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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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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Abstract:

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

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

1. Introduction

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

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

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

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

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

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

In most marine bivalves, the life cycle is characterized by a sessile adult phase, high fecundity, external fertilization, and extensive larval dispersal. The early stages of development are the most vulnerable in the life cycle of bivalves (Pechenik, 1999). Exposure to pollutants during this period of development can be particularly damaging to the organism (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 wasfirstly 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.

2. Material and methods

2.1. Study site and sampling

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

2.2. Chemicals and seawater

Reference contaminants ($CuSO_4 \cdot 5H_2O$ and $AgNO_3$) were analytical grade and were purchased from Sigma-Aldrich (St. Quentin Fallavier, France). Reference seawater was collected from “Banc d'Arguin” (Arcachon Bay, SW of France). This site is a natural reserve and it is considered as a reference site because of the very low concentrations of chemicals and was already used to furnish water for multiple mussel experimentations (Boukadida et al., 2019, 2017, 2016). Immediately after sampling, seawater was filtered using a filter system equipped with three filtration membranes 5, 1, and 0.2 μm. The filtered seawater (FSW) was stored at 4 °C in the dark.

2.3. Metal solutions

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

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

2.4. Mussel species genotyping

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

In both cases, a piece of gill was collected from each musseland stored at -20 °C until analysis. Genomic DNA was extracted from gill tissue using the NucleoSpin® Tissue according to the manufacturer instructions. DNA was then quantifiedby spectrophotometry and 5µgwas used for PCR. Me15and Me16primers were used to amplify a highly variable region of the Glu-5'gene (GenBank accession no D63778) that encodes the polyphenolic adhesive protein. This locus was previously validated as a genetic marker for the identification of three morphologically similar species within the *Mytilus* genus including *M. edulis* and *M. galloprovincialiss* (Inoue et al., 1995; Kijewski et al., 2011). After electrophoresis, gels were scanned in the Zoom BrowserX imaging system. Me15/Me16 PCR bands were identified on 2 % agarose gels. PCR fragments vary in length from 180 base pairs (bp) for *M. edulis* and 126 bp for *M. galloprovincialis*.

2.5. Embryotoxicity assay

Mature mussels used for embryotoxicity assays were purchased from Spain for *M. 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 microscopeat 200 ×
163 magnification.The solution containing the oocytes was then filtered using a 100µm sieveand
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 $p<0.05$.

3. Results

3.1. Genotyping of mussel species

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

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

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

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

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

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

3.3. Comparison of inter-species sensitivity to temperature

The embryo-larval sensitivity to temperature was evaluated for the 4 intraspecific and interspecific matings. The development of mussel larvae at 18 °C is presented in Figure 2. Our results show that *M. edulis* larvae were more sensitive to this temperature than *M. galloprovincialis* larvae with respectively a mean percentage of malformed larvae of 37.1 ± 9.6% and 15.8 ± 2.7%. Mating female *M. Galloprovincialis* and male *M. Edulis* resulted in 12 ± 3.8% abnormal larvae, while hybrid larvae from female *M. Edulis* and male *M. galloprovincialis* larvae had44.2± 19.4% of abnormality. Our results showed no significant difference between pure *M. edulis* larvae and hybrid larvae of a female *M. edulis* with a male *M. galloprovincialis*.

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

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

243 2.7%). Furthermore, there wasalso a significant and marked difference of malformation rate
244 between hybrid larvae from a female *M. galloprovincialis* (39.6 ± 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 ± 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 forhybrids 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 developmentof 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 ± 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 reaching61.4 ± 9.6%.

270

4. Discussion

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

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

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

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

hybridsby mating *Mytilus edulis* and *Mytilus galloprovincialis* in controlled laboratory conditions.

