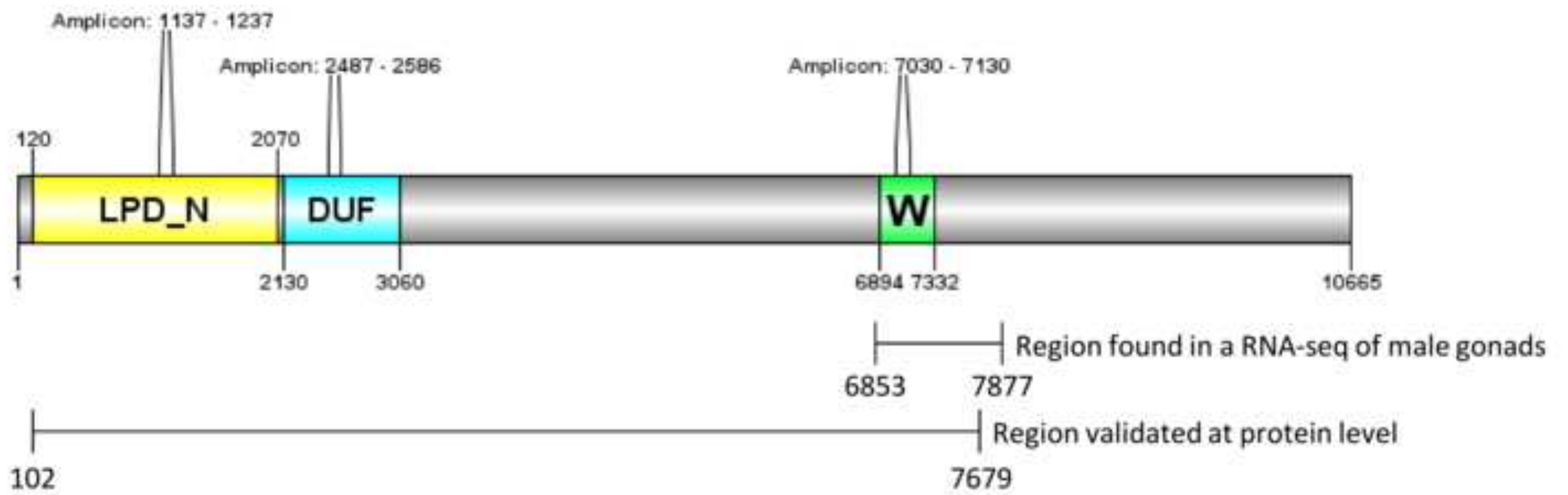


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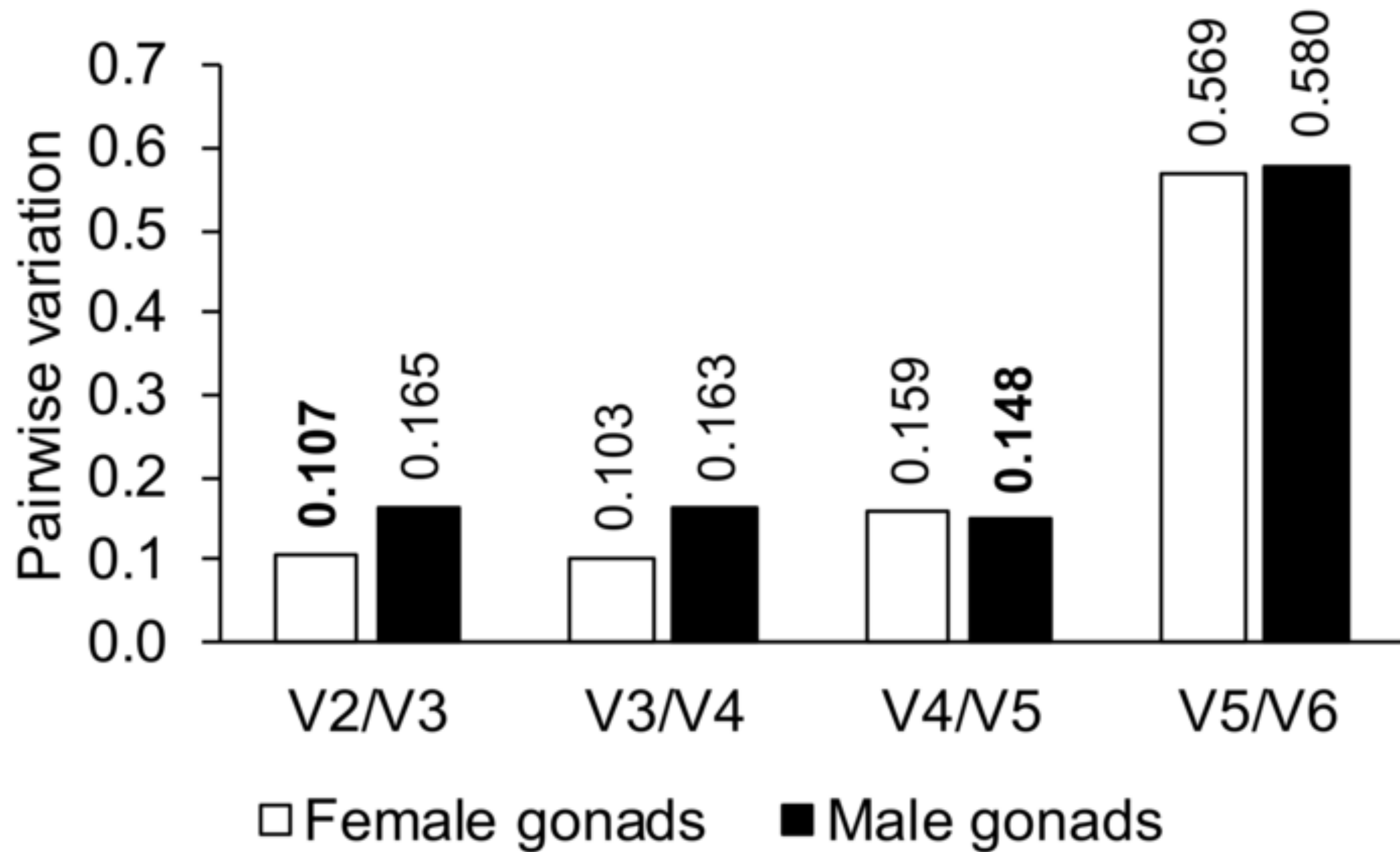


