RESEARCH ARTICLE

Adaptive plastic responses to metal contamination in a multistress context: a field experiment in fish

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Abstract

Wild populations often differ in their tolerance to environmental stressors, but intraspecific variability is rarely taken into account in ecotoxicology. In addition, plastic responses to multiple stressors have rarely been investigated in realistic field conditions. In this study, we compared the responses to metal contamination of gudgeon populations (*Gobio occitaniae*) differing in their past chronic exposure to metal contamination, using a reciprocal transplant experiment and an immune challenge mimicking a parasite attack to test for potential effects of multiple stressors across biological levels. We measured fish survival and traits involved in metal bioaccumulation, oxidative stress, immunity, cell apoptosis, and energy management to decipher underpinning physiological mechanisms across biological levels (i.e., gene expression, cell, organism). Fish from the two replicate High Contamination sites had higher survival when transferred into contaminated sites, suggesting a local adaptation to the contaminated site, possibly explained by higher levels of detoxification and antioxidant capacity but with potential higher apoptosis costs compared to their naïve counterparts. We found no evidence of co- or maladaptation to the immune stressor, suggesting no specific costs to face pathogens. In the emerging field of evolutionary ecotoxicology, this study underlines the need to consider intraspecific variability to better understand the effects of pollution in heterogeneous populations.

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Highlights
● The effects of multiple stressors on wild fish populations are difficult to predict
● Intraspecific variability is neglected during multiple stressors effects assessment
● Fish from High Contamination sites have higher survival than their naïve counterparts
● The difference in survival could be driven by detoxification and antioxidant capacity
● Better tolerance to contamination did not entail clear costs in the short term

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Introduction

In the wild, aquatic vertebrates are exposed to numerous stress factors which can interact and result in unexpected effects (Christensen et al. 2006; Petitjean et al. 2019b; Reid et al. 2019). Accordingly, a growing number of studies investigating the combined effect of multiple stressors have emerged over the past decades (Birk et al. 2020; Jackson et al. 2016). Under increasing anthropogenic activities and related global changes, both contaminations of the aquatic environment by numerous pollutants (Altenburger et al. 2018; Celander 2011) and novel infections by introduced

and emerging pathogens increase (Adlard et al. 2015), especially in fish (Johnson and Paull 2011; Reid et al. 2019). Furthermore, in the wild, pollutants and pathogen infections often co-occur with potentially deleterious effects on aquatic wildlife, yet, their combined effects on fish health have only been investigated recently (Marcogliese and Pietrock 2011).

Contaminants exposure and infection by pathogens may affect the same physiological pathways and have complex interactive consequences on health. Indeed, both stressors, as single sources, can alter oxidative balance (Dautremepuits et al. 2003; Lushchak 2011; Sanchez et al. 2005), immunity, inflammatory response (Davis et al. 2008; Jiaxin et al. 2020; von Gersdorff Jørgensen 2016), and energy management (Pi et al. 2016; Schaufler et al. 2008), which can sometimes result in apoptosis and tissue damage (Gao et al. 2013; Hoole et al. 2003) as well as reduced growth and survival (Johnson and Dick 2001; Wood et al. 2012, 2011). In addition, because these traits are closely interrelated, combined exposure to contaminants and pathogens infections have a substantial likelihood of triggering cascading effects affecting fish's ability to face stressors and, ultimately, survive in the wild. For instance, immune response and oxidative stress are mechanistically linked, the latter having a role in cellular communication and killing pathogens during inflammation, but can also be involved in substantial side effects (e.g., tissue damage) when immune defenses are misdirected or overexpressed (Costantini 2022; Sorci and Faivre 2009). Also, due to the energetic cost incurred by the detoxification of contaminants (Calow 1991) and immune responses (Lochmiller and Deerenberg 2000; Sheldon and Verhulst 1996; Sorci and Faivre 2009), stressors exposure may lead to energy trade-off among functions, with potentially detrimental consequences on fish health. Therefore, exposure to pollutants is thought to increase host susceptibility to pathogens (Lafferty and Holt 2003). For instance, organic contaminants can cause immunodeficiency and increase infection in Grass carp (*Ctenopharyngodon idellus*) (Zhao et al. 2020), and result in decreased survival and fitness, as in Roundhead galaxias (*Galaxias anomalus*) (Kelly et al. 2010). In contrast, while exposure to various metals is recognized to alter fish defense systems, especially immune response (Bols et al. 2001; Segner et al. 2012; Zelikoff 1993), few studies have investigated the combined effect of persistent polymetallic contamination and parasite in fish (but see Le Guernic et al. 2016; Marcogliese et al. 2005; Petitjean et al. 2020c).

In addition, the responses to stressors can strongly vary among individuals and populations, resulting in contrasted sensitivity to contamination depending on their exposure history (Reid et al. 2016; Whitehead et al. 2017). Indeed, natural selection imposed by stressors can lead to rapid evolutionary genetic, epigenetic, and plastic responses, leading to higher resistance and/or tolerance to stressors (Oziolor et al. 2016, 2017; Reid et al. 2016). For instance, killifish

(*Fundulus sp.*) populations having evolved in highly polluted sites have a higher tolerance to contamination by organing pollutants such as polychlorinated biphenyls (PCB) and polycyclic aromatic hydrocarbons (PAH) compared to populations from reference sites due to genetic differences (Oziolor et al. 2014; Reid et al. 2016; Whitehead et al. 2017). In addition, plasticity is often crucial to ensure survival in changing environments (Fox et al. 2019; Westneat et al. 2019). However, it is still challenging to predict the likelihood and consequences of adaptative responses to metal pollution and decipher its underlying costs and physiological mechanisms, especially in a multistress context (Loria et al. 2019; Petitjean et al. 2019b). Indeed, plasticity can occur at different biological levels, from molecules to the whole organism (multidimensional plasticity, Westneat et al. 2019). This underlines the need to further develop the promising area of evolutionary ecotoxicology under more realistic field conditions and decipher the underpinning molecular and physiological mechanisms in more complex environmental scenarios (Brady et al. 2017a, 2017b; Oziolor et al. 2016; Petitjean et al. 2019b).

Indeed, adaptation to a given stressor could either positively affect the capacity of organisms to respond to a second stressor (i.e., co-tolerance or cross-tolerance; Vinebrooke et al. 2004) or reduce it (i.e., maladaptation; Brady et al. 2019). For instance, Atlantic salmon (*Salmo salar)* adapted to high temperatures are also more tolerant to hypoxia due to increased ventricular size and myoglobin level (Anttila et al. 2013). Also, daphnia (*Daphnia magna*) adapted to high temperatures have a higher ability to cope with Zn contamination (Zhang et al. 2018), suggesting adaptive co-tolerance or cross-tolerance to multiple stressors (Vinebrooke et al. 2004). However, other studies suggest that plasticity is not always beneficial and that adaptation to a stressor could incur specific costs and be detrimental depending on environmental conditions (e.g., other stressors) (Agra et al. 2010; Hua et al. 2017; Jansen et al. 2011; Lavergne et al. 2015). For instance, populations of wood frogs (*Lithobates sylvaticus*) adapted to pesticide exposure had higher susceptibility to parasite infection and higher mortality risk (Hua et al. 2017), suggesting that evolved pesticide tolerance might be maladaptive to face pathogens (i.e., decreased fitness, Brady et al. 2019). However, studies investigating the costs and benefits of pollution adaptation in a multistress context are still scarce, limiting our ability to predict the effects of evolutionary responses of wildlife to anthropogenic changes (Hendry et al. 2017).

To tackle such questions, *in-situ* reciprocal transplant experiments in the field are powerful tools. By sampling populations from different environments and exposing them to differential stress levels in field enclosures or cages, this type of experiment enables testing for local adaptation/maladaptation to stressors and the associated plastic changes in

physiology under semi-natural conditions (Kawecki and Ebert 2004; Whitlock 2015) which is crucial to understand the consequences of realistic multistress exposure on wild populations (Bougas et al. 2016).

In this study, we compared the short-term plastic responses to metal contamination (mostly Cd, Cu, and Zn) in 4 populations (i.e., 2 from High Contamination and 2 from Low Contamination sites) of wild gudgeon (*Gobio occitaniae*) using a field reciprocal transplant experimental design. These populations were chosen based on previous studies showing contrasted water, sediment, and tissue contamination levels in the Adour-Garonne watershed, as well as high levels of phenotypic and physiological divergence among gudgeon populations from South-West of France (Côte et al. 2021; Petitjean 2019; Petitjean et al. 2020b, c). In a previous study, we showed high levels of intraspecific variability of responses to metals among populations in controlled laboratory conditions (Petitjean et al. 2020b). In the present study, we aimed to test plastic responses to changes in contamination levels under realistic field conditions and across biological levels: from molecules to the whole organism. We used two sets of replicate populations from two hydrographic sub-basins to test for parallelism in the plastic response of populations exposed to similar contamination conditions in different streams. More specifically, we measured survival as a proxy of fitness, and traits involved in metal bioaccumulation, oxidative stress, apoptosis, immune responses, and energy management (i.e., gene expression, cellular, and whole-organism responses). In addition, we tested the effect of a second stressor, an immune challenge mimicking parasite attack, on fish responses to contamination in a multistress context. We expect that fish populations originating from high-contamination sites outperform those from low-contamination sites when transplanted into contaminated environments (i.e., local adaptation). However, we also expect them to be less performant in low-contamination sites due to potential physiological or fitness costs incurred by contaminant tolerance. We also expect combined pollution and immune stressors to affect fish responses interactively because they affect the same physiological pathways. As evidence of co- or maladaptation to multiple stressors is very limited in the current literature, we have uncertain expectations about the outcome of the stressor's interactions on survival and fitness. However, because we expect that contaminant adaptation is costly, populations from contaminated sites may be less able to face concomitant exposure to an immune stressor.

