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Genotoxicity and activation of cellular defenses in transplanted zebra mussels *Dreissena polymorpha* along the Seine river

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Abstract :

The aim of the present study was to confirm the relevance of studying DNA adduct formation in a field study. In that context, freshwater mussels Dreissena polymorpha, collected in a reference station, were transplanted in different sites with a pollution gradient. After one and two months, mussels were collected and DNA adduct formation was analyzed using the ^{32}P post labelling technique on both gills and digestive glands. In addition, the expression of genes involved in the detoxification system (catalase (CAT), superoxide dismutase (SOD), glutathione S-transferase (GST), HSP70, aryl hydrocarbon receptor (AHR), P glycoprotein (PgP), metallothionein (MT)) was assessed by RT-PCR. DNA adducts were observed at amount comparable to data from literature. Increase of DNA adducts after two months of transplantation could be correlated with strong modulation of gene expression implicated in detoxification processes. Indeed, PgP and HSP70 gene expressions were similarly induced in gills and digestive glands while SOD and CAT expressions were down regulated in both tissues. AHR, GST and MT genes were differently regulated depending upon the tissue studied and the level of contamination in the different sites. We demonstrated that mussels transplanted in the different stations with pollution gradient were able to biotransform PAHs, assessed by DNA adduct formation and the high decrease of detoxification genes. Specific DNA adducts pattern obtained after one and two month mussel transplantations demonstrated the relevance of DNA adduct as biomarker of environmental pollution.

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Highlights

►*Dreissena polymorpha* mussels were transplanted in polluted sites along the Seine river. ►Expression of detoxification genes depended upon the tissue and the level of pollution. ►DNA adducts were persistent for one and two months of exposure to pollutants. ►Mussels were able to detoxify PAHs assessed by the low DNA adduct level.

Keywords : Biomarker, Pollution, DNA adducts, Detoxification, *Dreissena polymorpha*, RT-PCR

1. Introduction

The freshwater mussel *Dreissena polymorpha* (Pallas, 1771) has been largely used for biomonitoring in lakes and in rivers (Binelli et al., 2001, Guerlet et al., 2007, Bacchetta and Mantecca, 2009 and Bourgeault et al., 2010). Indeed, those animals present some advantages as bioindicator species: (i) they are sessile filter feeders capable of accumulating large amount of pollutants and especially metallic and hydrophobic contaminants such as polycyclic aromatic hydrocarbons (PAHs) (Fisher et al., 1993 and Bourgeault et al., 2010); (ii) they are widespread along the world (Morton, 1997) and the Seine river (Akopian et al., 2001); (iii) the fact that they live attached to rocky surfaces make the sampling easy.

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57

58 Seine basin is subjected to urban development since the last two centuries leading to an
59 increase of metals and PAHs in water, derived from domestic, industrial wastewater and
60 agricultural activities (Chevreuil and Garnier, 1991; Meybeck, 1998; Boët et al., 1999;
61 Tusseau-Vuillemin et al., 2007). Some compounds, like PAHs, are metabolized by zebra
62 mussels into electrophilic metabolites that have a high affinity to macromolecules such as
63 DNA and form bulky DNA adducts (Le Goff et al., 2006). These bulky DNA adducts have
64 been recognized to play a role in the initial step of chemical carcinogenesis in vertebrates
65 (Van deer Oost et al., 1996; Pfohl-Leszkowicz, 2008).

66

67 In that context, so as to evaluate the impact of a contaminant mixture on biota health,
68 molecular biomarkers have demonstrated their interest in biomonitoring studies. Biomarkers
69 of DNA alteration (DNA strand breaks, micronuclei or DNA adducts) have been shown to be
70 reliable indicators of genotoxic impact caused by pollutants, in zebra mussels (Rocher et al.,
71 2006; Binelli et al., 2007; Bourgeault et al., 2010). Indeed, a few studies in fluvial or lake
72 areas have underlined the interest of using DNA adducts (Pfohl-Leszkowicz et al 1993;
73 LeGoff et al., 2006; Rocher et al., 2006; Al-Subiai et al., 2012; Cachot et al, 2013) and DNA
74 strand breaks (Binelli et al. 2007; Jha, 2008; Michel et al., 2013) for biological effects
75 evaluation of chemical contamination. DNA adducts formation was observed in both marine
76 mussels (Akcha et al 2000 ; Skarpheoinsdottir et al., 2003; Pisoni et al., 2004; Amat et al,
77 2004; Bocquéné et al, 2004 ; Rocher et al., 2006) and freshwater mussels (LeGoff et al., 2006
78 and Rocher et al., 2006; Châtel et al., 2012), exposed to model PAHs which indicate that
79 these species are able to metabolize benzo[a]pyrene (B[a]P) into reactive metabolites that can
80 bind to DNA and form DNA adducts. Moreover, field experiments have demonstrated a
81 correlation between level of PAHs pollution and formation of DNA adduct in freshwater
82 mussels (Le Goff et al., 2006; Rocher et al., 2006).

83

84 It has been highly described in Mammals that PAHs enter the cells through the Aryl
85 hydrocarbon receptor (AHR) and then are metabolized by phase I and II enzymes. Very
86 recently, a battery of genes implicated in biotransformation and detoxification processes were
87 sequenced in *D. polymorpha* (Navarro et al., 2010; Contardo-Jara et al., 2011) : the
88 glutathione S-transferase (GST), the phase I biotransformation enzyme ; the superoxide
89 dismutase (SOD), responsible for the reduction of the superoxide radical to hydrogen
90 peroxide; the catalase (CAT), catalysing the breakdown of hydrogen peroxide to water and

91 oxygen (van der Oost et al., 2003; Bard, 2000); the HSP70 and the transmembrane protein
92 transporter P-gp1, which acts as efluxing xenobiotics out of cells (Pain and Parant, 2003;
93 2007; Minier et al., 2006). In Invertebrates, metallothionein (MT) is also widely thought to
94 play an important role in metal detoxification and in protecting cells against oxidative stress
95 (Viarengo et al., 2000; Cavaletto et al., 2002). Rocher et al. (2006) have observed an
96 induction of the antioxidant enzymes GST, catalase and SOD in gills of zebra mussels
97 collected in sites along the Seine River compared to reference site and a clear relationship
98 between SOD and GST activities and amounts of bioaccumulated metals and PAHs. Regoli
99 and Principato (1995) have also demonstrated a strong induction of SOD and Gpx activities in
100 *M. galloprovincialis* exposed to a complex mixture of metals in field conditions. Seasonal
101 changes and especially temperature combined with pollution level in different collection sites
102 were demonstrated to affect expression of genes encoding HSP70, SOD, MT in the oyster
103 *Crassostrea gigas* (Farcy et al., 2007).
104
105 The aim of this study was to get an insight into the presence of DNA adduct in zebra mussels
106 transplanted into Seine river sites along a pollution gradient so as to validate the interest of
107 using DNA adduct as biomarkers of contamination in *D. polymorpha*. Because only few
108 studies have compared the adduct levels in various tissues, we choose to compare those
109 amounts in gills and digestive glands of zebra mussels. In order to confirm the persistence of
110 DNA adduct observed in laboratory experiments (Châtel et al., 2012), mussels were
111 transplanted into three sites of the Seine river and also on the reference site, which correspond
112 to the sampling site, for 1 and 2 months. Moreover, expression of some genes implicated in
113 phase I and II detoxification mechanisms as well as biomarkers involved in different
114 metabolic pathways related to xenobiotic exposure were analysed in these mussels.

115 **2. Material and methods**

116 ***Mussel sampling and maintenance conditions***

117 Adult zebra mussels *Dreissena polymorpha* (Pallas, 1771) 18-22mm long, were collected in
118 Mars 2011 in a reference site Vertuzey (France) (48°45’33’’N, 5°36’05’’W). This site is
119 located, far away from every city, not subjected to urban contamination, in the Meuse river
120 North-eastern of France. Animals were transferred to laboratory, cleaned of all fouling
121 organisms and kept in tank containing raw water originating from Vertuzey site (16 °C) until
122 field deployment.
123

124 ***Mussel caging and study sites***

125 Mussels were placed into polyethylene 5mm-mesh experimental cages as previously
126 described (Bourgeault et al., 2010). Thirty mussels were transplanted for 1 or 2 months on
127 each of the three sites of the Seine river basin (France) (Fig.1). Thirty mussels were also
128 transplanted at the reference site of Vertuzey during the same time. The site of Marnay-sur-
129 Seine was located 200 km upstream Paris while the two other sites, Bougival and Triel-sur-
130 Seine were located downstream Paris and subjected to urban pollution as described in
131 previous studies (Fernandes et al., 1997; Bourgeault et al., 2010). The site of Triel is
132 subjected to both urban contamination and waste water discharge from domestic and
133 industrial sewages. After one and two months of transplantation, mussels were collected;
134 digestive glands and gills were dissected and stored at -80°C for further analysis.
135
136 ***^{32}P postlabeling Analysis of DNA adducts***
137
138 *DNA isolation*
139 DNA isolation has been done as described by Pfohl-Leszkowicz & Castegnaro 2005. In brief,
140 digestive glands or gills were homogenized in a solution containing NaCl (0.1 M), EDTA (20
141 mM) and Tris-HCl, pH 8 (50 mM) (SET). Proteins were precipitated by addition of SDS and
142 of potassium acetate (6 M, pH 5). The supernatant, which contained nucleic acids, was
143 collected and nucleic acids were precipitated overnight at -20 °C by adding 2 volumes of cold
144 ethanol. RNAs were eliminated following treatment by RNase A and RNase T1. Samples
145 were then treated with proteinase K solution (20 mg/mL SET) for 1 h at 37 °C. After
146 digestion, DNA was extracted by rotiphenol (phenol saturated by Tris). The aqueous phase
147 was collected after two extractions. After a final extraction with one volume of
148 chloroform/isoamyl alcohol (24:1), the aqueous phase was collected. The DNA was
149 precipitated by the addition of two volumes of cold ethanol overnight at -20°C. The purity of
150 the DNA was checked by recording UV spectra at 220 and 320 nm.
151
152 *^{32}P postlabeling*
153 DNA adducts were detected using the validated nuclease P_1 enrichment method (Philipps &
154 Castegnaro, 1999) and the separation was done after contact transfer (Pfohl-Leszkowicz &
155 Castegnaro, 2005). In brief, DNA (4µg) was digested at 37 °C for 4 h with 10 µL of the mix
156 containing of micrococcal nuclease and spleen phosphodiesterase. The digested DNA was
157 then treated with nuclease P1 at 37 °C for 45 min. The DNA adducts were labeled as follows.
158 To the NP1 digest, 5 µL of the reaction mixture containing 2 µL of bicine buffer [Bicine (800

