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**Natural distribution of pure and hybrid *Mytilus sp* along the south Mediterranean and North-east Atlantic coasts and sensitivity of D-larvae stages to temperature increases and metal pollution**

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1 **Abstract:**

2 The distribution of the Mediterranean mussel *Mytilus galloprovincialis* extends more and  
3 more northwards in the Atlantic. Crossings are frequently observed with the blue mussel  
4 *Mytilus edulis* along the French and English coasts. The aim of this study is firstly to identify  
5 the co-presence of *M.galloprovincialis*, *M.edulis*, and their hybrids in different sites of the  
6 Mediterranean and Atlantic coasts, and to provide insights for the thermal tolerance and  
7 toxicant susceptibility of *Mytilus edulis*, *Mytilus galloprovincialis* and their hybrids. Mussels  
8 were collected from the shore at 20 sampling sites in Europe and Tunisia and identified using  
9 Me 15/16 primers targeting the adhesive protein gene sequence. Samples were screened for  
10 the presence of *Mytilus edulis*, *Mytilus galloprovincialis*, and hybrids alleles using PCR. To get  
11 more information on hybrids sensitivities to temperature and metals, freshly fertilized eggs of  
12 the two species and their hybrids were reared at four temperatures 18, 20, 22, and 24 °C and  
13 exposed to concentrations of Cu, Ag, and a mixture of both metals. Arrests of development and  
14 malformations were recorded after 48 hours of exposure. The genotypic identification of the  
15 two species on 20 sites of the Mediterranean and Atlantic coasts carried out during this study  
16 confirms the presence of pure and hybrid species of mussel. Our results highlighted that hybrid  
17 larvae from a female of *M. galloprovincialis* are significantly more tolerant to temperature  
18 increases than pure larvae of *M. galloprovincialis* and pure and hybrid larvae of *M. edulis*. No  
19 significant interspecies-differences of sensitivity were noted for metal exposure alone.  
20 However, a co-exposure of larvae to both metal and high temperature highlighted the higher  
21 tolerance of hybrid larvae from a female of *M.galloprovincialis* to both stresses. The overall  
22 results could allow the prediction of the future evolution of mussel populations facing  
23 environmental changes.

24 **Keywords:** *Mytilus sp*; early life stages; hybrids; thermal stress; metal pollution.

## 25 **1. Introduction**

26 Several species, despite their close relatives and morphological similarities, are  
27 physiologically distinct, resulting in different but overlapping distributions (Parmesan and  
28 Yohe, 2003; Root et al., 2003). This is the case for *Mytilus galloprovincialis* and *Mytilus*  
29 *edulis*. These two species (or subspecies) seem to occupy the same biogeographical area in the  
30 Atlantic Ocean. *M. edulis* and *M. Galloprovincialis* are ecologically and commercially  
31 important and are bred along most of European coasts (Smaal, 2002). The Mediterranean  
32 mussel, *M. galloprovincialis*, is a warm water species, found mainly in the Mediterranean,  
33 extending northwards to the Atlantic coasts of France (Gosling, 1992; McDonald et al., 1991;  
34 Skibinski et al., 1983). The blue mussel, *M. Edulis*, is distributed in temperate and cold  
35 regions along the European Atlantic coasts from northern Europe to the French-Spanish  
36 border in the Bay of Biscay (Hilbish et al., 2012). The overlap of the distribution areas of the  
37 two sister species has created a large hybridization zone extended from the Bay of Biscay to  
38 northern Scotland, with alternating hybrid populations and pure parental populations (Bierne  
39 et al., 2003; Coustau et al., 1991; Hilbish et al., 2012). Inside this hybridization zone, the  
40 frequency of hybridization can range from low to very high levels (Dias et al., 2009).

41 Studies on mussel hybrids have focused to date on hybridization zone delimitation,  
42 mechanisms and evolution of reproductive isolation, and genetic exchange between hybrid  
43 populations (Arnold, 1997, 1992; Bierne et al., 2003; Coustau et al., 1991; Mallet, 2005).  
44 However, the effects of environmental factors on hybridization efficiency and hybrid  
45 selection have, for the most part, been ignored. Environmental factors are powerful selective  
46 agents for the evolution of living organisms (MacColl, 2011). It is not surprising that  
47 hybridization between two species with different histories of interactions with environmental  
48 factors can modify their sensitivity to physicochemical factors. Several factors can influence

49 the extent of this hybridization zone, such as spawning timing (Toro et al., 2002) and  
50 environmental conditions (Riginos and Cunningham, 2005).

51 Sea surface temperature (SST) is recognized as one of the most important determinants of  
52 geographic distributions of mussel species (Seed, 1976; Suchanek et al., 1997). Global  
53 warming is set to speed up in the coming century (IPCC, 2014) and its effects on aquatic  
54 organisms are worrying (Byrne, 2011). Following increases in sea surface temperatures, the  
55 range of cold-adapted species tends to shrink more and more while heat-adapted species tend  
56 to expand to the pole (Helmuth et al., 2006; Herbert et al., 2007; Root et al., 2003; Wetthey et  
57 al., 2011). Mussel distribution areas are also under a consistent increase in temperature. In the  
58 Mediterranean Sea, a consistent warming trend for SST has been found in the 1982-2016  
59 period with a mean total increase of  $1.27 \pm 0.12$  °C (Pastor et al., 2018). On a seasonal scale,  
60 the Mediterranean SST differs significantly with an interval going from 9.7-17.7°C in winter,  
61 15.8-22.1°C in spring, 20.8-28.3°C in summer, and 15.1-23.4°C in autumn (Shaltout and  
62 Omstedt, 2014). The North Atlantic Ocean has also been warming over the past 30  
63 years (Knight et al., 2006; Ting et al., 2009). Dye et al., (2013) studied the changes in SST of  
64 the North Atlantic, around Ireland, and the UK during the last three decades (1982-2010) and  
65 results showed that they warmed rapidly by 0.1 to 0.5 °C per decade.

66 Coastal ecosystems, such as Mediterranean and Atlantic coastal ecosystems, are experiencing  
67 the synergistic effects of multiple environments and anthropogenic stressors such as  
68 metal pollution (Cabral et al., 2019; Lu et al., 2018a, 2018b; Prada et al., 2019). Metal  
69 pollution has greatly accelerated since the industrial revolution (Förstner and Prosi, 1979) and  
70 has severely degraded the quality of coastal marine waters over the past 30 years.  
71 Consequently, metallic contamination of aquatic environments exerts a diffuse and chronic  
72 pressure on living organisms, resulting in modifications of biodiversity and disturbances of  
73 environmental processes (Roussel et al., 2008). Among the large variety of metal pollutants

