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Controversial use of vitellogenin as a biomarker of endocrine disruption in crustaceans: New adverse pieces of evidence in the copepod *Eurytemora affinis*

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Céline Boulange-Lecomte, Benoît Xuereb, Gauthier Trémolet, Aurélie Duflot, Nathalie Giusti, et al.. Controversial use of vitellogenin as a biomarker of endocrine disruption in crustaceans: New adverse pieces of evidence in the copepod *Eurytemora affinis*. *Comparative Biochemistry and Physiology - Part C: Toxicology and Pharmacology*, 2017, 201, pp.66 - 75. 10.1016/j.cbpc.2017.09.011 . hal-01918573

HAL Id: hal-01918573

<https://hal.science/hal-01918573v1>

Submitted on 14 Jun 2023

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1 **CONTROVERSIAL USE OF VITELLOGENIN AS A BIOMARKER OF ENDOCRINE**
2 **DISRUPTION IN CRUSTACEANS: NEW ADVERSE PIECES OF EVIDENCE IN THE**
3 **COPEPOD *EURYTEMORA AFFINIS***

4

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11 **Running title:** Vitellogenin characterization in the copepod *Eurytemora affinis*

12 Ms. has 25 pages, 2 tables, 5 figures

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20

21 **Abstract**

22 In recent years, the interest in the use of vitellogenin (*VTG*) as a biomarker of endocrine
23 disruption in fish has led to *VTG* being considered as a potential tool in invertebrates. Among
24 aquatic invertebrate models in ecotoxicology, the copepods are considered as reference
25 species in marine, estuarine and freshwater ecosystems. In this context, we identified a *VTG*
26 cDNA in *Eurytemora affinis*. The *Ea-VTG2* cDNA is 5,416 bp in length with an open reading
27 frame (ORF) of 5,310 bp that encodes a putative protein of 1,769 amino acids residues.
28 Phylogenetic analysis confirmed the hypothesis of a *VTG* duplication event before the
29 emergence of the copepod species. The analysis of the *Ea-VTG2* expression by qPCR in
30 males and females according to their reproductive stages allowed transcript basal levels to be
31 determined. The expression pattern revealed a gradual increase of transcript levels during
32 maturation in females. Important inter-sex differences were observed with a *VTG* level in
33 males ranging from about 1,900- to 6,800-fold lower than in females depending on their
34 stage. Moreover, the protein was only detected in ovigerous females. The inducibility of *Ea-*
35 *VTG2* by chemicals was studied in males exposed to either a model of endocrine disruptor in
36 vertebrates i.e. 4-nonylphenol (4-NP) or a crustacean hormone i.e. Methyl Farnesoate (MF),
37 and in males sampled from a multi-contaminated estuary. No induction was highlighted. The
38 *VTG* should not be considered as an appropriate biomarker in *E. affinis* as previously
39 suggested for other crustaceans.

40

41 **Key words:** 4-nonylphenol, biomarker, crustaceans, endocrine disruption, estuary, Methyl
42 Farnesoate, *VTG*

43

44 1. INTRODUCTION

45 Among aquatic invertebrate models in ecotoxicology, the copepods constitute an interesting
46 group of organisms since they occupy a key position in the aquatic food web, contributing to
47 the transfer of contaminants to higher trophic levels. Over the past few years, they have been
48 considered as reference species in marine, estuarine and freshwater ecosystems (Kulkarni et
49 al., 2013; Kwok et al., 2015). *Eurytemora affinis* is a calanoid copepod widely distributed in
50 the northern hemisphere estuaries (Lee, 1999). *E. affinis* is an appropriate test organism
51 because of its small size, sexual dimorphism, short generation time, and ease of culturing in
52 the laboratory (Katona, 1970; Katona, 1971). In recent years, research studies have mainly
53 focused on developing tools to evaluate the effects of environmental contaminants on *E.*
54 *affinis* larval development, mortality, swimming behaviour, enzymatic activities or
55 transcriptional alterations (Boulangue-Lecomte et al., 2014; Cailleaud et al., 2011b; Forget-
56 Leray et al., 2005; Forget et al., 2002; Legrand et al., 2016; Lesueur et al., 2015; Lesueur et
57 al., 2013). In spite of growing interest in using *E. affinis* as an ecological and an
58 ecotoxicological model during the past decade, many aspects of its physiology are still
59 puzzling, especially concerning reproduction. Indeed, only a few studies have contributed so
60 far to better understanding of reproduction by depicting copulation, developmental cycle,
61 reproductive stages in females and individual-based modelling (Boulangue-Lecomte et al.,
62 2014; Dur et al., 2009; Katona, 1971; Katona, 1975). Molecular aspects of reproduction are
63 also poorly understood since only one paper has supplied information, which was
64 fragmentary, by depicting molecular alteration induced by endocrine disrupter (ED) pesticides
65 using a next-generation sequencing approach (Legrand et al., 2016).

66 Recently, several papers have reported the characterization of vitellogenin (VTG) cDNA in
67 copepods (Dalvin et al., 2011; Hwang et al., 2010; Hwang et al., 2009; Lee et al., 2016). In
68 female macrocrustaceans, VTG is synthesized in the hepatopancreas and/or ovaries, released

69 in hemolymph and then deposited onto oocytes where it is taken up by receptor-mediated
70 endocytosis (Lee and Chang, 1999; Li et al., 2006; Mak et al., 2005; Yang et al., 2005). The
71 VTG, present in all oviparous organisms, is the precursor of egg yolk vitellins which provide
72 resources for embryos. This protein was firstly identified in vertebrates, in particular in fish,
73 where it has been widely studied. In fish, VTG was shown to be synthesized in the maternal
74 liver and then transferred to growing oocytes via the blood (Hara et al., 2016). In male fish,
75 the *VTG* gene is present but silent except after activation by EDs, in particular by estrogenic
76 compounds. The gender-specific expression pattern and the induction of VTG in males by
77 chemicals has led the scientific community to consider VTG induction as a useful biomarker
78 of exposure to EDs in vertebrates (Hara et al., 2016; Scholz and Mayer, 2008; Sumpter and
79 Jobling, 1995). In recent years, the numerous studies reporting the use of VTG as an ED
80 biomarker in fish has led to VTG being considered as a potential tool in invertebrates
81 (Matozzo et al., 2008). However, the studies dealing with VTG variations in aquatic
82 invertebrates in response to both experimental and environmental exposures to EDs have not
83 been as clear-cut as those described in vertebrates, since both increases and decreases were
84 highlighted (Matozzo et al., 2008; Xuereb et al., 2011).

85 In this context, the present study aims to identify a *VTG* cDNA in *E. affinis* in order to (i)
86 improve knowledge of *E. affinis* reproduction, especially vitellogenesis and, (ii) explore the
87 possibility of using *E. affinis* in bioassessment using *VTG* induction as a biomarker of EDs.
88 Even though the literature reports *VTG* variations in both sexes, we have chosen to focus our
89 study on *VTG* induction in males in order to consider the use of *VTG* induction as an “on-off”
90 biomarker which makes interpretation unambiguous, particularly in field approaches. Indeed,
91 fluctuations in females – which display VTG expression unlike males or larvae – raise the
92 issue of the threshold at which to consider transcript variation as a disrupting event. We thus
93 report cloning and sequence analysis of a *VTG2* cDNA, and the phylogenetic relationship of

94 *Ea-VTG2* with other arthropod species. *Ea-VTG2* expression was analysed in males and
95 females according to their reproductive cycle in order to determine basal levels. Finally, the
96 inducibility of *Ea-VTG2* by chemicals was studied in males exposed to either a model of
97 endocrine disruptor in vertebrates i.e. 4-nonylphenol (4-NP) or a crustacean hormone i.e.
98 Methyl Farnesoate (MF), and in males sampled from a multi-contaminated estuary (Tappin
99 and Millward, 2015). 4-NP was chosen for its ED proprieties in vertebrates and MF hormone
100 for its potential to regulate the endocrine system in crustaceans (LeBlanc, 2007; Soares et al.,
101 2008).