## Material and methods

Model species

The gudgeon (*Gobio occitaniae*) is a relatively sedentary benthic freshwater fish species from the South-West of France (Keith et al. 2011). Indeed, gudgeon (*Gobio sp.*) are mostly bound to a home range of about 100 m (Stott 1967; Stott et al. 1963), ensuring they are exposed to the same local conditions (e.g., pollution) throughout their life. This is particularly interesting since, in the wild, gudgeon populations can live under different levels of metal contamination, as shown by a previous field study (Petitjean et al. 2020c). Furthermore, gudgeon are also prone to parasite infection, suggesting that they are exposed to immune challenges in the wild (Loot et al. 2007). In addition, in our study area, gudgeon populations display high phenotypic divergence levels that are not only linked to genetic drift (Côte et al. 2021). Indeed, the results of a previous study underlined that phenotypic divergence (i.e., Qst) was significantly higher than neutral genetic divergence (i.e., Fst), especially for morphological traits (i.e., body elongation and lateral shape and fins and jaw size) (Côte et al. 2021). Also, a previous experimental study showed different populations' sensitivity to metals in controlled laboratory conditions (Petitjean et al. 2020b). Therefore, the present study investigated whether this variability could translate into different responses to transplant into contaminated streams in a realistic multistress context.

Contamination levels in study sites

Based on previous studies (Petitjean 2019; Petitjean et al. 2020b, c), we selected 4 wild gudgeon populations (ARIMAS, CELFIG, AUSCOR, and RIOU) originating from study sites located in the Garonne hydrographic regions and belonging to 2 hydrographic sub-basins (i.e., La Garonne, 28 927km^2: ARIMAS and AUSCOR and Le Lot, 11 518km^2: AUSCOR and RIOU, see Fig. 1A). Within each hydrographic sub-basins, selected pair of study sites are located on different streams which are relatively distant from each other (Fig. 1A). For instance, ARIMAS, located on the Arize stream and AUSCOR located on the Aussonnelle stream, both reach the Garonne river but are distant from 120 km in length. Also, CELFIG, located on the Célé, and RIOU, located on the Riou Mort stream, both reach the Lot river but are distant from 133 km in length. Thus, given the small home range of gudgeon (about 100 m, Stott 1967; Stott et al. 1963) and distance between the study sites, we expect none or very low gene flow among the selected populations.

The four study sites have been selected because they display the most different levels of trace metal elements, especially Cd, Cu, and Zn, among several study sites regularly monitored by the Adour-Garonne water agency (AEAG 2022) and previously studied in our group (see Petitjean et al. 2020b, c and Table 1 for detailed measures of toxicity: Toxic Unit – TU values). Therefore, ARIMAS and CELFIG sites were chosen as Low Contamination sites (LC), while AUSCOR and RIOU sites were selected as High Contamination sites (HC). Hence, HC sites have been chosen
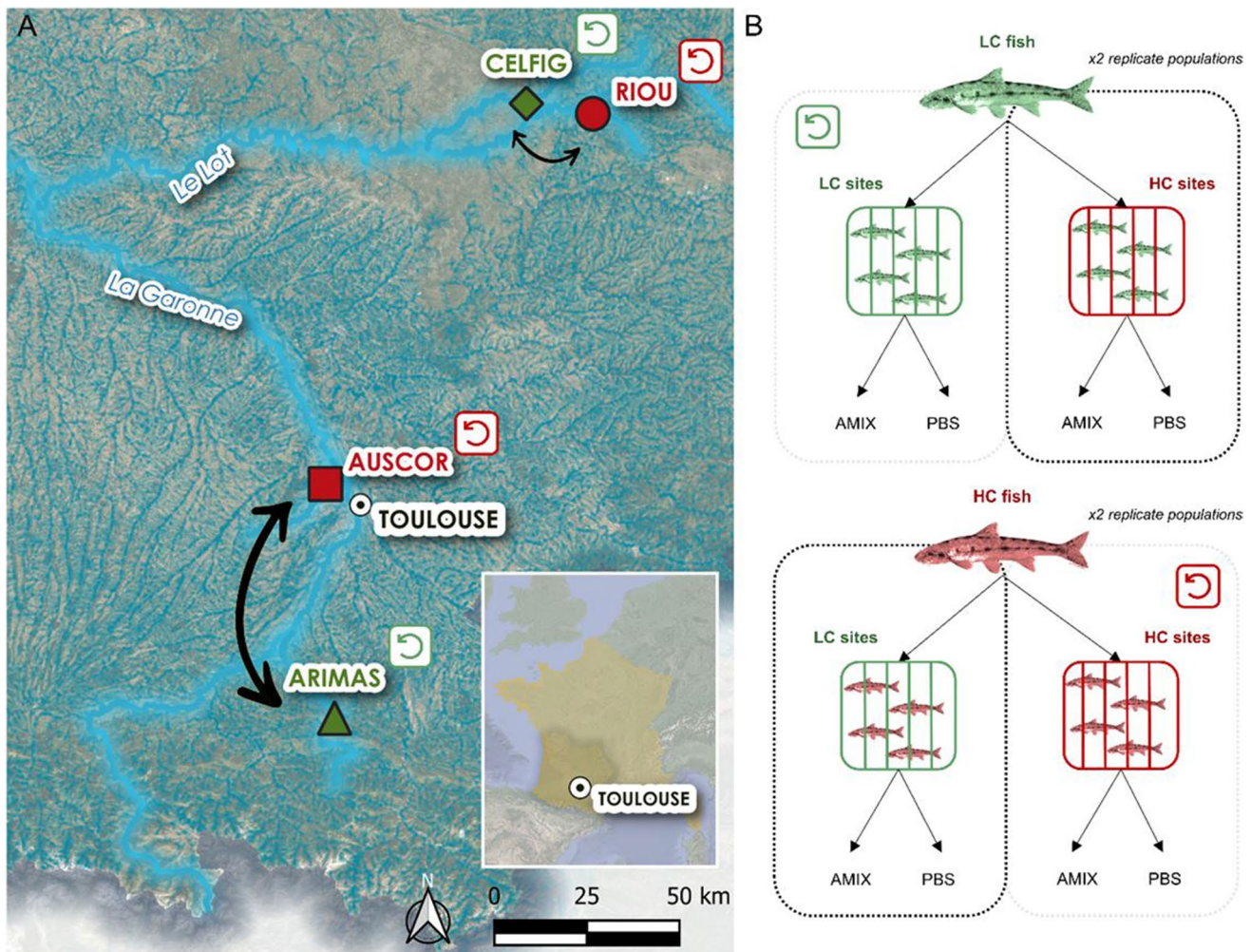
Fig. 1 A Study sites location categorized according to metal contamination levels (Low Contamination sites – LC in green and High Contamination sites in red), and **B** reciprocal transplant design. For each study site, half of the sampled fish were caged in their site of origin (green and red circular arrows for LC fish and HC fish, respectively), and the other half was transplanted in the associated transplant site with contrasted contamination levels (black arrows) to obtain 4 experimental groups (LC-LC: $n=66$, LC-HC: $n=72$, HC-HC: $n=63$ and HC-LC: $n=67$) for each set of replicate populations (ARIMAS and AUSCOR, CELFIG and RIOU). In each group, 3 replicate cages containing 10 to 12 fish were set up for 14 days. 7-days after the transplant, half of the individuals within each cage were injected with an antigen mixture (AMIX) to trigger an immune challenge mimicking a parasite attack seven days after the transplant. The other half was injected with a control-saline solution (PBS). Detailed sample sizes are provided in Table 2

according to their contamination levels and history. RIOU corresponds to a station located on the Riou Mort stream (station #05093550, AEAG 2022), a Lot river tributary, which is historically contaminated by trace metal elements mainly released by former metallurgical and mining activities that started in 1871 (Blanc et al. 1999; Say 1978; Schäfer et al. 2006). AUSCOR corresponds to a station located on the Aussonnelle stream (station #05159000, AEAG 2022), a Garonne river tributary for which the contamination has been recorded since 1976. AUSCOR presents a polymetallic contamination profile originating from multiple sources over the last decade (e.g., surrounding industries, road runoff, and domestic and wastewater release) that is related to

growing urbanization occurring over the last decades in the Toulouse area (AEAG 2022). On the contrary, the two LC sites have been chosen for their overall good ecological, physico-chemical, and low level of contamination over the last decades. Indeed, ARIMAS and CELFIG correspond to stations located on the Arize (station #05176150, AEAG 2022) and Célé (station #05091000, AEAG 2022) streams, a Garonne and Lot rivers tributaries, respectively, which are surrounded by more natural habitats and fewer pollution sources, thereby presenting a historically low level of metallic contamination.