74 that can contribute to aquatic pollution, copper (Cu) and silver (Ag) have been widely  
75 recorded on the Mediterranean and Atlantic coastal waters. Copper and silver are among the  
76 most toxic metals to aquatic species (Ratte, 1999). Copper, although an essential trace  
77 element (Szczytkas et al., 1994), can be toxic at high doses of exposure (Negri et al., 2013);  
78 silver is toxic even at low doses (Tappin et al., 2010). Copper is currently used throughout  
79 the world in antifouling paint (Liu et al., 2017; Schiff et al., 2004) and as a fungicide (Bisson  
80 et al., 2005). Silver and copper have been widely used in bimetallic combination Cu-Ag as  
81 bactericidal agents (Valodkar et al., 2011). The Cu mean concentration in seawater of the Bay  
82 of Biscay has an average of 0.12 to 0.15  $\mu\text{g/L}$ . In the coastal waters of Brittany, the copper  
83 concentrations were around 0.16  $\mu\text{g/L}$  for salinities of 35 to 35.5‰. In the outer estuary of  
84 the Loire, the dissolved copper concentrations are around 0.7  $\mu\text{g/L}$ . In Portuguese estuarine  
85 waters, copper concentrations ranged from 2  $\mu\text{g/L}$  in the Ria Formosa to 6  $\mu\text{g/L}$  in the Sado  
86 estuary (OSPAR Commission, 2000). Ag is mainly used in metallurgy, electroplating,  
87 electronics, and until very recently, in photography and therefore in radiology (Maillard and  
88 Hartemann, 2012). The Ag contamination in La Corogne was around 61.6 ng/L (Tappin et al.,  
89 2010) and in Galveston Bay (USA) it reached 8.9  $\mu\text{g/L}$  (Howe and Dobson, 2002). Previous  
90 studies have shown an additive effect of Cu and Ag in combination on the early stages of  
91 bivalve development (Boukadida et al., 2016; Coglianesi and Martin, 1981). Cu and Ag ions  
92 appear to act in the same way by affecting membrane permeability and ion channels (Coskun  
93 et al., 2012).

94 In most marine bivalves, the life cycle is characterized by a sessile adult phase, high  
95 fecundity, external fertilization, and extensive larval dispersal. The early stages of  
96 development are the most vulnerable in the life cycle of bivalves (Pechenik, 1999). Exposure  
97 to pollutants during this period of development can be particularly damaging to the organism  
98 (Gilbert, 2003). Thermal tolerance can vary significantly between the different life stages of

99 the same species of marine invertebrates (Mestre et al., 2009). The temperature may either  
100 accelerate or slow down the metabolic rate, thus affecting the spawning, the growth,  
101 development, and the survival of organisms (Clarke, 2003). Assessing the impact of  
102 environmental factors on larval performance of hybrids versus pure species is crucial to  
103 understand the dynamics of hybridizing species. This makes it possible to assess the potential  
104 selective pressures that favor or disadvantage hybridization (Bierne et al., 2003). In this  
105 context, the purpose of the present study was firstly to investigate the spatial distribution of *M.*  
106 *galloprovincialis*, *M. edulis*, and their hybrids in 20 sampling sites between Tunisia, Portugal,  
107 France, and the South of the United Kingdom and secondly to assess the sensitivity of hybrid  
108 and pure larvae of *Mytilus edulis* and *M. galloprovincialis* to heat stress along with Cu and Ag  
109 exposure.

## 110 **2. Material and methods**

### 111 **2.1. Study site and sampling**

112 This study was carried out at 20 different sites: 4 in Tunisia, 14 in France, 1 in Portugal, and 1  
113 in the South of the United Kingdom (Table 1). Twenty individuals of *Mytilus* sp. adult mussels  
114 were collected from the shore at the 20 sampling sites. Sampling was stratified by size to  
115 ensure that sufficient numbers of mature individuals of each genotype were obtained for the  
116 reproductive analysis. The samples were transported to the laboratory in identified plastic  
117 storage bags (collection site, number of mussels collected, and date of collection) and kept  
118 wet in a cooler at 4 °C to prevent drying. Back in the laboratory, the soft body of the mussels  
119 was dissected and the gills were removed and stored in 1 ml of 100% ethanol at -20 °C until  
120 genotype analysis.

### 121 **2.2. Chemicals and seawater**

122 Reference contaminants ( $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$  and  $\text{AgNO}_3$ ) were analytical grade and were  
123 purchased from Sigma-Aldrich (St. Quentin Fallavier, France). Reference seawater was  
124 collected from “Banc d'Arguin” (Arcachon Bay, SW of France). This site is a natural reserve  
125 and it is considered as a reference site because of the very low concentrations of chemicals  
126 and was already used to furnish water for multiple mussel experimentations (Boukadida et al.,  
127 2019, 2017, 2016). Immediately after sampling, seawater was filtered using a filter system  
128 equipped with three filtration membranes 5, 1, and 0.2  $\mu\text{m}$ . The filtered seawater (FSW) was  
129 stored at 4 °C in the dark.

### 130 **2.3. Metal solutions**

131 Stock solutions of copper (250 mg/L) and silver (100 mg/L) were prepared in pure Milli-Q  
132 water (Millipore). Working solutions were then obtained by diluting the stock solutions in  
133 FSW. The experimental concentrations of copper and silver were chosen based on preliminary  
134 studies (Boukadida et al., 2016). Three concentrations of exposure were selected for metals:



135 one concentration of copper ( $EC_{50} = 17.6 \mu\text{g/L}$ ), silver ( $EC_{50} = 6.58 \mu\text{g/L}$ ) and the mixture  
136 of both metals ( $\text{Cu } EC_{25} = 10.83 \mu\text{g/L}$  and  $\text{Ag } EC_{25} = 3.11 \mu\text{g/L}$ ). These concentrations were  
137 chosen to compare the sensitivity of hybrid and pure mussels to metals relative to the *M.*  
138 *galloprovincialis* species. The choice of concentrations for the mixture effects was based on a  
139 previous study about the sensitivity of *M. galloprovincialis* to both metals (Boukadida et al.,  
140 2016).

#### 141 **2.4. Mussel species genotyping**

142 In this study, two types of genotyping were carried out, the first to verify the percentages of  
143 pure and hybrid mussels in the wild in the different study sites and the second to confirm the  
144 species used during cross-breeding carried out in the laboratory.

145 In both cases, a piece of gill was collected from each mussel and stored at  $-20 \text{ }^{\circ}\text{C}$  until  
146 analysis. Genomic DNA was extracted from gill tissue using the NucleoSpin® Tissue  
147 according to the manufacturer instructions. DNA was then quantified by spectrophotometry  
148 and  $5 \mu\text{g}$  was used for PCR. Me15 and Me16 primers were used to amplify a highly variable  
149 region of the Glu-5' gene (GenBank accession no D63778) that encodes the polyphenolic  
150 adhesive protein. This locus was previously validated as a genetic marker for the  
151 identification of three morphologically similar species within the *Mytilus* genus including *M.*  
152 *edulis* and *M. galloprovincialis* (Inoue et al., 1995; Kijewski et al., 2011). After  
153 electrophoresis, gels were scanned in the Zoom BrowserX imaging system. Me15/Me16 PCR  
154 bands were identified on 2 % agarose gels. PCR fragments vary in length from 180 base pairs  
155 (bp) for *M. edulis* and 126 bp for *M. galloprovincialis*.

