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Parental exposure to the herbicide diuron results in oxidative DNA damage to germinal cells of the Pacific oyster Crassostrea gigas

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Abstract :

Chemical pollution by pesticides has been identified as a possible contributing factor to the massive mortality outbreaks observed in *Crassostrea gigas* for several years. A previous study demonstrated the vertical transmission of DNA damage by subjecting oyster genitors to the herbicide diuron at environmental concentrations during gametogenesis. This trans-generational effect occurs through damage to genitor-exposed gametes, as measured by the comet-assay. The presence of DNA damage in gametes could be linked to the formation of DNA damage in other germ cells. In order to explore this question, the levels and cell distribution of the oxidized base lesion 8-oxodGuo were studied in the gonads of exposed genitors. High-performance liquid chromatography coupled with UV and electrochemical detection analysis showed an increase in 8-oxodGuo levels in both male and female gonads after exposure to diuron. Immunohistochemistry analysis showed the presence of 8-oxodGuo at all stages of male germ cells, from early to mature stages. Conversely, the oxidized base was only present in early germ cell stages in female gonads. These results indicate that male and female genitors underwent oxidative stress following exposure to diuron, resulting in DNA oxidation in both early germ cells and gametes, such as spermatozoa, which could explain the transmission of diuron-induced DNA damage to offspring. Furthermore, immunostaining of early germ cells seems indicates that damages caused by exposure to diuron on germ line not only affect the current sexual cycle but also could affect future gametogenesis.

Keywords : *Crassostrea gigas*, Oxidative stress, DNA damage, Germ cell, Pesticides

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1. Introduction

The living cell is constantly exposed to potentially-damaging free radical species of endogenous origin, such as those arising from normal cellular metabolism, or exogenous origin, resulting from exposure to ultraviolet radiation, ionizing radiation or xenobiotics (Evans et al., 2004). Exposure of aquatic organisms to chemical pollutants can hence promote an increase in the production of reactive oxygen and nitrogen species (ROS/RNS) (Alves de Almeida et al., 2007). When the rate of ROS/RNS production exceeds the efficiency of antioxidant defences and repair systems, an imbalance occurs in the redox status and oxidative stress can arise, leading, inter alia, to the oxidation of key cell components such as proteins, fatty acids and DNA, thereby contributing to toxicity (Sies, 1993). Among

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59 chemicals, certain pesticides are known to induce oxidative stress (Lushchak, 2011).
60 Pesticides are ubiquitous pollutants of aquatic systems. In France, 93% of rivers are
61 contaminated by pesticides (SoeS, 2013). In addition to inputs by rivers, pesticides used
62 mainly for agricultural purposes can be dispersed to coastal waters through various processes,
63 including run-offs, leaching and spray drift. Marine environments and, in particular, coastal
64 ecosystems, are often considered as the end receptacle of chemical pollutants. Coastal areas
65 are usually characterized by high primary production, supporting the development of
66 numerous species, e.g. shellfish farming zones. The Pacific oyster, *Crassostrea gigas*
67 (Thunberg), was introduced into France in 1966 (Grizel and Héral, 1991) and is now the
68 most-cultivated bivalve species. With annual production reaching 82,000 tons in 2012 (FAO,
69 2014), France is currently Europe's leading country for oyster production. For several years,
70 this species has been facing mass summer mortality events; the causes of these are undefined,
71 but appear to be multifactorial and include physiological stress, infection by pathogenic
72 organisms and environmental conditions (Dégremont et al., 2010; Huvet et al., 2010; Renault
73 et al., 1994; Samain and McCombie, 2008). Chemical pollution by pesticides has been
74 identified as one of the possible factors involved in this mortality phenomenon, due to their
75 toxic effects on oysters (Akcha et al., 2012; Buisson et al., 2008; Gagnaire et al., 2007; His
76 and Seaman, 1993; Mai et al., 2012; Mottier et al., 2014; Ochoa et al., 2012; Wang et al.,
77 2009). In the Ebro delta (Spain), Köck et al. (2010) found a correlation between pesticide
78 concentrations in water and shellfish flesh and episodes of mortality. Agrochemical inputs are
79 known to display seasonal variations: in France, high pesticide concentrations have been
80 detected in spring in a major shellfish farming zone, the Marennes Oleron basin,
81 corresponding to the highly-sensitive oyster gametogenesis period (Burgeot et al., 2008;
82 Munaron, 2004; Soletchnik et al., 2005). Pesticides not only have direct toxic effects on adult
83 oysters, but also indirect trans-generational effects on their offspring (Barranger et al., 2015,

84 2014; Bouilly et al., 2007, 2003). In a previous experiment conducted by our laboratory,
85 oyster genitors in gametogenesis were subjected to short exposures (two 7-day pulses) of
86 environmental concentrations of the herbicide diuron (0.3 $\mu g.L^{-1}$). The genotoxicity of diuron
87 was demonstrated in genitor haemocytes. Moreover, DNA damage was also detected for the
88 first time in genitor spermatozoa (strand breaks) and in offspring (DNA aneuploidy in spat),
89 highlighting the vertical transmission of DNA damage further to parental exposure (Barranger
90 et al., 2014). The genotoxicity of diuron could result from oxidative stress. The oxidation of
91 DNA by ROS/RNS can actually produce strand breaks and a variety of modified DNA bases.
92 Base oxidation is probably the foremost source of DNA damage. Among the four normal
93 nucleobases, guanine (Gua) is the most susceptible to oxidation due to its low oxidation
94 potential. The interaction of HO• (the most reactive oxygen-free radical) with DNA strand
95 nucleobases, such as guanine, leads to the formation of C8-hydroxyguanine (8-OHGua), or its
96 nucleoside form deoxyguanosine (8-hydroxy-2'-deoxyguanosine). 8-oxodGuo is the most
97 abundant oxidized nucleobase found in DNA and is widely used as a marker of DNA damage,
98 carcinogenesis and oxidative stress in humans (Halliwell and Aruoma, 1991). If it is not
99 removed by DNA repair systems, 8-oxodGuo can result in mutagenesis by G:C to T:A
100 transversions during DNA synthesis (Shibutani et al., 1991). Its formation has also been
101 reported in bivalves following exposure to pollutants or exposure in the field. It has been put
102 forward and used in ecotoxicology as an efficient marker of both oxidative stress and
103 genotoxicity (Akcha et al., 2000a; Almeida et al., 2005; Aloisio Torres et al., 2002; Alves de
104 Almeida et al., 2007; Canova et al., 1998; Charissou et al., 2004; Lemiere et al., 2005).
105 Our previous results suggest that the vertical transmission of DNA damage occurs through
106 damage to genitor-exposed gametes. In order to further our understanding of diuron
107 genotoxicity, gonad tissue of genitors originating from our previous experiment (Barranger et
108 al., 2014) were analyzed for the detection of 8-oxodGuo. Two different methods were used