To ensure that contamination levels remained consistent with expectations and over the experiment, we performed

Table 1 Mean concentrations, standard deviation (SD), and minimum and maximum values (between square brackets) of the most representative trace metals (Cd, Cu, and Zn) measured in water ($\mu g.L^{-1}$) and sediment ($mg.kg^{-1}$dry weight) of each study site during the experiment. Water samples were collected at the start ($n=3$) and the end of the experiment ($n=3$) and averaged. Sediments were collected at the start of the experiment ($n=3$). Global TU_{water} was computed using mean metal concentrations (Al, As, Cd, Co, Cr, Cu, Ni, Pb, Zn) measured in the water before and after the experiment, as the sum of the mean concentrations of each metal divided by its toxicity on fish (LC50 in *Pimephales promelas*). All detailed concentrations and contributions to the TU value are available in Supporting Material 1

Mean concentration $\pm$ SD [Min; Max] ($\mu g.L^{-1}$ for water and $mg.kg^{-1}$dw for sediment)	Study sites	ARIMAS	CELFIG	AUSCOR	RIOU
	Lat.	1°22′26.1494″ E	1°59′14.5741″ E	1°19′38.2922″ E	2°12′41.3680″ E
	Long.	43°5′2.4677″ N	44°35′17.6546″ N	43°39′11.3598″ N	44°33′55.4580″ N
Cd	Water ($n=6$)	0.013 ± 0.008 [0.004; 0.024]	0.036 ± 0.025 [0.006; 0.074]	0.761 ± 1.783 [0.020; 4.40]	8.31 ± 2.07 [5.41; 10.85]
	Sediment ($n=3$)	0.294 ± 0.08 [0.217; 0.377]	0.18 ± 0.084 [0.09; 0.24]	0.10 ± 0.013 [0.09; 0.11]	4.96 ± 2.782 [2.3; 7.85]
Cu	Water ($n=6$)	1.47 ± 1.01 [0.50; 3.15]	0.98 ± 0.39 [0.46; 1.55]	3.00 ± 1.15 [2.21; 5.21]	5.26 ± 2.17 [2.88; 9.29]
	Sediment ($n=3$)	24.09 ± 5.96 [19.92; 30.91]	3.86 ± 2.10 [1.68; 5.87]	4.43 ± 0.50 [3.90; 4.90]	57.84 ± 47.06 [20.91; 110.83]
Zn	Water ($n=6$)	25.32 ± 14.82 [11.08; 48.87]	15.32 ± 9.73 [2.83; 31.81]	47.07 ± 46.85 [11.08; 122.67]	150.96 ± 82.43 [64.37; 272.30]
	Sediment ($n=3$)	84.88 ± 20.66 [66.21; 107.08]	44.97 ± 32.17 [11.13; 75.15]	21.41 ± 1.70 [19.58; 22.94]	459.92 ± 154.03 [282.76; 562.25]
Global TU_{water}		−0.4	−0.6	0.2	1.0
Contamination level		Low Contamination (LC)	Low Contamination (LC)	High Contamination (HC)	High Contamination (HC)

complementary *in-situ* measurements of trace metal contamination in water, suspended materials, and sediments (Table 1 and Supporting Material 1). Analyses show that all TUs were relatively consistent across studies (Chi-square test: $\chi^2=0.56$ $p=0.9$) and throughout the experiment (Supporting Material 1) (Chi-square test: $\chi^2=0.14$ $p=0.99$), ensuring that fish were indeed exposed to contrasted and relatively constant metal contamination levels over the experiment. We also measured other environmental parameters (i.e., temperature, pH, conductivity, and dissolved oxygen) over the exposure duration, and all parameters were relatively similar among study sites (Supporting Material 2).

Experimental design

In each study site, approximately 60 fish (7 to 15 cm) were caught by electrofishing (EFKO-FEG 1500). Half of the sampled fish (approximately 30 fish) were caged in their site of origin to control for caging stress while the other half (approximately 30 fish) was transferred into the associated transplant site with contrasted higher or lower contamination levels (see Fig. 1A and B). In other words, half of the LC fish were caged in an LC transplant site and the other half in an HC transplant site and vice versa resulting in 4 experimental groups (LC-LC: $n=66$, LC-HC: $n=72$, HC-HC: $n=63$, and HC-LC: $n=67$, see Table 2 for detailed final sample sizes).

Fish were placed in groups of 10 to 12 individuals per cage (mean biomass density 1.1 ± 0.5 $g.L^{-1}$) in cages ($70 \times 48 \times 39$ cm, $\approx$ 130L, 5 mm mesh) for 14-days. We chose this duration based on previous studies showing significant physiological responses but limited mortality and flood risks (Côte et al. 2019; Oikari 2006). Cages were placed on the bottom of the stream with rocks and litter to limit fish stress and allow foraging on macro-invertebrates prey that could pass through the mesh. Fish were marked using visible implant elastomers (Northwest Marine Technologies, Inc., Shaw Island, Washington).

To assess fish response to a second stressor, we injected half of the fish (approximately 15 fish per group) with a mixture of antigen (AMIX) 7 days after the transfer in each cage to mimic a parasite attack. The other half were injected with a control-saline solution (Phosphate Buffered Saline or PBS) (see Table 2 for detailed sample sizes). The antigen mixture included lipopolysaccharide (LPS, *Escherichia coli*, serotype: O111:B4, L2830 Sigma-Aldrich) and phytohemagglutinin (PHA, Phaseolus vulgaris phytohemagglutinin-L, L8754 Sigma-Aldrich) following previous studies in vertebrates (Petitjean et al. 2020a; Toomey et al. 2010). More particularly, we selected this combination of antigens because they are widely used in ecophysiological studies to mimic the effects of a broad range of pathogen infections, triggering an innate immune response in vertebrates, including fish (e.g., Ardia and Clotfelter 2006; Le

Table 2 Experimental design and final sample sizes (6 escaped fish removed). *LC* Low Contamination, *HC* High Contamination, *AMIX* Injection with a mixture of antigens, *PBS* control injection of Phosphate Buffered Saline. See also Fig. 1 for the location of study sites and detailed experimental design

Origin site	Transplant site	Contamination level in origin site	Contamination level in the transplant site	Immune challenge	Sample size
ARIMAS	ARIMAS	LC	LC	AMIX	18
ARIMAS	ARIMAS	LC	LC	PBS	15
ARIMAS	AUSCOR	LC	HC	AMIX	20
ARIMAS	AUSCOR	LC	HC	PBS	18
AUSCOR	ARIMAS	HC	LC	AMIX	18
AUSCOR	ARIMAS	HC	LC	PBS	17
AUSCOR	AUSCOR	HC	HC	AMIX	15
AUSCOR	AUSCOR	HC	HC	PBS	14
CELFIG	CELFIG	LC	LC	AMIX	17
CELFIG	CELFIG	LC	LC	PBS	16
CELFIG	RIOU	LC	HC	AMIX	17
CELFIG	RIOU	LC	HC	PBS	17
RIOU	CELFIG	HC	LC	AMIX	17
RIOU	CELFIG	HC	LC	PBS	15
RIOU	RIOU	HC	HC	AMIX	18
RIOU	RIOU	HC	HC	PBS	16
Total sample size					268

Guernic et al. 2016; Petitjean et al. 2020a). Briefly, LPS is an endotoxin isolated from the membrane of Gram-negative bacteria which triggers local and humoral innate immune responses, mimicking bacterial infection (Swain et al. 2008). PHA is a plant protein triggering a cell-mediated reaction (e.g., local inflammatory response and T-cells proliferation), mimicking parasites infection (Martin et al. 2006; Tella et al. 2008). Immune-challenged fish were injected in the caudal peduncle with 10 μL of the antigenic mixture (AMIX group: PHA at 90 μg.10 μL^{-1} and LPS at 90 μg.10 μL^{-1}), which corresponds to $\approx$ 9 mg.kg^{-1} of each antigen according to a previous study (Petitjean et al. 2020a). Control fish (the control saline group—PBS) were injected with the same volume of a neutral Phosphate Buffered Saline (PBS) to control injection stress.

There was no difference in fish sex ratio ($\chi^2 = 7.74$, $p = 0.35$), mass (12.9 ± 6.9 g; LMM Transplant: $\chi^2 = 0.44$, $p = 0.51$, Origin: $\chi^2 = 0.29$, $p = 0.59$, $p = 0.51$, Inj: $\chi^2 = 0.35$, $p = 0.55$), nor size (9.96 ± 1.59 cm; LMM Transplant: $\chi^2 = 0.45$, $p = 0.50$, Origin: $\chi^2 = 0.18$, $p = 0.67$, Inj: $\chi^2 = 0.02$, $p = 0.88$) among treatments. During the experiment, some fish ($n = 6$) escaped during cage cleaning, so that final sample sizes can vary between treatments (see Table 2 for detailed sample sizes).

Measurements

At T = 0, T = 7, and T = 14 days fish were measured and weighed. At the end of the experiment, fish were euthanized using an anesthetic overdose (Benzocaïne, 150 mg.L^{-1}) to collect tissues and organs for further analyses (see Supporting Material 3 for detail about sample collection). Then different traits across biological levels (organismal, cellular, and molecular) were measured to reflect metal bioaccumulation (cellular level: metal accumulation in muscle; molecular level: expression of metallothionein genes), oxidative stress (cellular: plasmatic oxidative damage, antioxidant capacity; molecular: expression of catalase and glutathione peroxidase genes), apoptosis (molecular level: caspase-3 gene expression), immune responses (cellular level: circulating neutrophils/lymphocytes ratio and local inflammatory response) as well as energy management (organism level: daily mass change and condition indices; cellular level: muscle lipids, proteins, and carbohydrates; molecular level: expression of pyruvate carboxylase and glycogen phosphorylase genes). Due to differential survival among treatment groups and difficulties in blood collecting, a few fish could not be sampled, and final sample sizes may slightly vary among treatments depending on the physiological trait considered.