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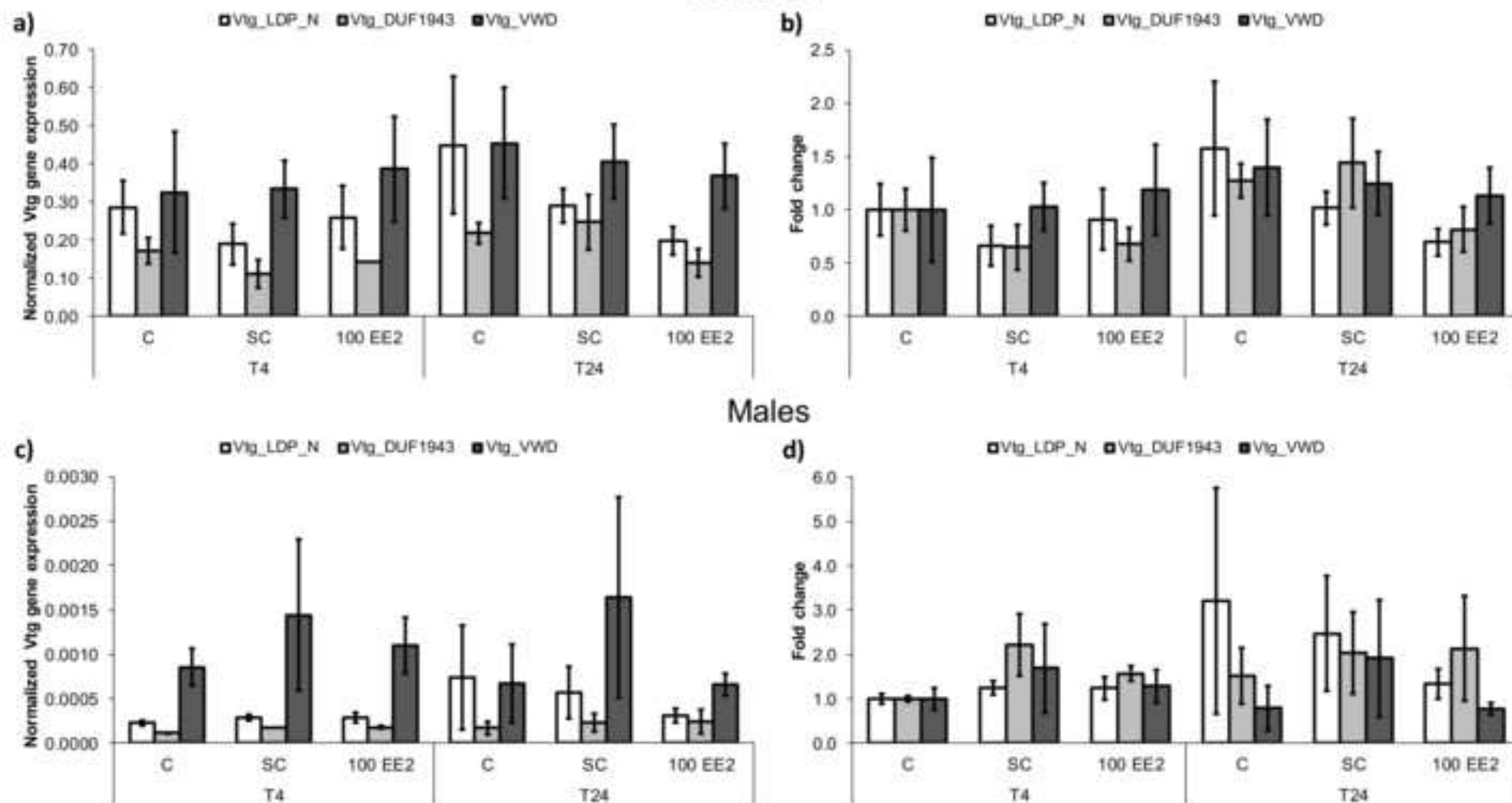

