Intraspecific variability of responses to combined metal contamination and immune challenge among wild fish populations☆

Quentin Petitjean [a, b, c, *], Lisa Jacquin [b, c], Louna Riem [a, b], Mathilde Pitout [a], Annie Perrault [a], Myriam Cousseau [a], Pascal Laffaille [a, c], Séverine Jean [a, c]

[a] Laboratoire Écologie Fonctionnelle et Environnement, UMR5245, Université de Toulouse, CNRS, 118 Route de Narbonne, 31062, Toulouse, France
[b] EDB, Laboratoire Évolution et Diversité Biologique, UMR5174 EDB, Université de Toulouse, CNRS, IRD, 118 Route de Narbonne, 31062, Toulouse, France
[c] LTSER France, Zone Atelier PYGAR « Pyrénées-Garonne », Auzeville-Tolosane, France

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ABSTRACT

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

1. Introduction

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

Among the most frequent stressors identified in aquatic systems, contamination by organic and inorganic compounds is recognized as a major threat to wild fish populations (Hamilton et al., 2017). More particularly, trace metal elements such as Cadmium (Cd), Copper (Cu) and Zinc (Zn) used for industrial purposes and pest management are continuously released into the environment. Metal contamination of water and sediments has deleterious effects on fish physiology (e.g., immune response, oxidative stress, energy use) (Guardiola et al., 2013; Lushchak, 2011; Pretto et al., 2014; Xu et al., 2018), body condition (Hopkins et al., 2004), behavior (Jacquin et al., 2020; Scott and Sloman, 2004) and ultimately survival (Wood et al., 2011, 2012). In addition, global changes (e.g., climate change, invasive species, contamination) can also affect the occurrence and severity of infection by emerging and re-emerging pathogens in aquatic environments (Mennerat et al., 2010; Peeler and Feist, 2011). For instance, temperature increase due to climate change and the introduction of new disease agents due to invasive species might increasingly expose fish to pathogens (Marcogliese, 2001; Peeler and Feist, 2011) and hence to costly immune challenges (Lochmiller and Deerenberg, 2000; Sheldon and Verhulst, 1996). Although the effects of parasites and their associated immune challenges have been extensively studied in fish (Magnadottir, 2010), their combined effects with contamination are still overlooked (but see Defo et al., 2019; Marcogliese et al., 2005; Renick et al., 2016; Zhao et al., 2020), thereby hindering our ability to reliably predict the impact of multiple stressors in the wild.

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

Fish behavior is also expected to be very sensitive to multiple stressors (Scott and Sloman, 2004; Wong and Candolin, 2015). Indeed, contamination can alter fish swimming activity and sociability (Martin et al., 2017; Tomkins et al., 2018; Zhou and Weis, 1998) due to neurotoxic, endocrine-disrupting effects and energy reallocations (Clotfelter et al., 2004; Jacquin et al., 2020; Zhao et al., 2020). Likewise, immune challenges generally trigger a "sickness behavior" (Adelman and Martin, 2009; Dantzer, 2004; Johnson, 2002), resulting in a reduction of investment in routine behaviors such as swimming activity, foraging, and sociability, which could save energy for costly immune responses (Kirsten et al., 2018; Volkoff and Peter, 2004). The combination of contamination and immune challenges is thus likely to result in complex interactive effects on behavior, depending on stress levels, individuals' energy state, and past exposure to stressors (Barber and Wright, 2005; Montiglio and Royauté, 2014).

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

In contrast, under acute or multiple stressors, the energetic demand for physiological defenses could exceed the organism's aerobic capacity, resulting in metabolic depression (Guppy and Withers, 1999; Petitjean et al., 2019b; Sokolova, 2013). This "conservation strategy" is characterized by an inhibition of physiological defenses, a reduction in metabolic activity, and limited depletion of energy reserves, with potentially detrimental consequences on cell damage and fish survival on a longer time scale (Petitjean et al., 2019b; Sokolova et al., 2012; Sokolova, 2013). However, few empirical studies experimentally tested these predictions, especially in a multistress framework (but see Gandar et al., 2017, 2016).