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

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

Our results show that *M. edulis* larvae are more sensitive to temperature increasesthan *M. galloprovincialis* larvae. This is in accordance with the spatial distribution of both species. Brenko and Calabrese (1969), showed that the survival of *M. edulis* larvae was optimal at 10 °C and 40‰ salinity and that larval growth was faster at 15 °C in a salinity range of 25-35‰. Bayne (1965), reported that *M. edulis* larvae can survive at 16 °C for 26 days without food. Ruiz et al. (2008), reported a significant increase in the growth rate of *M. galloprovincialis* larvae at temperatures between 16 and 20 °C. Several studies confirm that 18 °C is an optimum temperature for the embryonic development of this latter species (His et al., 1997; Boukadida et al., 2016). Our results indicated that the larval malformation ratereached a maximum for both species when temperatures reach 24 °C. It seems likely that at 24 °C, the effect on survival is the combined result of a decrease in larval viability and increased 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 arethe result of genetic variations between individuals. However, this
356 canalso be due to maternal effects. Maternal effects can include both transient modifications
357 of developmente .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 inheritanceand present two types of mitochondrial
361 genomes. Females are homoplasmic for thematernal genome (F), while males are
362 heteroplasmic for the paternalgenome (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 speciescan 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

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

Early life stages of bivalves are particularly sensitive to pollutants, especially heavy metals(Boukadida et al., 2016, 2017; Gamain et al., 2016, 2017; Mai et al., 2012; Nadella et al., 2009; Prato and Biandolino, 2010). In the present study, mussel larvae were exposed to copper an essential metal, and to silver a non-essential one. The main contribution of copper in waters is soil erosion (Georgopoulos et al., 2001). Cu also comes from copper sulfate used as a fungicide in agriculture and also for wastewater discharges(Bisson et al., 2005). In aquatic ecosystems, Cu concentration varies depending on the compartment and the location: for example in oysters from the Arcachon Bay its concentration is 400 mg/kg d.w.(Ifremer, 2014), in waters from the Bay of Biscay it ranges between 0.12 and 0.15 µg/L (OSPAR Commission, 2000) and in the superficial sediments of the Bizerte lagoon it ranges from 1 to 67.4 mg/kg (BenGarali et al., 2010). The ROCCH monitored the concentration of copper accumulated in mussels and oysters for the period 2003-2007. The data showed high concentrations of copper on the entire French coasts with the significant contamination recorded at the mouth of the Gironde estuary (up to 7.26 times the national median).Ag^{+} is one of the most toxic heavy metals for aquatic invertebrates (Lam and Wang, 2006).The concentration of silver in water ranges between 0.01 µg/L in unpolluted areas and 0.01-0.1 µg/L in urban and industrialized areas. For example, the concentration of silver in seawater is 1.73 ng/L at Gullmar Fjord (Sweden) and 61.6 ng/L atCoruña (Tappin et al., 2010). In the Basque Country, the concentration of Ag is 1.84 mg/kg d.w. in mussels and 6.3 mg/kg d.w. in oysters (Borja A and Collin, 2004). The results reported in this work have highlighted the effects of copper and silver on the embryo-larval development of *M. edulis* and *M. Galloprovincialis* and their hybrids. Our results show no significant difference of sensitivity 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 tosilver compared to other larvae whatever the temperature tested. It was also noted
397 that for hybrids derived from femalesof *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 assaywith*M.*
401 *galloprovincialis* embryos and obtained an EC50 of 17.6 µg/L for Cu and 6.6µg/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 factorsare 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 asmall 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 inmetal embryotoxicity between species reared at 22 °C
416 with a lower percentage of malformations in hybrids from femalesof *M. galloprovincialis*.
417 The percentages of malformations in pure *M. edulis* larvae and hybrid larvae of *M. Edulis*
418 femalewere significantly higher than those recorded in *M. galloprovincialis* larvae. Hybrid
419 larvae froma female of *M. galloprovincialis* had a lower rate of malformations than pure