159 μM), dithiothreitol (400 mM), MgCl2 (400 mM), and spermidine (400 mM) adjusted to pH
160 9.8 with NaOH], 9.6 U of polynucleotide kinase T4, and 100 μCi of [^{32}P]ATP (specific
161 activity 6000 Ci/mmol) was added and incubated at 37 °C for 45 min. Normal nucleotides,
162 pyrophosphate, and excess ATP were removed by chromatography on PEI/cellulose TLC
163 plates in 2.3 M NaH_2PO_4 buffer, pH 5.7, overnight (D1). The origin areas containing labeled
164 adducted nucleotides were cut out and transferred to another PEI/cellulose TLC plate, which
165 was run in 5.3 M lithium formate and 8.5 M urea (pH 3.5) for 3 h (D2). A further migration
166 was performed after turning the plate 90° anticlockwise in 1 M LiCl, 0.5M Tris and 8 M urea
167 (pH 8) for 2 h (D3). Finally, the chromatogram was washed in the same direction in 1.7 M
168 NaH_2PO_4, pH 6, for 2 h (D4). The N2 dG B[a]P adduct obtained during the EU project
169 (Philipps and Castegnaro 1999) was run in the same conditions and serve as standard for
170 quantification.
171 Radioactive spots were detected by autoradiography on Kodak super X-Ray film.
172 Autoradiography was carried out in the presence of an intensifying screen at -80 °C for 48 h.
173 (Amat-Bronnert et al., 2007). The radioactivity was measured by a phosphor imager as
174 described below.
175
176 *Quantitation of total DNA Adducts*
177 For the quantification of total DNA adducts, the TLC plates were then placed in a storage
178 phosphor cassette containing a storage phosphor screen (Amersham) and exposed overnight.
179 Results are digitized using a storage phosphor imaging system (Typhoon™ 9210, Amersham)
180 and quantitated using ImageQuant™ 5.0 software. After background subtraction, the levels
181 of DNA adducts were expressed as relative adduct labelling (RAL) in total nucleotides. To
182 calculate the levels of screen response (screen pixel) in dpm (disintegration per minute),
183 samples of ^{32}P-ATP at different concentrations from 10 to 500 dpm were appropriately
184 diluted and spotted on TLC plate. This TLC plate was then analysed on the Typhoon with the
185 samples to obtain a radioactivity scale. The sensitivity allows detection of B[a]P adduct as
186 low as 0.1 nucleotide/ 10^{10} nucleotides.
187
188 ***RNA extraction, RT-PCR and quantitative RT-PCR analysis***
189 Total RNA from control and exposed mussels was extracted using TRIzol Reagent as
190 described by Grebenjuk et al., 2002. RNA concentration and purity was measured by
191 spectrophotometric absorption at 260 and 280 nm. First strand cDNA synthesis was carried
192 out on 1 μg of total RNA extract with oligo-dT primers according to Improm II Reverse

193 Transcriptase kit (Promega). Preparations of digestive glands and gills cDNA were used to
194 quantify specific transcripts in LightCycler 480 Real Time PCR System (Biorad) using SYBR
195 Green Power Master Mix (Invitrogen) with the specific primer pairs (Table 1). Relative
196 mRNA abundances of different genes were calculated from the second derivative maximum
197 of their respective amplification curves (Cp). Cp values for target genes (TG) were compared
198 to the corresponding values for a reference gene (ribosomal S3 gene) to obtain ΔCp values
199 (ΔCp = Cpref - CpTG). PCR efficiency values for reference and tested genes were calculated
200 as described (Pfaffl, 2001). Results of gene expression from mussels collected in the different
201 sites were chosen to be compared to those of mussels from Vertuzey site as they were native
202 from this site; moreover this site is considered as the reference according to chemical analysis
203 (Michel et al., 2012).
204
205 ***Statistical analysis***
206 Adduct and RT-PCR results are given as mean values ± S.D. of 3 repetitions of each test
207 (10 mussels per condition pooled for DNA adduct analysis and 3 mussels per condition
208 pooled for RT-PCR measurements). The measured values were compared among different
209 locations using an Analysis of Variance (ANOVA) followed by a Tukey post hoc test.
210 Statistical significance was accepted at a $P < 0.05$ (*), $P < 0.01$ (**) and $P < 0.001$ (***).
211

211 **3. Results**

212 ***3.1 DNA adduct level***

213 An example of DNA adducts pattern is presented figure 2. Depending on the location, the
214 number of individual adducts ranged from 1 to 6 adducts. In reference location a faint adduct
215 numbered #a, was observed, and does not correspond to the other adducts. Comparison of
216 DNA adducts pattern from the three sites on Seine river showed that pollutants were not the
217 same everywhere. One adduct (#1) was present in the three locations, whereas the other ones
218 were specific to Marnay site (# 5; 6; 7) or to Triel (# 2; 3). The adduct number # 4 was
219 common to Bougival and Triel sites. Total DNA in gills and digestive glands are shown in
220 figures 3A and 3B respectively.

221

222 Concerning the gills (Fig. 3A), highest amounts of total DNA adducts were detected in
223 mussels encaged for 1 and 2 months in Bougival site (13.5 and 16.7 adducts/10^9 nucleotides,
224 respectively). Mussels from Triel station depicted the lowest quantity of DNA adducts for
225 both time exposure (3 and 5.2 adducts/10^9 nucleotides, respectively). After 2 months of
226 caging, quantity of DNA adducts increased in mussels collected from the three different sites.
227 As regard to mussels transplanted in Vertuzey site, they did not present any significant DNA
228 adducts (below limit of quantification) for both time exposure tested.

229

230 The highest amounts of DNA adducts in digestive gland was detected in mussels transplanted
231 to Marnay for one month (11.7 adducts/10^9 nucleotides). It is necessary to mention that this
232 high amount was mainly due to one specific adduct (# 7) observed at this site (Fig.3B).
233 Higher amount of DNA adducts was observed in digestive gland of mussels from Triel site
234 transplanted one month compared to Bougival site (6.8 versus 5.5 adducts/10^9 nucleotides,
235 respectively). After 2 months of transplantation, a significative decrease of total amount of
236 DNA adducts was observed at Marnay site, whereas it increased in Triel and Bougival sites.

237

238 ***3.2 Gene expression***

239 In order to investigate some of the mechanisms of detoxification, we performed gene
240 expression analysis in the freshwater zebra mussel using quantitative RT-PCR. S3 ribosomal
241 gene was chosen as the reference gene for normalization as previously demonstrated
242 (Contardo-Jara et al. 2010; Châtel et al. 2012).

243 In gills, after one month of transplantation, AHR mRNA expression was 10 times
244 lower in mussels from Marnay, Bougival and Triel stations than those transplanted in

245 Vertuzey site (reference site). The same tendency was observed in the digestive glands, as
246 AHR mRNA expression was drastically reduced in mussels transplanted in the Seine river
247 sites as compared to Vertuzey (Fig. 5 A).
248 On the contrary, after 2 months of caging, mRNA level in gills increased significantly in all
249 mussels transplanted in the Seine river sites, with a seven times increase in animals
250 transplanted at Bougival (Fig. 4A), compared to Vertuzey.
251 Concerning SOD gene, its expression was about 10 times decreased in gills and from 2
252 to 100 times in digestive glands in mussels encaged for 1 and 2 months in Marnay, Bougival
253 and Triel compared to Vertuzey (Fig. 4 B; fig. 5 B).
254
255 The same profile was observed for CAT gene expression in gill of mussels which
256 reduction was significant in all stations compared to control (Fig. 4 C). However, in the
257 digestive glands, only a significant CAT induction (about 1.5 fold) was noticed after 2 months
258 of caging in Triel compared to Vertuzey (Fig. 5 C).
259
260 GST mRNA level were only significant in gills of mussels from Marnay after 1 and 2
261 months (about 1.5 to 3 times). For all the other conditions, a significant decrease in GST
262 expression was observed (Fig. 4 and 5 D).
263
264 P-gP gene expression was induced in gills of mussels encaged in Bougival after 1 and
265 2 months, and only after 1 month in digestive glands (Fig.4 E). The highest induction was in
266 gills after one month of transplantation (9 times compared with Vertusey) (fig. 5 E). A slight
267 induction of P-gP mRNA level was also observed in gills of mussels from Marnay following
268 1 month of caging and 2 months of caging in both tissues.
269 A slight induction in PgP mRNA level was also noticed in mussels transplanted in Triel after
270 2 months of caging. For all of the other time points, a significant decrease of P-gP expression
271 was observed (Fig. 4 and 5 E).
272
273 As shown in figure 4 (F), HSP70 gene expression was significantly increased in gills
274 of mussels encaged in all stations for 1 month, with the highest level observed for mussels
275 collected in Marnay (4 fold increase compared to reference site). After 2 months of caging, a
276 decrease of its expression was observed in gills of mussels collected in all stations, compared
277 to Vertuzey. Digestive glands depicted the highest level of HSP mRNA in mussels encaged in
278 Marnay and Triel for 1 months, with a 4 and 13 fold increase compared to reference site,

279 respectively, whereas after 2 months, only an induction of HSP 70 mRNA expression was
280 noticed in digestive glands of mussels from Triel (16 fold increase compared to control
281 group) (Fig. 5 F).
282
283 Metallothionein gene expression was significantly decreased in all conditions tested
284 with the exception of a high induction in digestive glands of mussels transplanted in Marnay
285 and Triel for 2 months (Fig. 4 and 5 G). Interestingly, compared with others genes measured
286 in this study, the highest induction was observed with MT gene, with a 40 times induction in
287 the digestive glands of mussels transplanted at Marnay and Triel for 2 months (fig. 4 G).
288
289

289 **4. Discussion**

290 In the present study, formation of bulky DNA adducts and induction of genes
291 implicated in oxidative stress were investigated in zebra mussels *D. polymorpha* transplanted
292 at sites with different amount of organic and metallic pollution along the Seine river
293 (Tusseau-Vuillemin et al., 2007). This study aimed at getting a better understanding of the
294 relationship between genotoxicants exposure and biological responses in terms of DNA
295 damage and induction of genes implicated in detoxification mechanisms in transplanted
296 mussels so as to demonstrate the suitability of measuring DNA adducts for genotoxicant
297 monitoring in freshwater environments. All results obtained in this study are summarized in
298 table 2.

