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1 **Intraspecific variability of responses to combined metal contamination and immune**  
2 **challenge among wild fish populations**

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## 12 **Abstract**

13 Wild organisms are increasingly exposed to multiple anthropogenic and natural stressors that  
14 can interact in complex ways and lead to unexpected effects. In aquatic ecosystems,  
15 contamination by trace metals has deleterious effects on fish health and commonly co-occurs  
16 with pathogens, which affect similar physiological and behavioral traits. However, the  
17 combined effects of metal contamination and parasitism are still poorly known. In addition,  
18 the sensitivity to multiple stressors could be highly variable among different fish populations  
19 depending on their evolutionary history, but this intraspecific variability is rarely taken into  
20 account in existing ecotoxicological studies. Here, we investigated i) the interactive effects of  
21 metal contamination (i.e., realistic mixture of Cd, Cu and Zn) and immune challenge  
22 mimicking a parasite attack on fish health across biological levels. In addition, we compared  
23 ii) the physiological and behavioral responses among five populations of gudgeon fish (*Gobio*  
24 *occitaniae*) having evolved along a gradient of metal contamination. Results show that single  
25 stressors exposure resulted in an increase of immune defenses and oxidative stress at the  
26 expense of body mass (contamination) or fish swimming activity (immune challenge).  
27 Multiple stressors had fewer interactive effects than expected, especially on physiological  
28 traits, but mainly resulted in antagonistic effects on fish swimming activity. Indeed, the  
29 immune challenge modified or inhibited the effects of contamination on fish behavior in most  
30 populations, suggesting that multiple stressors could reduce behavioral plasticity.  
31 Interestingly, the effects of stressors were highly variable among populations, with lower  
32 deleterious effects of metal contamination in populations from highly contaminated  
33 environments, although the underlying evolutionary mechanisms remain to be investigated.  
34 This study highlights the importance of considering multiple stressors effects and intraspecific  
35 variability of sensitivity to refine our ability to predict the effects of environmental  
36 contaminants on aquatic wildlife.

37

38 **Main findings:** Fish responded to single stressors by inducing physiological and behavioral  
39 changes, while multiple stressors inhibited behavioral responses, but responses were highly  
40 variable among populations.

41

42 **Keywords**

43 Multiple Stressors; Evolutionary-Ecotoxicology; Metabolic strategy; Fish Behavior; Human-  
44 altered rivers

45 **Introduction**

46

47 Aquatic vertebrates such as freshwater fish are increasingly impacted by human activities  
48 (Harrison et al., 2018; WWF, 2018). Recent studies suggest that this could be related to the  
49 co-occurrence of multiple stressors in human-altered ecosystems (Reid et al., 2019), **such as**  
50 **contaminants and nutrients inputs, temperature increase and emerging pathogens** (Birk  
51 et al., 2020; Nöges et al., 2016; Schäfer et al., 2016; Schinegger et al., 2016). Indeed, multiple  
52 stressors can have complex interactive effects on individuals and ecosystems and result in  
53 more severe **or mitigated consequences** than single stressors, leading to **unexpected effects**  
54 (Christensen et al., 2006; Lange et al., 2018; Schinegger et al., 2016). **For instance,**  
55 **concomitant exposure to organic contaminants and parasites may synergistically**  
56 **decrease fish survival** (Kelly et al., 2010). **On the contrary, the negative effect of organic**  
57 **contaminants on amphibians' metamorphosis may be inhibited by nutrient enrichment**  
58 **resulting in antagonistic effects** (Boone and Bridges-Britton, 2006).

59 Among the most frequent stressors identified in aquatic systems, contamination by  
60 organic and inorganic compounds is recognized as a major threat to wild fish populations  
61 (Hamilton et al., 2017). More particularly, trace metal elements such as Cadmium (Cd),  
62 Copper (Cu) and Zinc (Zn) used for industrial purposes and pest management are  
63 continuously released into the environment. Metal contamination of water and sediments **has**  
64 **deleterious effects on fish physiology (e.g., immune response, oxidative stress, energy**  
65 **use)** (Guardiola et al., 2013; Lushchak, 2011; Pretto et al., 2014; Xu et al., 2018), **body**  
66 **condition** (Hopkins et al., 2004), **behavior** (Jacquin et al., 2020; Scott and Sloman, 2004)  
67 **and ultimately survival** (Wood et al., 2012, 2011). **In addition, global changes (e.g.,**  
68 **climate change, invasive species, contamination) can also affect the occurrence and**

69 **severity of infection by emerging and re-emerging pathogens in aquatic environments**  
70 (Mennerat et al., 2010; Peeler and Feist, 2011). **For instance, temperature increase due to**  
71 **climate change and the introduction of new disease agents due to invasive species might**  
72 **increasingly expose fish to pathogens (Marcogliese, 2001; Peeler and Feist, 2011) and**  
73 **hence to costly immune challenges (Lochmiller and Deerenberg, 2000; Sheldon and**  
74 **Verhulst, 1996).** Although the effects of parasites and their associated immune challenges  
75 **have been extensively studied in fish** (Magnadottir, 2010), their combined effects with  
76 contamination are still **overlooked** (but see Defo et al., 2019; Marcogliese et al., 2005; Renick  
77 et al., 2016; H. Zhao et al., 2020), thereby hindering our ability to reliably predict the impact  
78 of multiple stressors in the wild.

79 Because contaminants and parasitism **may** affect the same physiological and behavioral  
80 traits, they are expected to have complex interactive effects (Petitjean, 2019). For instance,  
81 metal contamination can either stimulate (Khangarot et al., 1999; Le Guernic et al., 2016;  
82 Prabakaran et al., 2006) or depress fish immune response (Aliko et al., 2018; Dautremepuits  
83 et al., 2004; Jolly et al., 2014), while parasitism generally has an immunostimulatory effect  
84 associated with a costly inflammatory response (Bonneaud et al., 2003; Magnadóttir, 2006;  
85 Sheldon and Verhulst, 1996). More globally, physiological defenses against both metal  
86 contamination and immune challenges induce oxidative stress and are energetically  
87 demanding (Lushchak, 2011; Marcogliese and Pietrock, 2011). The combination of  
88 contamination and immune challenge might thus result in complex interactive effects and  
89 induce synergistic (i.e., amplifying), or antagonistic (i.e., inhibiting) changes in immunity,  
90 oxidative stress and energy reserves, with ultimate consequences for fish survival and fitness.

91 Fish behavior is also expected to be very sensitive to multiple stressors (Scott and Sloman,  
92 2004; Wong and Candolin, 2015). Indeed, contamination can alter fish swimming activity and  
93 sociability (Martin et al., 2017; Tomkins et al., 2018; Zhou and Weis, 1998) due to

94 neurotoxic, endocrine-disrupting effects and energy reallocations (Clotfelter et al., 2004;  
95 Jacquin et al., 2020; J. Zhao et al., 2020). Likewise, immune challenges generally trigger a  
96 "sickness behavior" (Adelman and Martin, 2009; Dantzer, 2004; Johnson, 2002), resulting in  
97 a reduction of investment in routine behaviors such as swimming activity, foraging, and  
98 sociability, which could save energy for costly immune responses (Kirsten et al., 2018;  
99 Volkoff and Peter, 2004). The combination of contamination and immune challenges is thus  
100 likely to result in complex interactive effects on behavior, depending on stress levels,  
101 individuals' energy state, and past exposure to stressors (Barber and Wright, 2005; Montiglio  
102 and Royauté, 2014).

103       However, the type of interaction (e.g., synergistic, antagonistic) and the expected  
104 outcomes of multiple stressors for fish survival and fitness remain challenging to predict.  
105 Over the last decade, a conceptual framework has been proposed to characterize the effects of  
106 moderate or single vs. acute or multiple stressors on fish based on metabolism and energy  
107 management (McEwen and Wingfield, 2010; Petitjean et al., 2019b; Sokolova, 2013). First,  
108 under moderate or single stress, a "compensation strategy" could be set up resulting in energy  
109 reallocation towards physiological defenses (e.g., immunity and antioxidants) at the expense  
110 of energy reserves and routine behaviors (Bonneaud et al., 2003; King and Swanson, 2013; Pi  
111 et al., 2016). Although potentially costly, mounting efficient physiological defenses is  
112 expected to maintain homeostasis and limit the adverse effects of stressors on fish survival  
113 and fitness (Petitjean et al., 2019b; Sokolova, 2013).