102 **2. MATERIALS AND METHODS**

103 *2.1. Organism sampling*

104 Copepods were collected in the oligo-mesohaline zone of the Seine estuary (longitude
105 0°15'52"E, latitude 49°29'19"N; Haute-Normandie, France) using a horizontal plankton net
106 (200- μ m mesh size; 1-m diameter). Samples were directly passed through two sieves (500-
107 and 100- μ m mesh size) to eliminate large particles and predators. Immediately after sampling,
108 the copepods were placed into isotherm containers and were quickly brought back to the
109 laboratory.

110 Pools of 30 males (2 to 4 replicates) sorted using a stereomicroscope were collected just after
111 fishing in November 2015 and May 2016 to constitute the 'in-situ samples'. They were rinsed
112 with deionized RNase-free water before being frozen in liquid nitrogen and stored at -80 °C
113 until further investigation.

114 The other samples were collected after organism maintenance in the laboratory under
115 controlled conditions. Briefly, after acclimatization (salinity 10 PSU overnight), copepods
116 were cultured into 40-L aquariums filled with brackish water (a mixture of UV-treated filtered
117 (1 μ m) sea water and deionized water; Aquacaux, Octeville sur Mer, France) under optimal
118 culture conditions i.e. aeration, salinity 15 PSU, 15 °C, 12-h/12-h photoperiod (Devreker et

119 al., 2009). The copepods were fed *ad libitum* with a mixture of *Rhodomonas marina* and
120 *Isochrysis galbana* receiving a total of 20,000 cells.mL⁻¹. Algae cultures were grown at 20 °C
121 in 10-L tanks under 24-h fluorescent illumination and constant aeration in Conway medium.
122 In all cases, the copepods were depurated for at least 3 days in brackish water before any
123 analyses were conducted (Cailleaud et al., 2011a). Samples of 50 females were prepared for
124 *VTG* gene cloning. In order to measure the *VTG* basal levels, samples of 30 individuals (3
125 replicates per reproductive stage) or 100 individuals (2 replicates per stage) were collected for
126 qPCR or Western blot applications respectively (Boulangue-Lecomte et al., 2014). Copepods
127 were then quickly rinsed with deionized RNase-free water before being frozen in liquid
128 nitrogen and stored at -80 °C until further investigation. Finally, after depuration, copepods
129 (1 copepod / mL) were exposed either to 4-nonylphenol (4-NP; Sigma-Aldrich) i.e. 1 µg/L
130 and 5 µg/L for 24 h (3 replicates), or to (E,E)-Methyl Farnesoate (MF; Tebu-bio) i.e.
131 0.01 µg/L, 1 µg/L and 100 µg/L for 96 h (4 replicates). The stock solutions were prepared in
132 acetone (Sigma-Aldrich) in such a way that the final acetone concentration in the bioassays
133 was 0.01%. A solvent control (final acetone concentration of 0.01%) was conducted in the
134 same conditions as the contaminant exposures. The contaminated waters were renewed every
135 day. During the experiments, copepods were placed into a climatic chamber (aeration, salinity
136 15 PSU, 15 °C, 12-h/12-h photoperiod) and were fed once a day. The glassware was saturated
137 with contaminated waters for 24 h before exposures. At the end of exposures, samples of 15
138 to 30 males were quickly rinsed with deionized RNase-free water before being frozen in
139 liquid nitrogen and stored at -80 °C until further investigation.

140 2.2. *cDNA cloning*

141 Degenerate primers were designed from copepod *VTG* cDNA sequences available on the
142 GenBank database with the Primer 3 v4.0.0 software (<http://bioinfo.ut.ee/primer3/>; table 1).
143 PCR was performed using DyNAzymeTM II PCR master mix (Finnzymes) under the

144 following conditions: 95 °C / 4 min; 45 cycles of 95 °C / 30 s, 55 °C / 45 s, 72 °C / 1 min; and
145 72 °C / 5 min. After purification from 1.2 % agarose gel using the QIAquick Gel Extraction
146 Kit (QIAGEN), the PCR product was sequenced by the MilleGen® company (Labège,
147 France) with ABI3130XL sequencer (Applied Biosystems) by using Big-Dye® Terminator
148 v3.1 Cycle Sequencing Kit (Applied Biosystems™).

149 The full-length *VTG* cDNA was obtained by Rapid Amplification of cDNA Ends (RACE-
150 PCR) with Marathon™ cDNA Amplification Kit (Clontech) according to the user's manual.
151 The 3'-RACE product was amplified by PCR using the specific primer 3'-GSP1 (Table 1) and
152 the Adaptor Primer 1 (AP1, supplied with the RACE PCR Kit) as follows: 94 °C / 30 s; 5
153 cycles of 94 °C / 15 s, 65.5 °C / 1 min and 72 °C / 3 min; 5 cycles of 94 °C / 15 s,
154 62 °C / 1 min and 70 °C / 3 min; 25 cycles of 94 °C / 15 s, 62 °C / 1 min and 68 °C / 3 min;
155 and 72 °C / 10 min. The 5'-RACE product was amplified by PCR using the specific primer 5'-
156 GSP2 (Table 1) and AP1 as follows: 94 °C / 30 s; 5 cycles of 94 °C / 15 s, 60 °C / 1 min and
157 72 °C / 3 min; 5 cycles of 94 °C / 15 s, 59.9 °C / 1 min and 70 °C / 3 min; 25 cycles of
158 94 °C / 15 s, 60 °C / 1 min and 68 °C / 3 min; and 72 °C / 10 min. PCR products were
159 purified from 1.2 % agarose and sequenced as described above. The full-length *VTG* sequence
160 was obtained after cDNA assembly using the Geneious® 7.1.9 software
161 (<http://www.geneious.com>; (Kearse et al., 2012)).

162 *2.3. Sequence characterization and phylogenetic analysis*

163 The *E. affinis* *VTG* cDNA and its related protein were characterized using the Geneious®
164 7.1.9 software i.e. open reading frame (ORF), theoretical protein molecular mass (MM) and
165 isoelectric point (pI). Cellular localization was predicted by the PredictProtein server
166 (<https://www.predictprotein.org>). Putative signal peptides were researched using SignalP
167 (www.cbs.dtu.dk/services/SignalP) whereas protein domains were predicted using the

168 Conserved Domains Database (CDD, <https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>;
169 (Marchler-Bauer et al., 2015).

170 The phylogenetic analysis was performed after global alignment with GenBank amino acid
171 VTG sequences from 31 species (i.e. 15 crustaceans and 16 insects). The tree was constructed
172 by Jukes-Cantor genetic distance model and Neighbor-Joining method using the Geneious®
173 7.1.9 software. Blosum62 was used as the cost matrix. The gap open and gap extension
174 penalties were 12 and 3, respectively. The amino acid conservation was analysed comparing
175 percent identities generated from pairwise alignments (Clustal Omega; (McWilliam et al.,
176 2013).