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

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

2. Material and methods

2.1. Model species and sampling sites

The gudgeon (*Gobio occitaniae*) is a widely distributed fish species in the Garonne watershed (South-West France). Within this area, gudgeons populations are exposed to various levels of contaminants (mostly trace metal elements and, to a lesser extent,

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

| Sampling sites | Lat. | Long. | Trace metal concentration ($\mu g.L^{-1}$) ± 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)
FARM	2°20′9.1255″ E	45°38′50.0269″ N	0.007 ± 0.002	2.05 ± 0.96	5.95 ± 2.83	−0.9	20	20	20	20
ARIMAS	1°22′26.1494″ E	43°5′2.4677″ N	0.05 ± 0.06	1.49 ± 1.31	4.54 ± 2.35	−0.9	15	20	20	20
CELCAB	1°39′27.8050″ E	44°30′27.5836″ N	0.05 ± 0.02	0.99 ± 0.92	5.73 ± 5.27	−0.8	10	15	10	15
AUSCOR	1°19′38.2922″ E	43°39′11.3598″ N	0.05 ± 0.03	6.47 ± 12.7	25.6 ± 27.5	−0.3	30	30	35	45
RIOU	2°12′41.3680″ E	44°33′55.4580″ N	13.2 ± 8.87	2.57 ± 1.84	629 ± 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 $\mu g. L^{-1}$, 10 $\mu g. L^{-1}$ and 600 $\mu g. L^{-1}$, respectively. Immune challenge corresponds to a mixture of two antigens (90 μg of LPS and 90 μg of PHA in 10 μL) injected at the caudal peduncle of fish.

pesticides) (Petitjean et al., 2020b ; Shinn et al., 2015) and parasitism (mostly monogenean ectoparasites such as gyrodactylids and to a lesser extent copepods and nematodes) (Loot et al., 2007), which can contribute to phenotypic variability (Knapen et al., 2004; Shinn et al., 2015). We used the database from Adour-Garonne Water Agency (SIE, 2016) to calculate a Toxic Unit based on metals concentrations measured between 2012 and 2016 in a set of 16 sampling sites previously characterized (Petitjean, 2019; Petitjean et al., 2020b, and supporting material 1 and 2). Among them, we selected four sampling sites (ARIMAS, CELCAB, AUSCOR and RIOU) that differed in terms of metal contamination, mostly due to different levels of Cd, Cu and Zn contamination (Table 1 and supporting material 2). We also added a reference population originating from a fish farm where metal concentrations are low (Les Viviez de Haute Correze, VHC, Le Bourg, 19340 Courteix, France, named FARM) (Table 1).

2.2. Experimental design

A total of 516 gudgeons were caught and brought to the laboratory in early spring 2018. Among them, 169, 88, 94, and 68 gudgeons were caught by electrofishing using an EFKO-FEG 1500 in AUSCOR, ARIMAS, RIOU, and CELCAB, respectively, and 97 fish were collected in a fish farm (FARM). Fish were then acclimated for 30 days and treated using Praziquantel (Vetofish, France, purity: 1000 mg g^{-1}; concentration: 3 mg.L^{-1}) to eliminate most commonly detected parasites (i.e., monogenean) and standardize their immune status. After acclimation (30 days), 106 fish were not involved in the experiment because they were too small (< 6 cm) or died during acclimation or tagging. Hence, 410 remaining fish between 6 and 14 cm of length were involved in the experiment (140, 75, 65, 50, and 80 fish from AUSCOR, ARIMAS, RIOU, CELCAB, and FARM, respectively). During the experimental procedure, fish were held by groups of five individuals in experimental tanks (50 × 30 × 30 cm) at a constant temperature (17−18 °C) under a day: night cycle photoperiod of 12:12 h. Fish were fed daily (about 1% of biomass per tank) (Flammarion et al., 1998; Kestemont et al., 1991) with commercial fish food (JBL Propond Sterlet S pellets). Fish were randomly exposed to four different treatments (Table 1): The control group without stressor (NC-PBS: n = 90) was not exposed to contamination (NC) and was injected with a neutral Phosphate Buffered Saline solution (PBS); the contamination alone group (C-PBS: n = 100, see below for details); the immune challenge alone group, was challenged with a mixture of antigens (see below for details) (NC-AMIX: n = 105); and the multiple stressors group exposed to both contamination and immune challenge (C-AMIX: n = 115). There was no difference in fish sex ratio (χ^2 = 2.53,

p = 0.47), mass (8.64 ± 4.77 g; Anova: F = 0.21, p = 0.89) nor size (9.4 ± 1.7 cm; Anova: F = 0.57, p = 0.63) among treatments.