114       In contrast, under acute or multiple stressors, the energetic demand for physiological  
115 defenses could exceed the organism's aerobic capacity, resulting in metabolic depression  
116 (Guppy and Withers, 1999; Petitjean et al., 2019b; Sokolova, 2013). This "conservation  
117 strategy" is characterized by an inhibition of physiological defenses, a reduction in metabolic  
118 activity, and limited depletion of energy reserves, with potentially detrimental consequences

119 on cell damage and fish survival on a longer time scale (Petitjean et al., 2019b; Sokolova et  
120 al., 2012; Sokolova, 2013). However, few empirical studies experimentally tested these  
121 predictions, especially in a multistress framework (but see Gandar et al., 2017, 2016).

122 Besides, this theoretical framework does not take into account the intraspecific  
123 variability of stress responses among wild populations, especially when they have evolved in  
124 environments with contrasted contamination levels. For instance, Atlantic killifish (*Fundulus*  
125 *heteroclitus*) populations having evolved under various environmental conditions display  
126 different sensitivity to inorganic (Klerks and Weis, 1987) and organic contamination  
127 (Whitehead et al., 2017), temperature, salinity and pH (Schulte, 2014). These differences in  
128 sensitivity can be due to the selection of particular genotypes (Reid et al., 2016) and/or to  
129 different phenotypic plasticity, which can sometimes result in local adaptation to  
130 contamination (Mulvey et al., 2003; Nacci et al., 1999). However, intraspecific variability in  
131 stress responses is still rarely considered, and its consequences for adaptation to multiple  
132 stressors are largely unknown.

133 In this study, we investigated the interactive effects of a realistic metal contamination  
134 (i.e., mixture of Cd, Cu, Zn found in a contaminated river) and immune challenge (i.e.,  
135 antigen mixture mimicking a parasite attack) on several key physiological (i.e., metal  
136 bioaccumulation, immunity, oxidative stress, energy reserves) and behavioral traits (i.e.,  
137 swimming activity, sociability, foraging), as well as their consequences for fish survival using  
138 an experimental approach. We also tested the intraspecific variability of responses to these  
139 multiple stressors by comparing the responses of five gudgeon (*Gobio occitaniae*) populations  
140 having evolved along a gradient of metal contamination. Following the "metabolic strategy"  
141 framework, we expected single stressors to trigger a "compensation strategy", i.e., increased  
142 investment in physiological defenses at the expense of energy reserves or behavior. We also  
143 expected multiple stressors to trigger a "conservation strategy", i.e., inhibition of



144 physiological and/or behavioral adjustments, with deleterious effects on fish survival.

145 Besides, we expected fish from the most contaminated sites to be less sensitive to

146 experimental contamination.

147

148 **Material and Methods**

149 **Model species and sampling sites**

150 The gudgeon (*Gobio occitaniae*) is a widely distributed fish species in the Garonne watershed  
151 (South-West France). **Within this area, gudgeons populations are exposed to various**  
152 **levels of contaminants (mostly trace metal elements and, to a lesser extent, pesticides)**  
153 (Petitjean et al., 2020; Shinn et al., 2015) **and parasitism (mostly monogenean**  
154 **ectoparasites such as gyrodactylids and to a lesser extent copepods and nematodes)** (Loot  
155 et al., 2007), which can contribute to phenotypic variability (Knapen et al., 2004; Shinn et al.,  
156 2015). We used the database from Adour-Garonne Water Agency (SIE, 2016) to calculate a  
157 Toxic Unit **based on metals concentrations measured between 2012 and 2016** in a set of  
158 16 sampling sites previously characterized (Petitjean, 2019; Petitjean et al., 2020 and  
159 supporting material 1 and 2). Among them, we selected four sampling sites (ARIMAS,  
160 CELCAB, AUSCOR and RIOU) that differed in terms of metal contamination, mostly due to  
161 different levels of Cd, Cu and Zn contamination (**Table 1** and supporting material 2). We also  
162 added a reference population originating from a fish farm **where metal concentrations are**  
163 **low** (Les Viviez de Haute Correze, VHC, Le Bourg, 19340 Courteix, France, named FARM)  
164 (**Table 1**).

165 *Table 1. Characteristics of the sampling site and sample sizes in each population and experimental treatment. Mean water*  
 166 *Cd, Cu and Zn water concentrations over 4 years (corresponding to the life duration of gudgeon) were obtained from the*  
 167 *Water Agency (SIE database, 2016). The Toxic Unit (TU) was calculated as the logarithm of the sum of all-metal*  
 168 *concentrations (Al, As, Cd, Cu, Co, Cr, Ni, Pb, Zn) on 4 years divided by the LC50 ratio for a model fish species (Pimephales*  
 169 *promelas) and was used as a proxy of metal toxicity risk for fish (Petitjean, 2019).*

170

171

Sampling sites	Lat.	Long.	Trace metal concentration ( $\mu\text{g.L}^{-1}$ ) $\pm$ SD				Experimental treatment and sample sizes			
			Cd	Cu	Zn	TU	Control (NC-PBS)	Contamination alone (C-PBS)	Immune challenge alone (NC-AMIX)	Multiple stressors (C-AMIX)
<b>FARM</b>	2°20'9.1255" E	45°38'50.0269" N	0.007 $\pm$ 0.002	2.05 $\pm$ 0.96	5.95 $\pm$ 2.83	-0.9	20	20	20	20
<b>ARIMAS</b>	1°22'26.1494" E	43°5'2.4677" N	0.05 $\pm$ 0.06	1.49 $\pm$ 1.31	4.54 $\pm$ 2.35	-0.9	15	20	20	20
<b>CELCAB</b>	1°39'27.8050" E	44°30'27.5836" N	0.05 $\pm$ 0.02	0.99 $\pm$ 0.92	5.73 $\pm$ 5.27	-0.8	10	15	10	15
<b>AUSCOR</b>	1°19'38.2922" E	43°39'11.3598" N	0.05 $\pm$ 0.03	6.47 $\pm$ 12.7	25.6 $\pm$ 27.5	-0.3	30	30	35	45
<b>RIOU</b>	2°12'41.3680" E	44°33'55.4580" N	13.2 $\pm$ 8.87	2.57 $\pm$ 1.84	629 $\pm$ 521	1.3	15	15	20	15

Contamination treatment corresponds to a metal mixture of Cd, Cu and Zn with adjusted concentrations reaching the highest concentrations recorded at contaminated sites (RIOU and AUSCOR): 14  $\mu\text{g.L}^{-1}$ , 10  $\mu\text{g.L}^{-1}$  and 600  $\mu\text{g.L}^{-1}$ , respectively

Immune challenge corresponds to a mixture of two antigens (90 $\mu\text{g}$  of LPS and 90 $\mu\text{g}$  of PHA in 10 $\mu\text{L}$ ) injected at the caudal peduncle of fish

## 172 **Experimental design**

173 **A total of 516 gudgeons were caught** and brought to the laboratory **in early spring 2018**.  
174 **Among them, 169, 88, 94, and 68 gudgeons were caught by electrofishing using an**  
175 **EFKO-FEG 1500 in AUSCOR, ARIMAS, RIOU, and CELCAB, respectively, and 97**  
176 **fish were collected in a fish farm (FARM).** Fish were then acclimated for 30 days and  
177 treated using Praziquantel (Vetofish, France, purity: 1000 mg.g<sup>-1</sup>; concentration: 3 mg.L<sup>-1</sup>) to  
178 eliminate most commonly detected parasites (i.e., monogenean) and standardize their immune  
179 status. **After acclimation (30 days), 106 fish were not involved in the experiment because**  
180 **they were too small (< 6cm) or died during acclimation or tagging. Hence, 410 remaining**  
181 **fish between 6 and 14 cm of length were involved in the experiment (140, 75, 65, 50, and**  
182 **80 fish from AUSCOR, ARIMAS, RIOU, CELCAB, and FARM, respectively).** During  
183 the experimental procedure, fish were held by groups of five individuals in experimental tanks  
184 (50x30x30 cm) at a constant temperature (17-18°C) under a day: night cycle photoperiod of  
185 12:12h. Fish were fed daily (about 1% of biomass per tank) (Flammarion et al., 1998;  
186 Kestemont et al., 1991) with commercial fish food (JBL Propond Sterlet S pellets). Fish were  
187 randomly exposed to four different treatments (Table 1): The control group without stressor  
188 (NC-PBS: n = 90) was not exposed to contamination (NC) and was injected with a neutral  
189 Phosphate Buffered Saline solution (PBS); the contamination alone group (C-PBS: n = 100,  
190 see below for details); the immune challenge alone group, was challenged with a mixture of  
191 antigens (see below for details) (NC-AMIX: n = 105); and the multiple stressors group  
192 exposed to both contamination and immune challenge (C-AMIX: n = 115). There was no  
193 difference in fish sex ratio ( $\chi^2 = 2.53$ ,  $p = 0.47$ ), mass ( $8.64 \pm 4.77$  g; Anova:  $F = 0.21$ ,  $p =$   
194  $0.89$ ) nor size ( $9.4 \pm 1.7$  cm; Anova:  $F = 0.57$ ,  $p = 0.63$ ) among treatments.