299

300 ***DNA adduct formation***

301 DNA adducts are formed in gills and digestive glands of mussels transplanted either at
302 the reference site of Vertuzey or at three contaminated sites along the Seine River, Marnay,
303 Bougival and Triel. The total DNA adduct amount ranged from 3 to 16.7 adducts/10^9
304 nucleotides in gills and from 5.5 to 11.8 adducts/10^9 nt in the digestive glands. The highest
305 amount was measured in gills of mussels from Bougival (16.7 adducts/10^9 nt), mainly due to
306 one specific adduct. Skarphédinsdóttir et al. (2007) also showed higher levels of DNA
307 adducts in gills of marine mussels exposed to PAHs in Nordic sea sites compared to digestive
308 gland. Compared with other field studies, DNA adduct amount measured in this study was in
309 the same range. Indeed, zebra mussels collected in the Seine estuary presented DNA adduct
310 levels from 3.9 to 10 /10^9 nt (Le Goff et al., 2006). In the same way, Rocher et al. (2006) also
311 observed DNA adduct amounts in zebra mussels collected in stations from the Seine estuary
312 and the Seine Bay around 10 adducts/10^9 nt.

313

314 The same range of DNA adduct formation was previously noticed in laboratory experiments
315 where *D. polymorpha* exposed to Benzo[a]pyrene depicted DNA adducts ranging from 1.3 to
316 5.19 adducts/10^9 nucleotides in gills and from 0.9 to 2.29 adducts/10^9 nucleotides in the
317 digestive glands (Châtel et al., 2012). This was also comparable to marine mussels exposed to
318 the same xenobiotic (Canova et al., 1998; Skarpheoinsdottir et al., 2003; Akcha et al., 2000).
319 Michel et al. (2013) observed that Bougival and Triel presented highest concentrations of
320 PAHs in water in April and June compared to Marnay site, correlated with high concentration
321 of PAHs in soft tissues, indicating that mussels were not able to detoxify those compounds.
322 Formation of specific adducts site depending indicated that the nature of pollutants is

323 probably not exclusively related to PAHs. Some of specific adducts observed in Triel can be
324 due to waste water sewage for example. Two adducts were only observed at Bougival and
325 Triel and thus can be due to urban and industrial activities in Paris. DNA adducts are
326 dependent upon many factors such as activity of the organism, feeding, lipid content and
327 reproductive conditions (Skarpheoinsdottir et al., 2005) but also from biotransformation
328 pathway (Pfohl-Leszkowicz, 2008). In addition, the balance between activating and
329 detoxifying enzymes in cells such as glutathione S-transferase, cytochrome P450 have been
330 demonstrated to vary seasonally (Kirchin et al., 1992; Wooton et al., 1996; Shaw et al., 2000;
331 Rocher et al., 2006). Moreover, physico-chemical parameters such as temperature could also
332 affect the reactivity of mussels to contaminants (Buschini et al., 2003).
333
334 In our study, we observed that DNA adduct pattern was similar and in the same range after 2
335 months of caging, except in Marnay site. If we only take into account the adduct # 1, which is
336 common to the three sites, it appears that it increases at the both sites downstream Paris,
337 whereas it decreases at Marnay. The specific adduct found in organs of mussels from Marnay
338 could be due to other pollutants including pesticides or pharmaceuticals (Ginebreda et al,
339 2014). Globally, more individual adducts and higher amount of adduct # 1, which correspond
340 to B(a)P adducts, are formed in digestive glands rather than in gills as mostly demonstrated in
341 literature (Châtel et al., 2012; Skarpheoinsdottir et al., 2003). In a previous laboratory study,
342 we demonstrated that zebra mussels induced a tissue-specific formation of DNA adducts after
343 5 days of B[a]P exposure and that levels were persistent 28 days after the end of exposure
344 (Châtel et al., 2012). Skarpheoinsdottir et al. (2003) have proved that in *Mytilus*
345 *galloprovincialis* gills exposed to PAHs, DNA adducts were persistent after 2 weeks of
346 exposure (whereas PAHs tissue concentration was not detectable in gills at this time point)
347 indicating the interest of measuring DNA adduct levels rather than PAHs bioaccumulation in
348 tissues.
349
350 ***Metabolism gene expression***
351 This study also investigated induction of gene expression of enzymes implicated in cellular
352 defense processes that represent the initial steps of a cascade of events linking environmental
353 insults to ecological impact (Van der Oost et al., 2003).
354
355 In gills, an increase in AHR and GST gene expressions was noticed. AHR (Aryl Hydrocarbon
356 Receptor) is known to be recruited for biotransformation processes after exposure to PAHs

357 compounds (Genevois et al, 1998; Pfohl-Leszkowicz 2008). Once activated, AHR
358 translocated into the nucleus where it activated the expression of many genes implicated in
359 detoxification processes such as CYP450 (Whitlock, 1999; Hankinson, 1993). PAHs
360 metabolisation by CYP450 leads to the formation of reactive metabolites that covalently bind
361 to DNA forming DNA adducts (Newbold and Brookes, 1976; Osborne et al., 1981 ; Pfohl-
362 Leszkowicz, 2008). AHR expression was enhanced in gills of mussels collected in polluted
363 sites suggesting the presence of PAHs contaminants in those sites, as measured in water
364 (Michel et al., 2013). An induction of AHR expression was only detected in gills and not in
365 digestive glands which could be explained by the fact that gills is the first tissue in contact
366 with pollutants and could explain the high efficiency of gills to detoxify PAHs, assessed by
367 lower DNA adducts, compared to digestive glands. In a previous laboratory experiment
368 (Châtel et al., 2012), it has also been demonstrated a faster induction of AHR gene expression
369 in gills compared to digestive gland.
370
371 GST gene expression was only induced in gills of mussels transplanted in the different sites.
372 Glutathione S-transferase is a phase II enzyme that plays a role in the detoxification, by GSH
373 conjugaison, of electrophilic xenobiotic compounds. In this study, it appeared probably that
374 GST was precociously activated in digestive glands. Power and Sheehan (1996) have
375 observed a higher GST activity in gills than in digestive glands depending on the season.
376 It appeared that both in gills and digestive glands, HSP70 and PgP gene expression were
377 significantly increased whereas SOD and CAT gene expression were decreased. Tissue
378 specific differences were depicted in AHR, GST and MT gene expression levels. Indeed,
379 AHR and GST gene expression increased in gills and decreased in digestive glands while the
380 contrary was noticed for MT expression. In gills, the most induced genes were AHR (14
381 fold), PgP (10 fold), HSP70 and GST (4 fold) genes while MT (40 fold), HSP70 (16 fold),
382 PgP and CAT (2 fold) were more expressed in digestive glands.
383 All organisms from bacteria to mammals exposed to environmental stressors respond by
384 synthetizing heat-shock protein (HSP70) (Schlesinger et al., 1982). HSP70 gene expression is
385 implicated in protein repair, transport and protection from oxidative stress (Contardo-Jara et
386 al., 2010).
387 Our results showed that HSP70 mRNA expression was strongly induced (4 fold and 16 fold
388 increase compared to mussels from reference site) both in gills and digestive glands of *D.*
389 *polymorpha* transplanted at different sites with pollution gradient, this can be explained by
390 presence of xenobiotics such as PAHs. HSP70 protein expression was enhanced in marine

391 mussels exposed to PAHs (Werner et al., 1998), with a correlation to the formation of DNA
392 adducts and oxidative damage (Solé et al., 1996). Studies have demonstrated a variability of
393 HSP70 induction/duration depending on the stressor (temperature, salinity, xenobiotic,
394 metals…), dose, time of exposure and organisms. For example, in zebra mussels, an exposure
395 to heavy metals induced a transient increase in HSP70 mRNA level after 1 day (Navarro et
396 al., 2011) while exposure to B[a]P enhanced its induction from 12h exposure and remained
397 persistent for 28 days (Châtel et al., 2012). This increase in HSP70 mRNA levels could be
398 due to the fact that this gene possesses an anti-oxidant response element to which the
399 transcription factor Nrf2 binds as suggested in *D. polymorpha* for GST and CAT (Contado-
400 Jara and Wiegand, 2008).
401
402 However, in the present study, HSP70 mRNA expression was not correlated with the amount
403 of PAHs present in the different stations measured previously in the laboratory (Michel et al.,
404 2013). This may suggest that other parameters such as temperature and pH might also affect
405 HSP70 mRNA level induction, as demonstrated in other field studies (Hamer et al., 2004).
406
407 The transmembrane P-glycoprotein (P-gP) was as well induced in both tissues from mussels
408 transplanted in the different sites compared to reference site. P-gP is a part of the
409 multixenobiotic resistance (MXR) mechanism and has been demonstrated to be induced in
410 response to xenobiotics and especially PAHs (Bard, 2000).
411 Our results showed an induction of P-gP expression depending on the pollution gradient
412 (Michel et al., 2013) (a higher amount in mussels from Bougival than Marnay). PgP has been
413 demonstrated to be involved in excretion of PAHs from cells (Kurelec et al., 1995) and hence
414 indicated the process by which the mussel eliminates the pollutants from its cells, suggesting
415 a detoxification process in mussels transplanted. An up-regulation of P-gP mRNA was also
416 observed after mussel exposure to the β-blocker metroprolol (Contardo-Jara et al., 2010) and
417 to the propanolol (Franzellitti et al., 2011), thus demonstrating elimination process. However,
418 for the severely contaminated site located at Triel (Michel et al., 2013), P-gP expression was
419 lower. This can been explained by an induction of apoptosis, as they can be severely damaged
420 by pollutants (Châtel et al., 2011), and also by a lower DNA adduct quantity compared to the
421 two other sites.
422
423 Concerning the digestive glands, induction of CAT and MT gene expression was noticed in
424 mussels collected in the different sites. Catalase is an antioxidant enzyme that catalyses the