For the contamination treatment, we used a mixture of the three trace metal elements that contributed the most to the Toxic Unit in a set of 16 sampling sites previously studied (Petitjean, 2019; Petitjean et al., 2020b, and supporting material 1 and 2). The metal mixture was composed of Cd, Cu and Zn chloride (CdCl$_2$, CuCl$_2$ and ZnCl$_2$, Thermo fisher scientific, purity: 99.99%) and concentrations were adjusted to approximately reach the highest concentrations recorded at contaminated sites (RIOU and AUSCOR): 14 $\mu g. L^{-1}$, 10 $\mu g. L^{-1}$ and 600 $\mu g. L^{-1}$, respectively (Table 1). Half of the water was renewed every two days to ensure constant contamination levels under static exposure. The experimental contamination's reliability was checked by measuring exposure concentrations in random water samples for both non-contaminated (n = 29) and contaminated (n = 47) tanks. In non-contaminated tanks, Cd, Cu and Zn average concentrations reached 0.007 ± 0.005 $\mu g.L^{-1}$, 0.62 ± 0.15 $\mu g.L^{-1}$ and 4.06 ± 0.79 $\mu g.L^{-1}$ respectively. In contaminated tanks, contamination levels reached 13.5 ± 1.31 $\mu g.L^{-1}$, 9.96 ± 1.50 $\mu g.L^{-1}$ and 551 ± 45.2 $\mu g.L^{-1}$ respectively.

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

2.3. Cellular-level

2.3.1. Metal bioaccumulation in muscle

For metal assay, muscle was freeze-dried, homogenized using Fastprep®-24 homogenizer and analyzed by inductively coupled plasma mass spectrometry (ICP-MS Agilent 7500ce) after hot acidic mineralization in HNO$_3$ (67−69%, Trace Metal Grade, Fisher Chemical). We assessed the reliability of the analyses using TORT-3

certified reference material. Metal recovery reached an average of 105 ± 6.1%, 82 ± 15.2%, 107 ± 5.7% for Cd, Cu, and Zn, respectively.

2.3.2. Neutrophils/lymphocytes immune ratio

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

2.3.3. Local immune response

An important skin swelling caused by antigen injection reflects high investments in immune defense (i.e., inflammation and T-cells proliferation) and is recognized as a reliable measure of local immunocompetence (i.e., local immune response) in a variety of vertebrates, including fish (Ardia and Clotfelter, 2006; O'Connor et al., 2014; Tella et al., 2008). Skin swelling was assessed by measuring the caudal peduncle's thickness in each fish following previous studies in gudgeon (Petitjean et al., 2019a; Petitjean et al., 2020). The intra-individual repeatability of thickness measurement reached 99.6 ± 0.8% and 99.8 ± 1.3% before and after injection, respectively.

2.3.4. Oxidative stress index

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

2.3.5. Available energy in muscle

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

2.4. Individual-level

2.4.1. Condition index

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

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

2.4.2. Behavioral traits

Fish behavior was analyzed using BORIS software (Friard and Gamba, 2016) four days after the immune challenge (AMIX injection), according to a previous study investigating behavioral effects

of immune challenge in gudgeons (Petitjean et al., 2020). Three behavioral traits were selected because they are commonly affected by stressors: swimming activity, foraging and sociability (Barber et al., 2000; Jacquin et al., 2017; Kirsten et al., 2018; Koltes, 1985; Little and Finger, 1990) (see supporting material 6 for detailed method).

Because behavioral traits were partly correlated, we used a PCA to extract three synthetic indices. The first behavioral index was named "General activity" (PCA axis 1, 41% of variance explained, supporting material 7). Fish with a higher general activity index on the first PCA axis swam for a longer time and across more areas. The second behavioral index was named "Foraging" (PCA axis 2 multiplied by −1, 20.6% of variance explained, supporting material 7). Hence, fish with a higher foraging index displayed a higher motivation to feed (i.e., low latency to forage time) and a higher number of food items ingested. The third synthetic index was named "Sociability" (PCA axis 3 multiplied by −1, 16.7% of variance explained, supporting material 8). Hence, fish with a higher sociability index displayed a higher number of contacts with their counterparts and a lower ANND, reflecting higher sociability.

2.5. Statistics

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

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

Development Core Team, 2018).