109 for this study. Firstly, 8-oxodGuo levels in gonad tissue were measured using high-
110 performance liquid chromatography coupled with UV and electrochemical detection (HPLC-
111 UV-ECD). In a second step, immunohistochemical detection of 8-oxodGuo was performed to
112 locate base damage to the various types of cells present in gonadal tubules and, particularly, in
113 germ line. Our results should contribute to improving understanding of diuron genotoxicity in
114 oysters and how genitor exposure can significantly impact the DNA integrity of the following
115 generation, with potential effects on oyster physiology at a population level.
116

117 ## 2. Materials and Methods

118 ### 2.1 Genitor origin and diuron exposure

119 The adult Pacific oysters (*Crassostrea gigas*) used for this experiment were progenies of wild
120 oysters sampled in the Marennes-Oléron Bay (France). Oyster husbandry/broodstock
121 conditioning, and diuron exposure were performed as described by Barranger et al. (2014).
122 Briefly, the oysters were acclimatized for one month at the hatchery. Next, sea water
123 temperature (8°C ± 1°C) was gradually raised by two degrees per day for 1 week, to reach
124 19.8°C (± 0.3°C). Once gonad development had begun, the oysters were divided into three
125 experimental groups: a seawater control, a solvent control and a diuron-exposed group. Three
126 250-L tanks were used for each experimental group, each containing 240 oysters. Two 7-day
127 exposure periods took place at the start and mid-course of gametogenesis. Diuron - the
128 pesticide selected for our study - is a substituted urea herbicide used in agriculture for on-land
129 weed control. This herbicide is also used as an antifouling biocide (Thomas et al., 2001). In
130 France, its use as a phytosanitary product has been banned since 2008 (The Official Journal of
131 the French Republic no. 204 September 4, 2007), and as a biocide used in antifouling paints
132 since 2009 (The Official Journal of the French Republic - bylaw 21 August, 2008). However,
133 diuron is still the fourth most commonly-found pesticide in French rivers (SOeS, 2013) and

134 recent studies have reported its presence in various French coastal waters (Atlantic bays,
135 estuaries and Mediterranean Sea) (Buisson et al., 2008; Caquet et al., 2013; Munaron et al.,
136 2012). The oysters were exposed to nominal diuron concentrations of 0.4 and 0.6 µg/L,
137 respectively. However, the analysis of passive samplers (Polar organic chemical integrative
138 samplers, POCIS) used in the previous experiment showed oyster exposure to integrated
139 concentrations as low as 0.2 and 0.3 µg L^{-1}.

140 **2.2 Sampling program**

141 Various gonad samples were collected after completion of genitor exposure. For HPLC-UV-
142 ECD analysis, the gonads were sampled and stored in liquid nitrogen prior to analysis. 10
143 males and 10 females were analyzed in each experimental group. For histological analysis,
144 transverse sections (5 mm) cut in the vicinity of gill-palp junction were fixed in Davidson's
145 solution (48 h; 10% glycerol, 20% formaldehyde, 30% ethanol (95%), 30% sterile sea water,
146 10% acetic acid), then stored in 70% ethanol. 5 males and 5 females were analyzed in each
147 experimental group. For HPLC-UV-ECD and histological analyses, each sample/individual
148 was realized in duplicate.

149 **2.3 Quantification and location of oxidative DNA damage**

150 **2.3.1 Measurement of 8-oxodGuo levels in genitor gonads using HPLC/UV-ECD**

151 ***DNA extraction***

152 For each gonad sample, DNA was extracted from 100-150 mg of gonad tissue using the
153 chaotropic NaI method derived from Helbock et al. (1998), slightly modified by Akcha et al.,
154 (2000b). The samples were centrifuged at 1,500 g for 10 min at 4°C. Supernatants were
155 discarded and the pellets were suspended in 2 mL of Buffer A (320 mM sucrose, 5 mM
156 $MgCl_2$, 10 mM Tris-HCl, 0.1 mM deferoxamine mesylate, 1% Triton X-100, pH 7.5).
157 Following centrifugation (1,500 g, 10 min, 4°C), the pellets were recovered and resuspended

158 in 600 µL of Buffer B (5 mM EDTA-Na_2, 10 mM Tris-HCl, 0.15 mM deferoxamine
159 mesylate, pH 8). After addition of 35 µL of 10% SDS, RNA digestion was performed by
160 incubation with 120 µg of RNase A and 20 U of RNase T1 for 15 min at 50°C. Protein
161 digestion was performed by incubation with 600 µg of protease for 1hr at 37°C. The samples
162 were then centrifuged at 5,000 g for 15 min at 4°C, and supernatants were recovered in 15 mL
163 sterile tubes. After the addition of 1.2 mL of sodium iodide solution (20 mM EDTA-Na_2, 7.6
164 M NaI, 40 mM Tris-HCl, 0.3 mM deferoxamine mesylate, pH 8) and 2 mL isopropanol, the
165 tubes were centrifuged for 15 min at 5,000 g. The pellets were then recovered and
166 resuspended in 2 mL 40% isopropanol. After centrifugation (5,000 g, 15 min, 4°C), the pellets
167 were washed in 2 mL of 70% glacial ethanol and centrifuged at 5,000 g for 5 min at 4°C.
168 Ethanol was then discarded using a pipette, and the pellets were left to dry for 1 hr at room
169 temperature. DNA was finally resuspended in 100 µL of deferoxamine mesylate 0.1 mM and
170 left to dissolve overnight at 37°C. DNA quantification was performed by spectrophotometry
171 at 280, 260, and 230 nm wavelengths using a ND1000 NanoDrop (NanoDrop Technologies,
172 Inc). After quantification, the DNA samples were stored at -20°C prior to digestion.