195 For the contamination treatment, we used a mixture of the three trace metal elements  
196 that contributed the most to the Toxic Unit in a set of 16 sampling sites previously studied

197 (Petitjean, 2019; Petitjean et al., 2020 and supporting material 1 and 2). The metal mixture  
198 was composed of Cd, Cu and Zn chloride ( $\text{CdCl}_2$ ,  $\text{CuCl}_2$  and  $\text{ZnCl}_2$ , Thermo fisher scientific,  
199 purity: 99.99%) and concentrations were adjusted to approximately reach the **highest**  
200 **concentrations recorded at contaminated sites** (RIOU and AUSCOR):  $14 \mu\text{g. L}^{-1}$ ,  $10 \mu\text{g. L}^{-1}$   
201  $^1$  and  $600 \mu\text{g. L}^{-1}$ , respectively (Table 1). Half of the water was renewed every two days to  
202 ensure constant contamination levels under static exposure. The experimental contamination's  
203 reliability was checked by measuring exposure concentrations in random water samples for  
204 both non-contaminated ( $n = 29$ ) and contaminated ( $n = 47$ ) tanks. In non-contaminated tanks,  
205 Cd, Cu and Zn average concentrations reached  $0.007 \pm 0.005 \mu\text{g.L}^{-1}$ ,  $0.62 \pm 0.15 \mu\text{g.L}^{-1}$  and  
206  $4.06 \pm 0.79 \mu\text{g.L}^{-1}$  respectively. In contaminated tanks, contamination levels reached  $13.5 \pm$   
207  $1.31 \mu\text{g.L}^{-1}$ ,  $9.96 \pm 1.50 \mu\text{g.L}^{-1}$  and  $551 \pm 45.2 \mu\text{g.L}^{-1}$  respectively.

208         Seven days after the exposure to metal contamination, half of the fish were exposed to  
209 an immune challenge using  $10 \mu\text{L}$  of a mixture of antigens (AMIX) injected in the caudal  
210 peduncle to mimic parasite attack. These fish were injected with an antigen mixture (AMIX)  
211 of phytohaemagglutinin (PHA, *Phaseolus vulgaris* phytohaemagglutinin-L, L8754 Sigma-  
212 Aldrich -  $90 \mu\text{g.}10 \mu\text{L}^{-1}$ ) and lipopolysaccharide (LPS, *Escherichia coli*, serotype: O111: B4,  
213 L2830 Sigma-Aldrich -  $90 \mu\text{g.}10 \mu\text{L}^{-1}$ ) following previous studies in vertebrates, including  
214 fish (Ardia and Clotfelter, 2006; Otálora-Ardila et al., 2016; Toomey et al., 2010). Seven days  
215 later, fish were euthanized using an anesthetic overdose (benzocaine,  $150 \text{mg.L}^{-1}$ ) and blood  
216 and organs were collected for further analyses (see supporting material 3 for details about  
217 sample collection). In total, 10 to 45 fish per treatment and population were measured (Table  
218 1). However, due to differential survival rates among treatments and populations and  
219 difficulties in collecting blood or tissue on small individuals, sample sizes can vary across  
220 treatment (supporting material 4 for detailed sample size) and populations.

221

222 **Cellular-level**

223 *Metal bioaccumulation in muscle*

224 For metal assay, muscle was freeze-dried, homogenized using Fastprep®-24 homogenizer and  
225 analyzed by inductively coupled plasma mass spectrometry (ICP-MS Agilent 7500ce) after  
226 hot acidic mineralization in HNO<sub>3</sub> (67-69%, Trace Metal Grade, Fisher Chemical). We  
227 assessed the reliability of the analyses using TORT-3 certified reference material. Metal  
228 recovery reached an average of 105 ± 6.1%, 82 ± 15.2%, 107 ± 5.7% for Cd, Cu, and Zn,  
229 respectively.

230

231 *Neutrophils/Lymphocytes immune ratio*

232 We used the Neutrophils/Lymphocytes ratio (N/L immune ratio) in fish blood as a marker of  
233 immune response because neutrophils are important modulators of inflammation (Rosales,  
234 2018) and the number of neutrophils increases and lymphocytes decreases under stress and  
235 infection (reviewed in Davis et al., 2008). The relative abundance of neutrophils and  
236 lymphocytes was assessed using blood smears stained with May Grunwald-Giemsa solution  
237 as described in (Petitjean et al., 2020).

238

239 *Local immune response*

240 An important skin swelling caused by antigen injection reflects high investments in immune  
241 defense (i.e., inflammation and T-cells proliferation) and is recognized as a reliable measure  
242 of local immunocompetence (i.e., local immune response) in a variety of vertebrates,  
243 including fish (Ardia and Clotfelter, 2006; O'Connor et al., 2014; Tella et al., 2008). Skin  
244 swelling was assessed by measuring the caudal peduncle's thickness in each fish following a  
245 previous study in gudgeon (Petitjean et al., 2019a). The intra-individual repeatability of

246 thickness measurement reached  $99.6 \pm 0.8\%$  and  $99.8 \pm 1.3\%$  before and after injection,  
247 respectively.

248

#### 249 *Oxidative stress index*

250 We measured the antioxidant capacity and oxidative damage (OXY and DROM tests  
251 respectively, Diacron International, Grosseto, Italy) in fish plasma following previous studies  
252 in fish (Bagni et al., 2007; Hoogenboom et al., 2012; Petitjean et al., 2019a). For OXY test  
253 intra- and inter-plate repeatability were  $91.1 \pm 7.3 \%$  and  $89.7 \pm 6.7 \%$ , respectively. For  
254 DROM test intra- and inter-plate repeatability were  $84.9 \pm 12.5 \%$  and  $89.7 \pm 10.3 \%$ ,  
255 respectively. We then calculated an index of oxidative stress using the ratio between oxidative  
256 damage divided by the antioxidant capacity (x1000) (Costantini and Dell’Omo, 2006;  
257 Herborn et al., 2011; Petitjean et al., 2019a)

258

#### 259 *Available energy in muscle*

260 The available energy was assessed as the sum of total carbohydrates, lipid and protein  
261 contents in fish muscle following previous studies (De Coen and Janssen, 1997; Gandar et al.,  
262 2017, 2016; Smolders et al., 2004). Concentrations of carbohydrate, protein and lipid were  
263 then transformed into energetic values using their enthalpy of combustion (17, 24 and 39.5  
264  $\text{kJ.g}^{-1}$  respectively) and summed to obtain the available energy in muscle.

265

266 **Individual-level**

267 *Condition index*

268 Three condition indices were calculated to reflect fish general condition: the daily body mass  
269 change during the experiment, the hepatosomatic index (Chellappa et al., 1995), and the  
270 gonadosomatic index (Marentette and Corkum, 2008).

271 Because condition indices were partly correlated, we used a PCA to extract an integrative  
272 condition index from the first axis composed of the daily body mass change, HSI and GSI  
273 (51.5% of variance explained, supporting material 5). Hence, fish with a higher condition  
274 index had a lower body mass loss and a higher HSI and GSI.

