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1 **Effects of a chronic exposure to different water temperatures and/or to an**
2 **environmental cadmium concentration on the reproduction of the threespine stickleback**
3 **(*Gasterosteus aculeatus*).**

4

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31 **Abstract**

32 Knowledge about combined effects of chemicals and temperature on reproductive
33 capacity of fish are rare in literature, especially when it comes to the effects of chronic low-
34 dose chemical exposure combined to the thermal stress. The aim of the study was to evaluate
35 the single and combined effects of temperature (16, 18, 21 °C) and an environmentally
36 relevant concentration of waterborne cadmium (1 µg. L⁻¹, nominal concentration) on the
37 reproductive outputs of threespine stickleback (*Gasterosteus aculeatus*), and their
38 consequences on offspring survival parameters. The high temperature (21 °C) was the only
39 factor that affected parental parameters (gonadosomatic index “GSI”, and vitellogenin “VTG”
40 particularly). On females, 21 °C had a stimulating effect on gonadal development evaluated
41 by an early increase, followed by a sharp decrease of GSI, probably indicating gonadal
42 atresia. Promoting effect of temperature was corroborated by an early production of VTG. *In*
43 *vitro* fertilization assays showed interesting results, particularly cadmium effects. As it was
44 supposed, high temperature had a negative impact on offspring parameters (significant
45 decrease in survival and an increase of unhatched embryos). Parental exposure to the very low
46 concentration of cadmium had also negative consequences on mortality rate (significant
47 increase) and hatching rate (significant decrease). Our results indicate that in a global
48 warming context, high temperature and its combination with contaminant may impact
49 reproductive capacity of *G. aculeatus*, by decreasing parental investment (low eggs and/or
50 sperm quality).

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52 Keywords: cadmium stress; *Gasterosteus aculeatus*; *in vitro* fertilization, multiple stress;
53 reproduction impairment; temperature stress.

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65 **1. Introduction**

66 Freshwater habitats have been the most human mistreated and ignored natural
67 resources (Schindler, 2001). Anthropogenic activities are now largely recognized as the most
68 impacting factors in aquatic ecosystems (Meybeck, 2003), and are responsible for the
69 generation and the introduction in the aquatic ecosystems of chemical substances, many of
70 which are supposed to be present in trace amounts (*i.e.* heavy metals, PAHs, etc.) or even
71 absent in natural conditions (*i.e.* PCBs, DDT, pesticides, etc.) (Biziuk et al., 1996; Adams and
72 Greeley, 2000; Meybeck, 2003). A large number of man-made chemical substances are
73 known to date (over 130 million compounds reported on the CAS registry system) (CAS,
74 2017), many of them can be harmful for flora (Oukarroum et al., 2013), fauna (Bucke, 1993)
75 and even human health (Wang et al., 2005). The toxic effects of these chemical compounds
76 on aquatic organisms are largely studied and reported in literature (Reddy, 2013;
77 Chandurvelan et al., 2017; Jiang et al., 2017). In fish they may disturb energy metabolism
78 (Hani et al., 2018b), induce genotoxicity (Santos et al., 2014), immunotoxicity (Bado-Nilles et
79 al., 2014), oxidative damage (Livingstone, 2003), neurotoxicity and reproductive impairments
80 (Tierney et al., 2013), etc.

81 The reproductive processes are often considered to be a critical bottle-neck point in the
82 life history of living organisms, as they allow species to perpetuate themselves throughout
83 generations (Miller and Kendall, 2009), and therefore are an endpoint regularly used in
84 ecotoxicology studies (Jeziarska et al., 2009; Huang et al., 2017; Mennigen et al., 2017; Hou
85 et al., 2018). However, effects of chemical stressors are usually tested individually, but in
86 nature, organisms are not only exposed to anthropogenic stressors but to several stressors
87 simultaneously. Hence, several authors are increasingly realizing that single-stressor
88 experiments may not be appropriate in assessing the real effects of hazardous chemicals on
89 living organisms (Gunderson et al., 2016).

90 Generally, multiple stressor situations are characterized by an interaction between a
91 chemical and non-chemical stressor. The latter can be either an extrinsic abiotic (*i.e.* ionizing
92 radiation, temperature, pH, salinity, UV-radiation, etc.), or an extrinsic biotic (*i.e.* parasite,
93 bacteria, virus infections, etc.) factor, which could affect individual physiology and fitness
94 (*i.e.* growth and reproduction), and/or the population survival (Vinebrooke et al., 2004; Beyer
95 et al., 2014). One of the most important non-chemical stressors is the prospective of a

96 worrying global temperature rise, predicted by the Intergovernmental Panel on Climate
97 Change (IPCC, 2013), in the few next decades.

98 Temperature plays a strong and frequently dominant role in governing the
99 fundamental physiological processes of ectotherms (*i.e.* survival, growth and reproduction)
100 (Guderley, 1994), as their body temperature changes with the temperature of their
101 environment, resulting in corresponding alterations of the rates of all physiological and
102 biochemical reactions (Heugens et al., 2001; Sokolova and Lannig, 2008). As part of climate
103 change scenarios, the increase of temperature fluctuations and the frequency of extreme
104 temperatures, are expected as a result of a global temperature rise, which can be harmful or
105 even lethal to living aquatic organisms (Trenberth et al., 2007; Sokolova and Lannig, 2008).

106 In fish, temperature has influence on hormone synthesis, secretion and metabolism.
107 Consequently, higher than optimal temperatures can substantially impact reproductive
108 endocrine homeostasis, and its endpoint events (*i.e.* gametogenesis; spawning; fertilization),
109 with repercussions on offspring development and survival (Van Der Kraak and Pankhurst,
110 1997). For example, Dorts et al., (2012) exposed adult European bullhead (*Cottus gobio*) to
111 three temperature regimes (T1: 8, T2: 12 and T3: 16 °C, in mean), to assess the effect of
112 temperature on different reproductive parameters. Authors reported that an 8°C rise in water
113 temperature (T3) was responsible for a decrease in sex steroid levels, with an absence of
114 gonadosomatic index (GSI) peak, when compared with fish of the other temperature regimes.

115 While single effects of temperature and pollutants are well documented, there is a lack
116 of information (if not absent) about the effects of the interaction of chemical and thermal
117 stressors on the reproductive performance in fish, especially under environmentally realistic
118 exposure conditions. The focus on single stressors can lead to erroneous conclusions, while
119 multiple stressor approaches are of interest, for their potential to reveal interesting
120 information, which would be difficult to predict, based on single stressor studies (Tockner et
121 al., 2010).

122 Cadmium is a natural metal element found in mineral deposits and distributed widely
123 at low concentrations in the environment (US EPA, 2016). However, due to industrial uses, it
124 is considered as one of the most important and dangerous anthropogenic metal contaminant,
125 and classified as a “priority pollutant” in Europe (EE COMMISSION, 2013) and other
126 countries as the United States (US EPA, 2016). Its ability to cause adverse physiopathological
127 and toxicological effects, including reproductive toxicity in living organisms, is well
128 documented (de Angelis et al., 2017; Driessnack et al., 2017). In fish, cadmium exposure can

129 induce a decrease in spawning frequency and fecundity, as it was reported in fathead minnows
130 (*Pimephales promelas*) (Sellin and Kolok, 2006). The reproductive toxicity of cadmium may
131 be linked to its bioaccumulation capacity in fish gonads, resulting in cadmium-induced
132 impairment of gametogenesis in both male and female fish, or even gonad injuries (Luo et al.,
133 2015; Vergilio et al., 2015). Moreover, cadmium exposure is responsible for delayed or
134 premature hatching of fish eggs, and multiple skeletal and morphological deformities in larvae
135 (Jeziarska et al., 2009; Sfakianakis et al., 2015). Except a few studies like that of Wang et al.
136 (2014), the majority of studies reported in literature on the reproductive effects of cadmium,
137 were conducted using short-term exposure at sub-lethal concentrations, which do not reflect
138 nor a realistic exposure scenarios neither the real effects when it comes to the combination
139 with other stressors such as temperature.