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

5. Conclusion

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

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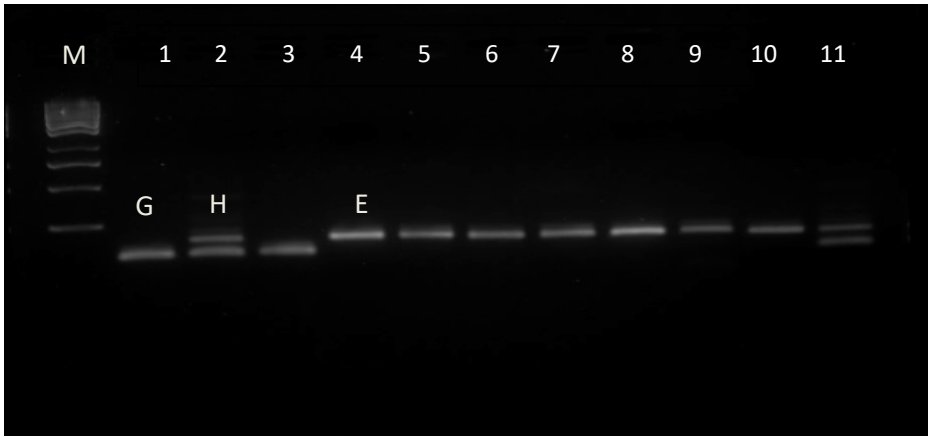


Figure 1: Separation on agarose gel (2%) and ethidium bromide coloration of the PCR amplification products of the DNA of the *Mytilus sp.* Collected from 11 different sampling sites using primers Me15 / Me16 (N = 20) for each site.

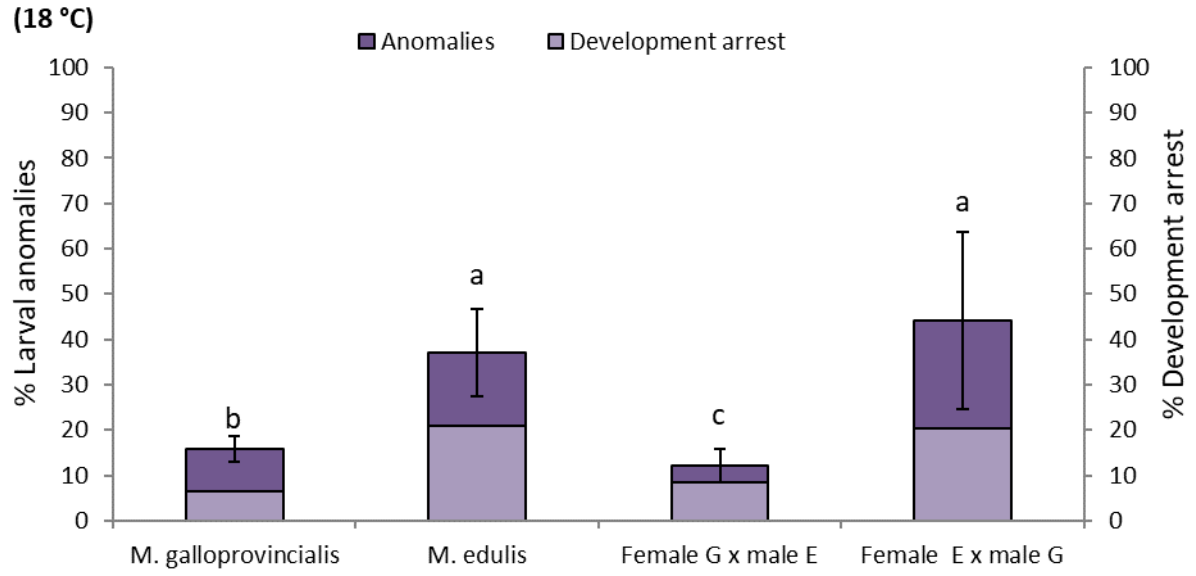


Figure 2: Developmental defects of D-larvae of *M. galloprovincialis*, *M. edulis,* and hybrids of both species at 18 °C for 48h. Different letters indicate significant differences ($p < 0.05$) between different pairings (Mean ± standard deviation, Tukey post-hoc test, For *M. galloprovincialis* N= 9,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