425 transformation of reactive oxygen species (ROS) into H_2O and hence indicate that
426 detoxification processes were strongly present in digestive glands of mussels transplanted in
427 the severe contaminated site.
428
429 MTs are cysteine rich peptides that mainly occur in cytosol, nucleus and lysosomes. They are
430 nonenzymatic proteins enable to bind to particular heavy metals. Tissues directly involved in
431 metal uptake, storage and excretion such as gills and digestive glands have a high capacity to
432 synthesize MTs (Sarkar et al., 2006). The high induction of MT gene in transplanted mussels
433 suggests that mussels are exposed to heavy metal, indicating the presence of metals in water.
434 The contamination by heavy metals has been largely described in the Seine river (Bourgeault
435 et al., 2010, Tusseau-Vuillemin et al., 2007) and dissolved Cd has been measured to be
436 around 37 ng/L. It is interesting to notice that MT gene is induced in mussels exposed to such
437 a low concentration of metal. Navarro et al. (2011) also demonstrated the strong induction of
438 MT gene in zebra mussels exposed to low Cd, Cu and Hg concentration (20µg/L).
439 All those data strongly converge towards the fact that mussels were able to activate their
440 detoxification mechanisms. However, this detoxification was not efficient enough to clear
441 PAHs compounds bioaccumulated in the different stations, as shown by the high amounts of
442 DNA adducts detected.
443
444 ***Conclusion – Perspectives***
445 Field studies showed the complexity of environmental impacts on freshwater organisms, and
446 made us realize about the importance of taking this into account to define new biomarkers.
447 This study was performed to validate the suitability of using DNA adduct measurement in
448 zebra mussels for genotoxicant monitoring of fresh water pollutants.
449 Our data highlighted the potential of the zebra mussel to detoxify pollutants. They also
450 showed significant DNA adducts formation in both tissues, correlated to high presence of
451 PAHs in water as in organisms (Michel et al., 2013). Moreover, a tissue specific induction of
452 genes implicated in detoxification processes was demonstrated. Difference in DNA pattern
453 allowed to confirm the presence of different kind of pollutants.
454
455
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459

459 **5. References**

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Table 1. Primer sequences (5'-3') used in qRT-PCR

Fig. 1. Localization of sampling sites along the Seine river basin.

Fig.2. Example of DNA adduct pattern of *D. polymorpha* transplanted in Vertuzey, Marnay, Bougival and Triel sites. The numbering of the individual adduct is depicted in the schemes.

Fig.3. Total DNA adduct in gills (A) and digestive glands (B) of *D. polymorpha* transplanted in Vertuzey, Marnay, Bougival and Triel sites for one or two months

Fig.4. Relative mRNA abundance values of AHR (A), SOD (B),CAT (C), GST (D), PgP (E), HSP70 (F) and MT (G) in gills of *D. polymorpha* transplanted in Vertuzey, Marnay, Bougival and Triel sites for one or two months, analysed by qRT-PCR. Results are normalised with the reference gene S3. (*): data significantly different compared to Vertuzey ($p<0.05$).

Fig.5. Relative mRNA abundance values of AHR (A), SOD (B),CAT (C), GST (D), PgP (E), HSP70 (F) and MT (G) in digestive gland of *D. polymorpha* transplanted in Vertuzey, Marnay, Bougival and Triel sites for one or two months, analysed by qRT-PCR. Results are normalised with the reference gene S3. (*): data significantly different compared to Vertuzey ($p<0.05$).

Table 2. Comparison of biomarker responses between gills and digestive glands of mussels transplanted in different sites along the Seine river

Table 1.

Gene	Short name	Forward primer	Reverse primer	Accession number
Ribosomal protein S3	S3	CAGTGTGAGTCCCTGAGATACAAG	AACTTCATGGACTTGGCTCTCTG	AJ517687
P-glycoprotein	P-gp1	CACCTGGACGTTACCAAAGAAGATATA	TCACCAACCAGCGTCTCATATTT	AJ506742
Aryl-hydrocarbon receptor	AHR	ATCACAGCGATGAGCCTCAG	AGACAGCATTGCGAGGTCAC	DQ159188
Superoxide dismutase	SOD	GACAGCATGGCTTCCATGTG	AGGAGCCCCGTGAGTTTTG	AY377970
Catalase	CAT	ATCAGCCTGCGACCAGAGAC	GTGTGGCTTCCATAGCCGTT	EF681763
Glutathione S-transferase	GST	ATGATCTATGGCAACTATGAGACAGG	GAAGTACAAACAGATTGTAGTCCGC	EF194203
Heat-shock Protein 70	HSP70	GCGTATGGACTTGATAAGAACCTCA	GAACCCTCGTCGATGGTCA	EF526096

Fig. 1.