177 *2.4. Quantitative real-time polymerase chain reaction (qPCR)*

178 Total RNA were extracted from samples using Tri-Reagent LS (Euromedex) according to the
179 procedure described in (Xuereb et al., 2012) after grinding copepods with micro-pestles
180 (Eppendorf) during 3 freeze/thaw cycles in liquid nitrogen, and homogenization in 250 µL of
181 nuclease-free ultrapure water (Sigma-Aldrich). Genomic DNA digestion and RNA
182 purification were conducted with TURBO DNA-free® kit (Ambion Applied Biosystems)
183 according to the manufacturer's recommendations. RNA integrity was checked by
184 electrophoresis on a 2 % agarose gel. Quantification and purity (A_{260}/A_{280} and A_{260}/A_{230}
185 ratios) were evaluated using a NanoDrop® ND-1000 spectrophotometer (NanoDrop
186 Technologies, Thermo Scientific). RNA samples were stored at -80 °C until further
187 experiments. Reverse transcription was performed from 1 µg of total RNA with M-MLV
188 reverse transcriptase RNase H minus (Promega) using oligo(dT)20 and Recombinant
189 RNasin® Ribonuclease Inhibitor (Promega). Complementary first-strand DNA (cDNA,
190 40 µL) were diluted in 60 µL of ultra-pure water and stored in 5-µL aliquots at -20 °C.
191 Quantitative real-time polymerase chain reaction (qPCR) was conducted as previously
192 described (Xuereb et al., 2012). Briefly, the specific primers (VTG-Ea-qS1 / VTG-Ea-qAS1)

193 were designed using the “Universal Probe Library” software (Roche Diagnostics, Table 1).
194 Amplifications were carried out in duplicate on the Rotor-Gene Q 2-plex HRM (QIAGEN)
195 from 5 μ L of cDNA using the QuantiTect® SYBR® Green Master Mix (1X, QIAGEN) and
196 the VTG-Ea-qS1 / VTG-Ea-qAS1 primers (0.5 μ M each). Non-template controls were
197 performed to ensure the absence of DNA contamination.

198 After an initial denaturation step at 95 °C/15 min, cDNA were amplified for 45 cycles of
199 94 °C / 15 s, 59 °C / 30 s, and 72 °C / 6 s. After thermocycling, cDNA were denatured by a
200 rapid increase to 95 °C and hybridized again for 20 s at 68 °C. The melting curve was finally
201 determined during a slow temperature elevation from 50 to 99 °C (1 °C.s⁻¹). The specificity
202 of the PCR product and the absence of primer dimers were checked using the dissociation
203 curve. The amplicon size was checked by a 1.2% agarose gel electrophoresis and its identity
204 was confirmed by sequencing (Biofidal, Vaulx-en-Velin, France). The expression levels of
205 target genes were calculated according to the absolute quantification method as previously
206 described (Xuereb et al., 2011). Briefly, the quantification cycle values (C_q; cycle number
207 from which fluorescence is detected above the noise threshold) were collected with the Rotor-
208 Gene Q series software (QIAGEN) using the comparative quantification method. To convert
209 C_q values into cDNA copy number, a specific standard curve was established from 10-fold
210 serial dilutions of the purified PCR product (from 10⁹ to 10¹ cDNA copies, in triplicate). *VTG*
211 was amplified from a cDNA sample with HotStar HiFidelity DNA Polymerase (QIAGEN)
212 according to the manufacturer’s manual and purified with QIAquick PCR® Purification Kit
213 (QIAGEN). Dilution series were performed in ultra-pure water after cDNA concentration
214 determination with a NanoDrop® ND-1000 spectrophotometer. The dilution of medium range
215 (10⁵ cDNA copies) called “standard point,” was distributed in 5- μ L aliquots and stored at -20
216 °C. This standard point was systematically amplified during the qPCR runs to confirm the
217 reliability of amplification and to correct the Y-intercept of standard curve equation. The

218 correlation coefficient of the standard curve and the PCR efficiency were 0.996 and 2.023
219 respectively.

220 2.5. Western blot

221 Copepods (100 individuals) were crushed and homogenized in Precellys® CK14 tubes
222 containing 200 µL of lysis buffer (50 mM TrisBase, 100 mM NaCl, 1 mM EDTA,
223 0.1 % Triton X-100, pH 7.8) and protease inhibitors (cOmplete™ Mini, Roche Applied
224 Science) using a Precellys® homogenizer (6,400 rpm, 2 x 10 s; Bertin Technologies). After
225 centrifugation for 15 min at 4 °C and 10,000 g, protein concentration of the supernatants was
226 assessed by the Bradford method using bovine serum albumin as a standard (Bradford, 1976).
227 A custom made VTG polyclonal antibody was obtained after immunization of rabbits with a
228 synthetic immunogenic peptide designed from the deduced *E. affinis* VTG sequence
229 (Millegen). This peptide ^{1,605}CGLCGNRDGDRRGDI^{1,619} was previously coupled to the
230 immunogenic carrier protein thyroglobulin to promote immunogenicity. A ‘GGG’ was added
231 between the VTG specific peptide and the thyroglobulin as a spacer. After 63 days, rabbit
232 serum was collected and the antibody was purified using Protein A.
233 The SDS-PAGE analysis was conducted from 25 µg of proteins after denaturation in 2X
234 Laemmli buffer by boiling for 5 min and separation on a reducing 10 % SDS-polyacrylamide
235 gel. Proteins were blotted to nitrocellulose membranes by electrotransfer. A Coomassie
236 staining of the gel was performed to ensure the transfer efficiency. Membranes were blocked
237 with 5 % non-fat dry milk in PBS 1X supplemented with 0.05 % Tween 20 overnight at 4 °C.
238 Blots were incubated for 1 hr at room temperature with the VTG primary antibody (1/5,000).
239 After washing five times with PBS 1X, membranes were incubated with a goat anti-rabbit
240 horseradish peroxidase-conjugated secondary antibody (1/5,000; Santa Cruz Biotechnology)
241 for 1 hr at room temperature. After washing five times in PBS 1X, detection was performed

242 by chemoluminescence (ECL SelectTM, Amersham). The immunogen peptide (1 µg) was used
243 as a positive control.

244 2.6. Statistical analysis

245 Statistical analysis of transcript levels was carried out using the SigmaStat 3.5 software
246 (Systat Software, Chicago, USA). Normality and equality of variances were checked using
247 the Kolmogorov–Smirnov and Levene median tests respectively. Statistical differences
248 between samples were assessed using the one way ANOVA coupled with the Student–
249 Newman–Keuls test as a post hoc analysis (p <0.05).