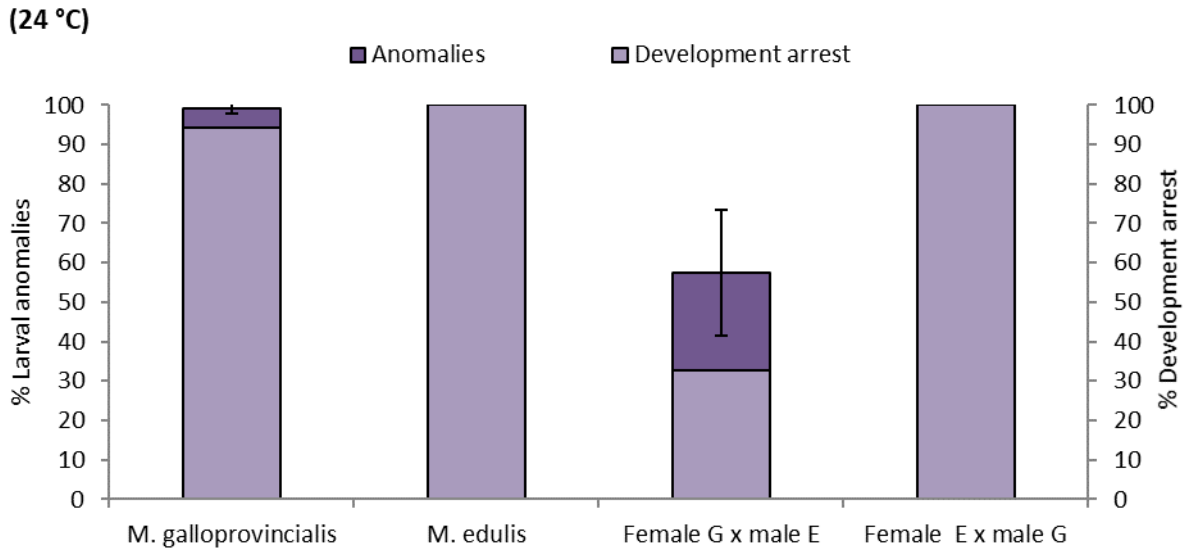


Figure 3: Developmental defects of D-larvae of *M. galloprovincialis*, *M. edulis,* and hybrids of both species at 24 °C for 48h. Different letters indicate significant differences ($p < 0.05$) between different pairings (Mean ± standard deviation, Tukey post-hoc test, For *M. galloprovincialis* N= 9,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