275

276 *Behavioral traits*

277 Fish behavior was analyzed using BORIS software (Friard and Gamba, 2016) four days after  
278 the immune challenge (AMIX injection), **according to a previous study investigating**  
279 **behavioral effects of immune challenge in gudgeons** (Petitjean et al., In press). Three  
280 behavioral traits were selected because they are commonly affected by stressors: swimming  
281 activity, foraging and sociability (Barber et al., 2000; Jacquin et al., 2017; Kirsten et al., 2018;  
282 Koltes, 1985; Little and Finger, 1990) (see supporting material 6 for detailed method).

283 Because behavioral traits were partly correlated, we used a PCA to extract three  
284 synthetic indices. The first behavioral index was named "general activity" (PCA axis 1, 41%  
285 of variance explained, supporting material 7). Fish with a higher general activity index on the  
286 first PCA axis swam for a longer time and across more areas. The second behavioral index  
287 was named "Foraging" (PCA axis 2 multiplied by -1, 20.6% of variance explained, supporting  
288 material 7). Hence, fish with a higher foraging index displayed a higher motivation to feed  
289 (i.e., low latency to forage time) and a higher number of food items ingested. The third



290 synthetic index was named "Sociability" (PCA axis 3 multiplied by -1, 16.7% of variance  
291 explained, supporting material 8). Hence, fish with a higher sociability index displayed a  
292 higher number of contacts with their counterparts and a lower ANND, reflecting higher  
293 sociability.

294

## 295 **Statistics**

296 To test the effects of treatments and populations, we used linear mixed-effect models (LMM;  
297 lme4 package, (Bates et al., 2015) on each trait: metal (i.e., Cd, Cu, Zn) bioaccumulation (log-  
298 transformed and scaled), N/L immune ratio (log-transformed), local immune response (skin  
299 swelling), available energy in fish muscle (log-transformed), general condition index (first  
300 condition PCA axis), oxidative stress index (sqrt-transformed), behavioral traits (i.e., general  
301 activity, foraging and sociability). The effect of treatments on survival was tested using  
302 generalized linear mixed-effects models with a binomial distribution (lme4 package, Bates et  
303 al., 2015). The trial and the replicate tank were included as random effects in models.  
304 Contamination treatment (Non-Contaminated - NC vs. Contaminated - C), immune challenge  
305 treatment (PBS vs. AMIX injection), the population of origin and third and second-order  
306 interactions were included as fixed factors. Best models were then selected by eliminating  
307 non-significant variables starting with interactions (stepwise model selection; see supporting  
308 material 9 for summary statistics). Body size and sex were also added as covariates in all  
309 models but removed from final models when non-significant. When interactions were  
310 significant, we used contrast posthoc analyses (emmeans package, Lenth et al., 2017) with  
311 False Discovery Rate adjustment to analyze pairwise differences between fish groups  
312 (Benjamini and Hochberg, 1995) with 5% as a significance threshold.

313 To compare the effects-size of single vs. multiple stressors, we computed standardized  
314 effect sizes (compute.es package) Hedge's g and 95% confident interval (CI) from LMM  
315 model outputs (e.g., z-values or t-value) following methods described in Hedges et al. (1999)  
316 and Del Re (2013). When the interaction between stressors was significant, we also compared  
317 the magnitude of interactive vs. additive effects of multiple stressors following methods  
318 described in (Lange et al., 2018) in order to characterize the type of interaction involved (i.e.,  
319 additive, synergistic or antagonistic) (see Petitjean et al., 2019a). When interactions were  
320 reversal, corresponding to "mitigating synergism" (sensu Jackson et al., 2016; Piggott et al.,  
321 2015) we considered them as antagonistic since in both cases, the interactions resulted in an  
322 inhibition of single stressors effects (Crain et al., 2008; Folt et al., 1999). When the interaction  
323 varied between populations (i.e., triple interaction: contamination x immune challenge x  
324 population), we characterized the type of interaction by running separate models for each  
325 population. All analyses were performed with R, version 3.5.2 (R Development Core Team,  
326 2018).

327

## 328 **Results**

329

### 330 **Cellular-level**

#### 331 *Metal bioaccumulation in muscle*

332 At the cellular level, metal bioaccumulation was affected by the **contamination alone in**  
333 **interaction with the population of origin** (Fig 1 and supporting material 9). Cu and Zn were  
334 not significantly accumulated (ANOVA, estimates (scaled log) =  $0.002 \pm 0.02$ ,  $F = 0.006$ ,  $p =$   
335  $0.94$  and ANOVA, estimates (scaled log) =  $0.001 \pm 0.05$ ,  $F = 0.0004$ ,  $p = 0.99$ , respectively).

336 However, Cd bioaccumulation was significant in fish exposed to the experimental  
337 contamination, but this effect depended on the population considered (ANOVA, estimates  
338 (scaled log) =  $0.15 \pm 0.06$ ,  $F = 5.78$ ,  $p = 0.02$ ) (Fig 1 and supporting material 9). More  
339 specifically, contrast posthoc analyses show that fish originating from the farm (FARM) and  
340 the least contaminated site (ARIMAS) displayed a significant increase of Cd concentration in  
341 their muscle when exposed to the experimental contamination compared to the control group  
342 (FARM-NC-PBS  $\bar{x}$  (scaled log):  $0.50 \pm 0.06$  vs FARM-C-PBS  $\bar{x}$  (scaled log):  $0.66 \pm 0.12$ ,  
343 estimates (scaled log) =  $0.48 \pm 0.15$ ,  $p = 0.01$  and ARIMAS-NC-PBS  $\bar{x}$  (scaled log):  $0.42 \pm$   
344  $0.05$  vs ARIMAS-C-PBS  $\bar{x}$  (scaled log):  $0.87 \pm 0.14$ , estimates (scaled log) =  $0.35 \pm 0.14$ ,  $p =$   
345  $0.046$ , respectively). In contrast, populations from the most contaminated sites (CELCAB;  
346 AUSCOR, RIOU) displayed no significant accumulation of metals in their muscle when  
347 exposed to the experimental contamination (Fig 2 A).

348

#### 349 *Neutrophils/Lymphocytes immune ratio*

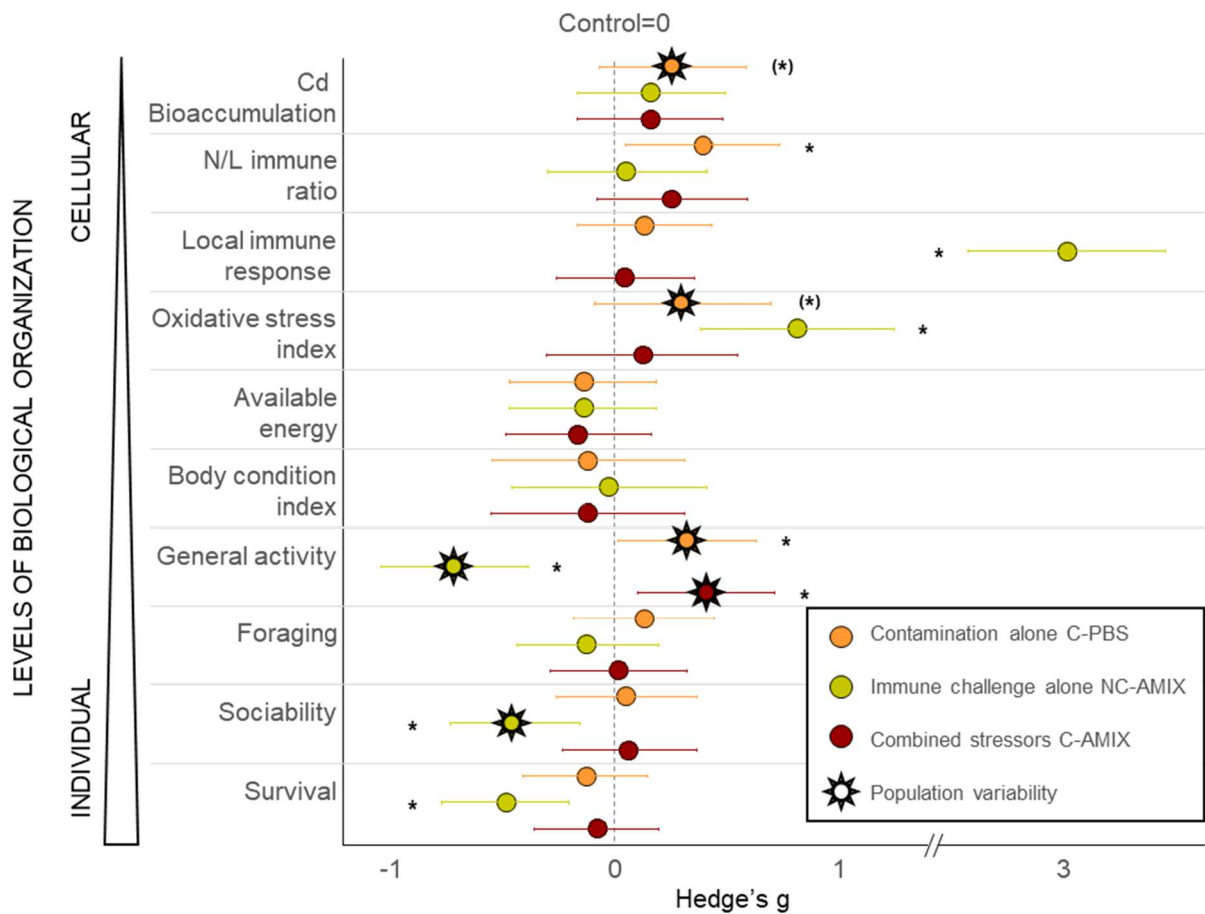
350 The N/L immune ratio was affected by the contamination alone (Fig 1 and supporting  
351 material 9). More particularly, fish exposed to contamination displayed an increase of  
352 neutrophils (ANOVA, estimate (log) =  $0.27 \pm 0.12$ ,  $F = 5.4$ ,  $p = 0.03$ ) and a decrease of  
353 lymphocytes count (ANOVA, estimate =  $-6.98 \pm 2.47$ ,  $F = 7.8$ ,  $p = 0.01$ ), resulting in a  
354 significant increase of the N/L immune ratio (ANOVA, estimate (log) =  $0.34 \pm 0.15$ ,  $F = 4.9$ ,  
355  $p = 0.03$ ).