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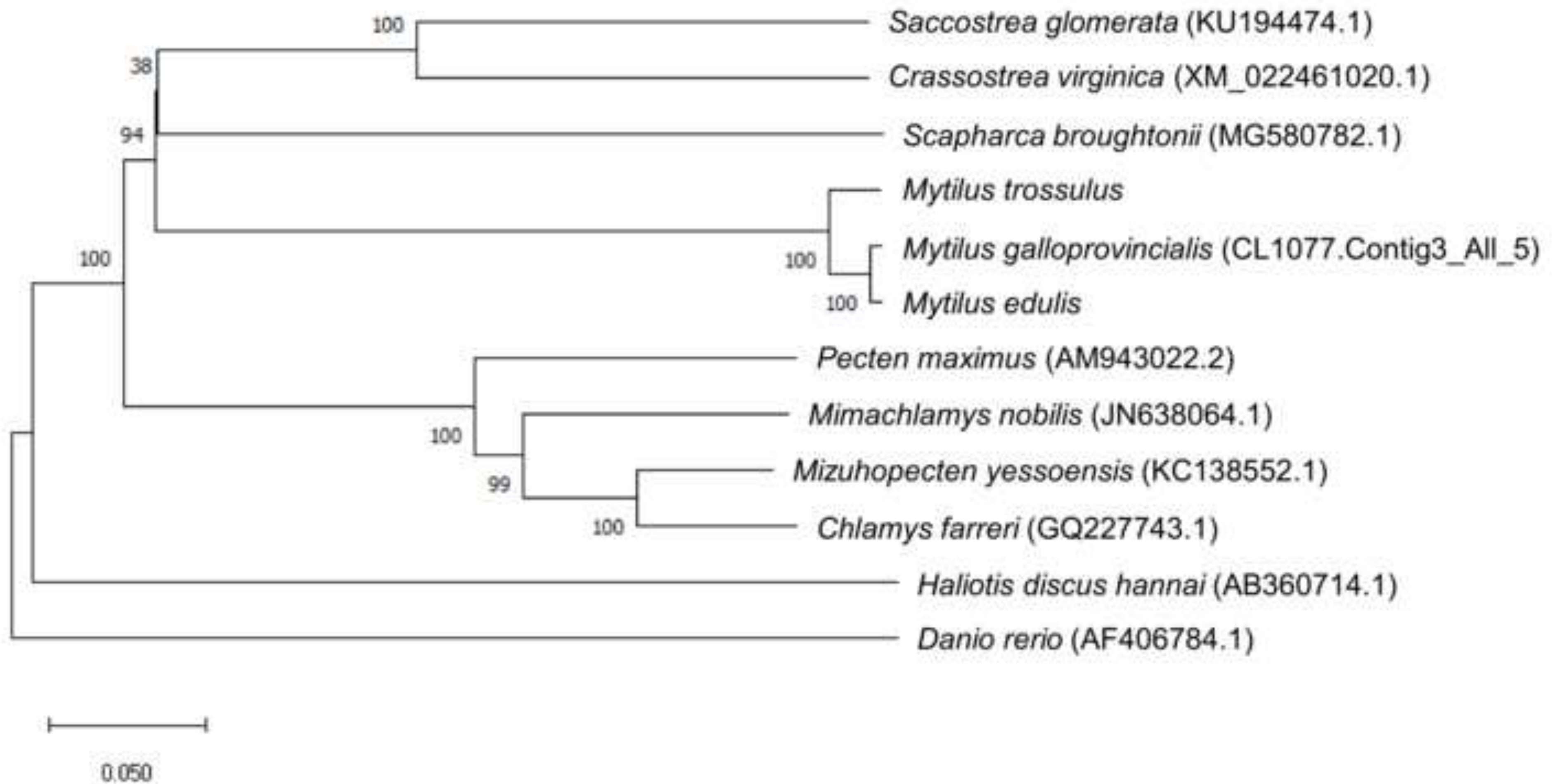


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