Gene expression: real-time PCR

To study fish response at the molecular level, we focused on the expression ratio of several key genes in the liver involved in metal sequestration (Metallothionein: Mtl) (Knapen et al. 2005), antioxidant capacity (i.e., Glutathione peroxidase: Gpx and Catalase: Cat) (Woo et al. 2009), and cellular apoptosis (Caspase 3: Casp3) (Gao et al. 2013), as well as energy metabolism, more particularly, synthesis of oxaloacetate, gluconeogenesis and glycogen breakdown

(Pyruvate carboxylase: Pcx and Glycogen phosphorylase: Pygl) (Gonzalez et al. 2005; Koglin et al. 2016). Because the model species (*G. occitaniae)* is not fully sequenced, we performed a multiple sequence alignment from a set of close fish species for each considered gene (Supporting Material 4). However, we failed to design primers for genes related to immunity (i.e., Interferon – IFN, Interleukin – Il1b, Immunoglobulin – IgM, and Tumor necrosis factor—TNF) and some others related to energy metabolism (i.e., Glucokinase – HK, Cytochrome C oxidase – Cox17) and antioxidant capacity (Superoxide dismutase – SOD) so that data are not available for these genes. Following RNA extraction and qPCR reaction (see Supporting Material 4 for detailed method), we used the relative standard curve method (Rutledge and Côté 2003). Relative gene expression was normalized to the expression of the reference gene β-actin (ACTB) using the delta Ct method (Pfaffl 2007). The expression of ACTB transcripts did not differ among transplant (LMM Transplant: $\chi^2 = 0.78$ $p = 0.38$) nor injection groups (LMM Inj: $\chi^2 = 0.031$, $p = 0.86$) indicating that this gene was suitable for normalization.

Trace metal analyses

Trace metal analyses were performed on water samples, suspended materials, sediments, and fish white muscle. Sample mineralization was performed using Trace Metal Grade acids (Fisher Chemical) and a block digestion system (DigiPREP) (see Supporting Material 5 for details). Trace metal concentrations were measured by inductively coupled plasma mass spectrometry (Thermo Scientific, iCAP TQ). Here we focused on Cd, Cu, and Zn because these metals mainly contributed to TU differences among study sites (Supporting Material 1). The reliability of the analyses was assessed using certified reference materials; metal recovery for the Cd, Cu, and Zn lie between 91.1 and 120.6% (see Supporting Material 5 for details). Using metal concentrations (Cd, Cu, and Zn) measured in fish white muscle, we computed an index of metal bioaccumulation by summing previously log-transformed, centered, and scaled metal concentrations.

Oxidative stress index

An oxidative stress index was calculated as the ratio between oxidative damage and antioxidant capacity multiplied by 1000 according to previous studies (Costantini and Dell'Omo 2006; Petitjean 2019). Oxidative damage (mMeqH$_2$O$_2$, plasmatic hydroperoxides corresponding to active oxidants capacity, and oxidative damage) and antioxidant capacity (mMeqHClO, overall non-enzymatic antioxidant ability of the plasma barrier), were measured using a d-ROM test

and OXY-adsorbent test (Diacron International, Grosseto, Italy), respectively. Both assays were performed following the method reported by Bagni et al. (2007) and Hoogenboom et al. (2012) and slightly modified by Petitjean et al. (2019a). In a nutshell, the d-ROM test was conducted using 8µL of plasma, 200 µL of 0.01 M acetic acid/sodium acetate buffer (pH 4.8), and 2µL of N, N-diethyl-p-phenylenediamine incubated in 96-well microplate for 75 min at 37 °C. After the incubation, the 96-well microplate was centrifuged at 3500 rpm for 2 min to retrieve a clarified supernatant before reading the absorbance at 490 nm using CLARIOstar (BMG LABTECH). The OXY-adsorbent test was conducted using 5µL of the diluted plasma (1:100) and 200 µL of HOCl solution incubated in a 96-well microplate for 10 min at 37 °C. After incubation, 5 µL of N, N-diethyl-p-phenylenediamine was added to each well before reading the absorbance at 490 nm using CLARIOstar (BMG LABTECH). For both assays, certified standards and blanks were made using the same relative volumes as samples. Due to limited blood quantity in some small fish, the sample size may vary among populations and treatments. For the d-ROM and OXY-adsorbent tests, intra-plate repeatability was $85.2 \pm 9.9\%$ and $89.3 \pm 6.0\%$, respectively, and inter-plate repeatability was and $92.4 \pm 7.7\%$ and $93.9 \pm 6.0\%$.

Immune response and inflammation

The local immune response was measured as the skin swelling after an antigen injection in the caudal peduncle following previous studies in wild vertebrates, including fish (Ardia and Clotfelter 2006; Petitjean et al. 2020a). The intensity of the local immune response was calculated as the difference between the mean peduncle thickness 48 h after the immune challenge minus the mean peduncle thickness before the immune challenge, divided by the mean peduncle thickness before the immune challenge × 100 as validated in a previous study conducted in gudgeon (Petitjean et al. 2020a).

We also measured the Neutrophils/Lymphocytes (N/L ratio) in fish blood as a marker of the inflammatory response following previous studies (Davis et al. 2008; Petitjean et al. 2020b, c). Indeed, previous studies on gudgeon showed that metal contamination triggered a significant inflammation associated with changes in the circulating N/L ratio in gudgeon (Petitjean et al. 2020b, c). Therefore, we assessed the relative abundance of each cell type on 100 leucocytes per blood smear previously stained with May Grunwald-Giemsa and calculated the N/L ratio reflecting the level of inflammation.

Energy status and available energy (Lipids, Proteins, Carbohydrates)

The energy status was assessed using body mass changes and organosomatic indices at the organism level and the available energy in fish muscle at the cellular level. First, we calculated the daily mass change of fish before and at the end of the experiment to measure changes in whole-body conditions. Second, we calculated the hepatosomatic index (HSI), as a proxy of energy content in the liver (Chellappa et al. 1995) and gonadosomatic index (GSI), as a proxy of reproductive investment (Marentette and Corkum 2008). Third, we measured the available energy in muscle following a protocol from De Coen and Janssen (1997) modified by Gandar et al. (2017) to measure the total carbohydrate, lipid, and protein contents in white muscle of fish at the end of the experiment. Total carbohydrate, protein, and lipid concentrations ($\mu g.mg^{-1}$ muscle) were transformed in their energetic values ($mJ. mg^{-1}$ of muscle) using their enthalpy of combustion (17, 24, and 39.5 $kJ.g^{-1}$, respectively) and summed to obtain the total available energy in muscle.

Statistics

All statistical analyses were performed using R version 4.0.3 (R Core Team 2020). We used linear mixed models (LMM, package lme4 Bates et al. 2015) with fish traits, i.e., survival, metal bioaccumulation, oxidative stress index, oxidative damage, available energy (log-transformed), antioxidant capacity, local immune response, N/L inflammatory immune response, daily mass change, HSI and GSI (power transformed) or relative gene expression (i.e., Mtl, Cat, Gpx, Pcx, Pygl, Casp3, power transformed) as response variables. The transplant site (i.e., Low Contamination LC or High Contamination HC transplant site), the site of origin (i.e., Low Contamination LC or High Contamination HC), and immune challenge treatment (AMIX or PBS), as well as their third and second-order interactions, were included as fixed factors. Fish size and sex were also included as covariates. The cage nested within the transplant site was added as a random effect, as well as the identity of the population of origin (i.e., ARIMAS, CELFIG, AUSCOR, and RIOU) to consider possible shared conditions within cages and transplant sites and shared characteristics of individuals originating from the same populations. For survival, because generalized linear mixed models (binomial distribution) did not converge, we ran additional GLM models without random effects. Best models were selected by backward selection procedure, eliminating non-significant interactions and variables (i.e., p-value > 0.05; see Table 3 for a summary of the best final models). When interactions were found significant, we analyzed differences between groups using pairwise t-test with false discovery rate adjustment (Benjamini and Hochberg 1995) or two-sample Fisher's exact test for count data (i.e., survival) (Agresti 2007).

In addition, we compared the parallelism of the plastic responses among the replicate population (i.e., ARIMAS, AUSCOR, or CELFIG, RIOU). For this, we compared the reaction norms (i.e., the slopes of the plastic response) between two different models according to a previous study (Jacquin et al. 2016). One model tested non-parallel responses to contamination (LC or HC in the transplant site) among replicate populations by incorporating a population-by-contamination interaction (population: contamination effect). The other model tested parallel response to contamination among replicate populations without this interaction (population + contamination effects). In other words, we compared GLMs with or without the interaction between the identity of the population of origin and the contamination level in the transplant site. Values are reported as mean ± SE. The data, R scripts, and Rmarkdown report (HTML and MD versions) supporting this study's findings are available in the following GitHub repository: https://doi.org/10.5281/zenodo.6845032.

Results

Survival

The contamination level of the transplant site significantly affected fish survival depending on the contamination level of the population of origin (Transplant: Origin, see Table 3). More specifically, fish originating from High Contamination sites displayed a high survival rate (above 94%), irrespective of the transplant sites (94 and 100% when transplanted into LC sites vs. 100% when transplanted into HC sites; two-sample fisher's exact test: $p = 0.50$, Fig. 2A). On the contrary, fish from Low Contamination sites displayed a significant decrease in survival when transplanted into High Contamination sites (94 to 97% when transplanted into LC sites vs. 78 to 79% when transplanted into HC sites; two-sample fisher's exact test: $p = 0.003$, Fig. 2A). In addition, figures and model comparisons performed on each population separately (i.e., HC: AUSCOR and RIOU populations, and LC: ARIMAS and CELFIG populations) show that the slopes were parallel and not significantly different among replicate populations (slope differences between HC origin populations AUSCOR and RIOU: estimate = -0.06, $p = 0.18$; LC origin populations ARIMAS and CELFIG: estimate = 0.06, $p = 0.59$).