#### 156 **2.5. Embryotoxicity assay**

157 Mature mussels used for embryotoxicity assays were purchased from Spain for *M.*  
158 *galloprovincialis* and from Ireland for *M. edulis*. In the laboratory, spawning was carried out

159 by thermal stimulation, mature mussels were placed separately and alternately in FSW at 15  
160 and 20 °C for 30 min each. Upon the release of their gametes, each spawning male and female  
161 was isolated in beakers containing 250 mL of FSW. Gamete quality (regular round-shaped  
162 oocytes and mobile and dense spermatozoa) was checked under a microscope at 200 ×  
163 magnification. The solution containing the oocytes was then filtered using a 100µm sieve and  
164 the sperm solution on a 50µm sieve. Egg suspension was fertilized by sperm suspension in the  
165 ratio of approximately 1:10. Four different matings were carried out:

166 (i) Female *M. galloprovincialis* × Male *M. galloprovincialis*

167 (ii) Female *M. edulis* × Male *M. edulis*

168 (iii) Female *M. galloprovincialis* × Male *M. edulis*

169 (iv) Female *M. edulis* × Male *M. galloprovincialis*

170 Fertilization success was checked briefly under a microscope. Approximately sixteen minutes  
171 after fertilization, fertilized eggs (250 to 300 eggs) were transferred into microplates of 24  
172 wells (Cellstar, GreinerBio-one) containing 2 ml of the contaminant solution. The number of  
173 exploitable pairs for each mating is shown in Table 2. The embryotoxicity assay used in the  
174 present study was described in detail by His et al., (1999), Quiniou et al., (2005) AFNOR,  
175 (2009). Fertilized eggs were incubated for 48h with the different contaminants to be tested at  
176 different temperatures (18, 20, 22, and 24 °C) in thermo-regulated chambers in the dark until  
177 reaching the D-larvae stage. These temperatures have previously been tested on the larvae of  
178 the Mediterranean mussel *M. galloprovincialis* (Boukadida et al., 2016). After 48h of  
179 incubation, 25 µL of 37% formalin was added to each microwell to stop development. The  
180 analysis of malformed larvae can be done immediately or later provided that the microplates  
181 are stored at 4 °C. The percentage of abnormal D-larvae was determined for each mating by  
182 observation and direct count of 100 larvae under an inverted microscope at 400X

183 magnification equipped with a digital camera and image acquisition software (Nikon, Inverted  
184 Eclips Microscope, TS 100 / TS100-F, TS100LED MV / TS100 LED-F MV). In our analysis,  
185 both mantle and shell malformations and arrest of development were recorded. Examples of  
186 embryo-larval anomalies observed throughout this study are shown in Table 3.

## 187 **2.6. Statistical analysis**

188 All data were processed statistically using the R software (<https://cran.r-project.org/>). The  
189 results (percentages of abnormal larvae) were first tested for normality (Shapiro-Wilk residue  
190 test with 1% risk) and variance equality (Levene residue test, 5% risk). Since data were not  
191 normally distributed ( $p > 0.05$ ), statistical analysis was performed by the nonparametric  
192 Kruskal-Wallis test. Differences between paired conditions were then tested using the Kruskal  
193 post hoc test (equivalent to the Tuckey HSD test for non-parametric data). The differences  
194 were considered significant when  $p < 0.05$ .

195 **3. Results**

196 **3.1. Genotyping of mussel species**

197 PCR amplification using Me15 and Me16 primers produced a unique band of 180bp, for  
198 *Mytilus edulis* and 126 bp for *Mytilus galloprovincialis*. The size of the bands is consistent  
199 with that obtained for these two species by Inoue et al. (1995).

200 For the wild mussels collected along the Mediterranean and the Atlantic coasts, a  
201 representative example of an electrophoresis gel with Me15/Me16 PCR products is presented  
202 in Figure 1. This analysis shows that the hybrids have only been identified at the Saint  
203 Nazaire and Brest sites. The co-presence of *M. galloprovincialis* and *M. edulis* was observed  
204 in the population of mussels collected in the Arcachon Bay, the Bay of Saint Brieuç, and in  
205 the Bay of Seine (Le Havre). The gel separation also indicates that the mussels population of  
206 the four Tunisian sites, Aveiro in Portugal and the Bay of Saint-Jean-de-Luz in France had  
207 only alleles of *M. Galloprovincialis* while the mussels sampled in Plymouth (England)  
208 contained only alleles of *M. edulis* (Table 4).

209 For farmed mussels used for the embryo-larval assay, a single Me15/Me16 PCR product was  
210 obtained for each individual that is consistent with pure species. Also, the size of the PCR  
211 product was in agreement with the origin of the mussels, i.e. *M. edulis* for Ireland and *M.*  
212 *galloprovincialis* for Spain (supplementary figure1).

213 **3.2. Inter-individual variability between different pairs of the same species**

214 Microscopic observation revealed very high levels of fertilization of eggs ( $\geq 90\%$ ) and 48h  
215 after fertilization, most of the embryos reach the larva-D stage. For all spawner couples, an  
216 important inter-spawners variability was shown (supplementary figures 2; 3; 4 and 5). For  
217 pairs of *M. galloprovincialis*, the percentage of abnormal larvae at 18 °C was systematically  
218 below 20% following the ISO guidelines for bivalve embryo-larval assay (ISO, 2015). Since

219 one of the objectives of this study is to evaluate the effect of temperature increase on mussel  
220 development, even conditions leading to more than 20% malformations were considered.

### 221 **3.3. Comparison of inter-species sensitivity to temperature**

222 The embryo-larval sensitivity to temperature was evaluated for the 4 intraspecific and  
223 interspecific matings. The development of mussel larvae at 18 °C is presented in Figure 2.  
224 Our results show that *M. edulis* larvae were more sensitive to this temperature than *M.*  
225 *galloprovincialis* larvae with respectively a mean percentage of malformed larvae of  $37.1 \pm$   
226  $9.6\%$  and  $15.8 \pm 2.7\%$ . Mating female *M. Galloprovincialis* and male *M. Edulis* resulted in  $12$   
227  $\pm 3.8\%$  abnormal larvae, while hybrid larvae from female *M. Edulis* and male *M.*  
228 *galloprovincialis* larvae had  $44.2 \pm 19.4\%$  of abnormality. Our results showed no significant  
229 difference between pure *M. edulis* larvae and hybrid larvae of a female *M. edulis* with a male  
230 *M. galloprovincialis*.

231 Incubation at 20°C increased the percentage of abnormal larvae for all matings in comparison  
232 to 18 °C (Figure S6). No significant differences of development were found between pure *M.*  
233 *galloprovincialis* larvae and hybrid larvae from *M. galloprovincialis* female and between pure  
234 *M. edulis* larvae and hybrid larvae from *M. edulis* female. In contrast, there was a significant  
235 difference in the malformation rate between pure *M. galloprovincialis* and *M. Edulis* larvae  
236 with respectively  $17.4 \pm 1.7\%$  and  $45.3 \pm 2.8\%$ . Besides, there was a significant difference  
237 between hybrid larvae derived from female *M. galloprovincialis* ( $18.8 \pm 1.4\%$ ) and those from  
238 a female *M. edulis* ( $52.7 \pm 14.8\%$ ).

239 Incubation at 22 °C had a significant impact on embryo-larval development for all matings  
240 (Figure S7). A 100% abnormal larvae including 93.4% developmental arrests was recorded in  
241 hybrid larvae from a female *M. edulis*. A significant difference in malformation rate was  
242 noted between pure larvae of *M. galloprovincialis* ( $66.9 \pm 11.8\%$ ) and *M. Edulis* ( $98.5 \pm$

243 2.7%). Furthermore, there was also a significant and marked difference of malformation rate  
244 between hybrid larvae from a female *M. galloprovincialis* ( $39.6 \pm 7.5\%$ ) and those from a  
245 female *M. edulis* (100%).

246 Finally, incubation at 24 °C prevented the development of larvae from for all matings (Figure  
247 3). Malformation rate reached a maximum and no significant difference between pure larvae  
248 from *M. galloprovincialis* (99%) and *M. edulis* (100%) and hybrid larvae from *M.*  
249 *edulis*(100%). In contrast, there were significantly less malformed larvae in hybrids from a  
250 female *M. galloprovincialis* ( $57.4 \pm 16\%$ ).