173 ***DNA digestion***

174 For each sample, 15 µg of DNA were filtrated (0.2 µm) and digested into 2'-
175 desoxyribonucleosides by incubation with 5 U of nuclease P1 for 2hrs at 37°C. Four units of
176 alkaline phosphatase were then added for an additional 1-hour incubation at 37°C. The sample
177 was centrifuged (5 min, 7,000g, 4°C) and the supernatant was recovered for injection.

178 ***HPLC analysis***

179 8-oxodGuo levels were determined by HPLC (Agilent 1200 series) coupled to
180 electrochemical (Coulochem III, ESA) and UV (Agilent 1200 series) detection. Separation of
181 8-oxodGuo and 2'- deoxyribosides was performed using an Ultrasphere pre-column (5C18,

182 Interchim) and an Uptisphere column (5ODB, Interchim). Elution was performed in isocratic
183 mode using a mobile phase composed of 10% methanol and 100 mM sodium acetate, at pH
184 5.2. The elution flow rate was set at 1 mL.min-1. The guard and measure cells were
185 respectively set at an oxidation potential of 460, 150 and 380 mV. The quantification of 8-
186 oxodGuo was performed in accordance with a calibration curve previously obtained with
187 known pmole amounts of authentic 8-oxodGuo. For a standard expression in the number of 8-
188 oxodGuo residues per 10^6 dGuo, deoxyguanosine was also quantified by fitting a UV detector
189 to the output of the HPLC column. The UV detector was set at a wavelength of 254 nm. A
190 calibration curve was also obtained for this compound within the nmole range. For the
191 described conditions, the retention times of both 8-oxodGuo and dGuo were respectively 14
192 and 10 min at 35°C. Each sample were analyzed in duplicate and results were expressed in
193 number of 8-oxodGuo per 10^6 dGuo.

194 **2.3.2 Immunohistochemical detection of 8-oxodGuo in genitor gonad cells**

195 As previously mentioned, cells naturally produce free radical species. A background level of
196 DNA damage, e.g. 8-oxodGuo, therefore exists in all living cells (Evans et al., 2004). As
197 immunostains are not stoichiometric, it is impossible to use immunohistochemistry such as
198 HPLC measurement to compare 8-oxodGuo levels across control and exposed groups.
199 Immunohistochemistry solely provides qualitative information on the location of 8-oxodGuo
200 in the various cell types of oyster gonads.

201 **2.3.3 Histology**

202 ***Slide preparation, gametogenesis stage and sex determination***

203 Each dehydrated sample was embedded in paraffin wax and several 4 µm sections were cut
204 and individually deposited on a slide for subsequent treatment. For each sample, one of the
205 slides was stained using the Prenant–Gabe trichrome method (Gabe, 1968) to allow individual

206 determination of sex and gametogenesis stage on the basis of criteria previously described by
207 Heude-Berthelin et al. (2001) for diploid *C. gigas*. Briefly, stage 0 corresponds to the sexual
208 resting stage. Stage I is defined as the early developmental stage (gonial mitosis). The
209 germinal lineage develops at stage II, whereas stage III is characterized as the ripe gonad.

210 ***Immunohistochemistry with anti-8oxo antibody***

211 The slides were deparaffinized (2x5min in Roti®-Histol), immersed in methanol–H_2O_2 3%
212 (v/v) for 30 min for inhibition of endogen peroxidases, rehydrated, and soaked in running
213 water (5 min). They were then incubated at 37°C for 1 h with 100µg/ml RNase and washed in
214 Buffer 1 (Tris 50 mM, NaCl 150 mM, pH 7.4) for 2x5 min. Proteins were removed from
215 DNA by digestion with proteinase K (10µg/ml in Tris buffer, at pH 7.5) at room temperature
216 for 5 min. To increase antibody accessibility to the antigen, a denaturation step in 1N HCl for
217 10 min was added. The slides were then rinsed in buffer 2 (Tris 50 mM, NaCl 150 mM,
218 gelatin 0.25%, Triton X-100 0.5%, pH 7.4) for 2×10 min (Franco et al., 2010) and incubated
219 overnight at 4°C with the primary antibody 8-OHdG (15A3, Santa Cruz Biotechnology)
220 diluted (1:100) in buffer 1. This antibody is recommended for the detection of 8-hydroxy-2'-
221 deoxyguanosine, 8-hydroxyguanine and 8-hydroxyguanosine. After two rinses in buffer 2, the
222 slides were incubated for 1 h at room temperature with horseradish peroxidase conjugated
223 polyclonal rabbit anti-mouse IgG (Dako®) diluted (1:100) in buffer 1, then rinsed in buffer 1.
224 Peroxidase activity was visualized after 10 min incubation with 200 µL DAB (3, 3′
225 diaminobenzidine, FastDAB, Sigma- Aldrich®) as a brown precipitate. Sections were
226 counterstained light green, then mounted in resin (Roti®-Histokitt Roth™). Controls without
227 primary or secondary antibodies were also performed. Identification of labelled cells in gonad
228 was based on Heude-Berthelin et al. (2001) and Franco et al. (2008).

229

230 **2.4 Statistical analyses**

231 In order to compare 8-oxodGuo levels detected with the HPLC method, statistical tests were
232 conducted using STATISTICA (StatSoft, Inc.,version 10). Normality was checked using
233 Lilliefor's test and variance homogeneity was evaluated using Bartlett's test. When necessary,
234 raw data were mathematically transformed (Ln) to achieve normality before proceeding with
235 an ANOVA. When significant, an a posteriori Tukey test was performed.