140 In this study, we were interested in this problematic. Hence, we used the threespine
141 stickleback (*Gasterosteus aculeatus*) –a recognized sentinel fish species, largely used as
142 animal model in ecotoxicology studies (Pottinger et al., 2002; Sanchez et al., 2007)–, to
143 evaluate the effect of a long-term exposure to temperature and/ or cadmium on the
144 reproductive success, and its consequences on the survival capacity of the offspring. Two
145 different exposure scenarios were tested, at two different stages of sexual maturity (adults or
146 juveniles). In the first experiment, we exposed adult reproductive sticklebacks for 120 days, to
147 three water temperatures (16, 18 and 21°C), and we aimed at evaluating the effects of this
148 single stress on reproductive outputs of sticklebacks, by evaluating some organosomatic
149 index (GSI, HSI and NSI), vitellogenin production, and the fertilization rate after standardized
150 *in vitro* fertilization trials (IVF). In the second experiment, we started the experiment with
151 juvenile non-reproductive sticklebacks, in order to explore the single or combined effects of
152 90-days of exposure to an environmental concentration of cadmium (nominal concentration of
153 1 µg. L⁻¹) and two different water temperatures (16 °C and 21 °C) on the reproductive
154 capacity of sticklebacks. For that we assessed, as for the first experiment, some
155 organosomatic index (GSI, HSI and NSI), vitellogenin quantities and the fertilization rate
156 after standardized *in vitro* fertilization trials (IVF).

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163 **2. Materiel and methods**

164 **2.1. Ethic statement**

165 All procedures described in the present paper were conducted in accordance with the
166 European directive 2010/63/UE for the protection of animals used for scientific purposes. The
167 INERIS registration number, where the experiments were conducted, is the C60-769-02. The
168 experimental protocols were submitted and reviewed by a French nationally recognized
169 ethical committee (CREMEAPS, registration number 96).

170 **2.2. Origin and maintenance of parental fish**

171 Sticklebacks reared in outdoor mesocosms of the French National Institute for
172 Industrial Environment and Risks (INERIS, Verneuil-en-Halatte, France) were used in this
173 study. Few months before the start of the experiments, fish were transferred for acclimation to
174 300- litter laboratory tanks with continuous freshwater renewal. *G. aculeatus* prefer relatively
175 cool temperatures ($< 18^{\circ}\text{C}$) (Guderley, 1994). Hence, for the two experiments of this study,
176 sticklebacks were acclimated to 16°C as control temperature, before the onset of the
177 experiments. All along the acclimation and the experimental periods, sticklebacks were daily
178 fed *ad libitum* with frozen commercial chironomids larvae, at 3 % of body weight/day
179 according to Leloutre et al (2016) recommendations.

180 **2.3. Experimental design**

181 2.3.1. Experiment 1: Effects of temperature on parental reproduction and its
182 consequences on the offspring quality

183 In this first experiment, 165 homogeneous adult reproductive sticklebacks ($955.80 \pm$
184 55.13 mg and 40.40 ± 0.49 mm) were transferred from the acclimation tanks into three new
185 300-litter tanks ($n=55/\text{tank}$), with continuous oxygenation and freshwater renewal. Each tank
186 corresponds to an experimental condition. Indeed, sticklebacks were exposed to three constant
187 water temperatures: (i) control condition (16°C), and experimental conditions as (ii) 18°C
188 which exceed slightly the control temperature, but remain in the range of optimal
189 temperatures of sticklebacks as reported by Guderley (1994), and (iii) 21°C as high-stressful
190 temperature condition (Hani et al., 2018a, 2018b). Water temperature of experimental
191 conditions was gradually increased at a rate of $0.5\text{-}0.8^{\circ}\text{C}\cdot\text{day}^{-1}$, to reach 18°C and 21°C .

192 Once water of the experimental tanks has reached the required temperatures, fish were
193 exposed continuously for 120 days. Throughout the experiment, water parameters
194 (temperature, pH, conductivity, dissolved oxygen) were daily monitored, and fifteen
195 individuals from each group were sampled on days 15, 60 and 120, during which vitellogenin,
196 gonadosomatic (GSI), hepatosomatic (HSI) and nephrosomatic (NSI) indices were evaluated.
197 Additionally, when a gravid female and a nuptially coloured male were observed in one of
198 tanks conditions, they were recovered (n=7 / sex/ condition) to evaluate the consequences of
199 the thermal exposure on their offspring survival, after standardized *in vitro* fertilization trials.

200

201 2.3.2. Experiment 2: Single or combined effects of temperature and/or
202 cadmium on parental reproduction and the consequences on their
203 clearwater-reared offspring

204 In this experiment, only two water temperatures were selected: 16 °C and 21 °C
205 (according to results of exp 1 see part 3.1.). Additionally to the thermic stress, we exposed
206 juvenile sticklebacks (n= 320, 608.4 ± 147.06 mg, and 35.47 ± 2.92 mm) to waterborne
207 cadmium in semi-static conditions with complete water renewal every other day. Fish were
208 transferred from acclimation tanks and randomly distributed into four 90-litter laboratory
209 glass tanks (n=80/tank), in which they were acclimated progressively to the desired
210 experimental temperatures (0.5-0.8 °C.day⁻¹). Each tank was continuously oxygenated and
211 corresponded to a condition group: one control (16 °C) and three experimental groups
212 (cadmium condition at 16 °C, temperature condition “21 °C” without cadmium, and
213 cadmium-temperature condition). The experiment lasted 90 days, and twenty-five individuals
214 from each group were sampled on days 15, 60 and 90 during which several biological
215 parameters were evaluated as for experiment 1. Throughout the experiment, water parameters
216 (temperature, pH, conductivity, dissolved oxygen) were assessed before and after water
217 replacement. Finally, in order to evaluate the consequences of the different stress on the
218 offspring, standardized *in vitro* fertilization trials were conducted from the second month,
219 when individuals (n=5/ sex/condition) were reproductively actives: ripe females and nuptially
220 coloured males (Wootton, 1984).

221 **2.4. Cadmium chloride (CdCl₂)**

222 Sticklebacks of experiment 2 were exposed to cadmium chloride (CdCl₂, Sigma-Aldrich
223 Chemicals, France). We chose 1 µg. L⁻¹ as nominal dose: a realistic environmental
224 concentration (Foran et al., 2002) which lines within the range of the Environmental quality

225 standards levels recommended by EE COMMISSION (2013) for the European Water
226 Framework Directive.

227 Throughout the experiment, water concentrations were monitored daily during the first
228 month of exposure and weekly for the rest of the study. For that, water samples were acidified
229 with nitric acid (2 %), and total cadmium concentrations were checked by electrothermal
230 atomic absorption spectrophotometry (SpectrAA, GTA110, Varian, Palo Alto, CA, USA),
231 according to the method of Geffard et al. (2010). Since exposure was conducted in semi-static
232 conditions, the actual cadmium concentration at which sticklebacks were really exposed was
233 calculated as time-weighted mean (TW Mean) according to OECD Test Guidelines (OECD,
234 2012), and was about $0.50 \pm 0.2 \mu\text{g. L}^{-1}$ (half the targeted nominal concentration).

235 Cadmium bioaccumulation assays were also conducted in liver and carcass, and were
236 published previously in Hani et al. (2018b). Briefly, liver, but not carcass had bioaccumulated
237 cadmium in fish exposed to cadmium and cadmium-temperature combination, whereas in
238 control and “temperature” groups, no bioaccumulation was observed.