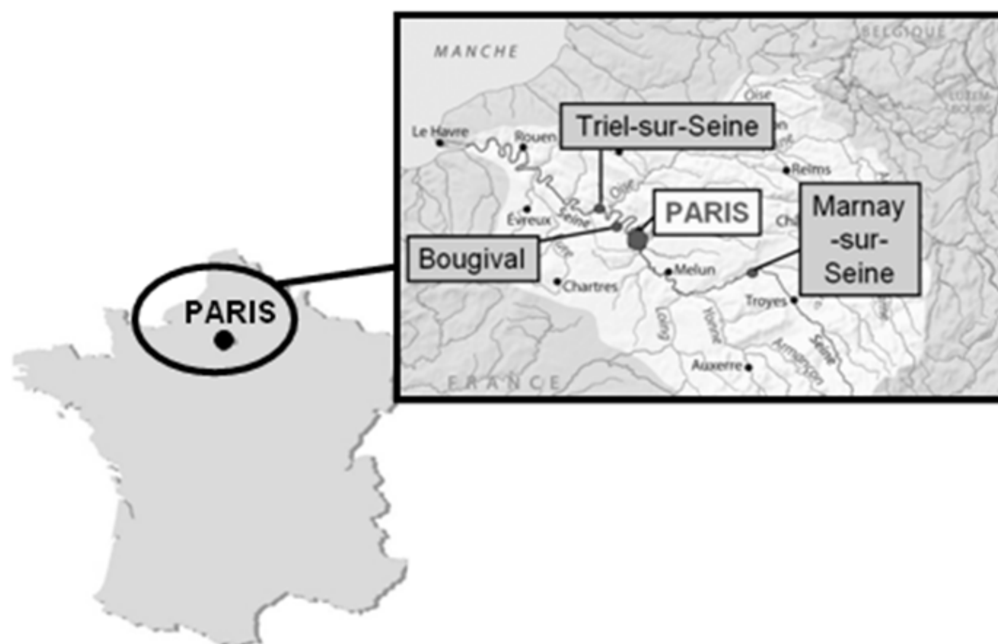


Fig.2

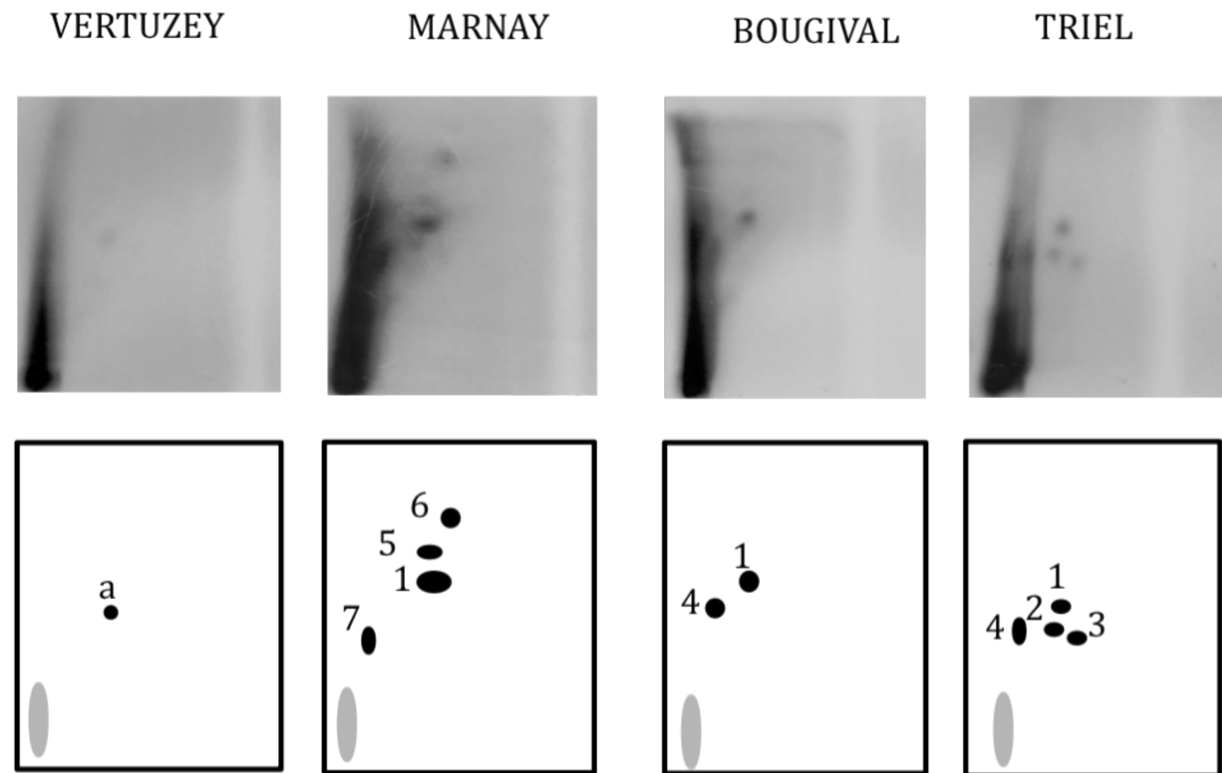


Fig.3

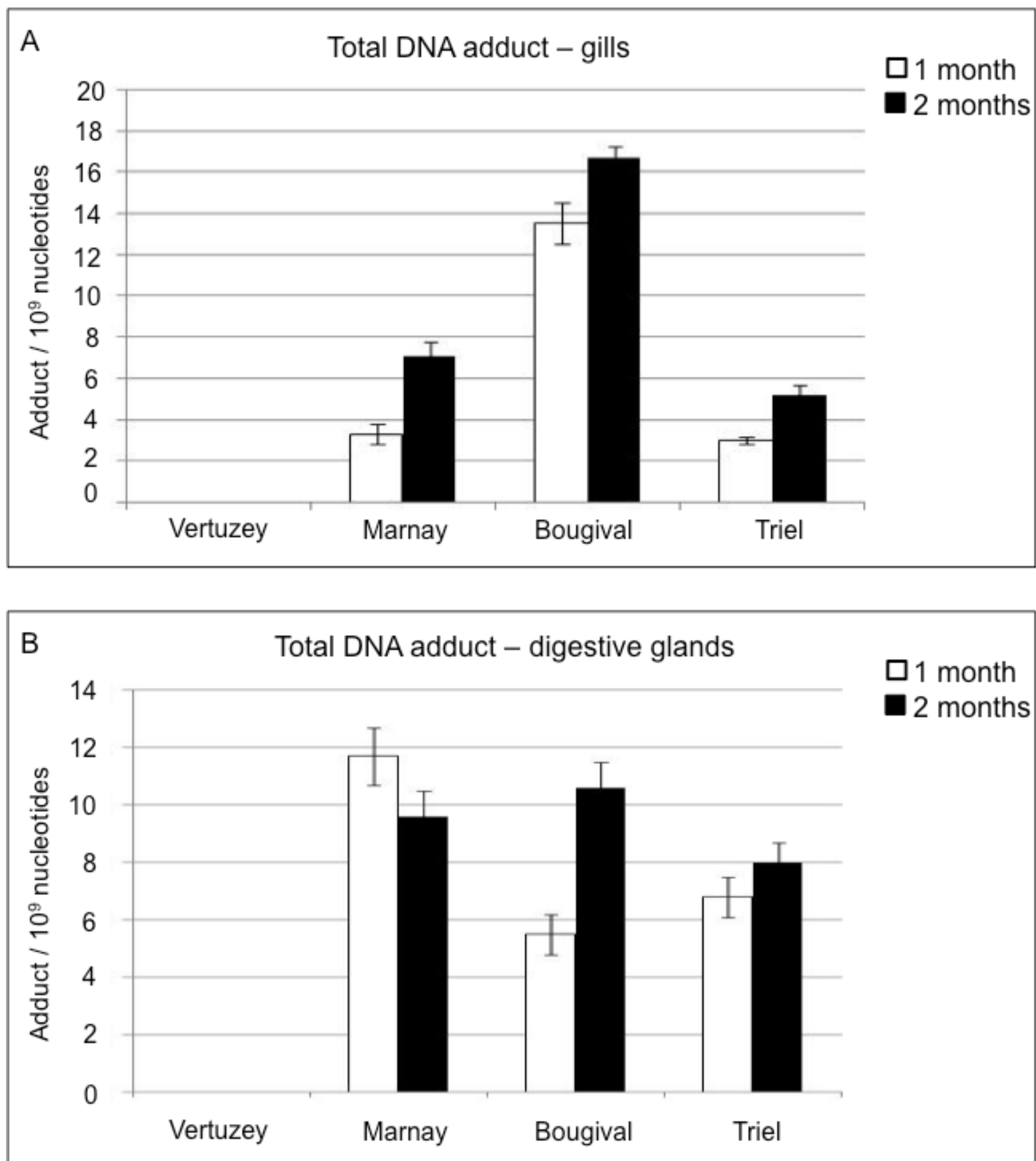
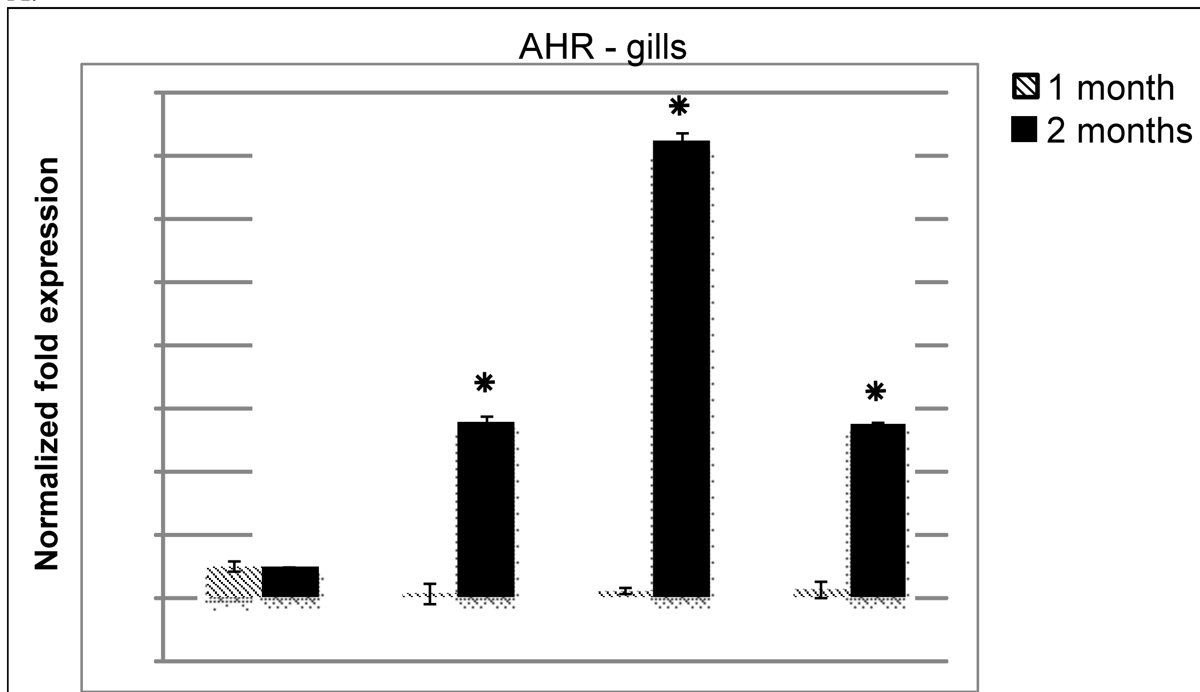
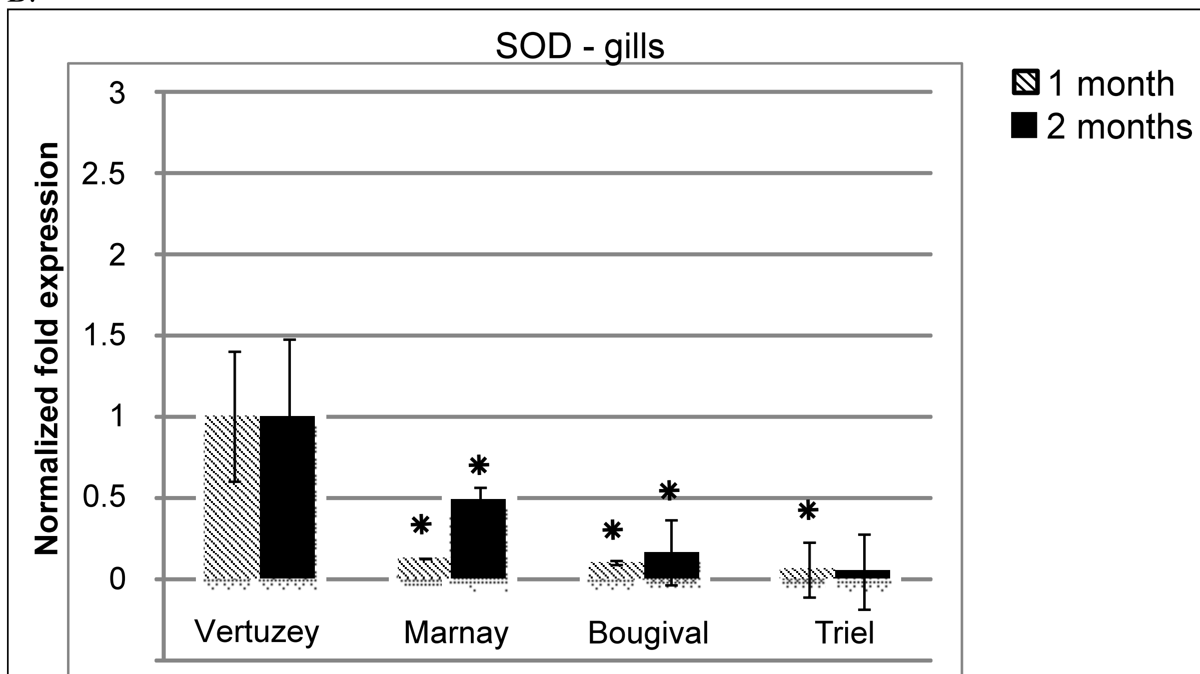


Fig.4

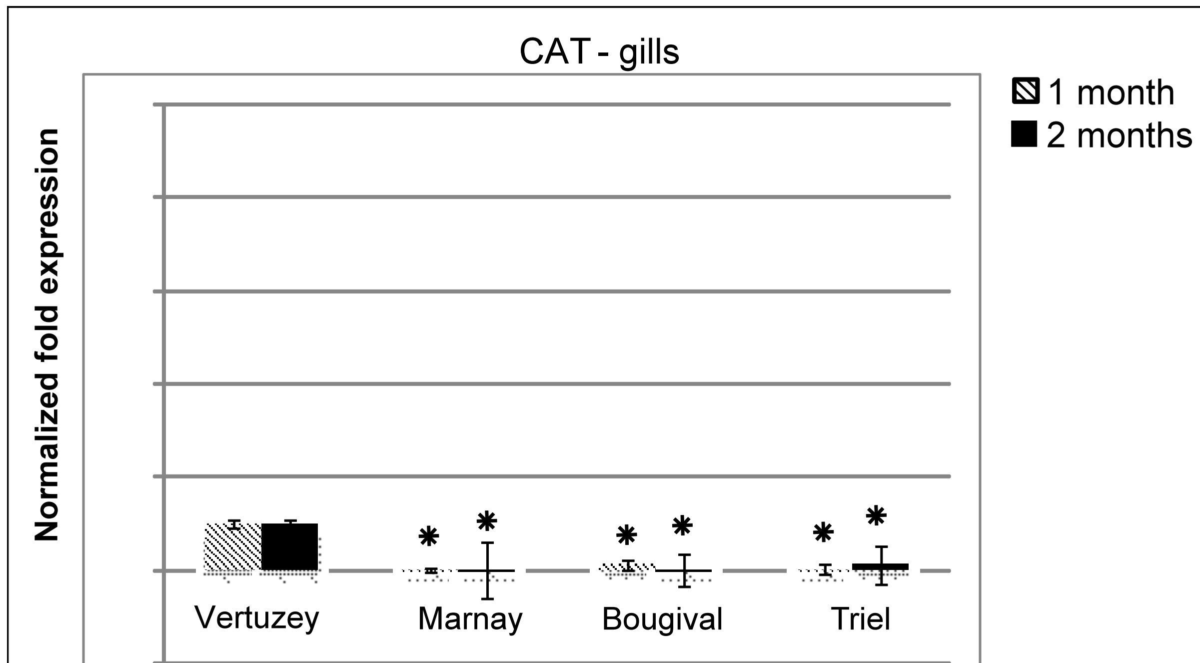
A.