Metal Bioaccumulation

The transplant site affected metal bioaccumulation in fish muscle depending on their origin (Transplant: Origin, see

Table 3 Summary of the results of the best linear mixed effect models (LMM) selected by backward selection procedure and testing the effects of the transplant, origin, and immune challenge on fish traits. Sample size and marginal and conditional R square are reported as n, R2m, and R2c, respectively

	Estimate	Std. Error	t or z value	df	Chisq	p.value
Survival | n=268						
Intercept	−19.6	1350	−0.0144	NA	NA	NA
Transplant (LC)	16.1	1350	0.0119	1	2.68	0.102
Origin (LC)	18.3	1350	0.0135	1	22	<0.0001
Transplant (LC): Origin (LC)	−17.9	1350	−0.0132	1	6.59	<0.05
Bioaccumulation | n=248 | R2m=0.0498 | R2c=0.385						
Intercept	0.00786	0.957	0.00821	1	0.0000675	0.993
Transplant (LC)	0.358	1.35	0.264	1	0.0699	0.791
Origin (LC)	0.499	0.352	1.42	1	2.01	0.156
Imm. challenge (PBS)	0.18	0.325	0.554	1	0.306	0.58
Transplant (LC): Origin (LC)	−1.09	0.491	−2.22	1	4.91	<0.05
Transplant (LC): imm. Challenge (PBS)	−0.972	0.452	−2.15	1	4.62	<0.05
Oxidative stress index | n=166 | R2m=0.0629 | R2c=0.258						
Intercept	3.54	0.355	9.97	1	99.4	<0.0001
Size	−0.101	0.0333	−3.03	1	9.21	<0.01
Oxidative damage | n=179 | R2m=0.0837 | R2c=0.34						
Intercept	2.07	0.246	8.41	1	70.7	<0.0001
Size	−0.0838	0.0227	−3.7	1	13.7	<0.01
Antioxidant capacity | n=169 | R2m=0.0502 | R2c=0.0661						
Intercept	11.3	0.163	69.5	1	4830	<0.0001
Origin (LC)	−0.558	0.236	−2.36	1	5.59	<0.05
Local immune response | n=249 | R2m=0.409 | R2c=0.492						
Intercept	26.2	2.43	10.8	1	117	<0.0001
Imm. challenge (PBS)	−23.4	1.66	−14.1	1	199	<0.0001
NL inflammatory immune response | n=229 | R2m=0.0714 | R2c=0.167						
Intercept	0.00754	0.0149	0.505	1	0.255	0.614
Transplant (LC)	−0.00736	0.00463	−1.59	1	2.53	0.111
Origin (LC)	−0.0109	0.0091	−1.2	1	1.44	0.23
Size	0.00365	0.00135	2.7	1	7.28	<0.01
Transplant (LC): Origin (LC)	0.0155	0.00674	2.3	1	5.28	<0.05
Available energy | n=243 | R2m=0.13 | R2c=0.256						
Intercept	8.38	0.0957	87.6	1	7670	<0.0001
Transplant (LC)	0.15	0.0877	1.71	1	2.93	0.087
Origin (LC)	−0.0398	0.117	−0.341	1	0.116	0.733
Transplant (LC): Origin (LC)	−0.302	0.0771	−3.92	1	15.3	<0.0001
Daily mass change | n=252 | R2m=0.028 | R2c=0.47						
Intercept	1.2	0.177	6.79	1	46	<0.0001
Transplant (LC)	0.074	0.25	0.295	1	0.0872	0.768
Origin (LC)	0.0535	0.0701	0.763	1	0.583	0.445
Transplant (LC): Origin (LC)	−0.217	0.0981	−2.22	1	4.91	<0.05
HSI | n=247 | R2m=0.0211 | R2c=0.407						
Intercept	0.102	0.234	0.434	1	0.189	0.664
Size	−0.0436	0.0199	−2.19	1	4.78	<0.05
GSI | n=244 | R2m=0.696 | R2c=0.753						
Intercept	0.63	0.103	6.11	1	37.3	<0.0001
Sex (M)	−1.41	0.0541	−26	1	674	<0.0001
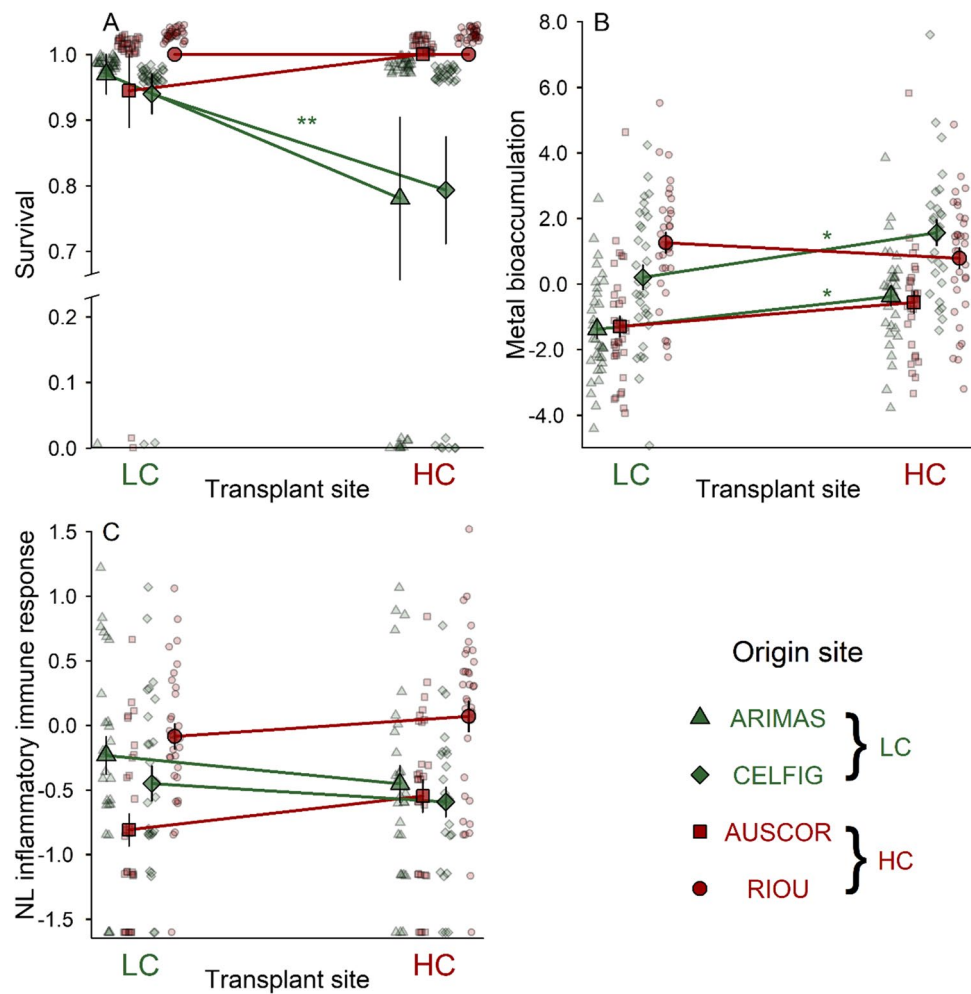
Fig. 2 Effect of the transplant site (Low Contamination—LC or High Contamination—HC) depending on the site of origin (populations from Low Contamination sites: ARIMAS and CELFIG in green; populations from High Contamination sites: AUSCOR and RIOU in red) on fish survival (**A**), metal bioaccumulation in muscle (sum of centered and scaled Cd, Cu, and Zn muscle content) (**B**) and N/L ratio reflecting the inflammatory immune response (power-transformed) (**C**). Small symbols represent individuals. Bigger symbols and black bars represent the mean value and standard error. Small symbols representing individuals are slightly jittered on the x-axis to improve the figure's readability. For survival (A), Small symbols are also slightly jittered on the y-axis. Asterisks represent significant differences between transplant sites in HC or LC origin sites (both replicates pooled) after post hoc tests: *** $p<0.001$; ** $p<0.01$; * $p<0.05$. In LC fish (in green), survival decreased when transplanted to HC sites while metal bioaccumulation increased and N/L ratio tended to decrease (significant interactive effect between transplant and origin but post-hoc test testing slopes between environment for each fish origin were not significant). In HC fish, on the contrary, survival, metal bioaccumulation, and immune N/L ratio did not change. Models comparison indicated that slopes were parallel among replicate populations (either ARIMAS and CELFIG or AUSCOR and RIOU) for all traits

Table 3) and the immune challenge (Transplant: Immune Challenge, see Table 3). More specifically, fish from Low Contamination sites accumulated significant amounts of metals in their muscle when transplanted into High Contamination sites (pairwise t-test with FDR adjustment: $p=0.014$, Fig. 2B), while fish originating from High Contaminated sites did not accumulate significant amounts of metals after transplantation into contaminated sites (pairwise t-test with FDR adjustment: $p=0.57$, Fig. 2B). In addition, replicate populations responded similarly to the transplant: model comparisons performed on each population separately

indicate that the slopes of the reaction norms were parallel among both replicate populations (slope differences between replicate HC populations: estimate $=1.21$, $p=0.06$; between replicate LC populations: estimate $=-0.35$, $p=0.59$).

In addition, the immune challenge changed the patterns of metal bioaccumulation depending on the transplant site: PBS-control fish exposed to contaminated sites bioaccumulated metals, while AMIX antigen-injected fish did not bioaccumulate metals (pairwise t-test with FDR adjustment: p-values $=0.011$ and 0.69 for PBS and AMIX injected fish, respectively, Fig. 3).