239

240 **2.5. Biological endpoint measurements in parental sticklebacks**

241 For both experiments and for each sampling date, fish were weighed, measured and
242 sacrificed by cervical dislocation after anesthesia with tricaine methanesulfonate MS222 (70
243 mg. L⁻¹, SIGMA-ALDRICH, France). Immediately after sacrifice, blood was collected,
244 diluted 10-fold in a buffer solution (Phosphate buffer “PBS” pH 7.4 + Glycerol 20% +
245 Phenylmethylsulfonyl fluoride “PMSF” 0,2 mM + Heparin 20%) and centrifuged at 9000 x g
246 for 10 min. The supernatant was frozen at -80°C before VTG analysis. Gonad, liver, kidney,
247 and other organs were rapidly dissected, weighed, and then frozen at -80°C, to analyse a
248 battery of biomarkers: digestive enzymes, antioxidant parameters and energy reserves
249 parameters (Hani et al., 2018a, 2018b) and calculate the corresponding somatic index (GSI,
250 NSI and HSI) according to the following equation: organ somatic index = (organ weight/fish
251 weight) × 100.

252 VTG concentration was measured in blood supernatant according to the modified
253 method of Sanchez et al. (2005a), adapted in microplate on the TECAN Freedom EVO®
254 automated platform. This assay is based on an indirect competitive ELISA using antibodies
255 anti-sticklebacks’ VTG at 2000-fold final concentration (GA-306, Biosense Laboratories,

256 Bergen, Norway), and purified stickleback VTG as standard. Results were expressed as ng.
257 ml⁻¹.

258 **2.6. Biological endpoint measurements in offspring (*in vitro* fertilization** 259 **trials)**

260 Artificial fertilization (IVF) was conducted according to the modified protocol of
261 Santos et al. (2013). For each trial, a reproductive male and a gravid female (apart from
262 individuals used to evaluate the direct effects of stress on parental reproduction) were used.
263 Animals were anesthetized (70 mg. L-1, MS222, SIGMA-ALDRICH, France), weighed and
264 standard body size was measured, before each operation.

265 After anesthesia, the reproductively active male was sacrificed with cranial
266 dislocation. The testes were carefully dilacerated using thin dissection blade in 400 µl of cold
267 Hank's Balanced Salt Solution (HBSS: 137 mM NaCl, 5.4 mM KCL, 0.25 mM Na₂HPO₄,
268 0.44 mM KH₂PO₄, 1.3 mM CaCL₂, 4.2 mM NaHCO₃), and then the sperm suspension was
269 collected in an Eppendorf tube, until fertilization. Eggs were obtained by gentle abdominal
270 stripping as described by Bakker et al. (2014). The egg clutch was gently mixed with the
271 totality of sperm suspension and let to fertilize into moistened PYREX™ Crystallizing dishes.
272 Two minutes after, fertilization had been stopped by pipetting three times 1000 µl of fresh
273 HBSS onto the egg clutch. After that, other series of washing were applied using 2000 µl of
274 hypochlorite solution (0.15% once during 2 min) and 1000 µl of NaCl solution (0.7%, three
275 times) (Santos et al., 2013). After fertilization and washing steps, egg clutch was immediately
276 transferred in incubation basket of 1 mm plastic mesh, to ensure egg immersion and aeration.
277 The clear water was continuously aerated and changed every other day.

278 The total number of eggs of each clutch and the number of fertilized/unfertilized eggs
279 were counted using a stereomicroscope with a DC digital imaging system (Leica MZ 12.5),
280 after 2h of the incubation period. At this moment, fertilized eggs become transparent and give
281 rise to the 2-cell stage, according to (Swarup, 1958). For each group, the fertilization rate was
282 calculated: [number of fertilized eggs/ total number of egg clutch * 100]. Offspring were daily
283 observed and counted after the dead ones were removed, until few days post hatching.
284 Throughout the embryogenic development, we were interested in several parameters to
285 evaluate offspring quality: (i) embryonic mortality rate [number of dead embryos/ number of
286 fertilized eggs * 100]; (ii) day of first hatching; (iii) hatching rate [number of hatched larvae/
287 number of fertilized eggs * 100].

2.7. Statistical analysis

Statistical analyses were performed using R statistical software (v3.3.1) at $\alpha = 0.05$. For organosomatic index and vitellogenin, two-way ANOVA test was performed after that normality and homogeneity were tested, using respectively Shapiro and Levene tests. When normality or homoscedasticity were not met, data were transformed using log, or square root method. We first analysed the effect of sex on the different parameters at each time-point independently, using two-way ANOVA. Sex and exposure conditions were considered as factors. Since there was a significant sex-effect, data were then analysed on males and females taken separately. For that, two-way ANOVA test was performed, using exposure and time conditions as factors. Tukey's HSD post-hoc test was performed to compare conditions at each time-point. For IVF essays, a nonparametric test (Kruskal-Wallis) was used followed by a non-parametric post-hoc test (Kruskal Nemenyi test), to determine which groups were different from the controls.

3. Results

3.1. Experiment 1: Effects of temperature on parental reproduction and its consequences on the offspring quality

3.1.1. Organosomatic index

GSI, HSI and NSI of parental sticklebacks from the different experimental groups are presented in Table 1. Unfortunately, NSI values were not recorded in both males and females at day 120 of the experiment. In males, a significant effect of exposure conditions (correspond to the different experimental groups: control, 18 °C and 21 °C) was recorded for GSI and HSI, but not for NSI. In fact, the higher temperature was responsible for a significant decrease on day 15 ($p=0.01$) and 60 ($p=0.02$) for HSI, and only on day 120 for GSI ($p<0.01$). Furthermore, time condition (correspond to the different times of exposure: days 15, 60 and 120) had a significant effect on males GSI and NSI, but not on HSI values. Indeed, when compared to day 15, a significant decrease in GSI values was recorded in control males on day 60 ($p=0.03$), and in males exposed to 21°C on day 120 ($p=0.0001$). However, for NSI, a significant increase was recorded in all male-groups on day 60 compared to day 15 ($p<0.001$). In females, exposure conditions had no effect on the three parameters throughout the experiment. Time condition had a significant effect on GSI but not on HSI and NSI values. Indeed, GSI values in females of the three groups, increased on day 60, and then decreased on

319 day 120. However, the temporal effect was significant only for females exposed to 18°C on
320 day 60 ($p=0.04$), and for the ones exposed to 21°C on day 120 ($p=0.02$).

321 3.1.2. Vitellogenin (VTG)

322 Vitellogenin production was assessed in males and females throughout the experiment.
323 VTG was not detected in males and thus results presented in Figure 1 concern only females.
324 Fish exposed to 21°C expressed the highest values of VTG until day 60 but was significant
325 only on day 15 compared to the other groups ($p=0.02$). Time condition had a significant effect
326 on VTG production and was recorded in females exposed to 16 and 18 °C, but not in females
327 of 21°C group. In fact, a significant increase was recorded in both 16°C and 18°C groups on
328 day 60 compared to day 15 ($p<0.01$), followed by a significant decrease on day 120,
329 compared to day 60 ($p<0.01$). In 21°C females group, VTG remained stable until day 60, and
330 decreased significantly on day 120, in comparison to day 15 ($p=0.02$) and day 60 ($p=0.01$).