250

251 3. RESULTS AND DISCUSSION

252 3.1. Sequence analysis of the *E. affinis vitellogenin*

253 The full length cDNA obtained after sequence assembly was analogous to the
254 *Pseudodiaptomus annandalei* vitellogenin 2 (GenBank accession no. [AGT28481](#); 37%
255 identity in overall; Blastx). It was thus called *Ea-VTG2* (GenBank accession no. [AGH68974](#)).
256 The *Ea-VTG2* cDNA is 5,416 bp in length with an open reading frame (ORF) of 5,310 bp.
257 The 5'-terminal untranslated region (UTR) of 15 bp contains a Kozak sequence
258 (CTCAATATGA), highly similar to the crustacean consensus sequence (ACCAACaugG/A;
259 (Mankad et al., 1998)). The 3'-UTR of 91 bp includes a stop codon (TAA), a signal sequence
260 for polyadenylation (AATAAA) and a poly(A) tail (Figure 1). The ORF encodes 1,769
261 amino-acids characterized by theoretical molecular mass (MM) and isoelectric point (pI) of
262 203.9 kDa and 8.79 respectively. These features are close to those of other copepod VTG
263 such as the *Tigriopus japonicus* VTG2 which displays MM and pI of 205.2 kDa and 8.21
264 respectively (Hwang et al., 2009). The localization of the protein was predicted as
265 extracellular as depicted by its cellular component ontology (GO:0005576). Otherwise, a
266 signal peptide was identified in position 1-21 and three functional domains were highlighted

267 i.e. vitellogenin_N domain (position 25-701), DUF1943 superfamily domain (position 750-
268 997) and von Willebrand factor type D domain (VWD; position (1,462-1,623). These three
269 domains permit VTG to be discriminated from other lipoproteins (Hayward et al., 2010). The
270 DUF1943 domain is specific to the VTG isoform 2 (Hwang et al., 2010). Like other
271 crustacean VTGs, no phosvitin and polyserine domains were detected. Phosvitin and
272 polyserine domains are involved in the VTG receptor binding during endocytosis and in
273 phosphate and metal ion transport related to bone formation in insects and vertebrates,
274 respectively (Wahli, 1988). The lack of phosvitin and polyserine domains in most crustacean
275 VTGs indicates the involvement of different mechanisms for VTG receptor binding during
276 endocytosis in this subphylum. Recently, Roth and colleagues have shown in *Macrobrachium*
277 *rosenbergii* that a specific β -sheet region in the vitellogenin_N domain is the receptor-
278 interacting site, with the rest of the protein serving to enhance affinity for the receptor (Roth
279 et al., 2013). The lack of phosvitin has been thought to make the VTG more susceptible to
280 oxidative damage induced by pollutants (Ishikawa et al., 2004; Matozzo et al., 2008). The
281 vitellogenin_N domain may also be involved in lipid transport (Anderson et al., 1998).
282 Whereas the functions of the C-terminal VWD domain and the DUF1943 domain are poorly
283 documented, a recent study suggested – in fish – that the DUF1943 and VWD domains may
284 contribute to the function of VTG as a pattern recognition receptor for non-self components,
285 and that DUF1943 may also contribute to the function of VTG as an opsonin by promoting
286 phagocytosis (Sun et al., 2013).

287 Phylogenetic analysis by the neighbor-joining method was used to examine the relationship
288 between the *E. affinis* sequence and VTG of 31 arthropod species (Figure 2). The branching
289 pattern demonstrated three groups corresponding to malacostraca, maxillopoda and insecta.
290 VTGs in copepods appeared close to VTGs in insects. Accordingly, Lee and colleagues
291 consider VTGs in copepods and insects as forming an ‘arthropod VTG-like clade’ distinct

292 from the ‘apolipoprotein B-like clade’ which includes decapod VTGs (Lee et al., 2016). This
293 pattern leads to consider VTG in *E. affinis* as orthologous with VTGs in insects. The
294 maxillopoda clade was divided into two clusters corresponding to VTG1 and VTG2 stressing
295 the hypothesis of a *VTG* duplication event before the emergence of the copepod species (Lee
296 et al., 2016). As expected, the *E. affinis* sequence was displayed into the maxillopoda group
297 and its orthology with the vitellogenin 2 was confirmed. The predicted protein sequence of
298 Ea-VTG2 shares the most identity (ID) with the *Pseudodiaptomus annandalei* vitellogenin 2
299 (36.8 %) whereas the ID percent was below 30 % with the others i.e. *Lepeophtheirus salmonis*
300 (26.8 %), *Paracyclops nana* (27.4 %), *Tigriopus japonicus* (27.9 %) and *Tigriopus*
301 *kingsejongensis* (28.5 %).

302 3.2. Vitellogenin expression during *E. affinis* reproductive cycle

303 The *Ea-VTG2* expression was analysed by qPCR in males and females according to their
304 reproductive stages i.e. stage 1 (immature), stage 2A (initiating maturation), stage 2B
305 (advanced maturation) and stage 3 (ovigerous; (Boulangue-Lecomte et al., 2014); Figure 3A).
306 In females, the transcript levels gradually increased during maturation (from 3.3×10^6 to
307 12×10^6). The number of transcripts in egg-bearing individuals was similar to mature females
308 (12×10^6 vs 9.4×10^6). The few studies that have explored the level of VTG transcripts were
309 conducted in macrocrustacean species; they demonstrated a similar gradual increase of VTG
310 during ovarian maturation (Phiriyangkul et al., 2007; Xuereb et al., 2011; Yu et al., 2015).
311 Among all copepod species for which a VTG cDNA was identified, the distribution of VTG
312 transcripts according to the sexual characteristics of individuals was only depicted in *T.*
313 *kingsejongensis* (Lee et al., 2016). The patterns of variation during female maturation were
314 similar in *T. kingsejongensis* and *E. affinis*. However, whereas the *T. kingsejongensis*
315 transcript levels were about 24-fold higher in mature / ovigerous females than in their
316 immature counterparts, the change was reduced to a factor of 3.6 for *E. affinis*. A similar

317 factor between immature and mature (growth secondary oocytes) females was characterized
318 in *Gammarus fossarum* (Xuereb et al., 2011). The difference between *T. kingsejongensis* and
319 *E. affinis* may lie in the distinct definition of the ‘immature stage’. Indeed, *E. affinis* immature
320 females were defined as adult females – characterised by the presence of a wing-like
321 expansion of the fifth pedigerous somite – without any observable oocyte in the oviducts. So,
322 whatever the investigated reproductive stages, all females were adult and thus shared the same
323 size. The immature females in *T. kingsejongensis* were characterised by their mating
324 behaviour after being caught by the antennule of a mature male; immature individuals were
325 reduced in size in comparison with mature individuals because they include copepodites (Lee
326 et al., 2016). Moreover, the *Ea-VTG2* cDNA in males was weak since only 1.7×10^3 copies
327 were detected (Figure 3A). Important inter-sex differences were thus observed with a *VTG*
328 level in males ranging from about 1,900- to 6,800-fold lower than in females depending on
329 their stage. In other copepod species, an important gap between sexes was also reported but it
330 was reduced e.g. from 250 to 800 for *T. japonicus* and *T. kingsejongensis* respectively.
331 Otherwise, the Western blot analysis showed specific *Ea-VTG2* expression in ovigerous
332 females (stage 3; Figure 3B). The MM of the detected protein was low i.e. about 30 kDa in
333 comparison with the calculated MM. It thus corresponded to a truncated form of the protein,
334 localized in the C-terminal region as ensured by the position of the immunogenic peptide used
335 for the antibody production. The existence of truncated forms in both vertebrate and non-
336 vertebrate species is well-documented. Indeed, VTG is known as a precursor protein (i)
337 mainly synthesized in extra-ovarian sites e.g. hepatopancreas, liver or adipose tissues, (ii)
338 taken up by endocytosis in oocytes (iii) in the form of cleaved proteins i.e. vitellins (Hara et
339 al., 2016; Hyne, 2011; Tufail and Takeda, 2008). A C-terminal peptide was particularly well
340 described in fish species (Hara et al., 2016). Otherwise, no *Ea-VTG2* protein was detected in
341 either males or immature females (stage 1; Figure 3B). The lack of protein in males supports

342 the VTG specificity of the custom-made antibody. To our knowledge, no antibody specific to
343 copepod VTG has become available so far. Moreover, the absence of detection in immature
344 females despite a basal level of transcripts may highlight the differential sensitivity between
345 detection methods. The present study is the first report examining the relationship between the
346 VTG protein level and reproductive characteristics in copepod species. It complements the
347 many studies conducted in macrocrustaceans which have also demonstrated an increase of the
348 VTG protein during ovarian and/or oocyte maturation highlighting a gap between immature
349 and mature stages (Jubeaux et al., 2012; Lee and Chang, 1997; Tsukimura et al., 2000).
350 The gender-specific pattern of *Ea-VTG2* expression makes it a potential biomarker of
351 endocrine disruption as in other species and prompts us to investigate the *Ea-VTG2*
352 expression in males after exposure to chemicals.