At the molecular level, the relative gene expression of Mtl was affected by the population origin, with fish from High Contamination sites having higher Mtl expression than fish from Low Contamination sites, irrespective of the transplant site (Table 4). In addition, females have higher Mtl relative expression than males (Table 4).

Oxidative stress index (oxidative damage and antioxidant capacity)

Fish size only affected the oxidative stress index, with larger fish having a lower oxidative stress index, mainly due to lower oxidative damage (Table 3). However, detailed analyses revealed that the antioxidant capacity (but not oxidative damage) varied according to the population of origin (Table 3), with significantly lower antioxidant capacity for fish originating from Low Contamination sites compared to fish from High Contamination sites irrespective of the transplant site (Table 3).

At the molecular level, genes linked to antioxidant activity (i.e., Gpx but not Cat) and cellular apoptosis (i.e., Casp3)
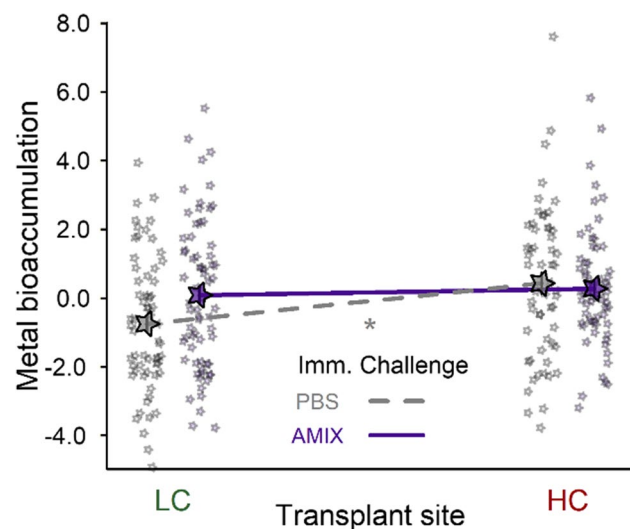
Fig. 3 Metal bioaccumulation in fish transplanted into Low Contamination (LC) or High Contamination (HC) sites depending on their immune treatment (control-saline – PBS-injected fish: dashed line and grey stars; immune challenged – AMIX-injected fish: solid line and purple stars) (sites of origin pooled). Muscle bioaccumulation is calculated as the sum of centered and scaled Cd, Cu, and Zn muscle content. Small stars represent individuals. Bigger stars and black bars represent the mean value and standard error. Small symbols representing individuals are slightly jittered on the x-axis to improve the figure's readability. Asterisks represent significant differences between transplant sites depending on immune challenge (pairwise t-test with FDR adjustment). *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$. The immune challenge altered metal bioaccumulation in fish depending on their transplantation site, irrespective of their origin (i.e., reduced depuration/excretion in AMIX injected fish transplanted into LC sites in grey)

were differently expressed depending on the contamination level of the origin site, with fish from High Contamination sites having higher Gpx and Casp3 expression than fish from Low Contamination sites (Table 4).

In addition, the immune challenge also affected the expression level of the antioxidant gene (Cat but not Gpx), with AMIX-injected fish having lower Cat expression than PBS-injected fish (Table 4).

Immunity: local immune response and N/L ratio

The immune challenge significantly affected the local immune response (i.e., skin swelling) (Table 3). Indeed, the AMIX solution triggered an effective local immune response compared to PBS-injected fish, irrespective of the transplant site and population of origin (Table 3).

The transplant site affected the circulating inflammatory response (N/L ratio) depending on the population of origin (Transplant: Origin, see Table 3). Indeed, when transplanted into High Contamination sites, LC fish tended to have a decreasing N/L ratio, while HC fish tended to have an increasing N/L ratio (Fig. 2C). However, post-hoc tests conducted in each population separately were not significant (all $p > 0.10$). In addition, replicate populations responded similarly to the transplantation: model comparisons performed on each population separately indicate that the slopes of the reaction norms were parallel among both replicate populations (slope differences between replicate HC populations: estimate = 0.002, $p = 0.83$; between replicate LC populations: estimate = -0.002, $p = 0.81$).

Available energy (Lipids, Proteins, Carbohydrates)

The amount of available energy was significantly affected by the transplant site depending on the population of origin (Transplant: Origin, see Table 3 and Supporting Material 6). More specifically, the available energy increased in fish originating from Low Contamination sites when transplanted into High Contamination sites (pairwise t-test with FDR adjustment: $p = 0.012$) and increased in fish originating from High Contamination sites when transplanted into Low Contamination sites (pairwise t-test with FDR adjustment: $p = 0.014$; Supporting Material 6A). Detailed analyses on lipid, protein, and carbohydrate contents indicated that this relationship was mainly driven by lipid and carbohydrate content in fish muscle (for lipids: LMM Transplant: $\chi^2 = 3.87$, $p = 0.05$, LMM Origin: $\chi^2 = 0.016$, $p = 0.90$, LMM Transplant: Origin: $\chi^2 = 17.28$, $p < 0.001$; for carbohydrates: LMM Transplant: $\chi^2 = 0.72$, $p = 0.40$, LMM Origin: $\chi^2 = 2.50$, $p = 0.11$, LMM Transplant: Origin: $\chi^2 = 9.64$, $p = 0.002$). However, model comparisons performed in each population separately indicated that the slopes were parallel between replicate HC populations AUSCOR and

Table 4 Summary of the results of the best linear mixed effect models (LMM) selected by backward selection procedure and testing the effects of the transplant, origin, and immune challenge on relative gene expression measured in fish liver. Overall, the origin of the population (either HC or LC) mainly explained the different levels of gene expression, with fish from HC site having higher basal expression levels of Mtl, Gpx, and Casp3 compared to LC fish. Also, fish injected with antigen mixture (AMIX) had lower Cat, Pcx, and Pygl expression levels than PBS control fish. Sample size and marginal and conditional R square are reported as n, R2m, and R2c, respectively

		Estimate	Std. Error	t or z value	df	Chisq	p.value
*Mtl—Metallothionein	n = 245	R2m = 0.113	R2c = 0.191*							
Metal sequestration	Intercept	1.32	0.237	5.6	1	31.3	< 0.0001
	Origin (LC)	−0.739	0.327	−2.26	1	5.12	< 0.05
	Sex (M)	−0.287	0.139	−2.06	1	4.24	< 0.05
*Cat—Catalase	n = 245	R2m = 0.0582	R2c = 0.523*							
Antioxidant—Cat	Intercept	−3.18	0.221	−14.3	1	206	< 0.0001
	Imm. challenge (PBS)	0.101	0.0381	2.65	1	7.04	< 0.01
	Size	0.0516	0.0169	3.05	1	9.31	< 0.01
	Sex (M)	−0.0958	0.0397	−2.41	1	5.83	< 0.05
*Gpx—Glutathione peroxidase	n = 245	R2m = 0.0918	R2c = 0.135*							
Antioxidant—Gpx	Intercept	−1.07	0.101	−10.6	1	113	< 0.0001
	Origin (LC)	−0.349	0.137	−2.54	1	6.45	< 0.05
	Sex (M)	−0.181	0.0788	−2.3	1	5.29	< 0.05
*Casp3—Caspase 3	n = 246	R2m = 0.253	R2c = 0.255*							
Apoptosis effector	Intercept	−5.19	0.316	−16.4	1	270	< 0.0001
	Origin (LC)	−0.442	0.0985	−4.48	1	20.1	< 0.0001
	Size	0.217	0.0311	7	1	49	< 0.0001
*Pcx—Pyruvate carboxylase	n = 239	R2m = 0.0289	R2c = 0.432*							
Energy metabolism (oxaloacetate synthesis & gluconeogenesis)	Intercept	−3.26	0.164	−19.8	1	392	< 0.0001
	Imm. challenge (PBS)	0.122	0.0502	2.44	1	5.95	< 0.05
	Sex (M)	−0.117	0.0526	−2.22	1	4.92	< 0.05
*Pygl—Glycogen phosphorylase	n = 226	R2m = 0.0369	R2c = 0.181*							
Energy metabolism (glycogen breakdown to produce glucose)	Intercept	−2.47	0.0749	−33	1	1090	< 0.0001
	Imm. challenge (PBS)	0.155	0.049	3.18	1	10.1	< 0.01

RIOU (slope differences between replicate HC populations: estimate = -0.19, $p = 0.13$) but not parallel among replicate LC populations ARIMAS and CELFIG (slope differences between replicate LC populations: estimate = -0.18, $p = 0.04$).

Daily body mass change was also significantly affected by the transplant site depending on the population of origin (Transplant: Origin, see Table 3). However, post-hoc tests indicate that slopes were not significant (pairwise t-test with FDR adjustment: $p = 0.12$ and pairwise t-test with FDR adjustment: $p = 0.53$; Supporting Material 6B), likely because responses were very different depending on the population considered (slope differences between replicate HC populations: estimate = -0.41, $p < 0.001$; between replicate LC populations: estimate = -0.53, $p < 0.001$).

Hepatosomatic and gonadosomatic indices were not affected by any treatment but rather by fish size and sex (Table 3).

At the molecular level, the relative expression of metabolism-related genes (i.e., Pcx and Pygl) was only affected by the immune challenge. Indeed, AMIX-injected fish displayed lower metabolism-related gene expression (Pcx and Pygl)

than PBS-injected fish (Table 4). In addition, larger fish and females had higher Pcx expression (Table 4).

All results are summarized in Table 5 for the sake of clarity.