356

#### 357 *Local immune response*

358 The local immune response was significantly affected by the immune challenge alone (Fig 1  
359 and supporting material 9). Indeed, fish injected with AMIX solution had a higher local

360 immune response (i.e., skin swelling) than fish injected with control saline solution (PBS)  
 361 whatever the population considered (ANOVA, estimate =  $44.4 \pm 2.2$ ,  $F = 389$ ,  $p < 0.001$ ).



362

363 *Figure 1. Effect size (hedge's g) and 95% confidence intervals (CI) of*  
 364 *experimental treatments (contamination alone in orange: C-PBS; immune*  
 365 *challenge alone in green: NC-AMIX; multiple stressors in red: C-AMIX) relative*  
 366 *to the control group (NC-PBS: dashed vertical line), across biological levels*  
 367 *from cells to the whole organism. When the 95% CI did not overlap 0 (control*  
 368 *group), the treatment's effect was significant, as indicated by an asterisk. The*  
 369 *asterisk between brackets indicates a marginally significant effect (lower*  
 370 *bound:  $-0.1 < 95\% \text{ CI} < 0.1$ ). Stars indicate significant variability among*  
 371 *populations (population-by-treatment interaction).*

372

373 *Oxidative stress index*

374 The oxidative stress index was significantly affected by single stressors (contamination and  
375 immune challenge alone) but not by multiple stressors (Fig 1 and supporting material 9). The  
376 oxidative stress index increased marginally and in a population dependent manner in fish  
377 exposed to contamination (ANOVA,  $F = 2.38$ ,  $p = 0.056$ ) and significantly in immune-  
378 challenged fish (ANOVA, estimate (sqrt) =  $0.44 \pm 0.97$ ,  $F = 15$ ,  $p = 0.001$ ).

379 More particularly, contamination increased the level of oxidative damage (ANOVA,  
380 estimates =  $0.16 \pm 0.03$ ,  $F = 24.9$ ,  $p < 0.001$ ) but had no significant effect on the level of anti-  
381 oxidant defenses in fish plasma (ANOVA, estimates (sqrt) =  $0.29 \pm 0.41$ ,  $F = 0.47$ ,  $p = 0.5$ ).  
382 In addition, the effect of contamination differed among populations (Fig 1 and supporting  
383 material 9). Indeed, only the FARM population displayed a significant increase of oxidative  
384 stress index **through an increase of oxidative damage** (Farm-NC-PBS  $\bar{x}$  (sqrt):  $1.46 \pm 0.12$   
385 vs Farm-C-PBS  $\bar{x}$  (sqrt):  $1.94 \pm 0.23$ , estimates (sqrt):  $0.79 \pm 0.22$ ,  $p = 0.002$ ) (Fig. 2 B). The  
386 immune challenge also increased the level of oxidative damage (ANOVA, estimates (log) =  
387  $0.15 \pm 0.03$ ,  $F = 18.1$ ,  $p < 0.001$ ) but also decreased the level of anti-oxidant defenses in fish  
388 plasma (ANOVA, estimates (sqrt) =  $-0.78 \pm 0.37$ ,  $F = 3.9$ ,  $p = 0.05$ ) and this effect was the  
389 same whatever the population considered (Fig 1 and supporting material 9).

390

391 *Available energy in muscle*

392 The amount of total available energy (sum of lipids, proteins and carbohydrates) in fish  
393 muscle was not significantly affected by any stressor (Fig 1 and supporting material 9).  
394 However, fish exposed to contamination alone had a higher amount of carbohydrates in their

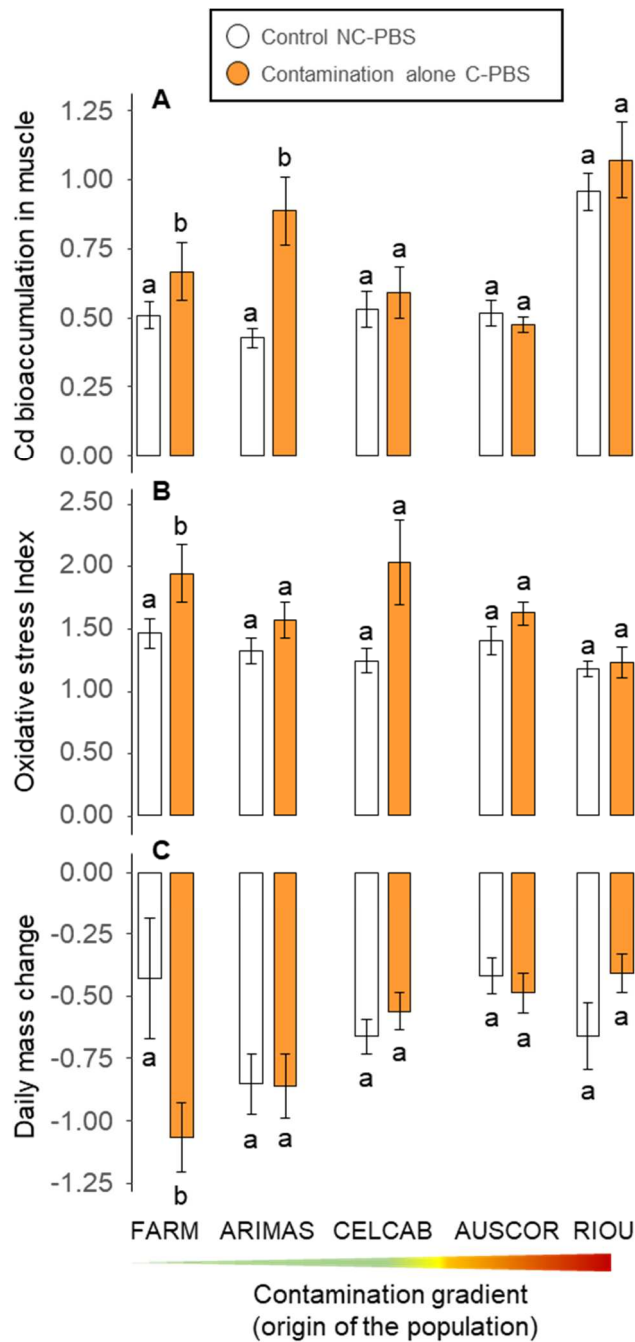
395 muscle (NC-PBS  $\bar{x}$  (log):  $3.5 \pm 0.09$  vs C-PBS  $\bar{x}$  (log):  $3.74 \pm 0.08$ , ANOVA, estimates (log)  
396 =  $0.27 \pm 0.08$ ,  $F = 12$ ,  $p = 0.002$ ) whatever the population considered.