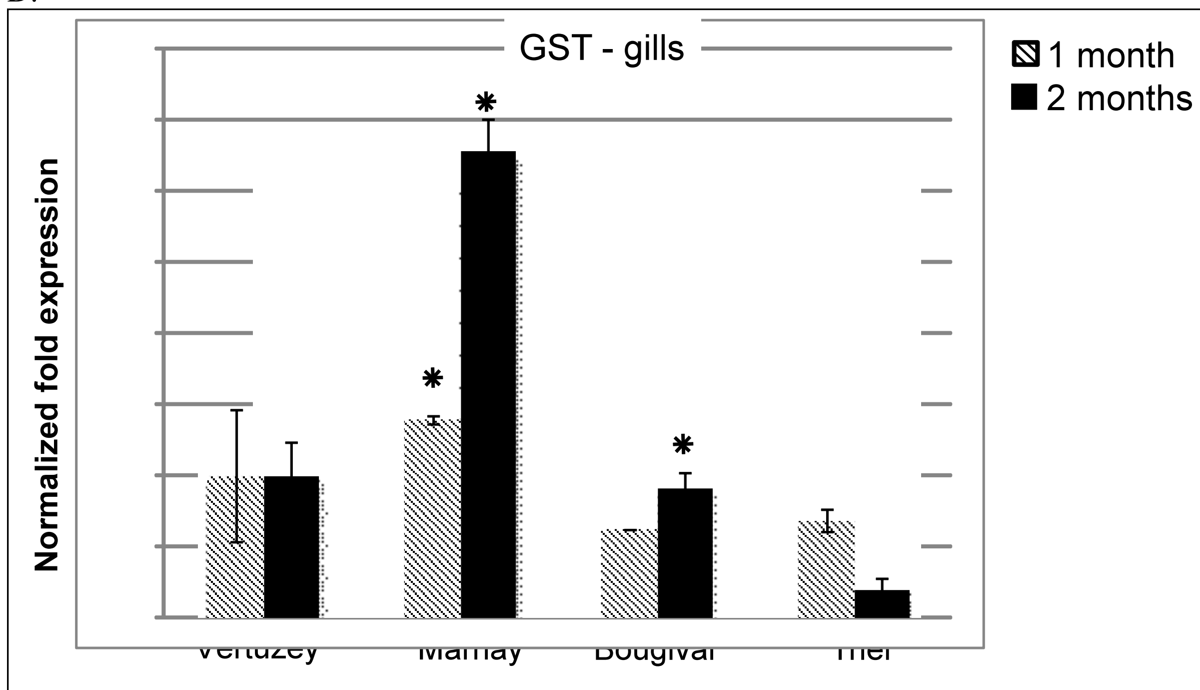
B.



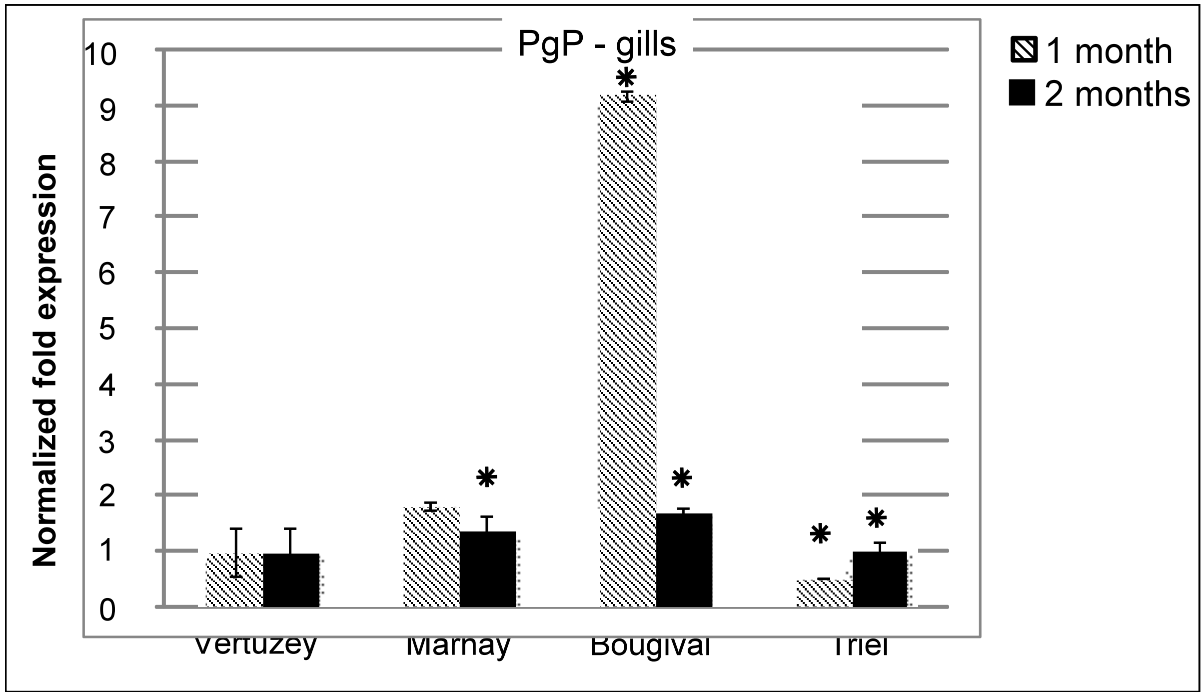
C.



D.



E.



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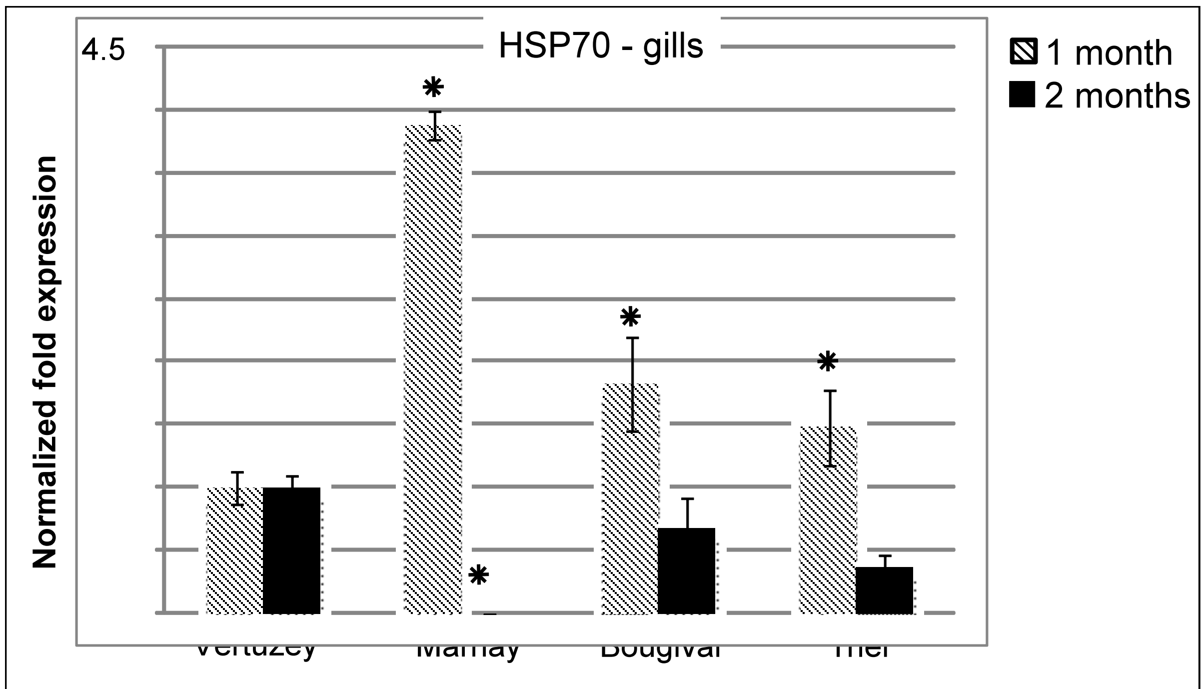