#### 251 **3.4. Comparison of inter-species sensitivity to metals with or without temperature** 252 **increase**

253 The effects of copper, silver, and Cu-Ag mixture exposures at 18 °C on the larval  
254 development of mussels are presented in Figure 4. Our results showed no significant  
255 difference between larvae from different mating pairs, whatever the metal considered, except  
256 for hybrid larvae from female *M. galloprovincialis* exposed to silver. The latter ones were  
257 significantly less sensitive to silver compared to the other three mating pairs. It was noted that  
258 the % of deformed larvae for hybrids from female *M. Galloprovincialis* was generally lower  
259 than for other cases of mating regardless of the contaminant used.

260 The combined effects of temperature and metals on the embryo-larval development of mussels  
261 are shown in Figure 5. Combined exposure to copper and a high temperature of 22 °C resulted  
262 in a lower percentage of malformations in hybrid larvae from *M. Galloprovincialis* female  
263 ( $61.4 \pm 9.7\%$ ) in comparison with other mating pairs. For silver or Cu-Ag mixture, no  
264 significant difference was observed between pure *M. edulis* larvae and hybrid larvae of a  
265 female *M. edulis*. The percentages of malformations in pure *M. edulis* larvae and hybrid  
266 larvae from a female *M. edulis* were significantly higher than those obtained for pure *M.*

267 *galloprovincialis* larvae. Hybrid larvae from a female *M. galloprovincialis* had a lower  
268 percentage of malformed larvae than those from *M. galloprovincialis* but this difference was  
269 only significant for Cu exposure with a malformation rate reaching  $61.4 \pm 9.6\%$ .

270

#### 271 4. Discussion

272 The genotypic identification of the two species *M. galloprovincialis* and *M. edulis* on 20 sites  
273 of the Mediterranean and Atlantic coasts carried out during this study confirms the presence  
274 of pure and hybrid species of mussel along the French Atlantic coast. The co-presence of *M.*  
275 *galloprovincialis* and *M. edulis* was observed in the Arcachon Bay, the Bay of Saint Brieuc,  
276 and the Bay of Seine (Le Havre). Our results also indicate that the mussel population of the  
277 four Tunisian sites, Aveiro in Portugal and the Bay of Saint-Jean-de-Luz in France, had only  
278 alleles of *M. galloprovincialis*. While the mussels sampled on the south side of England, in  
279 Plymouth, showed only alleles of *M. edulis*. Both species were encountered on the French  
280 Atlantic coasts and hybridizations were observed where their distributions overlap in  
281 particular in the Bay of Biscay. *Mytilus edulis* is probably the most abundant mussel species  
282 along the European coasts and in the North Atlantic. *M. galloprovincialis* is native to the  
283 Mediterranean Sea and its distribution extends north along the Atlantic coast to Scotland  
284 (Beaumont et al., 2008; Hilbish et al., 2012). The shift in *M. Galloprovincialis* spatial  
285 distribution was likely favored by the rise in surface water temperatures. The study of species  
286 hybridization is essential to understand the dynamic of evolution of species distribution as  
287 well as the ecological responses of marine organisms in the context of climate change (Taylor  
288 et al., 2015, 2014). Environmental variations are probably the main driver of biological  
289 evolution since organisms must continuously adapt to these changes for survival. But today  
290 the amplitude of variations imposed by man on the environment is much more marked and  
291 deeply influences the structure of biological communities, in particular by modifications of  
292 the distribution area of many marine species. Besides, human plays a central role in the  
293 introduction of non-native species into the marine environment (Pysek et al., 2010; Taylor and  
294 Irwin, 2004). This is done either directly and voluntarily through cultivation, trade, and  
295 breeding, or indirectly and involuntarily through ballast water discharges, fouling, and the



296 escape of aquaculture species. In most cases, these non-native species adapt poorly to their  
297 new environment and disappear quickly. Sometimes, if conditions are favorable and species  
298 are tolerant, these species can survive, reproduce, and settle. Foreign species can compete  
299 with native species and harm ecosystem functioning, habitats, and local biodiversity and can  
300 transfer certain diseases and parasites with negative consequences on the ecosystem and/or  
301 socio-economic and/or health(Bax et al., 2003; Mellin et al., 2016; Pyšek et al., 2009).  
302 Hybridization modifies in a complex way the genetic architecture of species and can produce  
303 hybrid offsprings likely to be better adapted than their parents (Breusing et al., 2017; Mallet,  
304 2005). According to Barton and Hewitt (1985),the term "hybrid zones" designates the narrow  
305 regions within which genetically distinct species meet, reproduce with each other, and  
306 produce hybrids. In the context of current climate change, and the change in the range of  
307 species, the displacement of hybrid zones under the effect of environmental changes (mainly  
308 anthropogenic pressures and global warming) affects the structure of biological communities  
309 and the adaptive capacities of species.

310 The second part of this work was undertaken to provide clues about the sensitivity of *M.*  
311 *galloprovincialis*, *M. edulis*, and their hybrids to temperature increase along with pollution.  
312 Indeed, thermal stress and contamination by heavy metals is an environmentally realistic  
313 scenario and their interactions can strongly affect physiological tolerance, limit survival, and  
314 change the distribution of ectothermic species, in particular mussels.

315 The results of this study confirm that hybridization between *M. edulis* and *M.*  
316 *galloprovincialis* produces normal D-larvae that survive at least 48 hours after fertilization.  
317 This is consistent with the widespread hybridizations observed between these species in the  
318 wild (Coustau et al., 1991; Wilhelm and Hilbish, 1998). Our results support those of Bierne et  
319 al. (2002) and Beaumont et al. (2004) who have successfully produced viable mussel

320 hybrids by mating *Mytilus edulis* and *Mytilus galloprovincialis* in controlled laboratory  
321 conditions.

322 The results of this study showed inter-spawners variability for the different species studied.  
323 Variation of reproduction success between couples is a well-known phenomenon in the living  
324 world. According to Toonen and Pawlik (2001), larvae are not all created equal. The fitness of  
325 certain organisms depends on particular environmental conditions, resulting in higher  
326 survival, growth, or reproduction (Koolhaas et al., 2010).

327 Environmental factors, including temperature, endured by early life stages have the potential  
328 to affect development causing irreversible phenotypic changes frequently affecting organism  
329 performances (Burton and Metcalfe, 2014). This study hypothesizes that the survival, growth,  
330 and development of mussel larvae vary according to species and environmental conditions.  
331 Our results support this hypothesis and suggest that the thermal tolerance but not the metal  
332 tolerance of mussel larvae are species-specific.

333 Our results show that *M. edulis* larvae are more sensitive to temperature increases than *M.*  
334 *galloprovincialis* larvae. This is in accordance with the spatial distribution of both species.  
335 Brenko and Calabrese (1969), showed that the survival of *M. edulis* larvae was optimal at 10  
336 °C and 40‰ salinity and that larval growth was faster at 15 °C in a salinity range of 25-35‰.  
337 Bayne (1965), reported that *M. edulis* larvae can survive at 16 °C for 26 days without food.  
338 Ruiz et al. (2008), reported a significant increase in the growth rate of *M. galloprovincialis*  
339 larvae at temperatures between 16 and 20 °C. Several studies confirm that 18 °C is an  
340 optimum temperature for the embryonic development of this latter species (His et al., 1997;  
341 Boukadida et al., 2016). Our results indicated that the larval malformation rate reached a  
342 maximum for both species when temperatures reach 24 °C. It seems likely that at 24 °C, the  
343 effect on survival is the combined result of a decrease in larval viability and increased  
344 proliferation of bacteriae.