397

## 398 **Individual-level**

### 399 *Condition index*

400 The integrative condition index was not significantly affected by any stressor (Fig 1 and  
401 supporting material 9). However, contamination decreased body mass compared to control  
402 fish, but in a population-dependent manner (ANOVA,  $F = 3.5$ ,  $p = 0.009$ ). More specifically,  
403 the FARM population was the only population displaying a higher body mass loss when  
404 exposed to the experimental contamination compared to the control group (FARM-NC-  
405 PBS  $\bar{x}$  (sqrt):  $-0.43 \pm 0.24$  vs FARM-C-PBS  $\bar{x}$  (sqrt):  $-1.06 \pm 0.14$ , estimates (sqrt):  $-0.14 \pm$   
406  $0.04$ ,  $p = 0.002$ ) (Fig 2 C).



407 *Figure 2. Effects of contamination alone (white bars, NC-PBS; orange bars, C-*  
 408 *PBS) in different fish populations along a gradient of contamination. The*  
 409 *contamination gradient in each population was determined using the toxic unit*  
 410 *(TU), which ranged from -0.9 for the least contaminated FARM population to 1.3*  
 411 *for the most contaminated RIOU population (Table 1). A. Cd bioaccumulation in*  
 412 *fish muscle (log-transformed and scaled  $\mu\text{g}\cdot\text{g}^{-1}$ ), B. oxidative stress index (sqrt*  
 413 *transformed index), and C. daily mass change of fish (percent changes per day).*

414 *Error bars correspond to the SEM. Different letters above **or below** bars indicate*  
415 *significant differences between control and contamination treatments within each*  
416 *population after contrast posthoc analyses.*

417

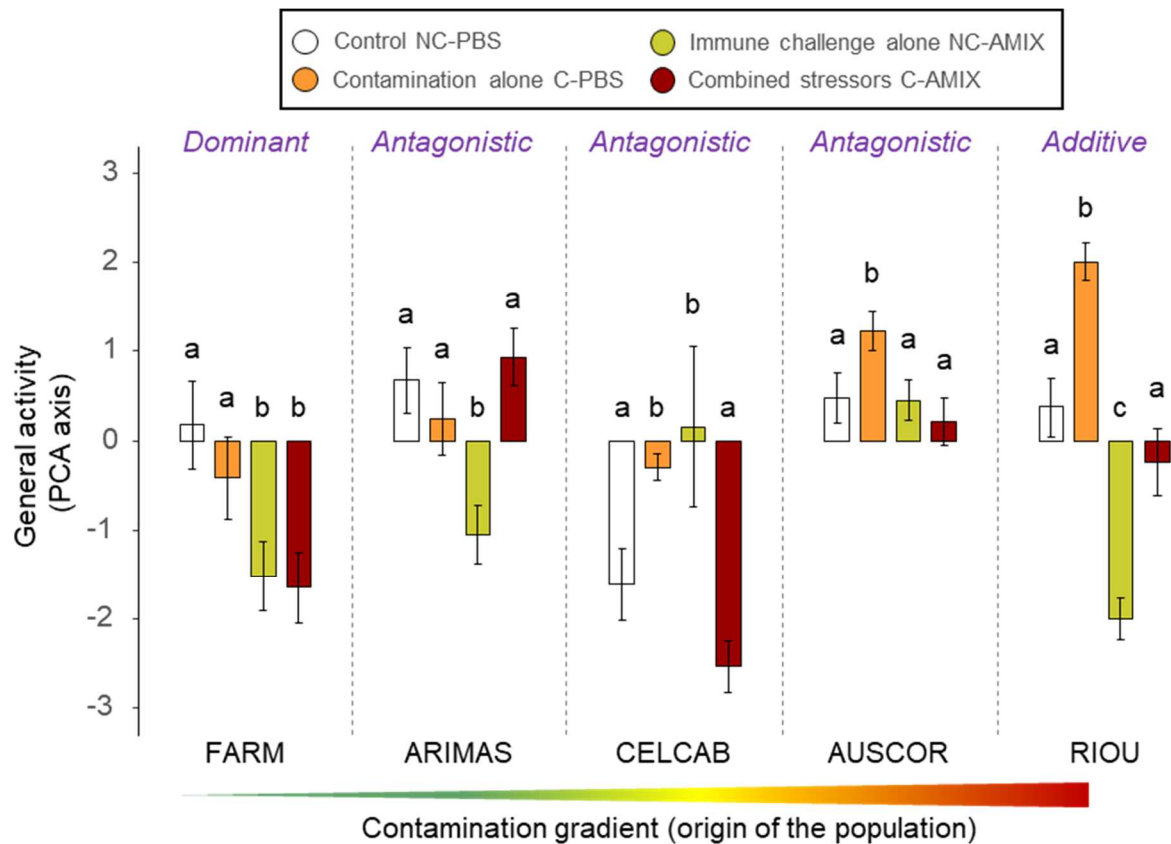
#### 418 *General activity*

419 The general activity index was significantly affected by single and multiple stressors, but  
420 behavioral responses depended on the population considered (contamination x immune  
421 challenge x population interaction, Fig 1 and supporting material 9). More particularly, the  
422 contamination alone (C-PBS) did not affect general activity in the least contaminated FARM  
423 and ARIMAS populations, but the experimental contamination increased fish general activity  
424 in CELCAB and the most contaminated AUSCOR and RIOU populations compared to the  
425 control group (Fig 3). The immune challenge alone decreased general activity in all  
426 populations except CELCAB and AUSCOR (Fig 3).

427 The effects of multiple stressors (C-AMIX group) were very variable depending on the  
428 population considered. There was no interactive effect of multiple stressors in FARM fish due  
429 to a dominant effect of the immune challenge (Hedge's  $g = 0.53$ , CI [-0.28; 1.34]) (Fig 3).  
430 There was antagonistic interactions of multiple stressors in the CELCAB and AUSCOR  
431 populations (CELCAB: Hedge's  $g = -5.23$ , CI [-7.18; -3.29]; AUSCOR: Hedge's  $g = -1.95$ ,  
432 CI [-2.54; -1.37]) and a "mitigating synergism" in ARIMAS (ARIMAS: Hedge's  $g = 3.4$ , CI  
433 [2.35; 4.50]), both resulting in a non-significant difference in general activity between fish  
434 exposed to multiple stressors compared to control (i.e., inhibiting effect), which is considered  
435 as antagonistic effect (Fig 3). Finally, there was an additive effect of multiple stressors in the  
436 RIOU population (Hedge's  $g = 0.23$  CI [-0.56; 1.02]). More specifically, both contamination  
437 and immune challenge had opposite effects on general activity in RIOU fish, resulting in



438 similar swimming activity levels between the control (NC-PBS) and multiple stress (C-  
 439 AMIX) group in RIOU fish (Fig 3).



440

441 *Figure 3. Mean ( $\pm$  SEM) general activity (first axis of PCA reflecting the time*  
 442 *spent swimming, the number of lines crossed and the number of visited areas) of*  
 443 *fish exposed to control condition (white bars, NC-PBS), contamination alone*  
 444 *(orange bars, C-PBS), immune challenge alone (green bars, NC-AMIX), or*  
 445 *combined contamination and immune challenge (red bars, C-AMIX) among fish*  
 446 *populations. The fish populations were ranked according to the level of*  
 447 *contamination recorded in their sampling site (contamination gradient*  
 448 *determined using the toxic unit, ranged from -0.9 (FARM) to 1.3 (RIOU), Table*  
 449 *1). Different letters indicate significant differences between treatments within*

450 *each population according to contrast analyses. The type of interaction (additive,*  
451 *antagonistic, synergistic) was tested following Lange et al. (2018) (see methods).*

452

#### 453 *Foraging behavior*

454 Foraging behavior index was not significantly affected by any treatment (Fig 1 and supporting  
455 material 9). However, additional analyses on each trait indicate that the latency to forage  
456 increased in immune challenged fish (NC-PBS  $\bar{x}$  (log):  $5.84 \pm 0.12$  vs NC-AMIX  $\bar{x}$  (log):  
457  $5.95 \pm 0.15$ , ANOVA, estimates (log) =  $0.35 \pm 0.13$ ,  $F = 6.4$ ,  $p = 0.01$ ) whatever the  
458 population considered. Similarly, the number of food items ingested by fish marginally  
459 decreased with the immune challenge (NC-PBS  $\bar{x}$  (log):  $1.02 \pm 0.10$  vs NC-AMIX  $\bar{x}$  (log):  
460  $0.82 \pm 0.09$ , ANOVA, estimates (log) =  $-0.19 \pm 0.09$ ,  $F = 3.9$ ,  $p = 0.054$ ).