Discussion

Local adaptation and adaptive plastic responses to environmental stressors can determine the ability to survive in a fast-changing world (Fox et al. 2019; Rolshausen et al. 2015). While there is growing evidence that chronic exposure to contamination can lead to genetic-based adaptive responses (Hamilton et al. 2017; Saaristo et al. 2018), we still have a limited understanding of the role of plasticity across biological levels (Hamilton et al. 2016; Westneat et al. 2019), especially in multistress conditions (i.e., co-tolerance/co-adaptation vs. maladaptation, Brady et al. 2017a; Vinebrooke et al. 2004). In this study, we compared the plastic responses of wild gudgeon (*G. occitaniae*) populations to metal contamination under semi-natural settings using an *in-situ* reciprocal transplant experiment between replicated

Table 5 Summary of main results

Trait	Effect	Main results	Summarized interpretation
Survival	Transplant x Origin	Higher survival in HC fish transplanted into HC sites	Local adaptation to contamination in HC fish Parallel plastic responses among replicate populations
Metal bioaccumulation (Cd, Cu, Zn)	Transplant x Origin	Bioaccumulation in LC fish transplanted into HC sites Constant metal levels in HC fish	Metal bioaccumulation in naïve LC fish and constant levels in HC fish Parallel plastic responses among replicate populations
	Transplant x Immune challenge	Altered metal bioaccumulation in immune-challenged fish exposed to LC sites	Multiple stress effect: immune challenge affected the response to metals
Metals bioaccumulation (Cd, Cu, Zn) Mtl relative expression	Origin	Higher expression of Mtl in HC fish	Suggests better metal detoxification in HC fish, potentially explaining higher tolerance to contamination
Oxidative stress antioxidant capacity	Origin	Higher non-enzymatic antioxidant capacity in HC fish	Better antioxidant defenses in HC fish potentially explaining higher tolerance to contamination but as a probable side effect of higher apoptosis
Oxidative stress Cat, Gpx and Casp3 relative expression	Origin	Higher basal Gpx and Casp3 expression levels in HC fish	
	Immune challenge	Lower Cat expression in immune challenged fish	Enzymatic antioxidant defenses (Cat expression only) decreased by Immune challenge
Immunity Local cellular immunity	Immune challenge	Higher skin swelling in immune challenge	Immune challenge triggered the local immune response
Immunity Circulating N/L ratio	Transplant x Origin	Marginally higher NL ratio in HC fish transplanted into HC sites	Marginally higher inflammatory response in HC fish Parallel plastic responses among replicate populations
Energy management Available energy and Daily mass changes	Transplant x Origin	Variable available energy and body mass changes	No parallel response among replicate populations
Energy management Pcx and Pygl relative expression	Immune challenge	Lower Pcx and Pygl expression in immune challenged fish	Immune challenge decreased carbohydrate metabolism

Low Contamination and High Contamination streams (see Fig. 1A), using a combination of biomarkers at several biological levels (molecular, cellular and whole organism). We also tested whether exposure to another stressor (i.e., an immune challenge mimicking a parasite attack) could affect response to metal contamination, reflecting a realistic multistress context.

Altogether, the results show that HC fish (i.e., fish originating from Highly Contaminated sites) could better survive after transplantation into contaminated sites compared to their LC naïve counterparts (originating from Low Contamination sites, see Fig. 2A). This higher survival was likely explained by enhanced detoxification ability and antioxidant defenses, with some potentially associated physiological costs linked to apoptosis (details below). In addition, most plastic responses were parallel among replicate populations suggesting potential patterns of parallel local adaptation to metal contamination, although the underlying evolutionary

mechanisms remain unknown. Second, contrary to what we expected, multiple stressors had few interactive effects (i.e., few interactions between the immune challenge and transplant site), although the immune challenge had substantial effects on immunity and affected metal bioaccumulation (see Table 5 for a summary of all results). Below we detail and discuss the results of each fish trait separately.

Variability in survival rate

Our results show that LC fish suffered higher mortality when transplanted into contaminated sites than HC fish. In addition, these survival differences were parallel among the replicate populations (i.e., LC: ARIMAS and CELFIG; HC: AUSCOR and RIOU) from the two different hydrographic sub-basins. Indeed, HC fish displayed higher survival than LC fish, with similar reaction norms (i.e., parallel slopes, see Fig. 2A). This response pattern suggests a local adaptation

to metal contamination (Kawecki and Ebert 2004). Interestingly, a recent study suggests that such divergence observed among gudgeon populations cannot be explained by genetic drift only (Côte et al. 2021), suggesting that natural selection could play a role in the observed phenotypic divergence. Accordingly, in the RIOU HC site, the contamination record is well documented, of longstanding duration (more than four decades) and high magnitude (see Sect. "Contamination levels in study sites"), suggesting that genetic adaptation to metallic contamination may occur in this population.

On the contrary, in the AUSCOR HC site, the level of contamination is lower and from various origins, and probably contemporary (over the last decade), mainly due to the growing urbanization of the area, resulting in more diffuse and occasional contaminants release. Together the results suggest that while contaminants' sources, levels, and age may differ between the replicate HC site, gudgeon populations display a similar pattern of adaptation to polymetallic contamination, suggesting parallel responses to natural selection by metals. However, the underlying evolutionary mechanisms (i.e., genetic, epigenetic, or plastic mechanisms) remain to be formally tested, for instance, by repeating this experiment in lab-reared F1 and F2 generations.

Our results are, nevertheless, consistent with previous studies showing local adaptation to contamination in various taxa (Dutilleul et al. 2017; Pedrosa et al. 2017), including fish (Hamilton et al. 2017; Oziolor et al. 2014; Reid et al. 2016). Interestingly, previous studies showed that pollution resistance or tolerance incurred some physiological and fitness costs (Meyer and Di Giulio 2003). For instance, fish (*Heterandria formosa*) selected for cadmium resistance displayed reduced fecundity, brood size, and longevity under uncontaminated conditions (Xie and Klerks 2004a). However, the existing literature brings mixed results on the existence and implications of such costs (Petitjean et al. 2019b; Shephard et al. 2021; Whitehead et al. 2017). In this experimental study, we found no evidence of short-term survival costs in HC fish since they also displayed a high survival when transferred to reference LC sites (Fig. 2A), although long-term studies are now needed. In addition, we found no evidence of associated costs to face pathogens since HC fish had a similar ability to face the immune challenge compared to their LC counterparts. However, we uncovered potential hidden physiological costs detailed below.

Variability in metal bioaccumulation

We found that fish from LC sites significantly accumulated metals when transplanted into HC sites, potentially explaining their lower survival rate. However, HC fish also had high levels of metals in their muscle after transplantation, reaching similar levels as LC fish (Fig. 2B). This suggests that the higher survival of HC fish was not explained by lower

metal bioaccumulation (i.e., resistance), contrary to previous studies (Jeffree et al. 2014; Xie and Klerks 2004b). However, further measurement of bioaccumulation in other fish tissues would be necessary to confirm this hypothesis (e.g., liver).

Alternatively, HC and LC fish could differ in their ability to tolerate metals through various mechanisms (Hamilton et al. 2017; Wood et al. 2012, 2011), such as a higher basal and/or inducible level of Metallothioneins (Mtl). Indeed, Mtls play an important role in detoxification by binding trace metals (including Zn, Cu, and Cd) and limiting their toxic effects on cells and organs (Coyle et al. 2002; Hamilton and Mehrle 1986; Klaassen et al. 1999). Accordingly, in our study, HC fish displayed a higher basal (but not inducible) expression of the Mtl gene (Table 4). This result is fully consistent with a previous experimental study showing that wild gudgeon (*Gobio gobio*) populations resistant to metal pollution had a similar metal accumulation rate but faster and more efficient production of Mtls (Knapen et al. 2004). Hence, our results further underline the essential role of Mtl in shaping the responses of wild fish populations to trace metals in semi-natural conditions. Nevertheless, complementary approaches studying Mtl's protein induction are needed to confirm this pattern. Indeed, Mtl's gene expression is not always consistent with observed Mtl's protein levels, probably related to effects on translation and/or protein degradation (Vasconcelos et al. 2002).

Irrespective of the underlying mechanisms, our study suggests that the higher survival of HC fish under contaminated sites (i.e., HC sites) should be mainly due to an improved detoxification capacity (i.e., higher basal Mtl's gene expression) rather than by lower metal bioaccumulation. This result suggests that HC fish's higher survival may be partly driven by adaptation to metal contamination.

Variability in oxidative stress responses

Antioxidant defenses are also crucial to face metal exposure and limit their effects on fish health and fitness. Indeed, increasing antioxidant defenses is an essential physiological pathway to counteract the deleterious effects of reactive oxygen species produced during contaminant exposure (Birnie-Gauvin et al. 2017; Lushchak 2011; Petitjean et al. 2020b). However, in our study, transplantation into HC sites did not affect oxidative stress markers (non-enzymatic antioxidant defenses, enzymatic antioxidant-related genes, and damage), irrespective of the population considered (Table 3). This is likely not due to low metal contamination levels because metal levels in polluted streams are comparable to several previous experimental studies (Bougas et al. 2016; Craig et al. 2007; Hansen et al. 2006). Nevertheless, in our study, antioxidant capacity differed among fish populations' origin. Indeed, HC fish displayed a higher basal (but not inducible) level of non-enzymatic antioxidant defenses in their blood (Table 3) and antioxidant enzymes (i.e., Gpx but

not Cat) gene expression (Table 4) in their liver, irrespective of the transplant site. HC fish might thus have a better antioxidant system through higher investment in antioxidant defenses, which could help them cope with environmental contamination, as shown in killifish populations exposed to contaminated sediments (Meyer et al. 2003).