345 Data from this study show that hybrids from an *M. Galloprovincialis* female and hybrids from  
346 an *M. Edulis* female display different sensitivities to temperature. Indeed, hybrids derived  
347 from a female of *M. galloprovincialis* were more resistant to temperature than hybrids derived  
348 from a female of *M. edulis*. Works on hybrid mussel larvae in laboratories are still very  
349 limited. Beaumont et al. (2004), performed studies on pure mussel species as well as their  
350 hybrids crossed in laboratories. They reported that hybrid larvae from an *M. Edulis* female  
351 incubated for 72 h at 14 °C had a significantly low percentage of abnormal larvae (36.3 to  
352 66.1%) than pure larvae of *M. galloprovincialis* (48.9 to 75%). At the same temperature, the  
353 malformation rate ranged from 53.4 to 60.9 % for hybrid larvae of a female *M.*  
354 *galloprovincialis*. We assume that the significant differences between these two species  
355 concerning temperature are the result of genetic variations between individuals. However, this  
356 can also be due to maternal effects. Maternal effects can include both transient modifications  
357 of development e.g. morphological and behavioral changes, etc. and permanent changes e.g.  
358 resistance to pollution, epigenetic modifications of gene expression, etc. (Uller, 2008).  
359 Mussels of the genus *Mytilus spp* exhibit an unusual mode of transmission of mitochondrial  
360 DNA (mtDNA) called doubly uni-parental inheritance and present two types of mitochondrial  
361 genomes. Females are homoplasmic for the maternal genome (F), while males are  
362 heteroplasmic for the paternal genome (M) (Skibinski et al., 1994; Zouros et al., 1994).

363 Adaptive responses of organisms to extreme temperatures may range from phenotypic  
364 changes to long-term evolutionary adaptation (Fischer and Karl, 2010). In a short time,  
365 ectothermic species can improve their thermal tolerance when exposed to sublethal  
366 temperatures (Angilletta, Jr. et al., 2010; Chown and Nicolson, 2004). A study by Hilbish et  
367 al. (2012), predicted that the population of *M. edulis* in the Bay of Biscay will disappear by  
368 2050 and that the entire region will be occupied by *M. galloprovincialis* or hybrid mussels  
369 dominated by a high frequency of alleles of *M. galloprovincialis*, such as that currently

370 observed along the western margins of Ireland and the United Kingdom (Skibinski and  
371 Roderick, 1991).

372 Early life stages of bivalves are particularly sensitive to pollutants, especially heavy  
373 metals (Boukadida et al., 2016, 2017; Gamain et al., 2016, 2017; Mai et al., 2012; Nadella et  
374 al., 2009; Prato and Biandolino, 2010). In the present study, mussel larvae were exposed to  
375 copper an essential metal, and to silver a non-essential one. The main contribution of copper  
376 in waters is soil erosion (Georgopoulos et al., 2001). Cu also comes from copper sulfate used  
377 as a fungicide in agriculture and also for wastewater discharges (Bisson et al., 2005). In  
378 aquatic ecosystems, Cu concentration varies depending on the compartment and the location:  
379 for example in oysters from the Arcachon Bay its concentration is 400 mg/kg d.w. (Ifremer,  
380 2014), in waters from the Bay of Biscay it ranges between 0.12 and 0.15 µg/L (OSPAR  
381 Commission, 2000) and in the superficial sediments of the Bizerte lagoon it ranges from 1 to  
382 67.4 mg/kg (BenGarali et al., 2010). The ROCCH monitored the concentration of copper  
383 accumulated in mussels and oysters for the period 2003-2007. The data showed high  
384 concentrations of copper on the entire French coasts with the significant contamination  
385 recorded at the mouth of the Gironde estuary (up to 7.26 times the national median). Ag<sup>+</sup> is  
386 one of the most toxic heavy metals for aquatic invertebrates (Lam and Wang, 2006). The  
387 concentration of silver in water ranges between 0.01 µg/L in unpolluted areas and 0.01-0.1  
388 µg/L in urban and industrialized areas. For example, the concentration of silver in seawater is  
389 1.73 ng/L at Gullmar Fjord (Sweden) and 61.6 ng/L at Coruña (Tappin et al., 2010). In the  
390 Basque Country, the concentration of Ag is 1.84 mg/kg d.w. in mussels and 6.3 mg/kg d.w. in  
391 oysters (Borja A and Collin, 2004). The results reported in this work have highlighted the  
392 effects of copper and silver on the embryo-larval development of *M. edulis* and *M.*  
393 *Galloprovincialis* and their hybrids. Our results show no significant difference of sensitivity  
394 between species regardless of the metal used, except for silver tolerance for hybrid larvae

395 from an *M. Galloprovincialis* female. These hybrid larvae appeared to be significantly more  
396 tolerant to silver compared to other larvae whatever the temperature tested. It was also noted  
397 that for hybrids derived from females of *M. galloprovincialis*, the percentages of malformed  
398 larvae are generally lower compared to other larvae regardless of the contaminant. Martin et  
399 al. (1981), exposed *M. edulis* embryos to various metals and reported an EC50 of 5.8 µg/L for  
400 Cu and 14 µg/L for Ag at 17 °C. Boukadida et al. (2016), performed the same assay with *M.*  
401 *galloprovincialis* embryos and obtained an EC50 of 17.6 µg/L for Cu and 6.6 µg/L for Ag at  
402 18 °C. To our knowledge, no studies have been conducted on the toxicity of metals on mussel  
403 hybrids.

404 Despite the effects of climate change on the distribution of species and the extent of  
405 hybridization areas have been demonstrated, it is unlikely that isolated factors are the only  
406 factors involved (Helmuth et al., 2006). The combination of stressors may have antagonistic,  
407 synergistic, and additive effects (Coors and De Meester, 2008), making predictions from  
408 single-stress studies difficult for the effects of climate change. The effects of temperature  
409 variations on the sensitivity of aquatic organisms to pollutants have already been documented  
410 in an ecotoxicological context (Attig et al., 2014; Boukadida et al., 2017, 2016; Cairns et al.,  
411 1975; Tomanek and Zuzow, 2010). However, only a few studies have described the  
412 interactive effects of temperature and trace metals on the early life stage of bivalves. Our  
413 previous studies reported that a small increase in water temperature significantly increases the  
414 toxicity of metals on *M. galloprovincialis* larvae (Boukadida et al., 2017, 2016). The present  
415 study shows a significant difference in metal embryotoxicity between species reared at 22 °C  
416 with a lower percentage of malformations in hybrids from females of *M. galloprovincialis*.  
417 The percentages of malformations in pure *M. edulis* larvae and hybrid larvae of *M. Edulis*  
418 female were significantly higher than those recorded in *M. galloprovincialis* larvae. Hybrid  
419 larvae from a female of *M. galloprovincialis* had a lower rate of malformations than pure

420 larvae of *M. galloprovincialis*, although it was not significant. These results highlight the  
421 greater resistance of the hybrid larvae of a female *M. galloprovincialis* to temperature  
422 increase. On the other hand, the larvae from the crossing of a male *M. galloprovincialis* and a  
423 female *M. edulis* show a temperature sensitivity comparable to that of a pure *M. edulis* larvae.  
424 We hypothesized that there is a maternal transmission via the oocyte of a pool of proteins  
425 (e.g. HSP) for hybrids derived from a female of *M. galloprovincialis* which could confer  
426 greater resistance to increases of temperature.