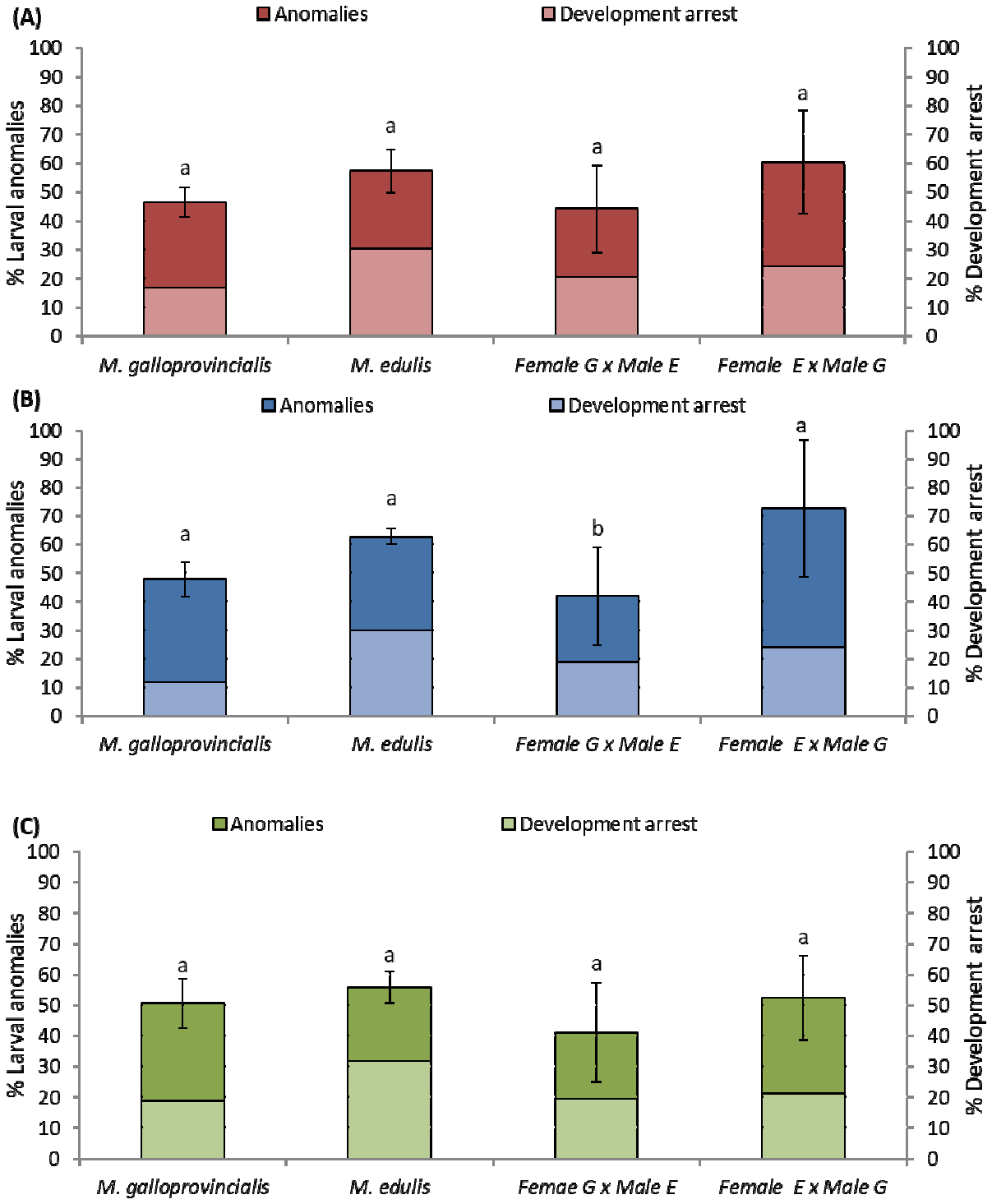


Figure 4: Toxicity of copper (A), silver (B), and a mixture of both metals (C) on the embryo-larval development of mussels *M. galloprovincialis*, *M. edulis,* and hybrids of both species at 18 ° C for 48h. Different letters indicate significant differences ($p < 0.05$, Tukey post-hoc test) between different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