331 3.1.3. IVF and offspring parameters

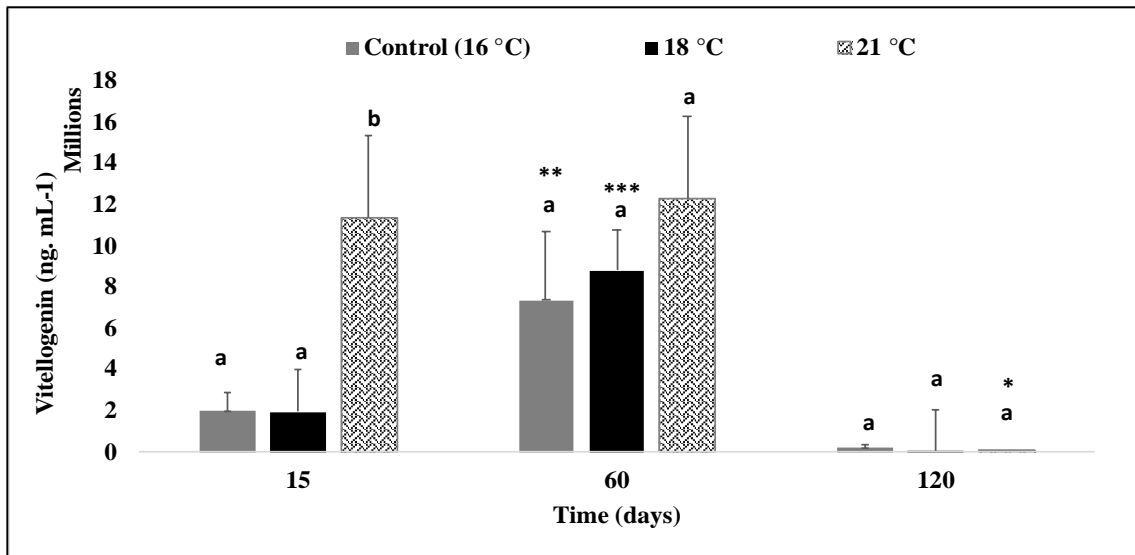
332 Results obtained from IVF assays are summarized in Table 2. Fertilization rate was
333 not significantly affected by any of the thermal treatments ($p>0.05$). Mortality and hatching
334 rates were the only parameters affected significantly by the treatment condition, when
335 compared to control group. On the one hand, an increase in the rate of mortality was recorded
336 in all groups but was significant ($p=0.01$) only in offspring from parents reared previously in
337 the highest water temperature (21 °C). On the other hand, a decrease was recorded in the
338 hatching success of experimental offspring, but was significant ($p=0.02$) only in individuals
339 from 21 °C parents group.

340 **Table 1. Organosomatic index (Gonadosomatic index “GSI”; Hepatosomatic index “HSI” and Nephrosomatic index “NSI”) of parental**
 341 **sticklebacks after 15, 60 and 120 days exposure to three thermal regimes (16, 18 and 21 °C). Fish reared at 16 °C are considered as**
 342 **control groups. Results are presented as mean ± S.E.** For a same time, different letters indicate significant differences between exposure groups (p<0.05). For the same
 343 exposure group, asterisks indicate significant temporal differences with respect to initial values of day 15 (*p<0.05; **p<0.01; ***p<0.001).

Conditions		GSI				HSI				NSI			
		♂		♀		♂		♀		♂		♀	
Day 15	Control (16 °C)	n=8	1.34 ± 0.45a	n=7	5.75 ± 2.95a	n=8	5.37 ± 0.36a	n=7	6.49 ± 0.36a	n=8	0.90 ± 0.15a	n=7	0.34 ± 0.04a
	18 °C	n=9	1.13 ± 0.30a	n=6	4.71 ± 3.50a	n=9	4.66 ± 0.14ab	n=6	5.83 ± 0.67a	n=9	0.72 ± 0.14a	n=6	0.42 ± 0.09a
	21 °C	n=8	1.27 ± 0.27a	n=7	7.63 ± 2.71a	n=8	4.27 ± 0.25b	n=7	5.51 ± 0.32a	n=8	1.05 ± 0.11a	n=7	0.57 ± 0.08a
Day 60	Control (16 °C)	n=8	0.83 ± 0.18a*	n=7	8.81 ± 3.85a	n=8	6.02 ± 0.41a	n=7	5.91 ± 0.43a	n=8	2.77 ± 0.23a***	n=7	0.54 ± 0.10a
	18 °C	n=7	0.96 ± 0.23a	n=8	10.71 ± 5.62a*	n=7	5.01 ± 0.45ab	n=8	5.77 ± 0.38a	n=7	2.32 ± 0.26a***	n=8	0.55 ± 0.07a
	21 °C	n=7	0.79 ± 0.21a	n=8	9.35 ± 3.16a	n=7	4.50 ± 0.19b	n=8	7.30 ± 0.33a	n=7	2.65 ± 0.42a***	n=8	0.88 ± 0.12a
Day 120	Control (16 °C)	n=6	1.00 ± 0.42a	n=9	2.02 ± 2.14a	n=6	5.60 ± 0.35a	n=9	8.18 ± 0.45a	n=6	N.R		N.R
	18 °C	n=7	0.84 ± 0.27a	n=8	2.59 ± 1.72a	n=7	6.18 ± 0.69a	n=8	7.58 ± 0.52a	n=7	N.R		N.R
	21 °C	n=8	0.42 ± 0.18b***	n=5	1.32 ± 0.45a*	n=8	6.30 ± 0.68a	n=5	7.74 ± 0.72a	n=8	N.R		N.R

344 N.R : Not recorded

345



346

347 **Figure 1. Evolution of the plasmatic vitellogenin (VTG) of female parental sticklebacks**
 348 **after 15, 60 and 120 days of exposure to three thermal regimes (16, 18, and 21 °C). Fish**
 349 **reared at 16 °C, are considered as control groups. Results are presented as mean ± S.E.**

350 For a same time, different letters indicate significant differences between exposure groups ($p < 0.05$). For the same exposure
 351 group, asterisks indicate significant temporal differences with respect to initial values of day 15 (* $p < 0.05$; ** $p < 0.01$;
 352 *** $p < 0.001$).

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365 **Table 2. *In vitro* fertilization parameters. The impact of the thermal stress on parental fertilization rate, and its consequences on**
 366 **clearwater-reared offspring. Results are represented as mean ± S.E.** Different letters indicate significant differences between treatment groups and
 367 control group (p<0.05). *Results in bold-italic characters should be taken with caution because of the high mortality rate observed in this group.*

Parental conditions	n	Fertilization rate (%)	Offspring conditions	Survivors (n)	Mortality rate (%)	Beginning of hatching (dpf)	Hatching rate (%)
Control (16 °C)	7	98.33± 0.80a	Non-exposed	109	64.00 ± 24.45a	8.00 ± 0.43a	34.26 ± 9.33a
18 °C	7	95.57 ± 2.18a	Non-exposed	29	91,60 ± 4.39ab	7.75 ± 0.36a	8.90 ± 4.32ab
21 °C	7	87.492 ± 1.98a	Non-exposed	5	98,77 ± 0.79b	<i>9.00 ± 0.53a</i>	<i>1.22 ± 0.79b</i>

368 **3.2. Experiment 2: Single or combined effects of temperature and/or**
369 **cadmium on parental reproduction and the consequences on their**
370 **clearwater-reared offspring**

371 3.2.1. Organosomatic index

372 Somatic index recorded throughout the experiment from parental sticklebacks, are
373 summarized in Table 3. Results of males and females were treated separately, since a
374 significant effect of gender ($p < 0.01$) was recorded for all parameters (except for HSI on day
375 15). In males, overall, no difference was recorded in treatment groups compared to control
376 groups, except for NSI on day 90 with significant decrease in temperature and cadmium-
377 temperature groups, compared to control males, $p < 0.05$. However, time condition (correspond
378 to the different times of exposure: days 15, 60 and 90) had a significant effect on the three
379 parameters of male sticklebacks. Indeed, for GSI, when compared to initial values of day 15, a
380 significant decrease was recorded in cadmium groups, on day 60 ($p = 0.001$) and day 90
381 ($p = 0.0007$). For HSI, a significant increase was recorded on day 60 ($p < 0.04$) and day 90
382 ($p < 0.01$), for all groups except for temperature groups ($p > 0.05$). Finally, for NSI values, a
383 significant increase in males of control groups ($p < 0.05$) was recorded on days 60 and 90, and
384 only on day 60 for cadmium ($p < 0.05$) and temperature ($p < 0.01$) groups.