236 **3. Results**

237 **3.1 Quantitative analysis of 8-oxodGuo levels in gonad tissue**

238 In the control group, 8-oxodGuo levels in gonad DNA were 11.8 ± 7.8 and 13.0 ± 1.2 per 10^6
239 dGuo in males and females respectively. In the solvent control group, 8-oxodGuo levels in
240 males were similar to those measured in the control group (12.2 ± 4.8 per 10^6 dGuo) (Figure
241 1A), but significantly higher in females (+89%; 24.6 ± 8.4 per 10^6 dGuo) (Figure 1B). Despite
242 the sex-specific effect of the solvent, a significant increase in 8-oxo-dGuo levels was detected
243 in both male (+73%) and female (+270%) gonads following diuron exposure ($p<0.05$). DNA
244 damage was significantly higher in females than in males: 48.1 ± 28.2 versus 20.4 ± 5.5 8-
245 oxodGuo per 10^6 dGuo (Figure 1).

246

247 **3.2 Immunodetection of 8-oxo-dGuo in gonad cells**

248 After exposure, analysis of the gonad histological slides showed all genitors, both males and
249 females, to be at stage III, corresponding to the mature reproductive stage. Controls without
250 primary or secondary antibodies showed no specific labelling (Figure 2 A, B). Of note, during
251 the methodological development of immunodetection, some slides were realized in males
252 without RNase treatment leading to a strong immunolabelling in particular in the cytoplasm of
253 spermatogonia (Figure 2 C, D).

254

255 ### 3.2.1 Male gonads

256 In male gonads, oxidized nucleosides were detected in all nuclei of spermatogonia I (pale
257 spermatogonia) and II (dark spermatogonia) (Figure 3 A, B, C, D) and in the nuclei of few
258 spermatocytes and spermatids (Figure 3 C). Immunolabelling was not systematically detected
259 in spermatozoids in individuals and across individuals (Figure 3 C, D). Oxidized nucleosides
260 were also labelled in intra-gonadal somatic cells (Figure 3 B) and in few haemocytes present
261 in the gonad (Figure 3 A, D). No differences were observed in terms of cell distribution of 8-
262 oxodGuo labelling across the experimental groups.

263 ### 3.2.2 Female gonads

264 In female gonads, oxidized nucleosides were observed in the nucleolus, nucleus and
265 cytoplasm of young oogonia and also in nucleus of undifferentiated germ cells (putative germ
266 stem cells) pressed against the inside of the tube (Figure 4 A, B, C), but were not detected in
267 oocytes (Figure 4 D, E). Similarly to male gonads, immunolabelling was also observed in
268 intra-gonadal somatic cells (Figure 4 C, D, E) and in interstitial conjunctive cells (Figure 4 C).
269 No differences were observed in terms of cell distribution of 8-oxodGuo across experimental
270 groups.

271 ## 4. Discussion

272 In this study, the levels and cell distribution of the oxidized base lesion 8-oxodGuo were
273 studied in the gonads of diuron-exposed genitor, in order to better characterize the
274 transgenerational effect observed in a previous study (Barranger et al., 2014). In bivalves, 8-
275 oxodGuo, as a marker of oxidative stress and genotoxicity, is essentially measured in gills,
276 digestive glands and mantles, but never, in our knowledge, in reproductive tissue. While
277 genotoxicity in somatic cells may have implications at the individual level, genotoxic events
278 occurring in germ cells may lead to genomic abnormalities in future generations.

279 In our study, 8-oxodGuo levels in the gonads of control genitors appeared to be in the range
280 of values reported in other bivalve tissues (Akcha et al., 2000a; Alves de Almeida et al., 2007;
281 Charissou et al., 2004; Lemiere et al., 2005). An increase in 8-oxodGuo lesions was observed
282 following exposure to diuron, with females shown to be more sensitive to base oxidation than
283 males. Oxidative stress induced by diuron could be one of the mechanisms responsible for
284 DNA damage to gonads of exposed genitors. Certain pesticides are known to induce oxidative
285 stress via various mechanisms. For example, the herbicide paraquat (N,N'-dimethyl-4,4'-
286 bipyridinium dichloride) is known to be directly responsible for enhancing free radical
287 generation, entering redox cycles and constantly generating ROS. Other herbicides (for
288 example diethyldithiocarbamate, DDC) are known to be inhibitors of antioxidant enzymes,
289 such as superoxide dismutase (SOD) and catalase (CAT) (Lushchak, 2011). In the case of
290 diuron, both mechanisms may be involved. Bouilly et al. (2007) reported an increase in ROS
291 production in oyster hemocytes following a 4-week exposure to 0.3 and 3.0 $\mu g.L^{-1}$ of diuron.
292 Moreover, diuron appears to decrease the activity of the antioxidant enzyme superoxide
293 dismutase (SOD) in oysters further to 6 and 24 h exposure to 1 $\mu g.L^{-1}$ of diuron (Luna-Acosta
294 et al., 2012). As diuron has a low bioconcentration factor (BCF) in oysters, with values
295 ranging from 7 to 17 (Luna-Acosta et al. 2012, Buisson et al. 2008), its biotransformation in
296 oyster can play a primordial rule in its ability to induce an oxidative stress.

297 High levels of 8-oxodGuo induced by acetonitrile, the solvent used for diuron dissolution,
298 were also observed in females. Acetonitrile is known to enhance oxidative stress in certain
299 organisms (Kirankumar et al., 2013) and is considered as the most toxic solvent used in
300 ecotoxicological studies (Barahona-Gomariz et al., 1994). Female genitors appeared more
301 prone to oxidative stress than males with regards to 8-oxodGuo levels in gonad tissue. Sex
302 differences in oxidative stress response have already been observed in humans, with higher
303 sensitivity shown by men or women according to the exposure context (Brunelli et al., 2014;