353 *3.3. Effect of chemicals on vitellogenin expression in male E. affinis*

354 The *Ea-VTG2* expression was analysed in males after exposure to 4-NP (1 µg/L, 5 µg/L) and
355 MF (0.01 µg/L, 1 µg/L, 100 µg/L) for 24 h and 96 h respectively. We opted to examine
356 transcript levels by qPCR in order to evaluate the early response to exposures. This approach
357 – which benefits from specificity and sensitivity – is particularly appropriate for studying
358 gene expression in microcrustaceans since only a small amount of biological material is
359 needed. Nonylphenol is known as a toxic xenobiotic compound classified as an ED capable of
360 interfering with the hormonal system of numerous vertebrate organisms (Soares et al., 2008).
361 In macrocrustaceans, literature data report 4-NP effects such as reduced testis weight, altered
362 sexual differentiation, changes in ecdysteroid levels, and disruption in growth and moulting
363 cycles (Brown et al., 1999; Hirano et al., 2009; Lye et al., 2008). However, in copepods, no
364 alteration in either sex ratio or fecundity of *Tisbe battagliai* was recorded whereas 4-NP
365 induced a growth inhibition in *E. affinis* even if no evidence for endocrine disruption or
366 general toxic effect was established (Bechmann, 1999; Lesueur et al., 2013). In the present

367 study, no *Ea-VTG2* induction was shown for all 4-NP concentrations (Figure 4A). In
368 crustaceans, most of the studies focusing on the effects of chemicals were conducted on
369 females, mainly quantifying the VTG protein. To our knowledge, only one study reported the
370 effect of nonylphenol on the level of *VTG* transcripts in crustacean males (Xuereb et al.,
371 2011). An induction was thus highlighted in *Gammarus fossarum* males after a 16-days of
372 exposure at the lowest concentration i.e. 0.05 µg/L, even if substantial inter-individual
373 differences were shown. Most of the studies investigating protein levels also demonstrated
374 inductions in males and larvae after exposures to 4-NP (0.01 to 1,000 µg/L) in shrimps and
375 crabs (Ricciardi et al., 2008; Sanders et al., 2005). The effects of 4-NP on *VTG* expression in
376 males appear rather consistent in crustaceans since inductions were widely shown. However,
377 as demonstrated in the present study and by Lye and colleagues, no fluctuation was
378 demonstrated in some species e.g. *E. affinis* and *Carcinus maenas*, (Lye et al., 2008).

379 In addition to using 4-NP as a vertebrate ED model, we investigated the effect of a crustacean
380 hormone. We opted for using MF which is a sesquiterpene hormone of the juvenile hormone
381 family synthesised by the mandibular organ, and which has been shown to be implicated in
382 reproductive maturation and moulting in decapods by increasing the production of VTG and
383 eggs (LeBlanc, 2007). MF is also considered as a sex determinant in cladocerans (Olmstead
384 and Leblanc, 2002). Most of the studies dealing with the impact of MF exposure on decapod
385 physiology have shown opposite effects, e.g. on oocyte growth or egg production (Borst et al.,
386 1987; Laufer and Biggers, 2001; Mak et al., 2005; Nagaraju, 2011; Rodriguez et al., 2002;
387 Tsukimura and Kamemoto, 1991). In the present study, no *Ea-VTG2* induction was shown
388 whatever the MF concentration (Figure 4B). To our knowledge, this is the first study to
389 examine the male *VTG* transcript levels in crustaceans after MF exposure. Otherwise, *VTG2*
390 transcript levels were decreased during the growth of *D. magna* neonates (15.6 and 156 nM)
391 (Tokishita et al., 2006). Regarding protein levels, Jubeaux and colleagues reported an increase

392 of VTG in males (Jubeaux et al., 2012). The effects of MF on the *VTG* expression therefore
393 seem to fluctuate according to species and endpoints i.e. transcript or protein.

394 The impact of a multi-contamination was also evaluated by measuring the *Ea-VTG2* levels in
395 males sampled from the Seine estuary without any depuration (Figure 5). Indeed, the Seine
396 estuary – supporting almost 40% of French economic activity, 30% of its agricultural activity
397 and 50% of its river traffic – is one of the most impacted estuaries, displaying a wide range of
398 contaminants (<http://www.naiades.eaufrance.fr/>). The *in-situ* sampling was conducted during
399 two distinct seasons i.e. November 2015 and May 2016, characterized by different flood and
400 low-flow events underlying potential different contamination patterns. No *Ea-VTG2* induction
401 was highlighted. Thus, contrary to what has been described in fish, whatever the contaminants
402 – be they chemicals with vertebrate- or invertebrate-specific MoA (Mode of Action; e.g.
403 estrogen or ecdysteroid pathways respectively), alone or in mixtures, in controlled or field
404 conditions – the *Ea-VTG2* levels remained low, far below the female transcript rates. The
405 differential response of vertebrates and invertebrates to EDs may be explained by
406 fundamental differences in their endocrine physiology, from hormones and receptors to
407 organs.

408 Overall, the studies dealing with the effects of EDs on *VTG* expression in male, female and
409 neonate crustaceans have frequently reported variable responses (Table 2). The *VTG* levels
410 fluctuate according to the species, the sex/stage of the organism and the conditions of
411 exposure (compound, concentration, duration). These data suggest that the *VTG* variations
412 may be a consequence of a global physiological impairment rather than a specific effect of ED
413 compounds. Accordingly, induced or reduced *VTG* levels have been reported after exposure
414 to non-ED compounds such as acetone or chloroform (Table 2). Moreover, besides its role in
415 reproduction, *VTG* may be implicated in the defence response of organisms, as demonstrated
416 in both vertebrates and invertebrates (Wu et al., 2015; Zhang et al., 2011).

417 In conclusion, we identified a *VTG2* gene in *E. affinis* whose transcript levels gradually
418 increased during maturation in females. These data contribute to the understanding of *E.*
419 *affinis* reproduction and to a greater extent, of the reproductive physiology in model copepod
420 systems. No induction of *VTG2* by chemicals has been highlighted in either controlled or field
421 conditions. The present study reinforces literature data suggesting that *VTG* may be an
422 unreliable biomarker in arthropods and stresses the requirement for appropriate taxon-specific
423 biomarker validation (Jubeaux et al., 2012; Short et al., 2014).