385 In females, when compared to control groups, the exposure condition had a significant
386 effect on all parameters. For GSI, female sticklebacks of temperature and cadmium-
387 temperature groups had a significant decrease on day 90 ($p < 0.001$). For HSI, a significant
388 increase was observed on day 60 ($p = 0.01$), when fish were exposed to cadmium alone,
389 however, on day 90 a significant decrease ($p = 0.04$) was reported in fish of cadmium-
390 temperature group. For NSI, the combination of cadmium and temperature was the only
391 exposure condition responsible for a significant increase, observed on day 90 ($p = 0.03$),
392 compared to control group. In female groups, somatic index (except NSI) were also impacted
393 significantly by time condition. Indeed, in all groups, when compared to initial values of day
394 15, GSI values increased significantly on day 60 ($p < 0.001$), and were higher (but not
395 significantly) in females exposed to temperature and the combination of cadmium-
396 temperature. In both groups (temperature and cadmium-temperature), the increase of GSI was
397 followed by a sharp decrease on day 90, while it remained higher in control and cadmium
398 groups ($p < 0.001$, compared to day 15). For HSI, as for males, a significant increase was
399 recorded on day 60 and day 90, for all female groups ($p < 0.01$), except for temperature groups
400 ($p > 0.05$).

401 3.2.2. Vitellogenin (VTG)

402 Since no production was recorded in males, VTG results are represented only for
403 female sticklebacks, in Figure 2. Exposure condition had a significant effect on VTG
404 production. In fact, until day 60 of the experiment, VTG production was higher in temperature
405 and cadmium-temperature groups, but was significant only on day 60 in fish exposed to the
406 double stress ($p=0.004$). On day 90, VTG production decreased significantly in temperature
407 group compared to control females ($p=0.0006$), but not when temperature was combined to
408 cadmium. Time condition impacted significantly VTG production. In control and cadmium
409 groups, VTG increased from day 60 with a peak at day 90. The increase was significant only
410 on day 90 for control females ($p<0.00001$), and on days 60 ($p<0.01$) and 90 ($p<0.00001$) for
411 cadmium groups. In temperature and cadmium-temperature groups, VTG production
412 increased (but not significantly) on day 60, and then decreased sharply on day 90 for
413 temperature group, while it remained stable for cadmium-temperature groups (not significant
414 compared to day 15).

415 3.2.3. IVF and offspring parameters

416 IVF results, obtained during experiment 2, are presented in Table 4. For the
417 fertilization rate, no significant differences were recorded using the Kruskal-Wallis test
418 ($p>0.05$), when compared to control groups despite a clear tendency. For offspring
419 parameters, only mortality and hatching rates were significantly impacted by the exposure
420 conditions. Indeed, all treatment groups were responsible for an increase in embryonic
421 mortality compared to control group. For hatching rate, a significant decrease was observed in
422 all experimental groups compared to control groups.

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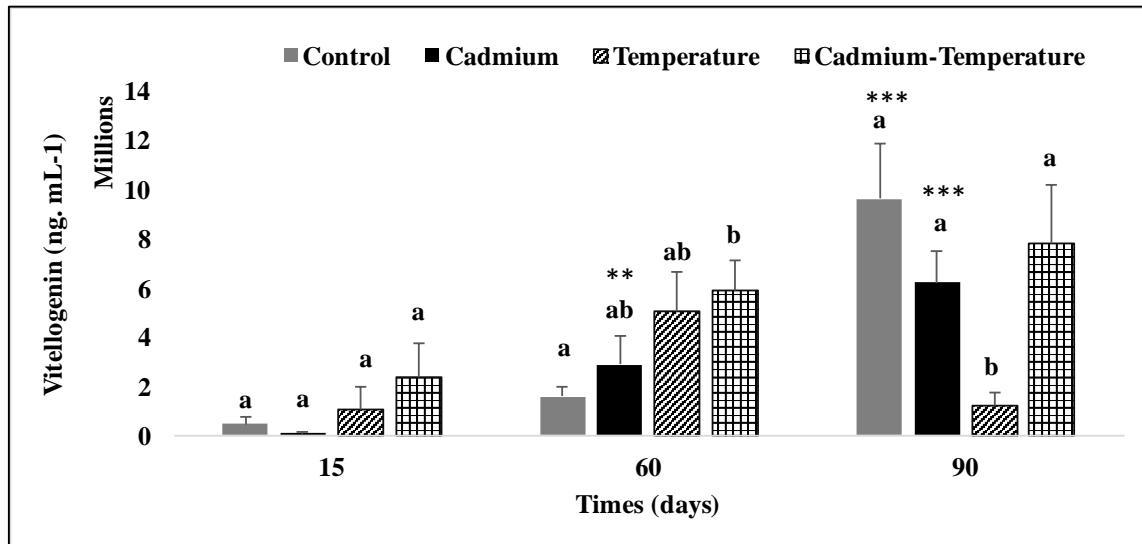
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426 **Table 3. Organosomatic index (Gonadosomatic index “GSI”; Hepatosomatic index “HSI” and Nephrosomatic index “NSI”) of parental**
 427 **sticklebacks after 15, 60 and 90 days exposure to cadmium and/or temperature of 21°C. Fish reared at 16 °C are considered as control**
 428 **groups. Results are presented as mean ± S.E.** For a same time, different letters indicate significant differences between exposure groups (p<0.05). For the same exposure
 429 group, asterisks indicate significant temporal differences with respect to initial values of day 15 (*p<0.05; **p<0.01; ***p<0.001).

Conditions		GSI				HSI				NSI			
		♂		♀		♂		♀		♂		♀	
Day 15	Control (16 °C)	n=12	1.34 ± 0.49ab	n=12	2.31 ± 1.74a	n=12	3.90 ± 0.17a	n=12	4.27 ± 0.22a	n=12	0.73 ± 0.05a	n=12	0.79 ± 0.08a
	Cadmium	n=16	1.79 ± 0.70a	n=9	2.22 ± 0.96a	n=16	4.14 ± 0.15a	n=9	4.57 ± 0.23a	n=16	1.02 ± 0.08a	n=9	1.02 ± 0.11a
	Temperature	n=20	0.83 ± 0.74b	n=5	1.80 ± 0.57a	n=20	4.65 ± 0.29a	n=5	5.17 ± 0.21a	n=20	0.82 ± 0.07a	n=5	0.88 ± 0.12a
	Cadmium-Temperature	n=15	0.87 ± 0.54b	n=10	1.92 ± 0.86a	n=15	4.04 ± 0.32a	n=10	4.24 ± 0.50a	n=15	0.94 ± 0.08a	n=10	0.96 ± 0.08a
Day 60	Control (16 °C)	n=12	1.16 ± 0.50a	n=13	7.35 ± 4.72a***	n=12	5.37 ± 0.21a***	n=13	5.79 ± 0.27a***	n=12	1.73 ± 0.35a*	n=13	0.90 ± 0.07a
	Cadmium	n=12	0.97 ± 0.34a**	n=13	9.79 ± 6.19a***	n=12	5.08 ± 0.52a**	n=13	6.88 ± 0.25b***	n=12	2.08 ± 0.32a*	n=13	0.89 ± 0.05a
	Temperature	n=15	0.87 ± 0.45a	n=9	11.89 ± 7.36a***	n=15	5.48 ± 0.23a	n=9	6.04 ± 0.25ab	n=15	1.90 ± 0.30a**	n=9	0.88 ± 0.06a
	Cadmium-Temperature	n=11	0.99 ± 0.57a	n=14	11.07 ± 5.13a***	n=11	5.10 ± 0.19a*	n=14	5.97 ± 0.23a**	n=11	1.54 ± 0.33a	n=14	0.76 ± 0.04a
Day 90	Control (16 °C)	n=11	0.90 ± 0.50a	n=14	12.36 ± 7.12a***	n=11	5.05 ± 0.27a**	n=14	6.76 ± 0.28ab***	n=11	2.00 ± 0.40a*	n=14	0.66 ± 0.06a
	Cadmium	n=11	0.92 ± 0.47a*	n=14	12.94 ± 7.09a***	n=11	5.42 ± 0.19a***	n=14	6.88 ± 0.21a***	n=11	1.59 ± 0.35ab	n=14	0.87 ± 0.05ab
	Temperature	n=12	0.40 ± 0.29a	n=12	2.05 ± 1.48b	n=12	5.27 ± 0.29a	n=12	6.16 ± 0.33ab	n=12	0.76 ± 0.07b	n=12	0.82 ± 0.08ab
	Cadmium-Temperature	n=9	0.54 ± 0.29a	n=16	4.63 ± 4.41b	n=9	5.17 ± 0.33a**	n=16	5.83 ± 0.27b**	n=9	0.92 ± 0.31b	n=16	0.89 ± 0.06b