304 Kamhieh-milz and Salama, 2014). Similar differences have also been observed in fish. In Nile
305 Tilapia, following paraquat exposure, males have been shown to have higher SOD activity
306 than females (Figueiredo-Fernandes et al., 2006). Despite the fact that female oysters showed
307 higher levels of 8-oxodGuo, it is interesting to note that immunohistochemistry analysis in
308 gonad revealed the cell distribution of oxidized nucleosides to be far more limited than in
309 males. Although 8-oxodGuo was only present in young oogonia and in some undifferentiated
310 germ cells pressed against the inside of the tube (Figure 4), it was detected in all the germ
311 lineage in male. Because in both sex, labelled early germ cells could correspond at least in
312 part to germ stems cells (which remain poorly characterized in this alternative
313 hermaphrodite), diuron exposure could affect not only the gametogenesis in course but also
314 future reproductive cycles. Of note, the results obtained in males corroborated previous
315 findings using the comet assay in sperm from the same genitors (higher levels of DNA strand
316 breaks) (Barranger et al., 2014). Spermatozoa of exposed genitors were potentially involved
317 in the transmission of damaged DNA, leading to the negative impact observed on oyster
318 recruitment (decreased hatching rate, higher levels of larvae abnormalities and reduced larvae
319 growth).
320 Little is known about DNA damage in germ cells and gametes of aquatic invertebrates. In
321 *Crassostrea gigas*, Akcha et al. (2012) showed that *in vitro* exposure to diuron leads to DNA
322 strand breaks in spermatozoa from concentrations as low as 0.05 μgL^{-1}. The sensitivity of
323 oyster sperm to herbicides was confirmed by Mai et al. (2014) following exposure to
324 metolachlor, with genotoxicity observed from concentrations of 0.01μgL^{-1}. In the freshwater
325 crustacean *Gammarus fossarum*, Lacaze et al. (2011) also found spermatozoa to be clearly
326 more sensitive to genotoxicants than oocytes. Moreover, they observed that after 5-day
327 exposure to methyl methanesulfonate (MMS) and 4-day recovery, spermatozoa showed no
328 decrease in DNA damage, contrary to oocytes, in which DNA damage was far lower than

329 values measured prior to the recovery period. In the blue mussel *Mytilus edulis*, significant
330 recovery of DNA integrity has been also demonstrated in sperm following exposure to MMS,
331 suggesting that male gametes have a capacity for repair, albeit more limited than that of
332 somatic cells (Lewis and Galloway, 2009). The higher sensitivity of spermatozoa to
333 genotoxicants may be explained by the fact that they are particularly prone to oxidative stress,
334 as most of the cytoplasm containing antioxidant enzymes is released during spermiogenesis
335 (Aitken and Curry, 2010). This information could explain the presence of damage at all stages
336 of germ cells in males, which is not the case for females, also probably due to their higher
337 DNA repair capability (Ménézo et al., 2010). Indeed, the consequences of pollutant-induced
338 DNA damage in germ cells and gametes are directly dependent on the efficiency of DNA
339 repair mechanisms. Base excision repair (BER) is likely to be the main mechanism involved
340 in the enzymatic restoration of oxidative base lesions within the DNA. In humans,
341 spermatozoa have been found to have a very low ability to respond to such an attack, as they
342 only possess the first enzyme in the BER pathway: 8-oxoguanine glycosylase 1 (OGG1)
343 (Smith et al., 2013). If the oocyte fails to complete the post- fertilization repair process
344 properly, or if the repair machinery is inadequate to repair DNA damage, de novo mutations
345 in offspring are potentially created (Aitken et al., 2014).
346 Our immunohistochemistry analysis offered additional information. Cytoplasm was also
347 labelled in oogonia. The detection of 8-oxodGuo in this cell area could indicate the presence
348 of DNA damage in mitochondrial DNA. HPLC analysis was actually performed on whole
349 DNA extraction, including both genomic and mitochondrial DNA.
350 In addition to germ cells, intra-gonadal somatic cells (ISC) were also stained in males and
351 females. These cells have a crucial role on the structuring of germinal epithelium in gonadal
352 tubule. In male they possess some ultrastructural characteristics founded in vertebrate Sertoli
353 cells (Franco et al., 2011). Therefore, these properties make ISC ideal candidates as paracrine

354 regulator of germ lineage development. DNA damage on these cells could have consequences
355 on gametogenesis progress.
356 Moreover, it is interesting to note that without RNase digestion, high immunolabelling was
357 observed in the cytoplasm of spermatogonia. This could indicate oxidized base presence in
358 RNA, which is likely more prone to oxidative damage than DNA for different reasons. RNA
359 is mainly single-stranded and more easily accessible to ROS. It has relatively less association
360 with proteins and an extensive sub-cellular distribution. Cytoplasmic RNA is in close
361 proximity to mitochondria where the majority of ROS is generated and up to now there is no
362 evidence of repair mechanisms of oxidatively-damaged RNA (Li et al., 2006). Oxidative
363 RNA damage could result in a loss or alteration of RNA function. In humans, evidence that
364 oxidized RNA is related to the pathogenesis of degenerative diseases is mounting (Kong and
365 Lin, 2010).

366 **Conclusion**

367 In this study, HPLC and immunohistochemical analysis allowed the detection of 8-oxodGuo
368 for the first time in gonads of the Pacific oyster. Diuron exposure resulted in oxidative DNA
369 damage in oyster reproductive tissue, probably due to the induction of oxidative stress. The
370 DNA damage revealed in germ cells explained the damage found in gametes of diuron-
371 exposed genitors in our previous study (Barranger et al, 2014), leading to the vertical
372 transmission of damaged genetic material to offspring. Male and female gonads both showed
373 high levels of 8-oxodGuo. However, sex differences were detected with regards to the number
374 and cell distribution of oxidized nucleosides. These differences need to be explored by
375 studying the various mechanisms in play - DNA repair, antioxidant defences and
376 biotransformation capacity - separately in males and females. An interesting and disturbing
377 find was the presence of oxidative DNA damage in early germ cells, suggesting a possible
378 long term effect on successive germ lines of the exposed animals. It would be useful to

379 quantify the oxidized base 8-oxo dGuo in RNA after exposure to pesticides in a future study,
380 to assess whether RNA, as well as DNA, can be damaged. Our results demonstrate the
381 significant impact of chemical pollution and especially pesticides on reproduction of the
382 Pacific oyster.
383

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388

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576 ## Legends

577 **Figure 1:** 8-oxodGuo levels in male (A) and female (B) gonads. Data marked with different
578 letters differed significantly ($p<0.05$).