3. Results

3.1. Cellular-level

3.1.1. Metal bioaccumulation in muscle

At the cellular level, metal bioaccumulation was affected by the contamination alone in interaction with the population (Fig. 1 and supporting material 9). Cu and Zn were not significantly accumulated (ANOVA, estimates (scaled log) = 0.002 ± 0.02, F = 0.006, p = 0.94 and ANOVA, estimates (scaled log) = 0.001 ± 0.05, F = 0.0004, p = 0.99, respectively). However, Cd bioaccumulation was significant in fish exposed to the experimental contamination, but this effect depended on the population considered (ANOVA, F = 2.35, p = 0.059) (Fig. 1 and supporting material 9). More specifically, contrast posthoc analyses show that fish originating from the farm (FARM) and the least contaminated site (ARIMAS) displayed a significant increase of Cd concentration in their muscle when exposed to the experimental contamination compared to the control group (FARM-NC-PBS x (scaled log): 0.50 ± 0.06 vs FARM-C-PBS x (scaled log): 0.66 ± 0.12, estimates (scaled log) = 0.48 ± 0.15, p = 0.01 and ARIMAS-NC-PBS x (scaled log): 0.42 ± 0.05 vs ARIMAS-C-PBS x (scaled log): 0.87 ± 0.14, estimates (scaled log) = 0.35 ± 0.14, p = 0.046, respectively). In contrast, populations

from the most contaminated sites (CELCAB; AUSCOR, RIOU) displayed no significant accumulation of metals in their muscle when exposed to the experimental contamination (Fig. 2 A).

3.1.2. Neutrophils/lymphocytes immune ratio

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

3.1.3. Local immune response

The local immune response was significantly affected by the immune challenge alone (Fig. 1 and supporting material 9). Indeed, fish injected with AMIX solution had a higher local immune response (i.e., skin swelling) than fish injected with control saline solution (PBS) whatever the population considered (ANOVA, estimate = 44.4 ± 2.2, F = 389, p < 0.001).

3.1.4. Oxidative stress index

The oxidative stress index was significantly affected by single stressors (contamination and immune challenge alone) but not by multiple stressors (Fig. 1 and supporting material 9). The oxidative
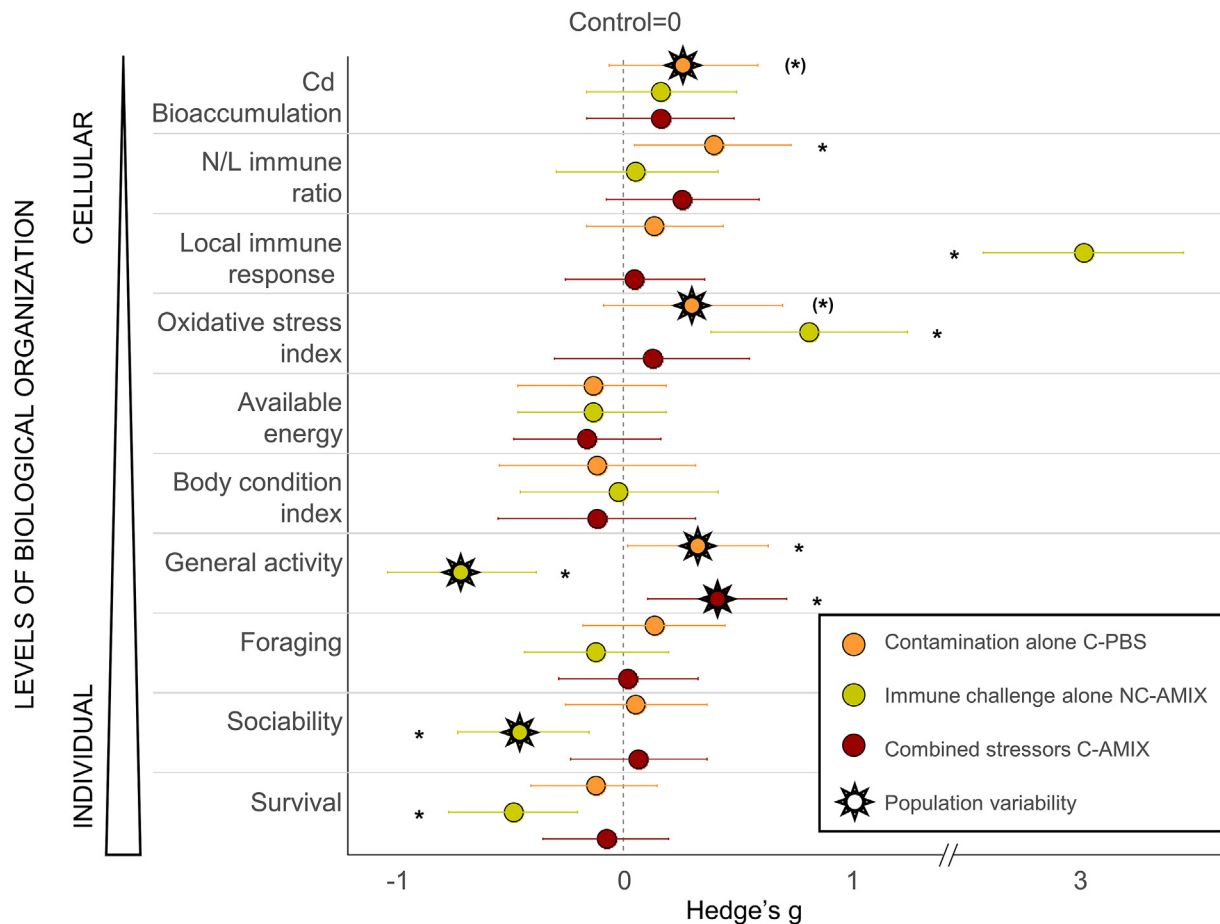