424 **ACKNOWLEDGMENTS.** This research was funded by the French Ministry of Ecology,
425 Energy, Sustainable Development, Territorial Development and the Sea (MEEDDM), through
426 the national research program on endocrine disruptors (PNRPE) and by the ECOTONES
427 project of GIP Seine-Aval, Haute-Normandie (France).

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672

673 **LEGENDS**

674 **Table 1. Primers used for degenerate, RACE and quantitative PCRs in *E. affinis*.**

675 **Table 2. Overview of literature data reporting VTG fluctuations in crustaceans after**
676 **chemical exposure.** Compounds were classified according to their mode of action (MoA) i.e.
677 ‘vertebrate- specific’ (e.g. estrogen pathway), ‘invertebrate-specific’ (e.g. ecdysteroid
678 pathway) or ‘others’ (unknown).

679 **Figure 1. cDNA full-length sequence of vitellogenin (*Ea-VTG2*) of *E. affinis* (GenBank**
680 **accession no. AGH68974).** The Kozak sequence (CTCAATATGA; position 10-19) and a
681 polyadenylation site (AATAAA; 5,367-5,371) are boxed with light shading. The asterisk (*)
682 indicates the position of the termination codon (TAA). The signal peptide (position 1-21) is
683 depicted as a dark shaded region whereas the VTG specific domains i.e. vitellogenin_N
684 domain (position 25-701), DUF1943 superfamily domain (position 750-997) and von
685 Willebrand factor type D domain (VWD; position 1,462-1,623) are highlighted as open
686 boxes.

687 **Figure 2. Phylogenetic analysis of the *E. affinis* VTG after global alignment with 31**
688 **GenBank amino acid VTG sequences from three arthropod classes i.e. malacostraca,**
689 **maxillopoda and insecta.** The tree was constructed by Jukes-Cantor genetic distance model
690 and neighbor-joining method. The GenBank accession numbers of sequences were indicated
691 between square brackets. The scale bar represents 0.07 substitutions per site.

692 **Figure 3. *Ea-VTG2* expression during the reproductive cycle of *E. affinis* i.e. sexual**
693 **differentiation (♂, male; ♀, female) and oogenesis stage (stages 1 to 3).** (A) Study of *Ea-*
694 *VTG2* expression by qPCR. Each bar represents the mean of cDNA copy number ± standard
695 deviation (SD) of independent biological samples. Significant differences are indicated by

696 different letters ($p < 0.005$). (B) Study of *Ea-VTG2* expression by Western blot. The primary
697 antibody was a custom-made polyclonal antibody obtained after immunization of rabbits with
698 a synthetic immunogenic peptide designed from the deduced *E. affinis* VTG sequence. The
699 immunogenic peptide was used as a positive control (+).

700 **Figure 4. Levels of *Ea-VTG2* transcripts measured in *E. affinis* males after chemical**
701 **exposure.** Copepods were exposed either to 4-nonylphenol (4-NP) for 24 h (A), or to (E,E)-
702 Methyl Farnesoate (MF) for 96 h (B). A solvent control was conducted in the same conditions
703 as the contaminant exposures. Each bar represents the mean of cDNA copy number \pm
704 standard deviation (SD) of independent biological samples. Significant differences are
705 indicated by different letters ($p < 0.005$).

706 **Figure 5. Levels of *Ea-VTG2* transcripts measured in *E. affinis* males sampled from a**
707 **multi-contaminated estuary.** Each bar represents the mean of cDNA copy number \pm
708 standard deviation (SD) of independent biological samples. Significant differences are
709 indicated by different letters ($p < 0.005$).

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Table 1

Step	Primer name	Sequence (5'->3') initiale	nucleotide position
degenerate PCR	D5F1	gctatggttgaacyaaccc	1,397-1,416
	D5R	cggcrgcaatwccgacttc	1,981-1,999
RACE-PCR	3'-GSP1	gccaccataacctcattatatcagag	1,487-1,511
	5'-GSP2	agagtgcctgagggagtagacagcaa	1878-1902
Real-time qPCR	VTG-Ea-qS1	actgcagagaaacagattgcat	5,156-5,177
	VTG-Ea-qAS1	ggctctcctcataatgtcttg	5,208-5230