579 **Figure 2**: Control slides. Control slide without primary antibodies in male gonad (A) and
580 control slide without secondary antibodies in female gonad (B). Sections show no specific
581 labeling. Immunohistological detection of 8-oxodGuo in male gonadal tubule without RNase
582 (C) and with RNase (D). Without RNase staining is detected around nuclei of spermatogonia

583 **Figure 3**: Immunohistological detection of 8-oxodGuo in male gonadal tubule of Crassostrea
584 gigas. Different views of male gonadal tubule (A, B, C, D), showing labelled spermatogonia
585 (A, B, C, D); spermatids (C), intragonadal somatic cells (B), haemocytes (A, D) and interstitial
586 conjunctive cells (B). sg I: spermatogonium I, sg II: spermatogonium II, sp: spermatid, sz:
587 spermatozoid, isc: intragonadal somatic cell, h: haemocyte, cc: interstitial conjunctive cell.
588

589 **Figure 4**: Immunohistological detection of 8-oxodGuo in female gonadal tubule of
590 *Crassostrea gigas*, showing the presence of 8-oxodGuo in nuclei, nucleoli and cytoplasm of
591 oogonia (A, B, C), in putative germ stem cells (B), in intragonadal somatic cells (C, D, E),
592 interstitial conjunctive cells (C) but not in oocytes (D, E). og: oogonia, o: oocyte, n: nucleus,
593 nu: nucleolus, cyt: cytoplasm, pGSC: putative germ stem cells, isc: intragonadal somatic cell,
594 cc: interstitial conjunctive cells.
595

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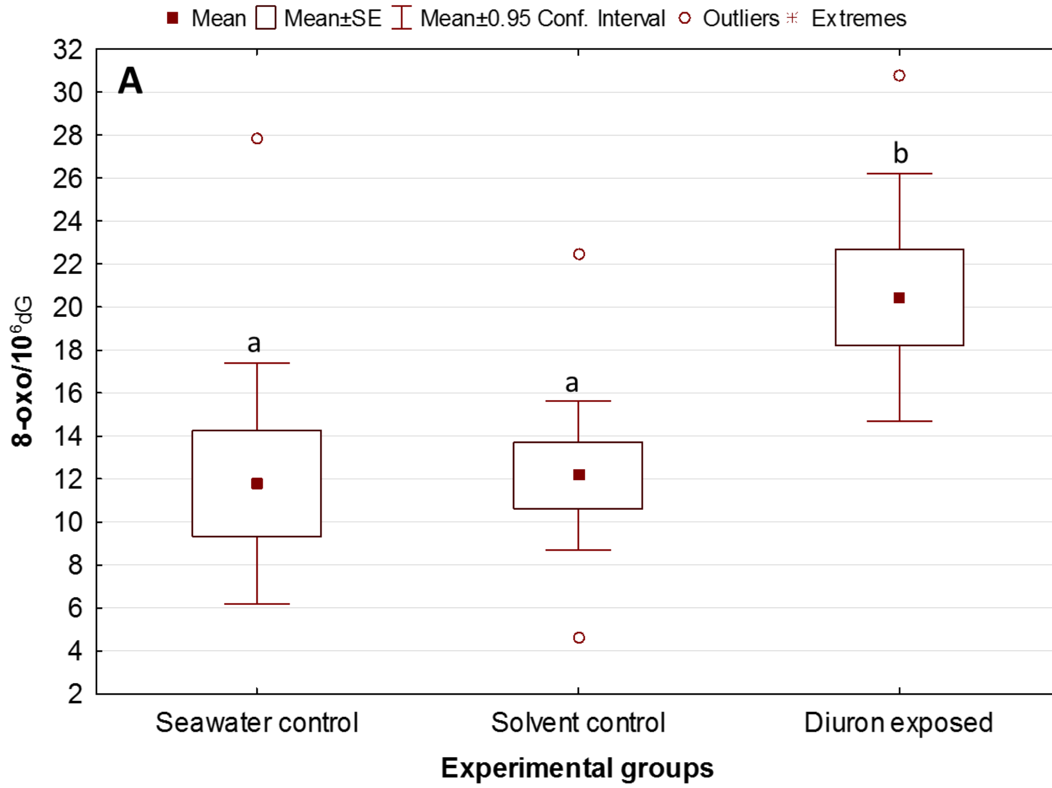
598 **Figure 1**



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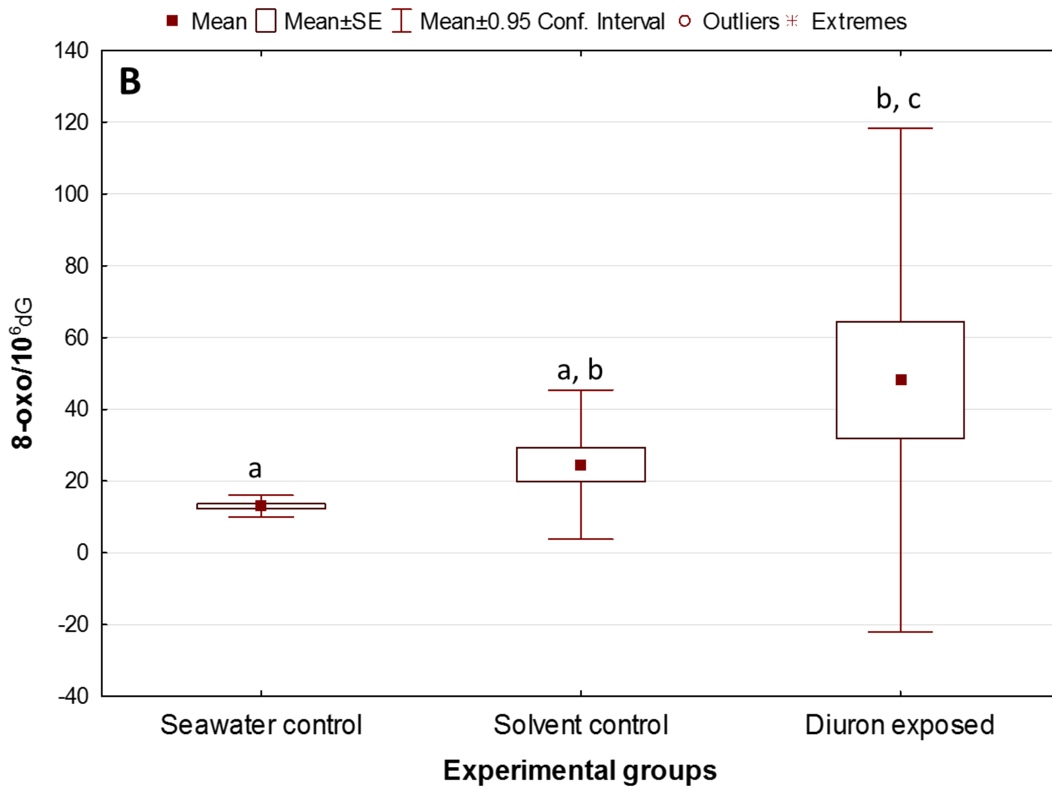