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432 **Figure 2. Evolution of the plasmatic vitellogenin (VTG) of female parental sticklebacks**
433 **after 15, 60 and 90 days of exposure to cadmium and/or temperature stress. Fish reared**
434 **at 16 °C, are considered as control groups. Results are presented as mean ± S.E.** For a same
435 time, different letters indicate significant differences between exposure groups ($p < 0.05$). For the same exposure group,
436 asterisks indicate significant temporal differences with respect to initial values of day 15 (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

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449 **Table 4. *In vitro* fertilization parameters. The direct effect of a multiple stress (cadmium and/ or temperature of 21°C) on parental**
 450 **fertilization rate, and its consequences on the clearwater-reared offspring. Results are represented as mean ± S.E.** Different letters indicate
 451 significant differences between treatment groups and control group (p<0.05). *Results in bold-italic characters should be taken with caution because of the*
 452 *high mortality rate observed in these groups.*

Parental conditions	n	Fertilization rate (%)	Survivors (n)	Mortality rate (%)	Beginning of hatching (dpf)	Hatching rate (%)
Control (16 °C)	4	95.86 ± 2.18 a	200	38.39 ± 15.19 a	8.66 ± 0.66 a	61.60 ± 15.19 a
Cadmium	5	97.61 ± 2.38 a	140	72.08 ± 9.50 b	9.50 ± 0.34 a	27.91 ± 9.50 b
Temperature	4	84.79 ± 7.09 a	15	87.04 ± 10.71 b	<i>10.50 ± 0.50a</i>	<i>12.95 ± 10.71b</i>
Cadmium- Temperature	4	77.26 ± 12.52 a	12	96.23 ± 2.42 b	<i>10.50 ± 0.50a</i>	<i>3.76 ± 2.42b</i>

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455 **4. Discussion**

456 Knowledge about biological effects of multiple stressors on living organisms is still
457 limited in literature. In this study, reproductive parameters of *G. aculeatus* were addressed as
458 biological outputs after a chronic exposure to a single or combined effect of temperature
459 and/or cadmium waterborne. Some authors have been interested in the effect of chronic
460 exposure to temperature (Breckels and Neff, 2013; Donelson et al., 2014; Cardoso et al.,
461 2017) or cadmium (Sellin and Kolok, 2006; Wang et al., 2014; Luo et al., 2015) alone on fish
462 reproduction. The combined effect of temperature and cadmium was reported by other authors
463 at different levels of biological organization in fish (Sassi et al., 2010; Fadhlaoui and Couture,
464 2016; Abdel-Tawwab and Wafeek, 2017; Fadhlaoui et al., 2018; Zhao et al., 2018) and
465 crayfish (Del Ramo et al., 1987). However, to our best knowledge, the study reported by
466 Hallare et al. (2005) is the only one that was interested in the effect of the combination of the
467 two stresses (cadmium and temperature) on the reproduction output of fish (*Danio rerio*).
468 Nonetheless, their study was interested only in the effects of the double stress on the
469 embryonic development of zebrafish embryos (Hallare et al., 2005). Hence, our study is the
470 first to address the impacts of a long exposure to cadmium and / or temperature on the
471 reproductive capacity of parental fish (*i.e.* *G. aculeatus*) and the consequences on their
472 offspring survival.

473 This study demonstrated that generally, whatever the stage of the beginning of
474 exposure (juvenile or adults), chronic exposures of parental sticklebacks to the different
475 stresses affected in the same way their reproduction capacity (effect of high temperature stress
476 particularly) and resulted in a negative impact on their offspring survival (effects of both high
477 temperature and cadmium and their combination).

478 **4.1. Effects on parental reproduction**

479 4.1.1. Organosomatic index

480 High temperature (alone or combined with cadmium) was the only factor that affected
481 reproductive parental parameters. Cadmium had no significant impact on any of these
482 parameters, and its combination with thermal stress did not modulate temperature effects.
483 Temperature is a potent physical stressor for fish, with major effects on the metabolism,
484 growth, and fundamental biochemical processes (Hovel et al., 2015). In sticklebacks
485 temperature is known to be one of the main environmental factor, which determine the
486 reproductive capacity of sticklebacks (Wootton, 1976, 1984). Somatic indexes (GSI, HSI and

487 NSI) and vitellogenin showed a strong gender difference, and the most temperature-impacted
488 parameter in both experiments were GSI (in both male and females) and VTG (in females).

489 The effect of temperature was evaluated in both experiments. In males, a decrease in
490 GSI was found with high temperature in both experiments. Similar results were reported in
491 male fathead minnow (*Pimephales promelas*) exposed to different water temperatures (18 and
492 26 °C; Shappell et al., 2018). The decrease of GSI in males exposed to the high temperature
493 may indicate a reduction in spermatozoa quantity as it was suggested by Dorts et al. (2012).

494 The effect of temperature on GSI of females was significant only in the second
495 experiment. In fact, at 21 °C (with or without cadmium exposure), the GSI peaked on day 60,
496 whereas in control group the peak was reached only after 90 days. These results suggest that
497 oocyte formation was promoted earlier in females raised at 21 °C. Temperature plays an
498 important role in stickleback reproduction, and high temperature has been reported to
499 augment gonadal development in teleost fish, including sticklebacks (Wootton, 1976;
500 Guderley, 1994; Acharia et al., 2014). Conversely, some authors observed a decline in GSI
501 values in fish exposed to higher temperatures: that is the case of the Argentinian silverside
502 (*Odontesthes bonariensis*) (Elisio et al., 2012), and zebrafish (*Danio rerio*) (Cardoso et al.,
503 2017). In our study, even if 21 °C had a stimulating effect on females GSI, a sharp decrease of
504 about 3-fold compared to control group, was observed on day 90. These results suggest that a
505 prolonged exposure to 21 °C is a limiting factor for gonadal development in sticklebacks.
506 Degeneration of gonad morphology following exposure to a constant high temperature has
507 been reported (Acharia et al., 2014), and can be a possible explanation of our results.
508 Sokolowska and Kulczykowska (2006) reported that temperatures as high as 20 °C could be a
509 limit factor to stickleback breeding and that a further increase in temperature could disturb
510 gametogenesis in both male and female sticklebacks.