## 427 **5. Conclusion**

428 The results of this study confirm the successful hybridization in controlled laboratory  
429 conditions between *M. edulis* and *M. galloprovincialis*. Hybrid embryos survived and  
430 developed normally at 18 °C at least for the first 48 h after fertilization. It has also been shown  
431 that the larvae of *M. galloprovincialis*, *M. edulis*, and hybrids have different sensitivity levels  
432 to temperature and in a lesser extent to metals. Our study also ranked the sensitivity of *Mytilus*  
433 sp to temperature and Cu and Ag as follows: hybrid from a female *M. edulis* and a male *M.*  
434 *galloprovincialis* = pure *M. edulis* > pure *M. galloprovincialis* > hybrid from a female *M.*  
435 *galloprovincialis* and a male *M. edulis*.

436

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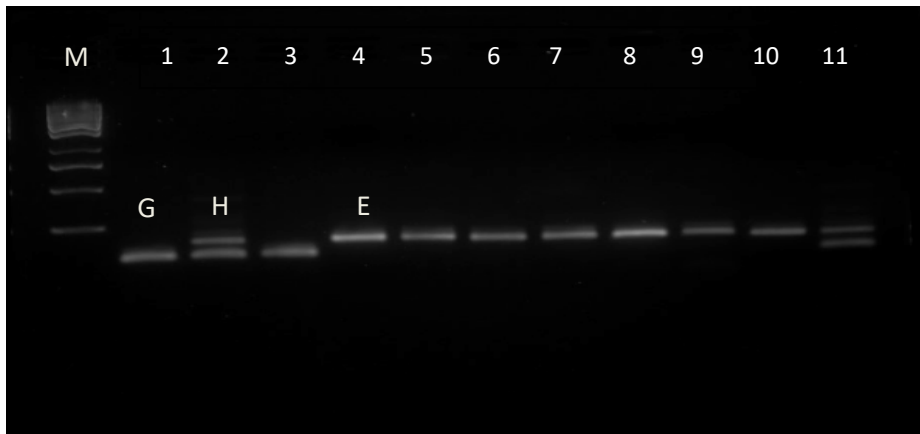


Figure 1: Separation on agarose gel (2%) and ethidium bromide coloration of the PCR amplification products of the DNA of the *Mytilus* sp. Collected from 11 different sampling sites using primers Me15 / Me16 (N = 20) for each site.

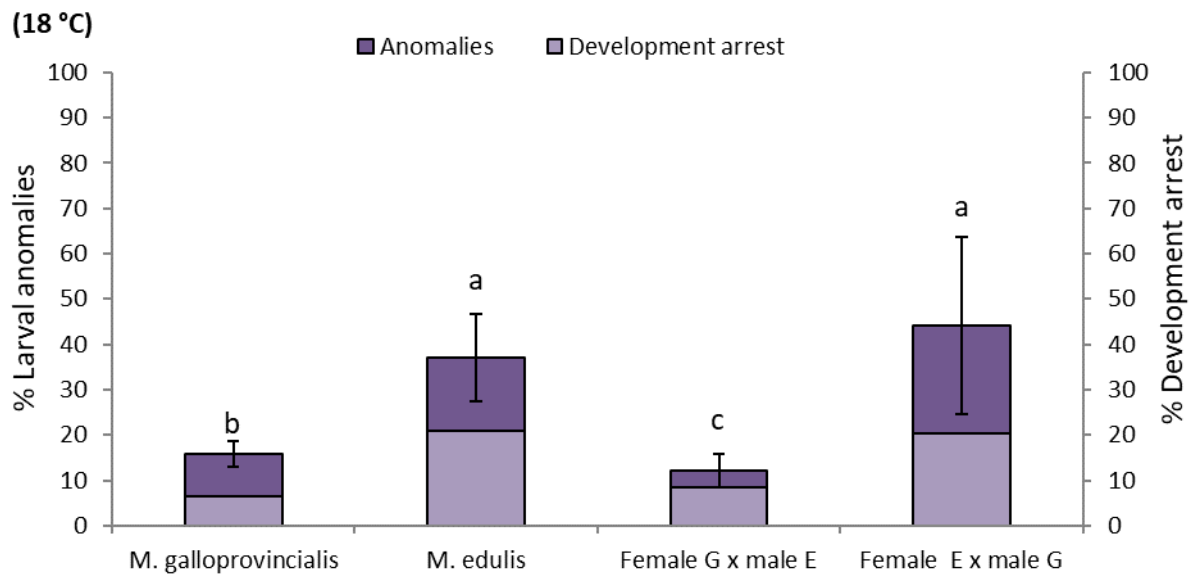


Figure 2: Developmental defects of D-larvae of *M. galloprovincialis*, *M. edulis*, and hybrids of both species at 18 °C for 48h. Different letters indicate significant differences ( $p < 0.05$ ) between different pairings (Mean  $\pm$  standard deviation, Tukey post-hoc test, For *M. galloprovincialis* N= 9, *M. edulis* N= 7, Female G x male E N= 7, Female E x male G N= 7).

(24 °C)

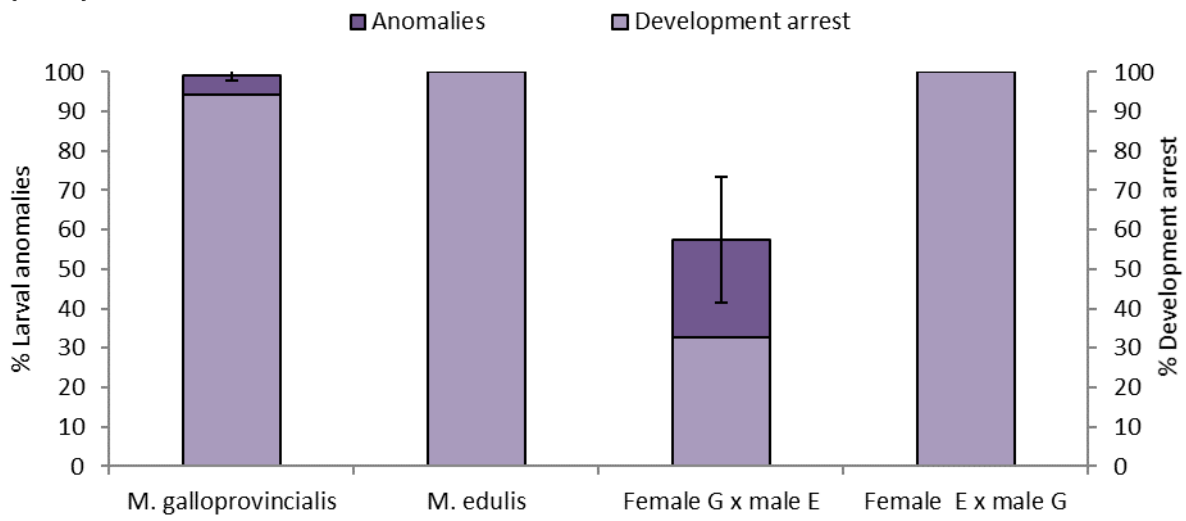


Figure 3: Developmental defects of D-larvae of *M. galloprovincialis*, *M. edulis*, and hybrids of both species at 24 °C for 48h. Different letters indicate significant differences ( $p < 0.05$ ) between different pairings (Mean  $\pm$  standard deviation, Tukey post-hoc test, For *M. galloprovincialis* N= 9, *M. edulis* N= 4, Female G x male E N= 7, Female E x male G N= 4).