Table 2

	Compound	Species	Gender/stage	Variations	Endpoint	Reference
Vertebrate-specific MoA	17b-estradiol	<i>Palaemon elegans</i>	larvae	reduced	protein	Sanders et al., 2005
		<i>Sicyonia ingentis</i>	female	unchanged	protein	Tsukimura et al., 2000
	4-nonylphenol	<i>Daphnia magna</i>	female	induced	transcript	Hannas et al., 2011
		<i>Carcinus maenas</i>	male	unchanged	protein	Lye et al., 2008
		<i>Charybdis japonica</i>	mixed gender	induced	transcript	Park et al., 2014
		<i>Palaemon elegans</i>	larvae	induced	protein	Sanders et al., 2005
		<i>Gammarus fossarum</i>	male	induced	transcript	Xuereb et al., 2011
		<i>Carcinus aestuarii</i>	male	induced	protein	Ricciardi et al., 2008
	Atrazine	<i>Daphnia magna</i>	female	reduced	transcript	Hannas et al., 2011
	Benzophenone	<i>Gammarus fossarum</i>	male	unchanged	protein	Jubeaux et al., 2012
	Bisphenol A	<i>Daphnia magna</i>	neonate	reduced	transcript	Jeong et al., 2013
		<i>Charybdis japonica</i>	mixed gender	induced	transcript	Park et al., 2014
	Carbamazepine	<i>Gammarus fossarum</i>	male	unchanged	protein	Jubeaux et al., 2012
	Chlordane	<i>Daphnia magna</i>	female	induced	transcript	Hannas et al., 2011
		<i>Neocaridina denticulata</i>	male	induced	protein	Huang et al., 2004
		<i>Neocaridina denticulata</i>	female	reduced	protein	Huang et al., 2006
	Chlordecone	<i>Macrobrachium rosenbergii</i>	male	unchanged	transcript	Lafontaine et al., 2016
		<i>Macrobrachium rosenbergii</i>	female	induced	protein	Lafontaine et al., 2016
		<i>Eurytemora affinis</i>	male	reduced	transcript	Legrand et al., 2016
		<i>Eurytemora affinis</i>	female	induced	transcript	Legrand et al., 2016
		<i>Macrobrachium rosenbergii</i>	post-larvae	induced	protein	Lafontaine et al., 2017
	Cyproterone	<i>Gammarus fossarum</i>	male	induced	protein	Jubeaux et al., 2012
		<i>Gammarus fossarum</i>	male	induced	transcript	Xuereb et al., 2011
		<i>Daphnia magna</i>	female	reduced	transcript	Hannas et al., 2011
	Estrogen	<i>Palaemon elegans</i>	larvae	reduced	protein	Sanders et al., 2005
	Estrone	<i>Neomysis integer</i>	juvenile	reduced	protein	Ghekiere et al., 2006
	Lindane	<i>Neocaridina denticulata</i>	male	unchanged	protein	Huang et al., 2004
<i>Neocaridina denticulata</i>		female	induced	protein	Huang et al., 2006	
Progesterone	<i>Sicyonia ingentis</i>	female	unchanged	protein	Tsukimura et al., 2000	
Propiconazole	<i>Gammarus fossarum</i>	male	induced	protein	Jubeaux et al., 2012	
R-propranolol	<i>Gammarus fossarum</i>	male	unchanged	protein	Jubeaux et al., 2012	
Triclosan	<i>Daphnia magna</i>	female	reduced	transcript	Hannas et al., 2011	
17a-hydroxyprogesterone	<i>Sicyonia ingentis</i>	female	unchanged	protein	Tsukimura et al., 2000	
Invertebrate-specific MoA	20-hydroxyecdysone	<i>Tigriopus kingsejongensis</i>	female	unchanged	transcript	Lee et al., 2016
		<i>Daphnia magna</i>	female	reduced	transcript	Hannas et al., 2011
		<i>Gammarus fossarum</i>	male	unchanged	protein	Jubeaux et al., 2012
		<i>Gammarus fossarum</i>	female	unchanged	protein	Jubeaux et al., 2012
	Farnesoic acid	<i>Tigriopus kingsejongensis</i>	female	induced	transcript	Lee et al., 2016
	Methoprene	<i>Neomysis integer</i>	juvenile	unchanged	protein	Ghekiere et al., 2006
	Methoxyfenozide	<i>Gammarus fossarum</i>	male	induced	protein	Jubeaux et al., 2012
		<i>Tigriopus kingsejongensis</i>	female	unchanged	transcript	Lee et al., 2016
	Methyl farnesoate	<i>Gammarus fossarum</i>	female	reduced	protein	Simon et al., 2010
		<i>Gammarus fossarum</i>	male	induced	protein	Jubeaux et al., 2012
		<i>Gammarus fossarum</i>	female	reduced	protein	Jubeaux et al., 2012
		<i>Daphnia magna</i>	female	unchanged	transcript	Hannas et al., 2011
	Piperonyl butoxide	<i>Daphnia magna</i>	female	induced	transcript	Hannas et al., 2011
	Ponasterone	<i>Daphnia magna</i>	female	reduced	transcript	Hannas et al., 2011
	Pyriproxyfen	<i>Eurytemora affinis</i>	female	reduced	transcript	Legrand et al., 2016
		<i>Gammarus fossarum</i>	male	reduced	transcript	Trapp et al., 2015
<i>Gammarus fossarum</i>		neonate	reduced	transcript	Tokishita et al., 2006	
Others	Acetone	<i>Daphnia magna</i>	female	reduced	transcript	Hannas et al., 2011
	Arsenic	<i>Paracyclopsina nana</i>	mixed gender	induced	transcript	Hwang et al., 2010
		<i>Tigriopus japonicus</i>	mixed gender	unchanged	transcript	Lee et al., 2008
		<i>Daphnia magna</i>	female	induced	transcript	Hannas et al., 2011
	Cadmium	<i>Paracyclopsina nana</i>	mixed gender	induced	transcript	Hwang et al., 2010
		<i>Tigriopus japonicus</i>	mixed gender	induced	transcript	Lee et al., 2008
		<i>Sinopotamon henanense</i>	female	reduced	transcript	Yang et al., 2017
	Chloroform	<i>Sinopotamon henanense</i>	female	reduced	protein	Yang et al., 2017
		<i>Daphnia magna</i>	female	induced	transcript	Hannas et al., 2011
	Copper	<i>Tigriopus japonicus</i>	mixed gender	unchanged	transcript	Lee et al., 2008
<i>Paracyclopsina nana</i>		mixed gender	induced	transcript	Hwang et al., 2010	
Silver	<i>Tigriopus japonicus</i>	mixed gender	unchanged	transcript	Lee et al., 2008	

1656 Q G A N S I N K Q V E K S P V S N I L R
5041 GGCAACCTTGAAAAGTGGCTCCAGATGAAGCATTGATTATCAGGCAATCTGACAAAGTC
1676 G N L E K C S Q M K H L I I S Q S D K V
5101 TGTTTCTCTCAACTTCCATTGTTGAAATGGAAGTGGATGTTCTCCAAGATCAACTGCA
1696 C F S Q L P I V E C G S G C S P R S T A
5161 GAGAAACAGATTGCATTCTCTGTCTTCCCTGACCGTAGAGTCAACAAGACATTATGAG
1716 E K Q I A F S C L P A D R R V T R H Y E
5221 GAGAGGCCAGACAAGGAGAAGTTCTTCTGAGCTCAGAAACATGGAGAAAACCTTCTCT
1726 E R A R Q G E V L P E L R N M E K T F S
5281 TCCAAGATAGAAATCCAAACCGATGCCCCACCTGGACTTAAATAACAATTATTGA
1756 S K I E I P T G C A H P G L -
5341 ACAAATGTTTTTCTGCAAAAATGGATAAAAAATACCATCCTAAAAA
5401 AAAAAAAAAAAAAA