461

#### 462 *Sociability behavior*

463 Fish sociability index was affected by the immune challenge alone, but this effect varied  
464 among populations (ANOVA,  $F = 8.3$ ,  $p < 0.001$ ) (Fig 1 and supporting material 9). More  
465 particularly, posthoc analyses show that sociability decreased in AMIX-injected fish from  
466 CELCAB (PCA axis 3, CELCAB-NC-PBS  $\bar{x}$ :  $0.25 \pm 0.15$  vs CELCAB-NC-AMIX  $\bar{x}$ :  $-1.11$   
467  $\pm 0.54$ , estimates =  $-1.58 \pm 0.34$ ,  $p < 0.001$ ) while it increased for fish from RIOU (PCA axis  
468 3, RIOU-NC-PBS  $\bar{x}$ :  $-0.52 \pm 0.36$  vs RIOU-NC-AMIX  $\bar{x}$ :  $0.32 \pm 0.13$ , estimates =  $0.87 \pm$   
469  $0.31$ ,  $p < 0.01$ ). Sociability was not significantly affected by any stressor in other populations.

470

471 *Survival*

472 Fish survival was significantly affected by the immune challenge alone but not by the  
473 contamination nor multiple stressors (Fig 1 and supporting material 9). Indeed, AMIX-  
474 injected fish had a lower survival compared to control fish whatever the contamination  
475 treatment and whatever the population considered (NC-PBS: 81.1%, C-PBS: 85.0%, NC-  
476 AMIX: 69.5%, C-AMIX: 71.3%; ANOVA, estimates =  $-1.47 \pm 0.32$ ,  $\chi^2 = 21.4$ ,  $p < 0.001$ ).

477

## 478 **Discussion**

479 In this study, we tested the effects of multiple stressors (i.e., metal contamination and immune  
480 challenge) on gudgeon health from cells to the whole organism in different populations  
481 having evolved along a gradient of metal contamination. Results show that each single  
482 stressors affected different key physiological and behavioral traits and that combined stressors  
483 had limited interactive effects excepted on behavior. In addition, there was a high  
484 interpopulation variability of physiological and behavioral responses to stressors (summarized  
485 in supporting material 10).

486

### 487 **Single stressor effects**

488 The theoretical framework of metabolic strategies predicts that organisms react to a moderate  
489 or single stressor by setting up a "compensation strategy" characterized by increased  
490 investment in physiological defenses (immunity and antioxidant defense) at the expense of  
491 depletion of energy reserves and/or decrease in routine behaviors (Petitjean et al., 2019b;  
492 Sokolova, 2013; Sokolova et al., 2012). **Our results do not show direct energetic costs of**

493 **stressors but show significant physiological and behavioral changes, although some**  
494 **responses varied according to the population.**

495 First, fish exposed to single stressors had increased immune defenses. Indeed, the  
496 experimental contamination alone increased the N/L immune ratio in all populations, as well  
497 as the bioaccumulation of Cd in the muscle of some populations. This suggests that the  
498 experimental contamination triggered an inflammatory response involving the recruitment of  
499 neutrophils due to the accumulation of toxic metals as shown in previous experimental studies  
500 (e.g., Aliko et al., 2018; Dautremepuits et al., 2004; Jolly et al., 2014; Xu et al., 2018) and in a  
501 previous field study in the same species (Petitjean et al., 2020). Similarly, the immune  
502 challenge alone triggered a significant local inflammatory response (i.e., skin swelling)  
503 reflecting a local infiltration of immune cells at the site of injection (Ardia and Clotfelter,  
504 2006). However, no effect was detected on the N/L immune ratio, maybe because we missed  
505 the peak of immune cell recruitment at 7 days (Afonso et al., 1998; von Gersdorff Jørgensen,  
506 2016). These results suggest that the exposure to a single stressor (i.e., experimental  
507 contamination or immune challenge alone) had an immunostimulatory effect and elicited a  
508 significant inflammatory response. This is likely to incur significant costs since inflammation  
509 is one of the most costly physiological defense (Bonneaud et al., 2003; Lochmiller and  
510 Deerenberg, 2000; Sheldon and Verhulst, 1996) in terms of oxidative stress production  
511 (Costantini and Dell’Omo, 2006; Costantini and Møller, 2009) and/or on energy reserves  
512 depletion (Bonneaud et al., 2003; Lochmiller and Deerenberg, 2000).

513 Accordingly, the fish exposed to both single stressors displayed a significant increase  
514 in oxidative stress index, mainly due to increased oxidative damage, which was consistent  
515 with previous studies (Costantini and Møller, 2009; Fatima et al., 2000; Le Guernic et al.,  
516 2016). We also found that antioxidant defenses decreased in immune-challenged fish,  
517 suggesting that the immune challenge **can trigger oxidative stress, with potential**

518 **detrimental effects on fitness. Further studies are now needed to measure specifically**  
519 **other enzymatic antioxidant components (e.g., Catalase, Super-Oxide Dismutase), but**  
520 **our results suggest that exposure to single stressors incur a significant oxidative cost**  
521 **with potential consequences for fish fitness.**

522 According to the “compensation strategy”, we expected depletion of energy reserves  
523 due to increased investment in physiological defenses. However, results were quite different  
524 depending on the stress considered. Indeed, the immune challenge alone had no effects on  
525 energy reserves and condition indices, **suggesting limited energetic costs.** In contrast,  
526 contamination alone caused a decrease in body mass, although only in FARM fish, without  
527 depleting available energy in muscle but instead increased carbohydrates. This might be  
528 explained by an increase in gluconeogenesis and/or glucose mobilization towards muscle to  
529 support locomotion under stressful conditions, as suggested in previous studies (Philp et al.,  
530 2005; Pretto et al., 2014; Weber et al., 2016).

531 Under a "compensation strategy", we also expected a decrease in routine behaviors to  
532 save energy for physiological defenses. Accordingly, the immune challenge decreased fish  
533 swimming activity and sociability, although in a population dependant manner, which could  
534 enable fish to save energy for immunity (i.e., "sickness behavior"; Adelman and Martin, 2009;  
535 Dantzer, 2004; Johnson, 2002). In contrast, contamination had the opposite effect and  
536 increased fish swimming activity, at least in some populations (i.e., CELCAB, AUSCOR,  
537 RIOU). **This increase in swimming activity under experimental contamination could be**  
538 **due to neurotoxic effects (Jacquin et al., 2020) but could also be linked to the increase in**  
539 **carbohydrates content in fish muscle and probably to cortisol secretions, as suggested in**  
540 **previous studies (Brun et al., 2019). Increased swimming activity could potentially have an**  
541 **adaptive value by increasing** food acquisition in the wild, potentially compensating for the  
542 costs of detoxification, but this hypothesis remains to be tested.

543 Overall, our results show an increased investment in immune and antioxidant defenses  
544 **with limited costs, which differ among the studied traits and the stress considered (i.e.,**  
545 **body mass for contamination, swimming activity for immune challenge alone). This**  
546 **brings only partial support for the “compensation strategy” hypothesis. In addition, fish**  
547 **exposed to the immune challenge had decreased survival, suggesting that stress-induced**  
548 **physiological changes had significant fitness costs despite potential compensation. Also,**  
549 **body mass changes related to contamination depended on fish populations and fish**  
550 **involved in all experimental treatments, including the control group, lost weight during**  
551 **the experiment, suggesting that captivity itself might be significantly stressful for fish**  
552 (Calisi and Bentley, 2009). **Further *in-situ* approaches within rivers would be useful to**  
553 **investigate the costs of stressors in a realistic context.**

554

### 555 **Multiple stressors effects**

556 Under multiple stressors exposure, the "conservation strategy" hypothesis predicts that the  
557 energy demand for physiological defenses would be too high, resulting in a metabolic  
558 depression. This "conservation strategy" is generally characterized by limited investment in  
559 immune and antioxidant defenses and few or no changes in energy reserves and behaviors,  
560 resulting in interactive antagonistic (i.e., inhibiting) effects of multiple stressors (Petitjean et  
561 al., 2019b; Sokolova, 2013; Sokolova et al., 2012). However, contrary to these predictions,  
562 combined metal contamination and immune challenge had few interactive effects excepted on  
563 behavior, but behavioral responses to multiple stressors were dependent on the population  
564 considered.