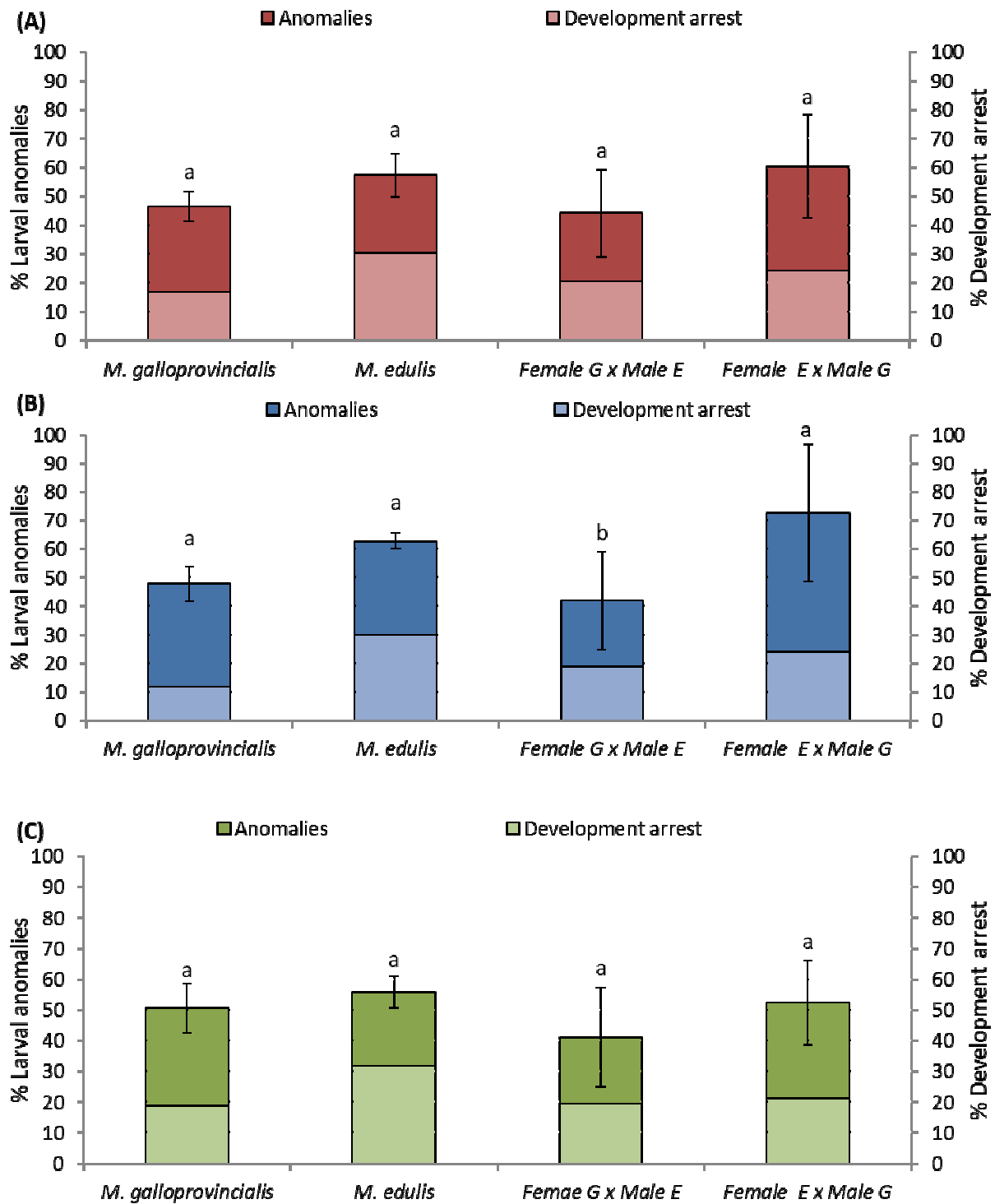


Figure 4: Toxicity of copper (A), silver (B), and a mixture of both metals (C) on the embryo-larval development of mussels *M. galloprovincialis*, *M. edulis*, and hybrids of both species at 18 °C for 48h. Different letters indicate significant differences (p < 0.05, Tukey post-hoc test) between different pairings (Mean ± standard deviation).