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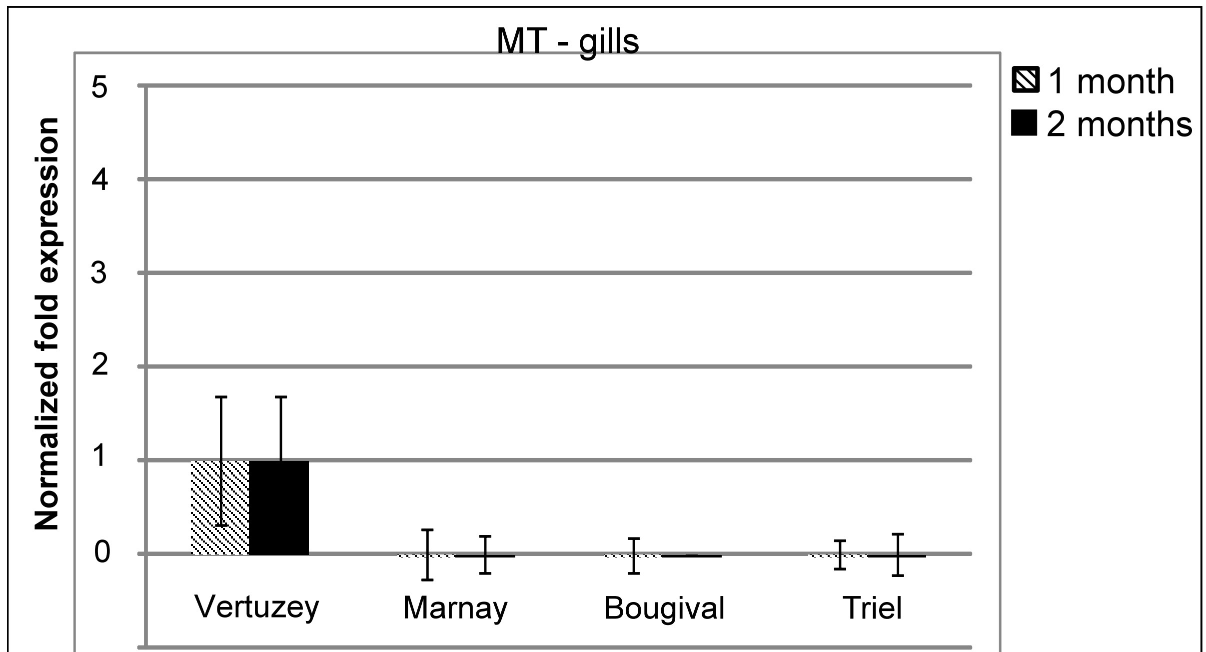

Fig.5.
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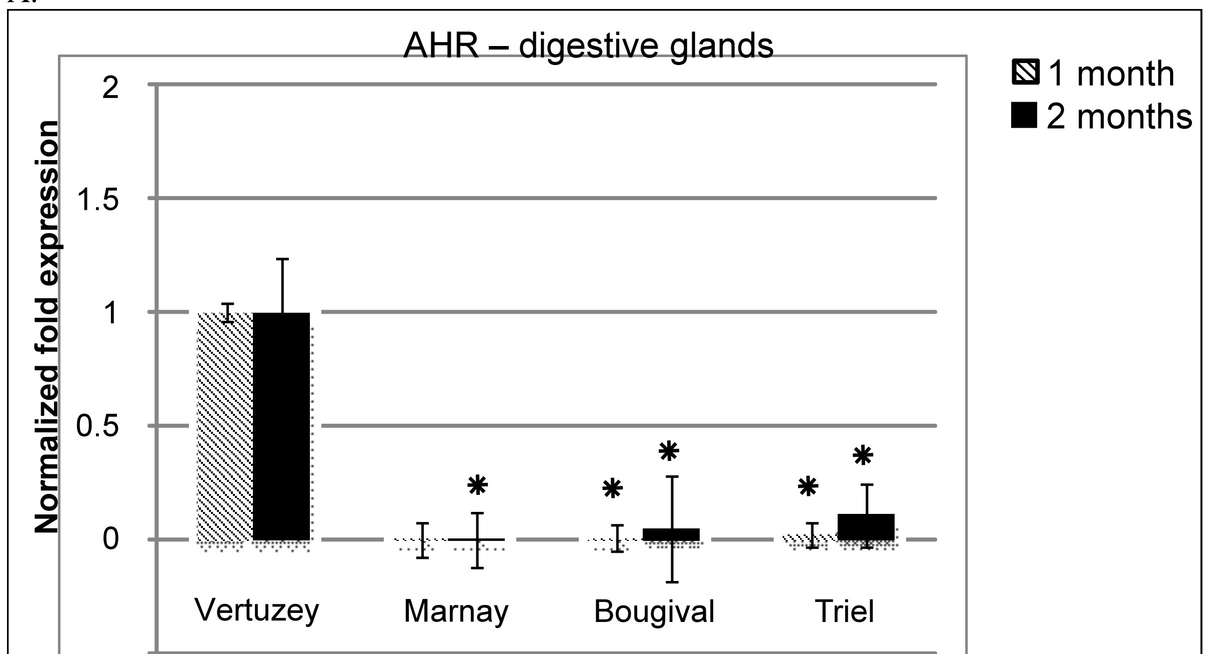

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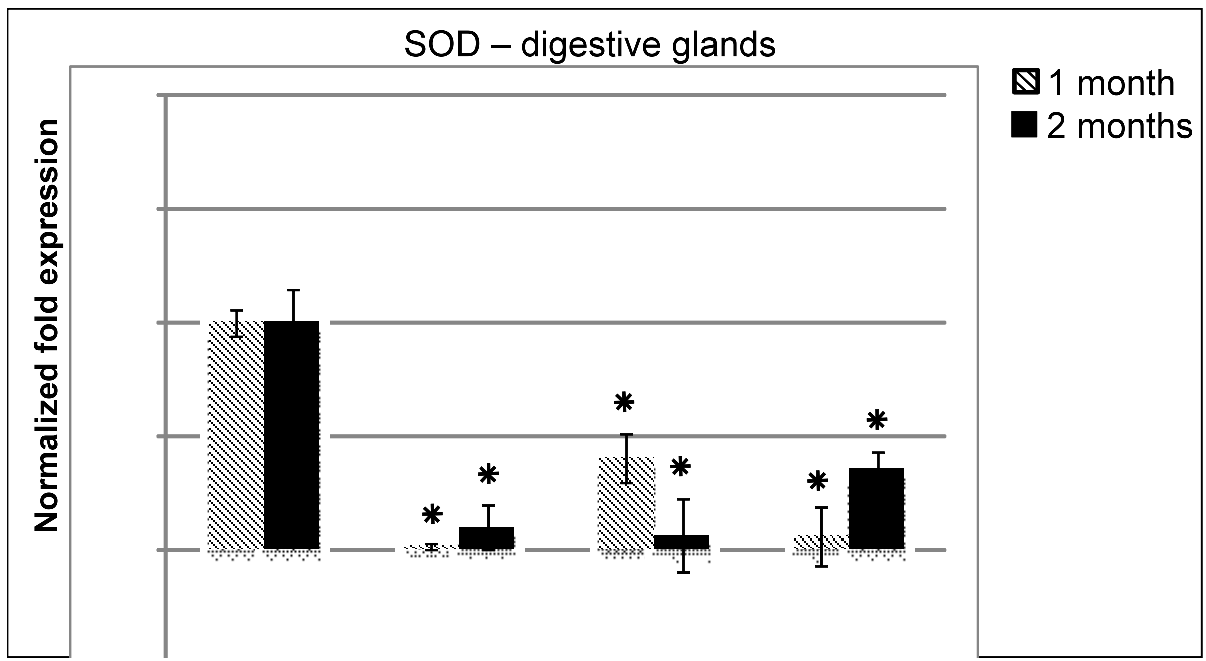

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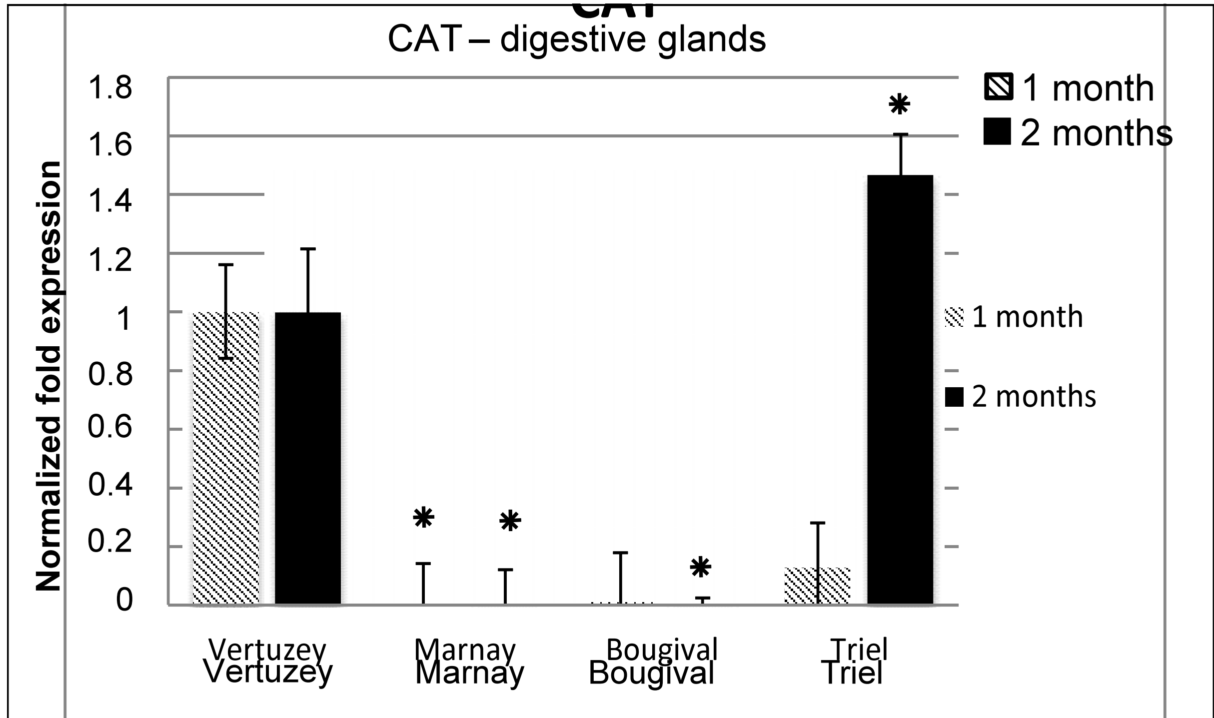