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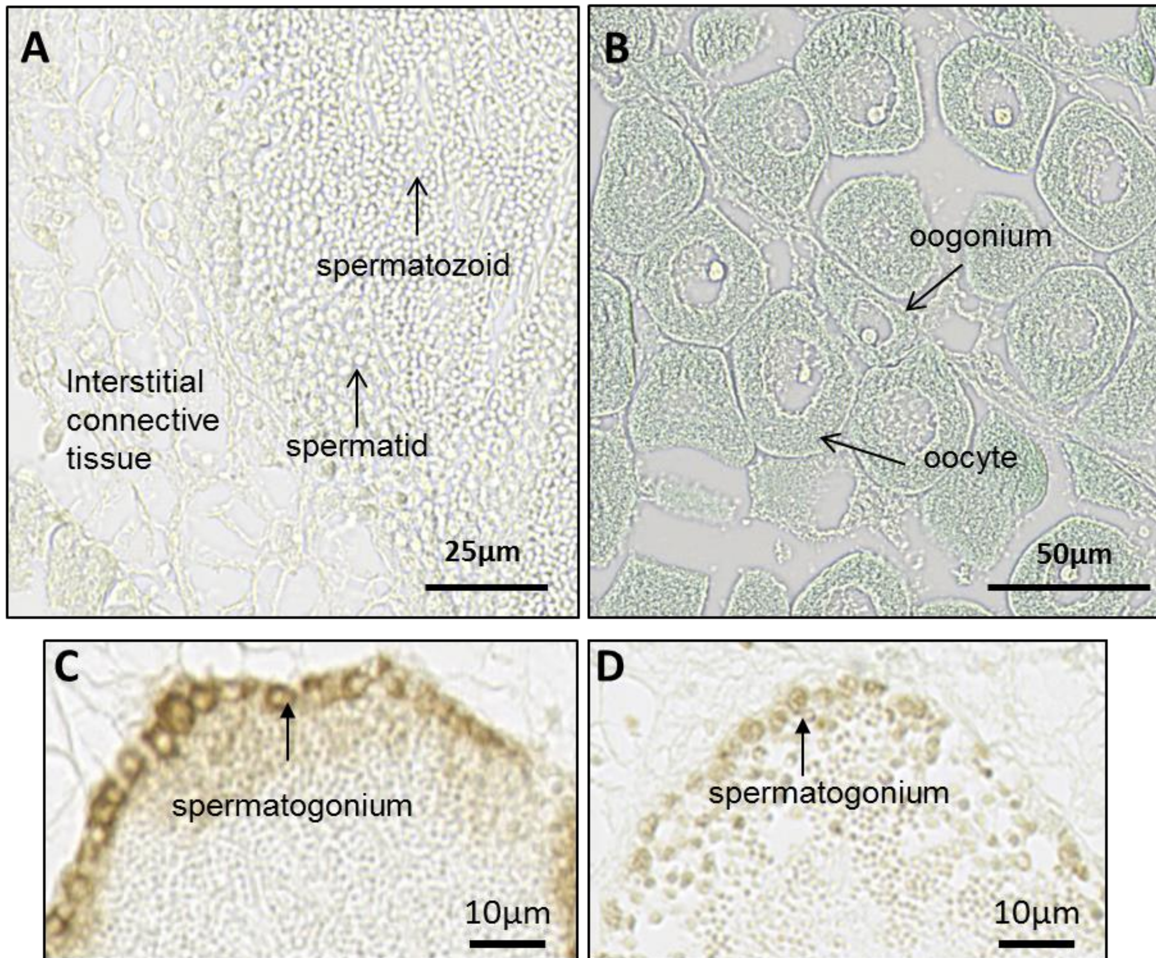
602 **Figure 2**