Fig. 1. Effect size (hedge's g) and 95% confidence intervals (CI) of experimental treatments (contamination alone in orange: C-PBS; immune challenge alone in green: NC-AMIX; multiple stressors in red: C-AMIX) relative to the control group (NC-PBS: dashed vertical line), across biological levels from cells to the whole organism. When the 95% CI did not overlap 0 (control group), the treatment's effect was significant, as indicated by an asterisk. The asterisk between brackets indicates a marginally significant effect (lower bound:-0.1 < 95% CI < 0.1). Stars indicate significant variability among populations (population-by-treatment interaction). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)
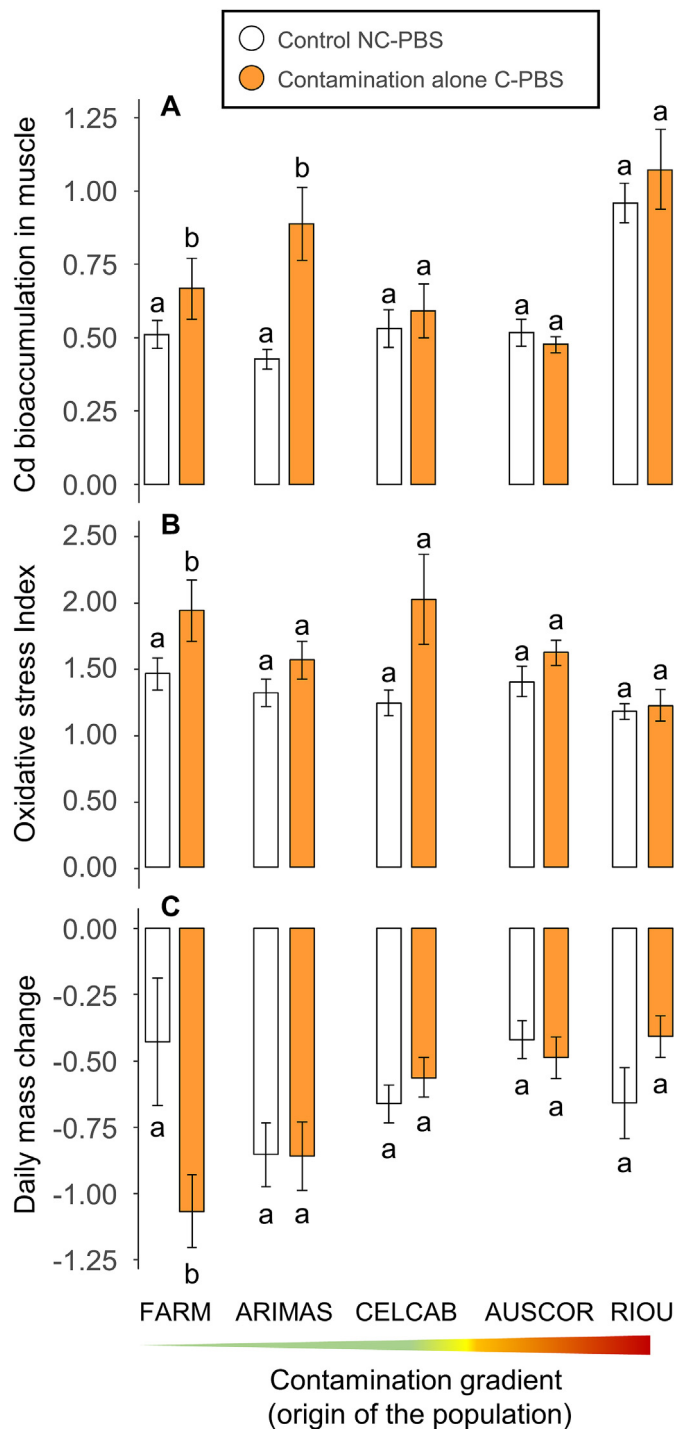
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Fig. 2. Effects of contamination alone (white bars, NC-PBS; orange bars, C-PBS) in different fish populations along a gradient of contamination. The contamination gradient in each population was determined using the toxic unit (TU), which ranged from −0.9 for the least contaminated FARM population to 1.3 for the most contaminated RIOU population (Table 1). A. Cd bioaccumulation in fish muscle (log-transformed and scaled μg.g⁻¹), B. oxidative stress index (sqrt transformed index), and C. daily mass change of fish (percent changes per day). Error bars correspond to the SEM. Different letters above **or below** bars indicate significant differences between control and contamination treatments within each population after contrast posthoc analyses. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