565 More particularly, we did not find any significant interactive effect of multiple  
566 stressors on immune and antioxidant defenses. For instance, local (i.e., skin swelling) and

567 circulating (i.e., N/L immune ratio in fish blood) immune responses were not affected by  
568 multiple stressors, likely because both stressors affected different immune traits. Further  
569 studies focusing on other components, such as the phagocytic capacity and the oxidative burst  
570 activity, would be necessary to decipher the underpinning immune effects of multiple  
571 stressors (Jolly et al., 2014; Le Guernic et al., 2016). In addition, multiple stressors had no  
572 interactive effects on oxidative stress, although changes in other antioxidants molecules that  
573 were not measured in our study are still possible (Lushchak, 2011). **Whatever the**  
574 **underlying mechanisms, we found no interactive effects of multiple stressors on fish**  
575 **physiological traits, suggesting that the metabolic capacity of fish is not overwhelmed by**  
576 **multiple stressors and/or that the selected traits were not integrative enough to uncover**  
577 **interactive effects.**

578 We also expected antagonistic (i.e., inhibiting) effects on energy reserves and/or  
579 behavior. We found no effect of multiple stressors on energy reserves nor condition indices,  
580 but the exposure to combined contamination and immune challenge inhibited the behavioral  
581 plasticity of fish. Indeed, we found no difference in swimming activity between groups  
582 exposed to multiple stressors and control groups due to inhibiting effects (i.e., "mitigating  
583 synergism" for ARIMAS, antagonism for CELCAB and AUSCOR) or opposite additive  
584 effects of combined stressors (i.e., RIOU). In other words, in most populations, the immune  
585 challenge inhibited the effects of contamination on fish behavior resulting in lower behavioral  
586 responses to multiple stressors compared to single stressors. **This result highlights the need**  
587 **to consider parasitism and other stressors in behavioral-based ecotoxicological tests to**  
588 **avoid misinterpretation.** In addition, it suggests that labile traits such as behaviors may be  
589 particularly sensitive to multiple stressors, with potential implications for fish fitness by  
590 altering reproduction or ability to escape predators in the wild (Scott, 2003; Wingfield, 2003;  
591 Wong and Candolin, 2015).

592           **Overall, only few results support the hypothesis that fish would follow a**  
593 **"conservation strategy" when exposed to multiple stressors. Indeed, only behavior was**  
594 **affected by multiple stressors, and effects had no consequences on physiology or**  
595 **survival, suggesting that multiple stressors are not more deleterious than single stressors**  
596 **for fish fitness,** although we only tested short term responses under controlled laboratory  
597 conditions. **Results also underscore that fish responses strongly depend on stressors type**  
598 **and the origin of the population considered.** Studies investigating fish metabolism and the  
599 long-term fitness consequences of multiple stressors in a more realistic context, such as cross  
600 transplantation in the field, are now needed to better understand the effects of multiple  
601 stressors in the wild.

602

### 603 **Population variability of responses to stressors**

604 There was high variability of response to single and multiple stressors among populations,  
605 underlining the need to take into account intraspecific variability to better predict the effects  
606 of environmental stressors on aquatic wildlife, especially in ecotoxicological tests. First, the  
607 bioaccumulation of Cd was significant only in fish from the least contaminated sites (i.e.,  
608 FARM and ARIMAS). One potential explanation is that populations from contaminated sites  
609 already had high levels of metals in muscle due to previous pollution exposure in their natural  
610 environment (Faucher et al., 2008; Goto and Wallace, 2010; Klaassen et al., 1999).  
611 Accordingly, fish from the most contaminated site (i.e., RIOU) displayed a higher basal level  
612 of Cd in their muscle than other populations. **Other organs such as liver and kidney were**  
613 **used for another study and could not be analyzed here. Although fish muscle is not the**  
614 **first recipient of metal accumulation** (Bervoets et al., 2009; Cretì et al., 2010; Rajotte and  
615 Couture, 2002), **our results indicate that the experimental contamination led to**



616 **significant bioaccumulation of Cd (but not Zn and Cu) in muscles. This suggests that Cd**  
617 **could reach a much higher concentration in target organs such as liver and kidney and**  
618 **explain the observed responses, especially in FARM fish.**

619         Second, oxidative stress increased and body mass decreased significantly in the least  
620 contaminated FARM population in response to contamination, but not in other populations.  
621 This suggests that fish from the least contaminated FARM site **may be** more sensitive to  
622 metal contamination than other populations, which is consistent with recent findings in  
623 several fish species (M. Oziolor et al., 2016; Oziolor et al., 2014; Reid et al., 2016).

624         Third, we found a strong variability in fish behavioral responses to stressors,  
625 especially on fish swimming activity. In our study, the experimental contamination alone had  
626 no effects on the two least contaminated populations (FARM and ARIMAS) but increased  
627 other populations' swimming activity (CELCAB, AUSCOR, RIOU). This suggests that  
628 contamination could induce plastic hyperactivity in fish from the most contaminated sites  
629 (Ellgaard et al., 1978; Steele, 1983), maybe because of neurotoxic or endocrine-disrupting  
630 effects (Drummond and Russom, 1990; Renick et al., 2016; Weis et al., 2001) and/or energy  
631 reallocations under stressful conditions, which could increase food acquisition (Pyke et al.,  
632 1977). Nevertheless, **anthropogenic activities and other** factors not accounted here could  
633 also explain these behavioral differences (e.g., **domestication in the FARM population:**  
634 **Johnsson et al., 2014, predator presence/absence in some populations:** Bell et al., 2010).  
635 Further studies on a higher number of replicate populations are hence necessary to specifically  
636 disentangle the environmental factors explaining these interpopulation differences. **Whatever**  
637 **the determinants of these interpopulation differences, our results underscore the need to**  
638 **consider the variability of behavioral responses among wild populations within the**  
639 **emerging field of behavioral ecotoxicology (Jacquin et al., 2020).**

640 To sum up, our results are in accordance with previous studies showing that different  
641 fish populations could differ in their sensitivity to stressors depending on their history of  
642 contamination in the wild (e.g., Bélanger-Deschênes et al., 2013; Bourret et al., 2008;  
643 Hamilton et al., 2017; Oziolor et al., 2014; Reid et al., 2016). Differences in genetic and  
644 plastic-based ability of detoxification, physiological, and behavioral adjustments could enable  
645 fish to better mitigate the effects of contaminants depending on past selection in stressful  
646 environments. Further analyses comparing the genetic structure of F0 fish and experiments on  
647 F1 and F2 generations would be necessary to investigate the potential genetic basis explaining  
648 this interpopulation variability. **Whatever the underpinning mechanisms, this variability**  
649 **of responses among fish populations could lead to misinterpretation of contaminants'**  
650 **threats since ecological risk assessment is generally derived from ecotoxicity tests**  
651 **performed on a single laboratory raised population (Brady et al., 2017).** Hence, our study  
652 suggests that ecotoxicology should take into account the evolutionary history of populations  
653 to better predict the effects of environmental stressors in an evolutionary ecotoxicology  
654 framework (M. Oziolor et al., 2016).

655

#### 656 **Compliance with ethical standards**

657 This research was conducted in compliance with French and European legislation for animal  
658 experimentation (European directive 2010/63/UE). Fish sampling was conducted under local  
659 authorities' authorization, and fish were treated for parasites according to the prescription  
660 2529, VetoFish. Experimental procedures were conducted under the establishment approval  
661 for vertebrate experimentation N°A3113002, and were approved by the ethical committee  
662 n°073 (authorization n°8538).

663

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