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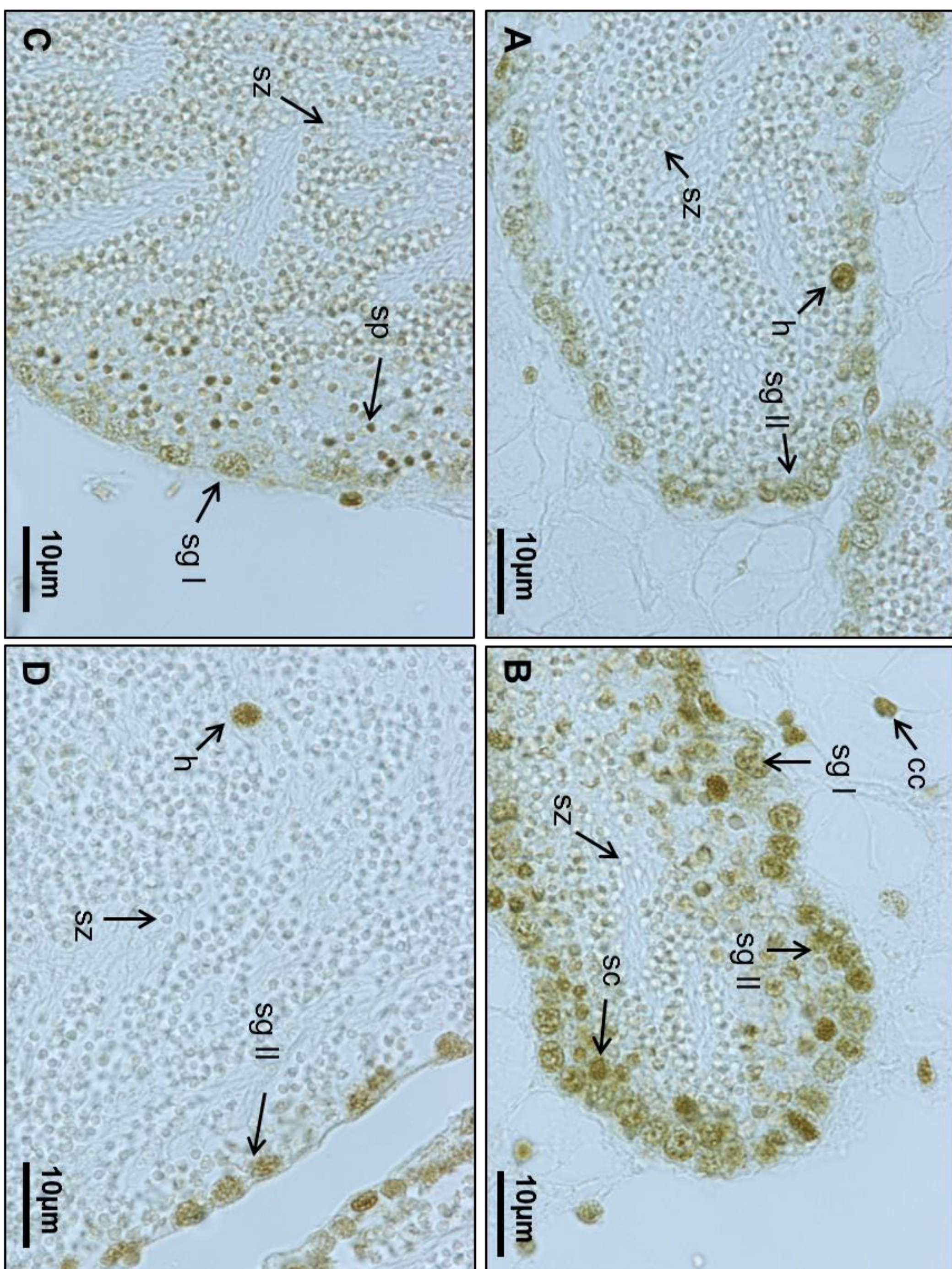
606 **Figure 3**



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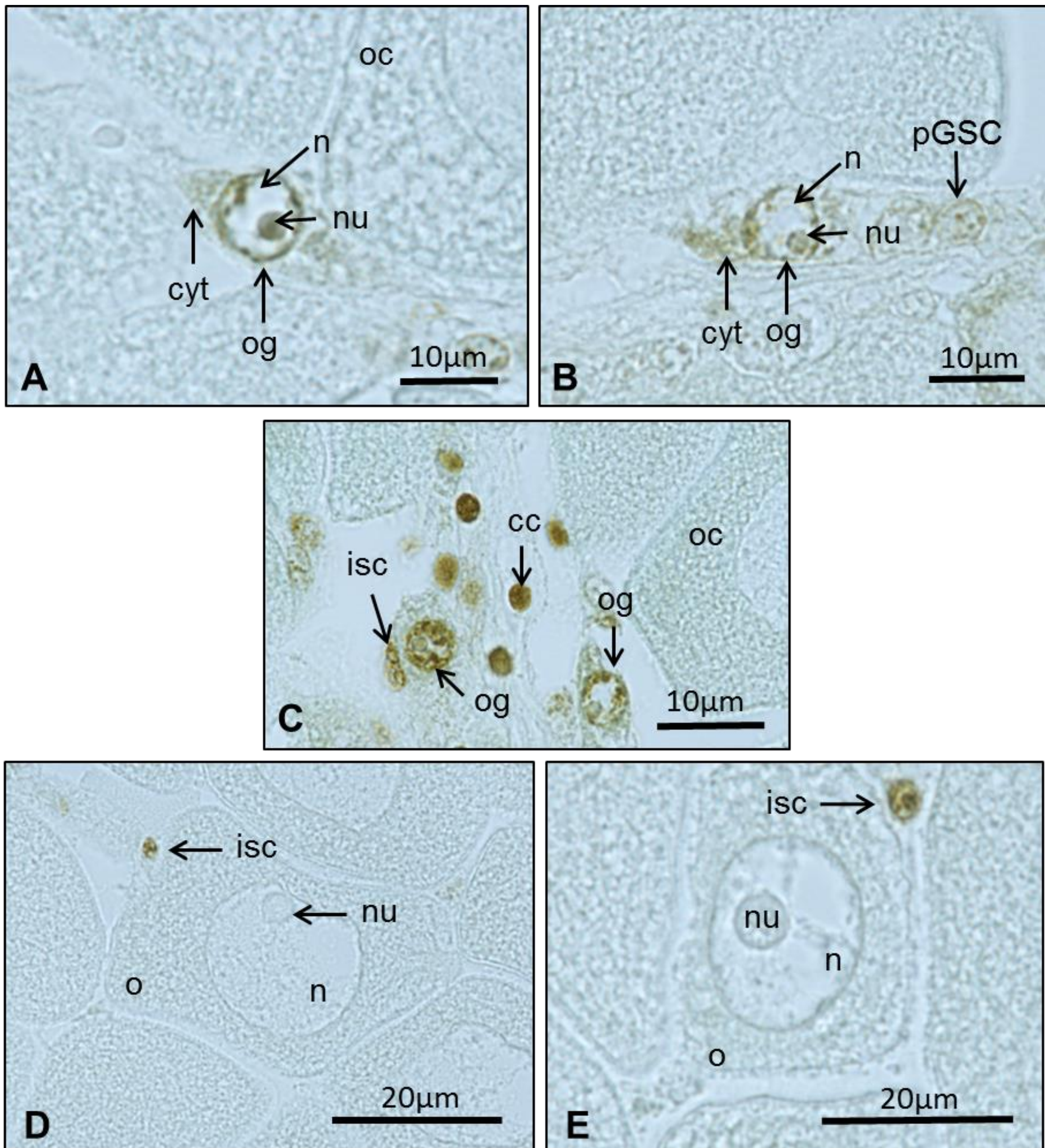
610 **Figure 4**



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