511 The effect of cadmium alone and in combination with temperature on parental
512 reproduction, was evaluated in the second experiment. In this experiment, the real water
513 cadmium concentration (*i.e.* 0.5 µg. L⁻¹) was 2-fold lower than the nominal concentration
514 targeted (*i.e.* 1 µg. L⁻¹). Therefore sticklebacks were exposed chronically to a very low
515 cadmium concentration, below the freshwater chronic criterion (CCC) recommended by the
516 US Environmental Protection Agency : 0.72 µg.L⁻¹(US EPA, 2016). At this very low
517 concentration of waterborne cadmium, no effect was observed on somatic indexes of male
518 and female sticklebacks. These results concord with what was reported by Aït-aïssa et al.
519 (2003), when they exposed for 21 days juvenile rainbow trout (*Oncorhynchus mykiss*) to 1.5
520 µg. L⁻¹ of cadmium.

521 4.1.2. Vitellogenin

522 In female sticklebacks, reproduction is initiated by an increase in gonadosomatic
523 index, which indicates a transition of fish to the vitellogenous stage. At this period, oocyte
524 diameters increased as a result of the deposition of a large amount of egg yolk (Sokolowska
525 and Kulczykowska, 2006; Zhang et al., 2015).

526 The hypothesis that high temperature could be responsible for an early gonadal
527 development, may be supported by vitellogenin results obtained during both experiments.
528 Indeed, in the first experiment females reared at 21 °C expressed the highest values of VTG
529 (6-fold increase) from day 15, while in control females, maximal VTG values were only
530 observed at 60 days of the experiment. In the second experiment, a high production of VTG
531 was recorded on day 60 in females of 21 °C (with or without cadmium, 2-fold increase), while
532 in control females the highest values of VTG was recorded on day 90. These results are
533 consistent with GSI evolution. Our results concord with those of literature. Sokolowska and
534 Kulczykowska (2006) reported that temperature has a stimulatory effect on vitellogenin
535 production in female sticklebacks. When Fathead Minnows (*Pimephales promelas*) were
536 exposed to different temperature treatments, a stimulation of spawning and an elevated
537 expression of mRNA VTG were observed in high temperature groups, compared to the low
538 temperature group (Firkus et al., 2017). Other studies have shown inverse effects of
539 temperature on yolk egg precursor. For example, prolonged exposure of Atlantic salmon
540 (*Salmo salar*) females to elevated temperature was responsible for a significant decrease in
541 VTG production, through the disruption of endocrine processes as shown by altered levels of
542 plasma oestradiol (King et al., 2003). During ovarian growth, VTG constitute an important
543 part of egg yolk contents (Sokolowska and Kulczykowska, 2006). The latter is considered by
544 developmental biologists as one of the intrinsic properties that determine eggs quality (Brooks
545 et al., 1997). High temperature could result in oocyte precocity, with an early VTG deposition
546 in the yolk. However, this early maturity may result in a quick egg yolk deposit that may be
547 finished at a small-oocyte size (Luo et al., 2017). Accordingly, impaired production and/or
548 uptake of VTG, can result in a reduction in the egg quality, and consequently it could be a
549 limiting factor for offspring survival once fertilization performed. For example, in the study
550 reported by King et al. (2003), VTG reduction in the Atlantic salmon (*Salmo salar*) at higher
551 temperature was accompanied by a significant decrease in egg size, and consequently a
552 reduction in fertility, and offspring survival. In our study, the early stimulation of
553 reproduction observed in 21°C females group, could result in a low eggs quality (*i.e.* small
554 eggs), that could consequently compromise reproduction success of sticklebacks (*i.e.*

555 fertilization success, offspring survival, etc.). Unfortunately, egg size and egg-VTG cotenant
556 were not evaluated in both experiments, to confirm this hypothesis.

557 Single exposure to cadmium had no effect on VTG production, which is in accordance
558 with what was reported by Aït-aïssa et al. (2003), when juvenile rainbow trout (*Oncorhynchus*
559 *mykiss*) were exposed to 1.5 µg. L⁻¹ of cadmium. However, when cadmium is combined to
560 high temperature, VTG remained higher at day 90, while it sharply decreased in females
561 exposed to temperature alone. It is well known that heavy metals such as cadmium could act
562 as endocrine disrupters in fish (Sfakianakis et al., 2015). Depending on metal concentration
563 and/ or fish species, cadmium could have a stimulating (Lizardo-Daudt and Kennedy, 2008)
564 or inhibiting (Chouchene et al., 2016) effect on some important endocrine parameters, such as
565 Oestradiol, or its gene expression, which are involved in the reproductive function and in
566 VTG production. In our study, cadmium seems to have a likely delayed effect on the end of
567 the reproductive cycle of *G. aculeatus* females, when it is combined to temperature. It could
568 be the result of a stimulating effect of this metal on oestradiol production (and indirectly on
569 VTG production), which is expressed under the effect of high temperature, since it is well
570 known that temperature could modified heavy metal toxicity (Hooper et al., 2013). Further
571 experiments are needed to explain this mechanistic.

572 4.1.3. Fertilization success

573 The fertilization success of parental sticklebacks was evaluated two hours after *in vitro*
574 fertilization assays were conducted. Due to the large variability in the different groups,
575 statistically, no difference was recorded between control and exposed groups in both
576 experiments. However, a tendency was observed, characterized by 1.1-fold decrease in
577 fertilization rate under the effect of high temperature in both experiments, and 1.2-fold
578 decrease in the second experiment, when sticklebacks were exposed to the combination of
579 cadmium-temperature condition. In the rare minnow (*Gobiocypris rarus*), fertilization rate
580 was significantly decreased at 30 °C compared to those at 24 °C and 27 °C (Luo et al., 2017).

581 **4.2. Impact on offspring survival**

582 Several parameters were assessed throughout experiments in order to evaluate the
583 consequences of parental exposure to thermal and/or cadmium stress on offspring quality. In
584 both experiments, embryonic mortality (1.4 to 2.2-fold increase) with hatching rate (6 to 20-
585 fold decrease) were significantly impacted by the higher temperature of 21 °C (in both
586 experiments) and by the combination of temperature with cadmium (in experiment 2).
587 Interestingly, in experiment 2, exposure of parental sticklebacks to the very low concentration

588 of cadmium ($0.5 \mu\text{g. L}^{-1}$) had a severe impact on offspring survival (1.8-fold increased
589 mortality, and 2.2-fold decrease in hatching rate, compared to control group). However,
590 results of hatching rate in individuals from parents exposed previously to 21 °C alone (in
591 experiment 1 and 2) or combined to cadmium (in experiment 2), have to be taken with
592 caution, because of the low number of individuals that survived until hatching ($n < 5$ in the first
593 experiment and $n < 16$ in the second experiment).

594 It has long been shown that reproduction and embryonic development are influenced by
595 temperature in many species including fish (Van Der Kraak and Pankhurst, 1997).
596 Reproductive success depends on the performance of males and females at the same time, and
597 the temperature can influence each other at different degrees. Several authors have reported
598 adverse effect on offspring quality after parental exposure to high temperatures (King et al.,
599 2003; Donelson et al., 2010; Luo et al., 2017). The maintenance of rainbow trout
600 (*Oncorhynchus mykiss*) at different water temperatures resulted in a decrease of eggs viability
601 with temperature increasing, and was probably related to the rapid ATP depletion within the
602 egg cytoplasm. Authors suggested that one of the effects of elevated temperature could be the
603 increase rate at which ATP stores in the egg are depleted before spawning (Pankhurst et al.,
604 1996). Once ovulated, fish eggs are known to be impermeable to nutrients (except water and
605 chemicals). Hence, all the contents of an egg (yolk proteins, nutrients, etc.) that will determine
606 its quality, must be incorporated during gametogenesis (Brooks et al., 1997). Incomplete or
607 impaired egg yolk deposit resulting from high temperature, may be one reason for poor
608 offspring quality (Luo et al., 2017). In our study, we hypothesized that early maturation and
609 VTG production observed under the high temperature, could result into small-poor-quality
610 eggs. The high mortality and unhatched embryos rates observed after IVF assays in both
611 experiments, corroborate this hypothesis. Our results concord with other studies reported in
612 literature. For example, in Atlantic salmon (*Salmo salar*) females held at a water temperature
613 of 22 °C, were characterized by a 2-fold decrease in VTG production, compared to females
614 reared at 14 °C. Decreased VTG values in females of higher temperature treatment had severe
615 repercussions on egg quality (1.1-fold decrease in oocyte diameter) and consequently on
616 reproduction success (2.8-fold decrease in fertility) and also on offspring survival (7-fold
617 increase in mortality) (King et al., 2007). Impairment in nutrients deposit into eggs during
618 gametogenesis, could also have repercussions on the offspring quality as it was discussed by
619 Luo et al. (2017). For example a decrease in free fatty acids and phospholipid levels were
620 reported in eggs of Brown trout (*Salmo trutta*) exposed to elevated temperature (Lahnsteiner
621 and Leitner, 2013). Impairment in nutrient deposit is viewed as a reduction in energetic