Interestingly, we also found that HC fish displayed higher Casp3 gene expression levels (Table 4), irrespective of the transplant site. This result suggests that potentially increased metal tolerance in HC fish may incur a long-term cost by triggering cell apoptosis and subsequent tissue damage through Casp3 activation in the liver (Gao et al. 2013). Although this hypothesis remains to be confirmed by long-term approaches, it is consistent with a previous correlative field study showing patterns of increased inflammation and liver lesions in fish originating from the same metal-contaminated areas (Petitjean et al. 2020c). Irrespective of the underlying mechanisms, all results thus lead towards a differential ability to tolerate metal exposure in fish populations from HC sites (i.e., higher survival, enhanced detoxification ability, antioxidant defenses), which could have important consequences for fitness and the response to other stressors such as pathogens and immune challenges.

Variability in immunity

The immune system plays a central role in response to environmental stressors such as pollution and pathogens. First, the immune challenge treatment efficiently triggered a local immune response. In addition, HC fish tended to have an increasing N/L ratio when transplanted into HC sites (Fig. 2C), suggesting that inflammation can be modulated by contaminant exposure. Accordingly, in a previous study conducted in the laboratory, we showed that a field-realistic mixture of Cd, Cu, and Zn enhanced the N/L ratio of wild gudgeon (*G. occitaniae*) (Petitjean et al. 2020b). Such discrepancies between laboratory and field studies (i.e., significant results in the lab. became trends in the field) have already been highlighted in a previous study investigating the combined effects of antigen injection and metal exposure in fish (Le Guernic et al. 2016). This could be explained by confounding factors that have not been measured in our study (e.g., infection by microparasites, resource availability) and/or which could fluctuate under field conditions (e.g., contaminants, temperature) while constant in the laboratory.

Besides, many contaminants have been reported to affect fish immune responses with immunosuppressive or immunostimulatory effects depending on contaminants and immune traits (Bols et al. 2001; Segner et al. 2012; Zelikoff 1993). For instance, exposure to Aluminium (Al) enhanced the leukocyte oxidative burst in Roach (*Rutilus rutilus*) (Jolly et al. 2014). On the contrary, exposure to Cd inhibited the serum complement activity and leucocyte oxidative burst but

promoted serum peroxidase activity and leucocyte phagocytosis in a time-dependent manner in gilthead seabream (*Sparus aurata*) (Guardiola et al. 2013). While a growing body of literature reports contaminants' immunomodulating or immunotoxic effects in fish, the mechanisms involved are still unclear (Desforges et al. 2016; Segner et al. 2021). Yet, contaminants are suspected of stimulating immune responses by promoting the release of pro-inflammatory cytokines and chemokines (Hossein-Khannazer et al. 2020) and/or through tissues injuries, resulting in the detection of damage-associated molecular patterns (DAMPs) (Segner et al. 2021). These hypotheses are in accordance with our results showing an increase in the neutrophils, which are important modulators of inflammation (Rosales 2018), under contaminated environments.

Irrespective of the mechanisms involved, it is still challenging to predict the immunomodulation effects of metals on parasite resistance. Indeed, we showed that exposure to contaminated sites tended to stimulate the inflammatory responses in fish. However, we could not measure the expression level of immune-related genes linked to other immune components (see Sect. ''Gene expression: real-time PCR'') or parasite load, providing an incomplete picture of fish immune responses.

Variability in energy management

Contrary to our expectation, we did not find any effect of the contamination on energy management at the molecular level (i.e., Pcx and Pygl, see Table 4). In addition, energetic responses at the cell and whole-organism levels were not parallel. Indeed, available energy in muscle and body mass changes were altered by the transplantation, but the responses were highly variable depending on the replicate population (Supporting Material 6). This result suggests that the plastic energetic response to transplant is not determined by stream contamination and/or might be blurred by differences in food availability depending on local conditions and/or macroinvertebrate assemblages in each site (Carlisle and Clements 2005, 2003; Woodcock and Huryn 2007). Measuring the fish feeding rate and, more globally, fish behavior within the cages would be helpful to confirm this hypothesis, although recording fish behavior in their natural habitat remains challenging. Further investigations using more integrative traits reflecting energy metabolisms, such as standard and maximal metabolic activity and aerobic scopes, may be useful in this context (Lindberg et al. 2017).

Multiple stressors effects

The immune challenge efficiently triggered a local immune response and strongly affected energy management. Indeed,

the immune challenge decreased the expression of genes involved in energy metabolism and, more particularly, gluconeogenesis and glycogen breakdown (i.e., Pcx and Pygl expression levels, see Table 4), irrespective of the transplant site and population considered. This result suggests a decreased energy metabolism due to the immune challenge, consistent with a "sickness syndrome" (i.e., decreased physiological and behavioral activity; Lopes et al. 2021). However, the immune challenge affected neither available energy in fish muscle nor daily mass change, suggesting limited impacts on fish energy reserves contrary to previous studies (Bonneaud et al. 2003; Ots et al. 2001; Petitjean et al. 2020a). One potential explanation is that energy expenditure incurred by the immune challenge is transient and may be rapidly counterbalanced by increased feeding rate and/or reduced behavioral activity, as suggested in a previous experimental study in controlled conditions (Petitjean et al. 2020a).

There were few interactive effects of contamination and immune challenge. However, the immune stressor altered the level of metal bioaccumulated in fish muscle after transplantation. Indeed, antigen injection (AMIX group) alleviated the decrease of metal bioaccumulation in PBS-injected fish transplanted into LC sites (Fig. 3). This suggests that the immune challenge decreased fish's ability to excrete metals. This could be explained by the reallocation of energy and/or essential metals, such as Cu and Zn, which can be mobilized during the inflammatory response and detoxication processes (Djoko et al. 2015). However, further work is needed to decipher the underpinning mechanisms.

Overall, we found no evidence of immunodepression linked to contamination and no support for a potential cost of metal adaptation to face pathogens, at least in the short term (14 days). However, interpretations are limited because we used an antigen injection to standardize the immune state of individuals and did not use real parasites with pathogenic effects; we hence could have underestimated multiple stressors' effects on fish populations (Seppala and Jokela 2011).

Interestingly, we found that females had higher basal gene expression related to metal sequestration (Mtl), antioxidant defense (Cat, Gpx), and energy management (Pcx). Indeed, sex-specific responses are understudied in fish ecophysiology, yet several lines of evidence exist regarding sexual dimorphism in stress responses (Campbell et al. 2021). For instance, several studies have underlined sex-specific responses to industrial effluent (Afonso et al. 2003), Endocrine-Disrupting Chemicals (i.e., 17α-ethinylestradiol) (Selmoni et al. 2019), handling stress (Eliason et al. 2020), or temperature (Missionário et al. 2022). Interestingly, responses to multiple stressors may also differ in a sex-specific manner. For instance, males three-spined sticklebacks (*Gasterosteus aculeatus*) exposed to copper displayed increased upper thermal tolerance than females (Mottola et al. 2022), suggesting that it is crucial to consider sex-specific

responses to better predict fish populations' persistence under global changes.

More generally, our results suggest that different sexes have alternative strategies to face stressors, although it is unclear whether this might benefit females under multiple stressor exposure. It is hence of primary importance to consider sex-specific responses to predict how (i.e., adaptive or maladaptive) potential adaptation to one stressor can affect phenotypic response under multiple stressors.

In sum, our results show that multiple stressors (i.e., exposure to contaminated environments and immune challenges) had only few interactive effects on fish traits. This suggests that potential adaptation to metal contamination did not result in specific costs (maladaptation, Brady et al. 2019) nor benefits (co-adaptation, Vinebrooke et al. 2004) to face an immune stressor, although combined effect with other stressors such as temperature or hypoxia cannot be excluded (see for instance, Gandar et al. 2017; Lavergne et al. 2015; Petitjean et al. 2019a).

Conclusion

Altogether, our results show that fish plastic responses depended on past exposure to contamination. More particularly, HC fish displayed higher survival, potentially explained by a higher detoxification ability and antioxidant capacity compared to their naïve LC counterparts. In addition, our results bring no clear evidence of a potential cost entailed by adaptive responses to contaminated environments, except a higher basal expression of a gene involved in apoptosis (i.e., Casp3), suggesting potential long-term consequences on cell integrity (Don Xavier et al. 2019; Petitjean et al. 2020c). Yet, our results do not support the hypothesis of co- or maladaptation to a secondary stressor (i.e., an immune challenge mimicking parasite attack), at least on a short-term timescale.

This study also outlines parallelism in plastic responses to contamination at different biological levels (i.e., survival, bioaccumulation and immunity, but not energy management), which suggests that contamination drives similar responses in independent replicate populations and at different biological levels (i.e., multidimensional plasticity, Westneat et al. 2019). However, this study focused on short-term plastic responses and physiological mechanisms; here, we did not explore potential genetic differences among populations. Hence, further long-term studies on F1 and F2 offspring and genomic studies are needed to test fitness consequences and decipher the underlying evolutionary processes explaining the observed population differences.

With this study, we hope to encourage future ecotoxicological studies to bridge the gap between molecular ecology, ecotoxicology, and evolutionary biology to unravel the complex effects of chronic contamination on wildlife

health and better predict wild population responses to current and future anthropogenic stressors.

Declarations Experimental procedures complied with French and European legislation for animal experimentation (European directive 2010/63/U.E.) and were conducted under the French animal handler's certificate (N° 31–103), the establishment approval for vertebrate experimentation N°A3113002, and were approved by the ethical committee n°073 (authorization n°8538). Fish capture was conducted under local authorizations (2018–0118, 300118, E-2018–15).

Consent to participate Not applicable.

Consent to publish All authors gave explicit approval to publish this version of the manuscript under the Creative Commons Attribution License 4.0 and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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