stress index increased marginally and in a population dependent manner in fish exposed to contamination (ANOVA, F = 2.38,

p = 0.056) and significantly in immune-challenged fish (ANOVA, estimate (sqrt) = 0.44 ± 0.97, F = 15, p = 0.001).

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

3.1.5. Available energy in muscle

The amount of total available energy (sum of lipids, proteins and carbohydrates) in fish muscle was not significantly affected by any stressor (Fig. 1 and supporting material 9). However, fish exposed to contamination alone had a higher amount of carbohydrates in their muscle (NC-PBS x (log): 3.5 ± 0.09 vs C-PBS x (log): 3.74 ± 0.08, ANOVA, estimates (log) = 0.27 ± 0.08, F = 12, p = 0.002) whatever the population considered.

3.2. Individual-level

3.2.1. Condition index

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

3.2.2. General activity

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

The effects of multiple stressors (C-AMIX group) were very variable depending on the population considered. There was no interactive effect of multiple stressors in FARM fish due to a dominant effect of the immune challenge (Hedge's g = 0.53, CI [-0.28; 1.34]) (Fig. 3). There was antagonistic interactions of multiple stressors in the CELCAB and AUSCOR populations (CELCAB: Hedge's g = −5.23, CI [-7.18; −3.29]; AUSCOR: Hedge's g = −1.95, CI [-2.54; −1.37]) and a "mitigating synergism" in ARIMAS (ARIMAS: Hedge's g = 3.4, CI [2.35; 4.50]), both resulting in a non-significant difference in general activity between fish exposed to multiple stressors compared to control (i.e., inhibiting effect), which is considered as antagonistic effect (Fig. 3). Finally, there was an
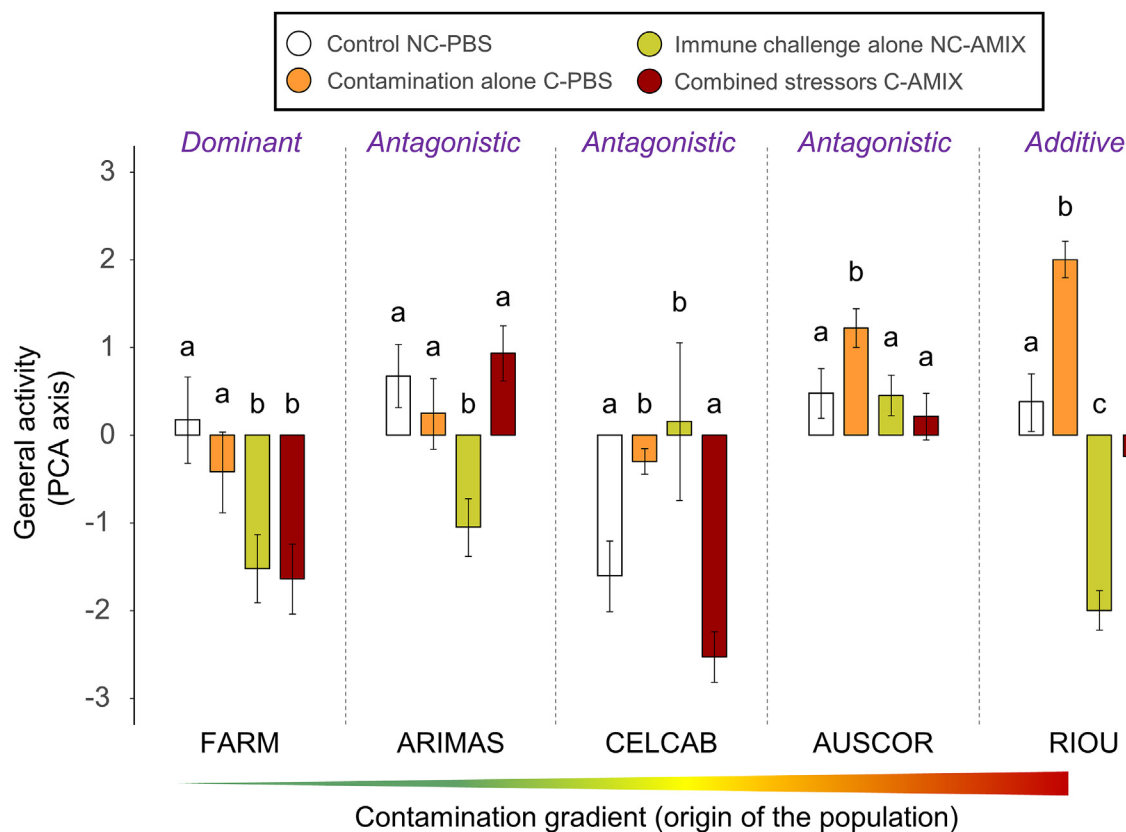
Fig. 3. Mean (±SEM) general activity (first axis of PCA reflecting the time swimming, the number of lines crossed and the number of visited areas) of fish exposed to control condition (white bars, NC-PBS), contamination alone (orange bars, C-PBS), immune challenge alone (green bars, NC-AMIX), or combined contamination and immune challenge (red bars, C-AMIX) among fish populations. The fish populations were ranked according to the level of contamination recorded in their sampling site (contamination gradient determined using the toxic unit, ranged from −0.9 (FARM) to 1.3 (RIOU), Table 1). Different letters indicate significant differences between treatments within each population according to contrast analyses. The type of interaction (additive, antagonistic, synergistic) was tested following Lange et al. (2018) (see methods). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