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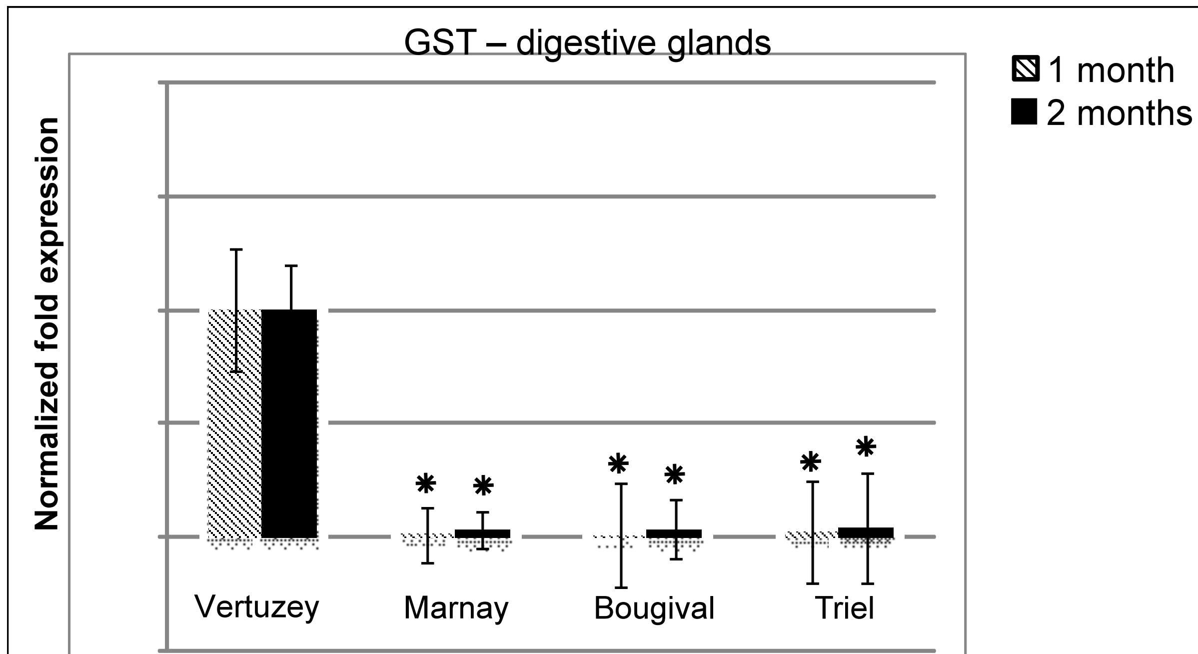

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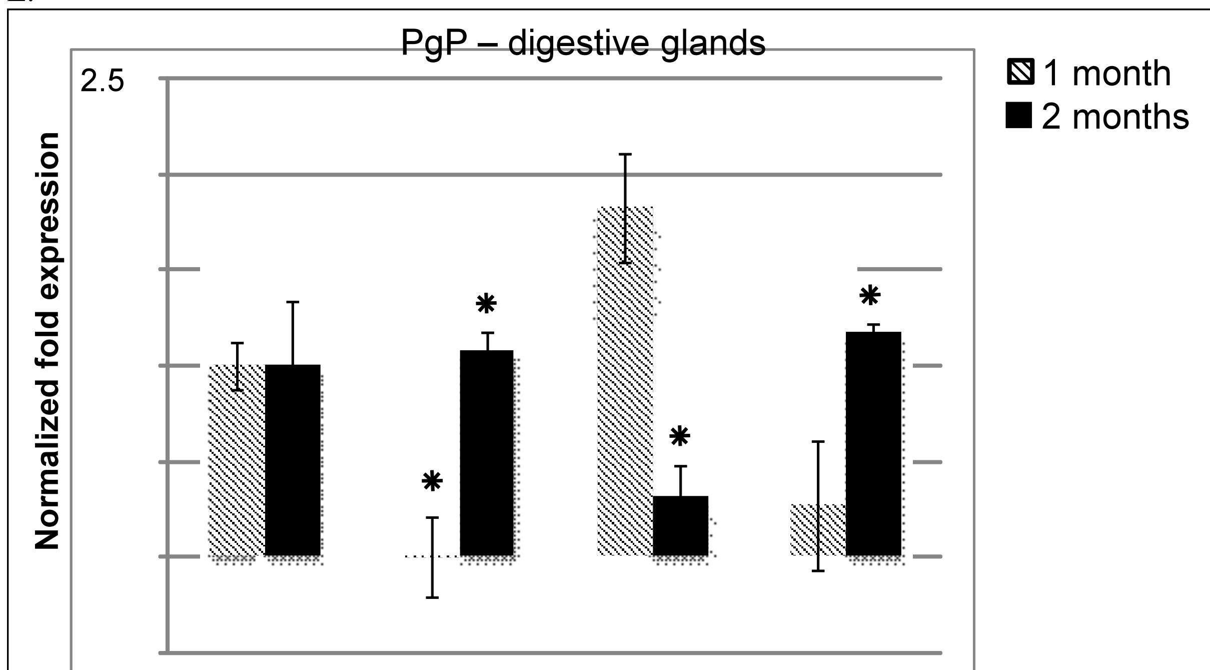

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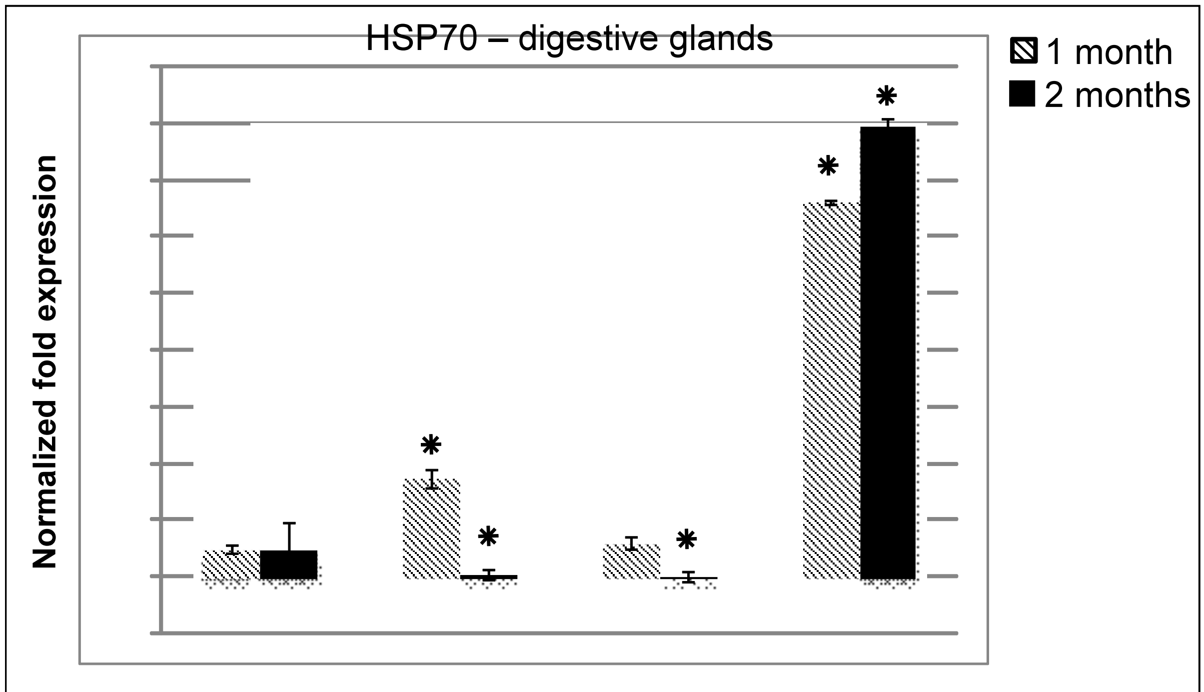

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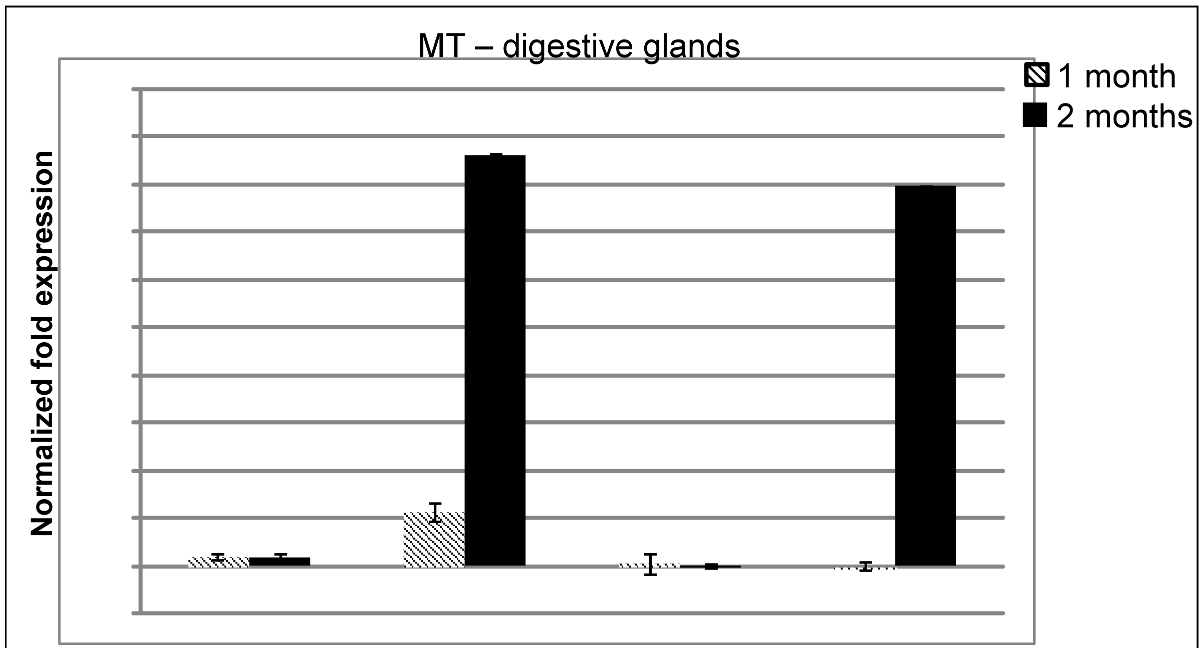

Table 2.

Assay	Gills	Digestive glands
ADDUCTS	Bougival>>Marnay> Triel Increase after 2 months	Marnay> Triel> Bougival Increase (except Marnay)
AHR	Bougival>Marnay=Triel Increase after 2 months	inhibited
SOD	Decrease vs Verturzey (reincrease at Marnay 2 months)	Decrease (marnay=Triel>>bougival)
CAT	Decrease	Decrease Marnay & Bougival Increase Triel
GST	Increase only Marnay	decrease
PgP	Increase only after 1 month bougival	No significative variation
HsP70	Increase only after 1 month Marnay> Bougival=Triel	Increase at marnay after 1 month only High increase at Triel
MT	Decrease	Increase Marnay & Triel