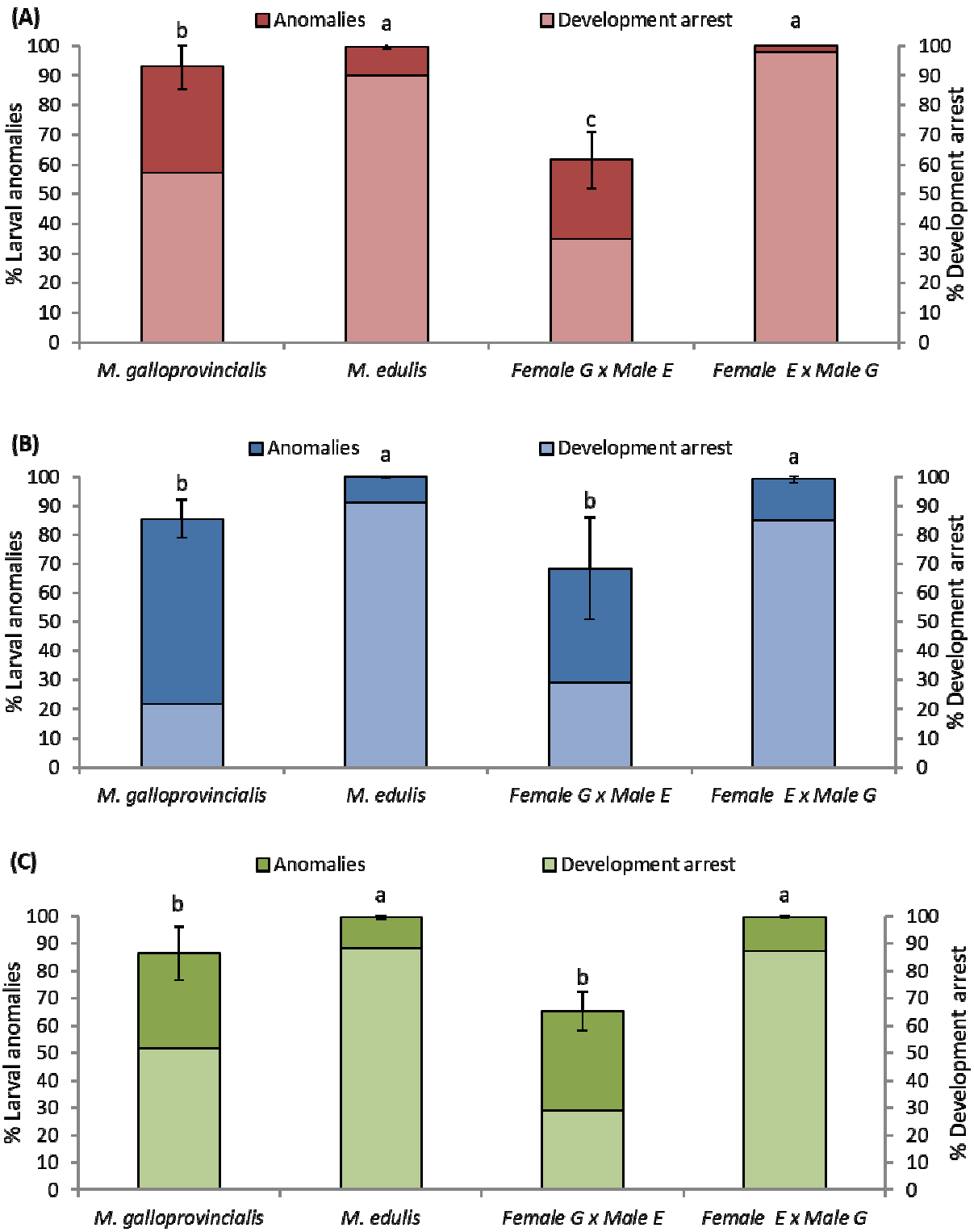


Figure 5: Toxicity of copper (A), silver (B), and a mixture of both metals (C) on the embryo-larval development of mussels *M. galloprovincialis*, *M. edulis,* and hybrids of both species at 22 °C for 48h. Different letters indicate significant differences ($p < 0.05$, Tukey post-hoc test) between different 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**
Pure M. galloprovincialis	15.8 ± 2.7	9	9	9	9
Pure M. edulis	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

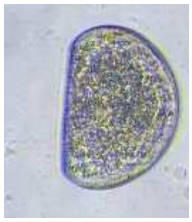
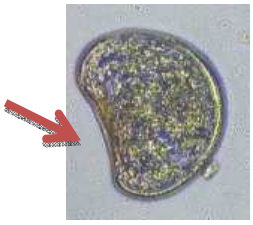
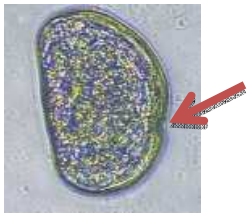
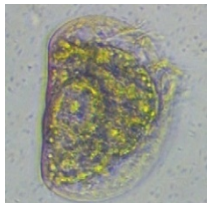
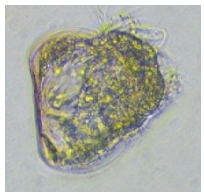
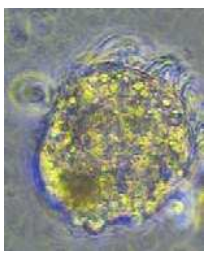
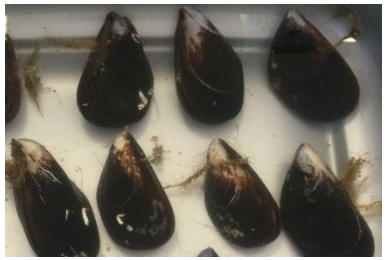
Descriptions		Observations
Normal D-Larva		
Shell anomalies	Concave hinge	
	Slitting	
Mantle anomalies	Mantle retracted	
	Hypertrophy with expulsion	
Development arrest		

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



Mussel sampling and genotyping

Pure species

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

Hybrids

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

Crossings

18 °C
20 °C
22 °C
24 °C

Embryos exposure for 48h

Ag
Cu
Cu + Ag

18 °C
22 °C

D-Larvae

Developmental arrests
Malformations