additive effect of multiple stressors in the RIOU population (Hedge's g = 0.23 CI [-0.56; 1.02]). More specifically, both contamination and immune challenge had opposite effects on general activity in RIOU fish, resulting in similar swimming activity levels between the control (NC-PBS) and multiple stress (C-AMIX) group in RIOU fish (Fig. 3).

3.2.3. Foraging behavior

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

3.2.4. Sociability behavior

Fish sociability index was affected by the immune challenge alone, but this effect varied among populations (ANOVA, F = 8.3, p < 0.001) (Fig. 1 and supporting material 9). More particularly, posthoc analyses show that sociability decreased in AMIX-injected fish from CELCAB (PCA axis 3, CELCAB-NC-PBS x : 0.25 ± 0.15 vs CELCAB-NC-AMIX : −1.11 ± 0.54, estimates = −1.58 ± 0.34, p < 0.001) while it increased for fish from RIOU (PCA axis 3, RIOU-

NC-PBSx: −0.52 ± 0.36 vs RIOU-NC-AMIXx: 0.32 ± 0.13, estimates = 0.87 ± 0.31, p < 0.01). Sociability was not significantly affected by any stressor in other populations.

3.2.5. Survival

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

4. Discussion

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

4.1. Single stressor effects

The theoretical framework of metabolic strategies predicts that organisms react to a moderate or single stressor by setting up a "compensation strategy" characterized by increased investment in physiological defenses (immunity and antioxidant defense) at the expense of depletion of energy reserves and/or decrease in routine behaviors (Petitjean et al., 2019b; Sokolova, 2013; Sokolova et al., 2012). Our results do not show direct energetic costs of stressors but show significant physiological and behavioral changes, although some responses varied according to the population.

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

Accordingly, the fish exposed to both single stressors displayed a significant increase in oxidative stress index, mainly due to increased oxidative damage, which was consistent with previous studies (Costantini and Møller, 2009; Fatima et al., 2000; Le Guernic et al., 2016). We also found that antioxidant defenses decreased in immune-challenged fish, suggesting that the immune challenge can trigger oxidative stress, with potential detrimental effects on fitness. Further studies are now needed to measure specifically other enzymatic antioxidant components (e.g., Catalase, Super-Oxide Dismutase), but our results suggest that exposure to single stressors incur a significant oxidative cost with potential consequences for fish fitness.