622 investment, which could be a consequence of disorder in energy metabolism (*i.e.* altered
623 energy acquisition) under the effect of temperature.

624 Adverse effects of temperature may result in decreased reproductive performance of
625 males. Sperm quality is an important factor which determine successful fertilization, and
626 subsequently the normal development of embryos (Bobe and Labbé, 2010; Breckels and Neff,
627 2013). After fertilization, embryonic quality will also be determined by the contribution of the
628 paternal genes (Brooks et al., 1997). The effects of temperature could be expressed by
629 impaired DNA integrity of spermatozoa. For example, in Brown trout (*Salmo trutta*),
630 spermatozoal DNA integrity decreased in fish kept at elevated temperature (Lahnsteiner and
631 Leitner, 2013). In the threespine stickleback, high mortality and abnormal development of
632 offspring was in relation to a decrease in spermatozoal DNA integrity after *ex vivo* exposure
633 to an alkylating genotoxicant (Santos et al., 2013).

634 Embryonic and larval stages in fish are particularly sensitive to chemicals, especially
635 heavy metals (Sfakianakis et al., 2015). These disturbances can result from a direct effect of
636 heavy metals on the offspring, as they can be the consequence of an influence on parental
637 parameters (Jeziarska et al., 2009). In the second experiment, parental sticklebacks were
638 exposed to cadmium alone and in combination with temperature, and their offspring were
639 reared in clear water. In this experiment, cadmium exposure (alone or with temperature)
640 impacted negatively offspring survival. In their review, Jeziarska et al. (2009) reported a
641 detailed synthesis of the effects of various heavy metals (*i.e.* cadmium) on embryonic
642 development in fish. Heavy metals can accumulate in the male and female gonads (Szarek-
643 Gwiazda, 1999; Vergauwen et al., 2013), causing effects through different mechanisms:
644 osmotic disruption, endocrine disruption, or alteration of metabolic processes. These different
645 disturbances are responsible for the harmful effects on fertilization, hatching, and the
646 development of the offspring (mortality and malformations) (Jeziarska et al., 2009).

647 **5. Conclusions**

648 In this study, reproductive parameters were addressed after chronic exposures of
649 sticklebacks of two different reproductive stages (adults and juveniles) to different
650 temperatures. Whatever the developmental stage at the beginning of the experiments (adult or
651 juvenile), the high temperature stress impacted in the same way the reproductive capacity of
652 *G. aculeatus*. Regarding parental parameters, high temperature (21 °C) was the main
653 impacting factor on stickleback reproduction. The effects of high temperature alone or in

654 combination with cadmium, may be due to an effect on gamete quality, which was assessed
655 by effects on GSI in both sexes and VTG in females. Parental exposures to high temperature
656 of 21 °C (in both experiments) had negative repercussions on offspring survival (high
657 mortality rate and low hatching rate), and confirmed that effects on stickleback progeny may
658 be due to an impairment in parental investment (low egg and/or sperm quality). In addition,
659 offspring parameters (viability and hatching) were impacted by cadmium and its combination
660 with temperature, causing significant increase in mortality and decrease in hatching rates.

661 Our results indicate that reproductive capacity of *G. aculeatus* may be mostly impacted
662 by temperature elevation in a global change context, but also by cadmium exposure at this
663 very low concentration (0.5 µg. L⁻¹), which is below the freshwater chronic criterion (CCC) of
664 0.72 µg. L⁻¹ recommended by the US Environmental Protection Agency (EPA). Hence, we
665 recommend considering both of parental and offspring parameters, when evaluating the
666 ecotoxicological effects of chemicals on the reproductive function of fish.

667

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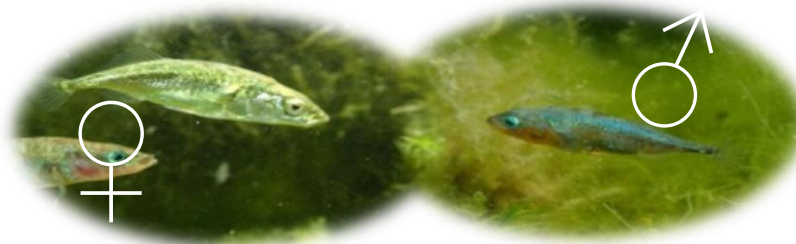
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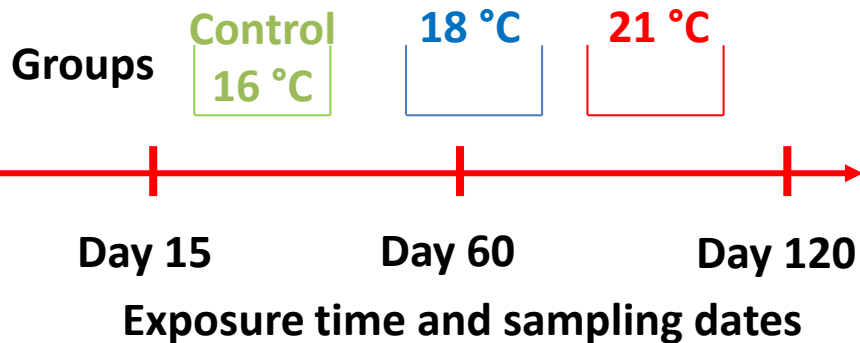


Threespine stickleback
(*Gasterosteus aculeatus*)

I. Chronic exposure to different temperatures and/ or cadmium

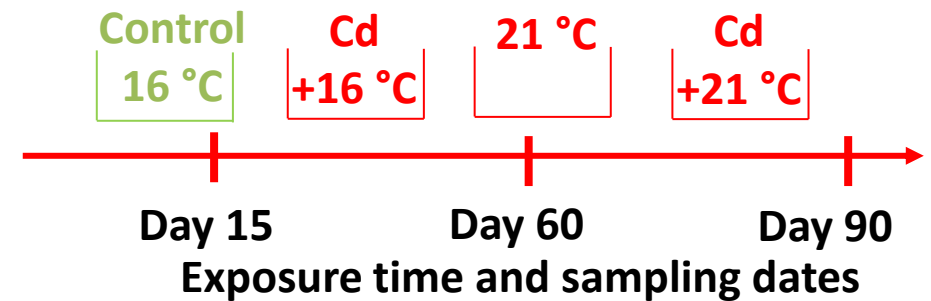
Experiment 1 (adult fish):

Single exposure to temperature



Experiment 2 (juvenile fish):

Single and double exposure to temperature and cadmium



II. Evaluation of parental reproductive parameters and offspring survival

Parents

- Major effects of high temperature (especially on females)
- Promoting effect followed probably by a gonad atresia

Offspring

- Negative consequences of cadmium and high temperature
- ↗ Embryonic mortality
- ↘ Hatching rate