Figure 2

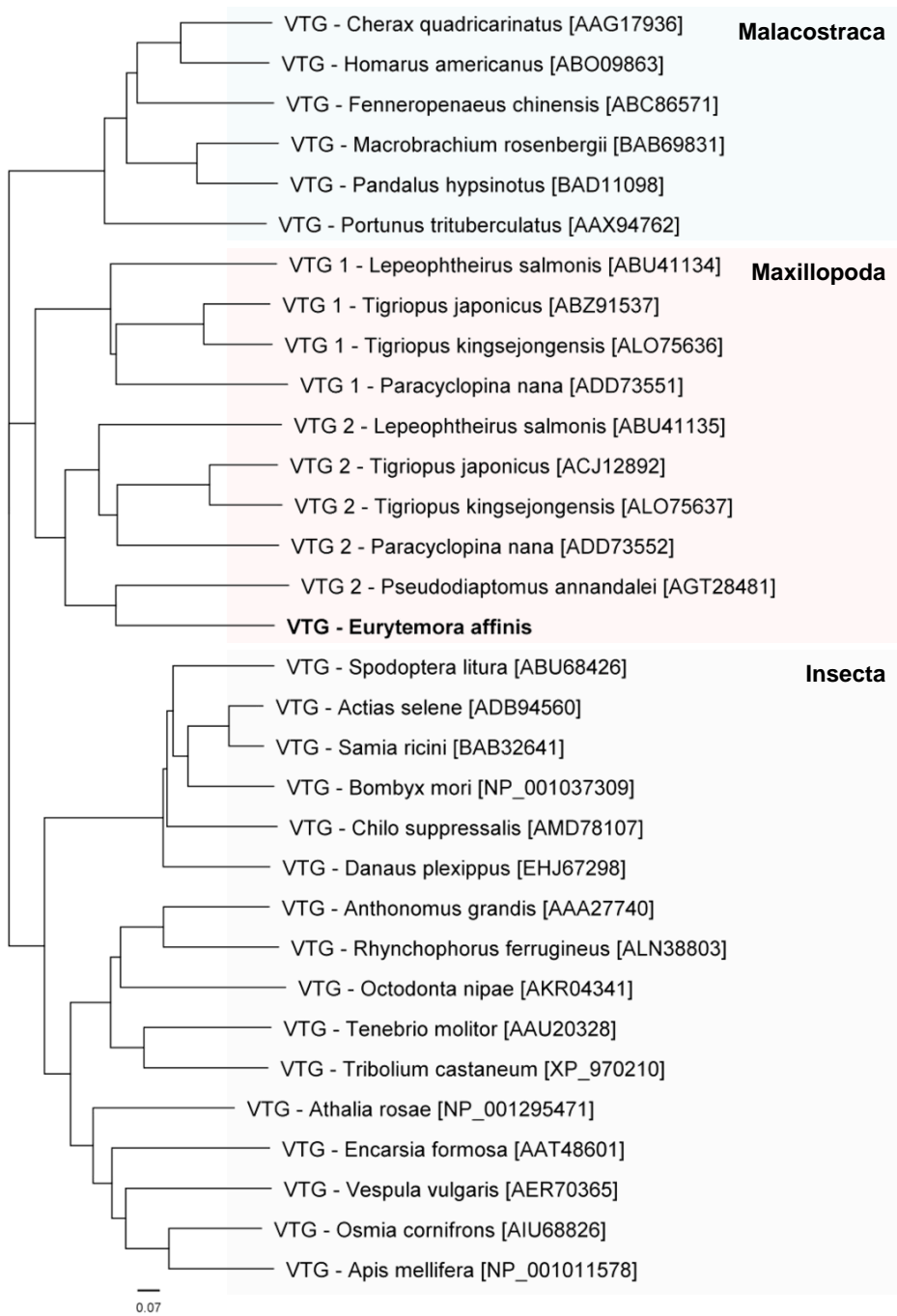
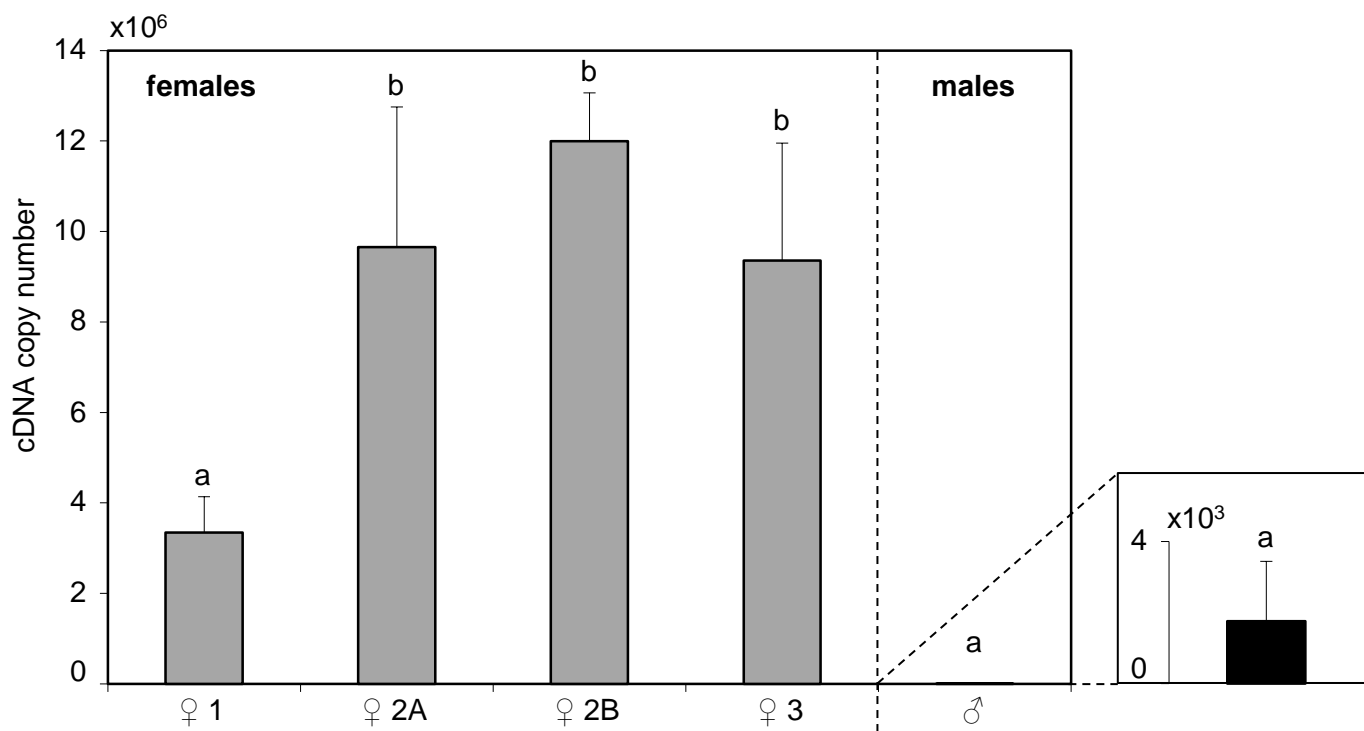


Figure 3

A



B

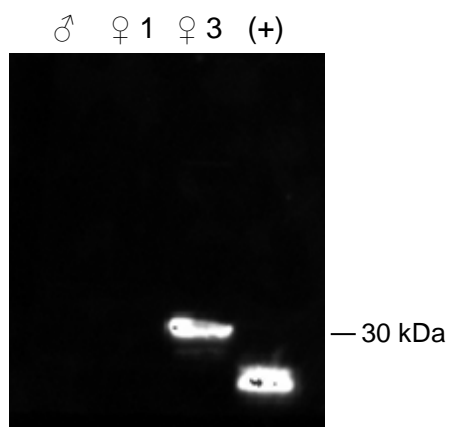
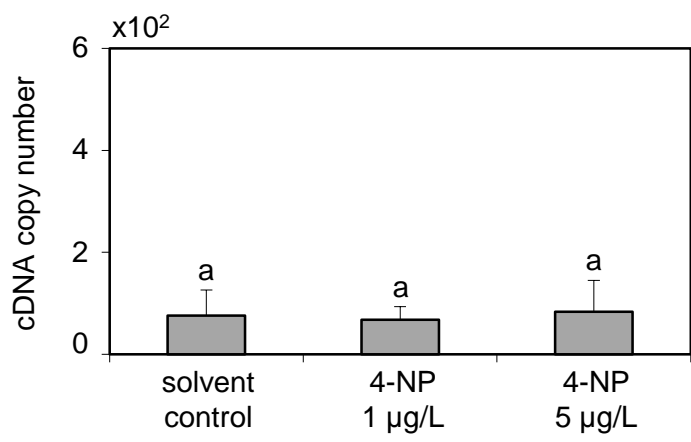


Figure 4

A



B

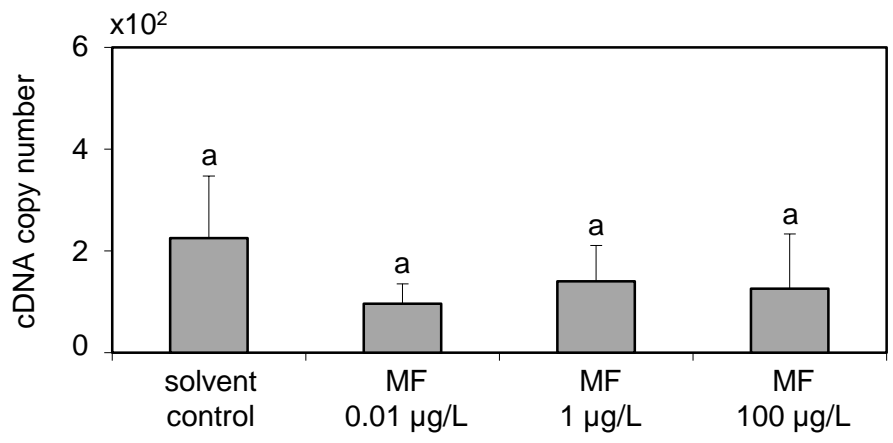


Figure 5