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

Under a "compensation strategy", we also expected a decrease in routine behaviors to save energy for physiological defenses. Accordingly, the immune challenge decreased fish swimming activity and sociability, although in a population dependant manner,

which could enable fish to save energy for immunity (i.e., "sickness behavior"; Adelman and Martin, 2009; Dantzer, 2004; Johnson, 2002). In contrast, contamination had the opposite effect and increased fish swimming activity, at least in some populations (i.e., CELCAB, AUSCOR, RIOU). This increase in swimming activity under experimental contamination could be due to neurotoxic effects (Jacquin et al., 2020) but could also be linked to the increase in carbohydrates content in fish muscle and probably to cortisol secretions, as suggested in previous studies (Brun et al., 2019). Increased swimming activity could potentially have an adaptive value by increasing food acquisition in the wild, potentially compensating for the costs of detoxification, but this hypothesis remains to be tested.

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

4.2. Multiple stressors effects

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

More particularly, we did not find any significant interactive effect of multiple stressors on immune and antioxidant defenses. For instance, local (i.e., skin swelling) and circulating (i.e., N/L immune ratio in fish blood) immune responses were not affected by multiple stressors, likely because both stressors affected different immune traits. Further studies focusing on other components, such as the phagocytic capacity and the oxidative burst activity, would be necessary to decipher the underpinning immune effects of multiple stressors (Jolly et al., 2014; Le Guernic et al., 2016). In addition, multiple stressors had no interactive effects on oxidative stress, although changes in other antioxidants molecules that were not measured in our study are still possible (Lushchak, 2011). Whatever the underlying mechanisms, we found no interactive effects of multiple stressors on fish physiological traits, suggesting that the metabolic capacity of fish is not overwhelmed by multiple stressors and/or that the selected traits were not integrative enough to uncover interactive effects.

We also expected antagonistic (i.e., inhibiting) effects on energy reserves and/or behavior. We found no effect of multiple stressors on energy reserves nor condition indices, but the exposure to combined contamination and immune challenge inhibited the behavioral plasticity of fish. Indeed, we found no difference in

swimming activity between groups exposed to multiple stressors and control groups due to inhibiting effects (i.e., "mitigating synergism" for ARIMAS, antagonism for CELCAB and AUSCOR) or opposite additive effects of combined stressors (i.e., RIOU). In other words, in most populations, the immune challenge inhibited the effects of contamination on fish behavior resulting in lower behavioral responses to multiple stressors compared to single stressors. This result highlights the need to consider parasitism and other stressors in behavioral-based ecotoxicological tests to avoid misinterpretation. In addition, it suggests that labile traits such as behaviors may be particularly sensitive to multiple stressors, with potential implications for fish fitness by altering reproduction or ability to escape predators in the wild (Scott, 2003; Wingfield, 2003; Wong and Candolin, 2015).

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

4.3. Population variability of responses to stressors

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

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

Third, we found a strong variability in fish behavioral responses to stressors, especially on fish swimming activity. In our study, the experimental contamination alone had no effects on the two least contaminated populations (FARM and ARIMAS) but increased other populations' swimming activity (CELCAB, AUSCOR, RIOU). This suggests that contamination could induce plastic hyperactivity in fish from the most contaminated sites (Ellgaard et al., 1978; Steele, 1983), maybe because of neurotoxic or endocrine-disrupting effects

(Drummond and Russom, 1990; Renick et al., 2016; Weis et al., 2001) and/or energy reallocations under stressful conditions, which could increase food acquisition (Pyke et al., 1977). Nevertheless, anthropogenic activities and other factors not accounted here could also explain these behavioral differences (e.g., domestication in the FARM population: Johnsson et al., 2014, predator presence/absence in some populations: Bell et al., 2010). Further studies on a higher number of replicate populations are hence necessary to specifically disentangle the environmental factors explaining these interpopulation differences. Whatever the determinants of these interpopulation differences, our results underscore the need to consider the variability of behavioral responses among wild populations within the emerging field of behavioral ecotoxicology (Jacquin et al., 2020).

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

Credit author statement

LJ, SJ, PL and QP conceptualized and designed the study. LJ, LR, PL and QP performed fieldwork. LJ, LR, MP, AP, MC, SJ, PL and QP performed the experiment and traits measurements. QP performed statistical analyses and wrote the original draft. All authors participated in the discussion of the results. All authors participated in the manuscript improvement and approved this version of the manuscript.

Declaration of competing interest

Acknowledgements

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2020.116042.

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