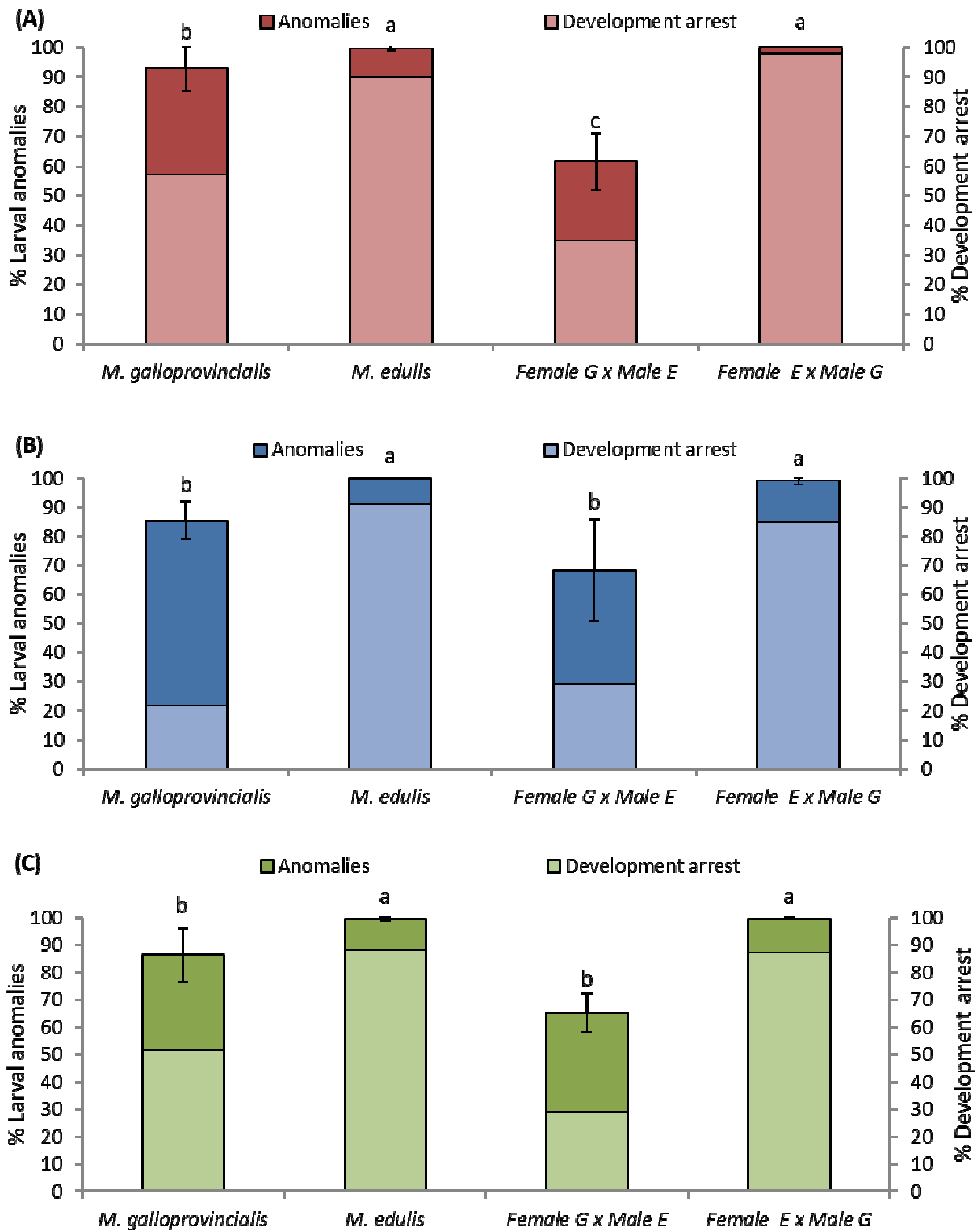


Figure 5: Toxicity of copper (A), silver (B), and a mixture of both metals (C) on the embryo-larval development of mussels *M. galloprovincialis*, *M. edulis*, and hybrids of both species at 22 °C for 48h. Different letters indicate significant differences (p < 0.05, Tukey post-hoc test) between different pairings (Mean ± standard deviation).

Table 1: Geographic coordinates of sampling sites

Sites	Country	Study sites	Sampling sites	Latitude	Longitude	
T 1	Tunisia	Beni khiar	-	36°26'56.34"N	10°46'50.77"E	
T 2		Rimmel	-	37°15'19.39"N	9°55'33.39"E	
T 3		Lagune de Bizerte I	-	37°11'31.84"N	9°52'6.93"E	
T 4		Lagune de Bizerte II	-	37°14'18.98"N	9°48'42.33"E	
F 1	France	Bay of Saint-Jean-de-Luz	Socoa port (inside)	43°23'36.09"N	1°40'57.13"O	
			Socoa port (outside)	43°24'2.13"N	1°40'37.02"O	
F 2		Arcachon Bay	Le Mouleaux	44°38'24.39"N	1°12'10.40"O	
			Arcachon city (Jetée d'Eyrac)	44°39'48.94"N	1° 9'49.41"O	
F 3		Saint-Nazaire	Loire estuary	47°16'22.84"N	2°12'5.72"O	
			La Grande Plage	47°16'13.59"N	2°12'24.68"O	
F 4		Rade de Brest	Le Conquet	48°21'28.47"N	4°46'54.12"O	
			Le Moulin Blanc	48°23'46.79"N	4°25'42.18"O	
F 5		Saint-Brieuc	Saint-Brieuc Bay	48°38'43.84"N	2°40'8.35"O	
			Le Plein (Port)	48°31'25.79"N	2°44'52.24"O	
F 6		Le Havre	Port (outside)	49°28'53.88"N	0° 5'51.96"E	
			Port (inside)	49°28'14.48"N	0° 8'49.60"E	
F 7		Boulogne	Port (outside)	50°36'58.48"N	1°33'55.24"E	
F 8		Wimereux	beach	50°46'13.59"N	1°36'15.32"E	
P		Portugal	Aveiro	Paredão beach	40°38'26.14"N	8°45'3.40"O
MF		England	Plymouth	May Flower Marina	50°21'52.00"N	4°10'10.00"O

Table 2: Number of exploitable pairs for each crossing and percentage of abnormal larvae at 18 °C

Crossings	Abnormal larvae at 18 °C (%)	Pair number at			
		18 °C	20 °C	22 °C	24°C
<i>Pure M. galloprovincialis</i>	15.8 ± 2.7	9	9	9	9
<i>Pure M. edulis</i>	37.1 ± 9.6	7	4	7	4
Female G x male E	12.0 ± 3.8	7	7	7	7
Female E x male G	44.2 ± 19.4	7	4	7	4

E: *Mytilus edulis*, G: *Mytilus galloprovincialis*

Table 3: Examples of embryo/larval anomalies observed throughout this study




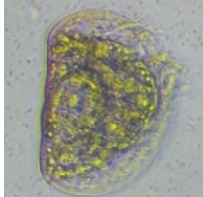

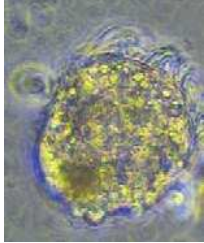
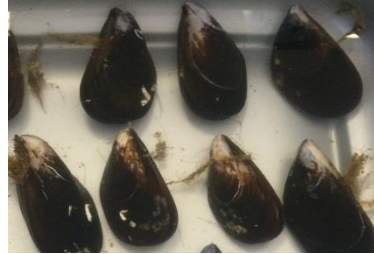
Descriptions		Observations
<b>Normal D-Larva</b>		
<b>Shell anomalies</b>	Concave hinge	
	Slitting	
<b>Mantle anomalies</b>	Mantle retracted	
	Hypertrophy with expulsion	
Development arrest		

Table 4: Distribution of the different species identified (in %), (n = 20).



Sites	Country	Study sites	Sampling sites	Identified species (%)
T 1	Tunisia	Beni khiar	-	100% <i>M. galloprovincialis</i>
T 2		Rimmel	-	100% <i>M. galloprovincialis</i>
T 3		Lagune de Bizerte I	-	100% <i>M. galloprovincialis</i>
T 4		Lagune de Bizerte II	-	100% <i>M. galloprovincialis</i>
F 1	France	Bay of Saint-Jean-de-Luz	Socoa port (inside)	100% <i>M. galloprovincialis</i>
			Socoa port (outside)	100% <i>M. galloprovincialis</i>
F 2		Arcachon Bay	Le Mouleaux	60% <i>M. galloprovincialis</i> 40% <i>M. edulis</i>
			Arcachon city (Jetée d'Eyrac)	100% <i>M. galloprovincialis</i>
F 3		Saint-Nazaire	Loire estuary	100% Hybrid
			La Grande Plage	100% <i>M. edulis</i>
F 4		Rade de Brest	Le Conquet	35% <i>M. galloprovincialis</i> 25% <i>M. edulis</i> 40% Hybrid
			Le Moulin Blanc	75% <i>M. galloprovincialis</i> 25% Hybrid
F 5		Saint-Brieuc	Saint-Brieuc Bay	100% <i>M. galloprovincialis</i>
			Le Plein (Port)	65% <i>M. galloprovincialis</i> 35% <i>M. edulis</i>
F 6		Le Havre	Port (outside)	55% <i>M. galloprovincialis</i> 45% <i>M. edulis</i>
			Port (inside)	100% <i>M. galloprovincialis</i>
F 7		Boulogne	Port (outside)	100% <i>M. edulis</i>
F 8		Wimereux	beach	100% <i>M. edulis</i>
P	Portugal	Aveiro	Paredão beach	100% <i>M. galloprovincialis</i>
MF	England	Plymouth	May Flower Marina	100% <i>M. edulis</i>



Mussel sampling  
and genotyping

**Pure species**

**Hybrids**

- (i) Female *M. galloprovincialis* × Male *M. galloprovincialis*
- (ii) Female *M. edulis* × Male *M. edulis*

- (iii) Female *M. galloprovincialis* × Male *M. edulis*
- (iv) Female *M. edulis* × Male *M. galloprovincialis*

Crossings

Embryos exposure for 48h

18 °C

20 °C

22 °C

24 °C

Ag

Cu

18 °C

Cu + Ag

22 °C

D-Larvae

Developmental arrests  
Malformations