Direct and indirect effects of multiple environmental stressors on fish health in human-altered rivers

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HIGHLIGHTS

- Multiple environmental stressors can affect fish health at different biological levels.
- Parasitism and water temperature had limited effects on gudgeon health.
- Trace metal contamination indirectly increased liver lesions through over-inflammation.
- Combined contamination and temperature decreased fish growth.
- Multiple stressors have complex interactive effects on fish health in human-altered rivers.

GRAPHICAL ABSTRACT
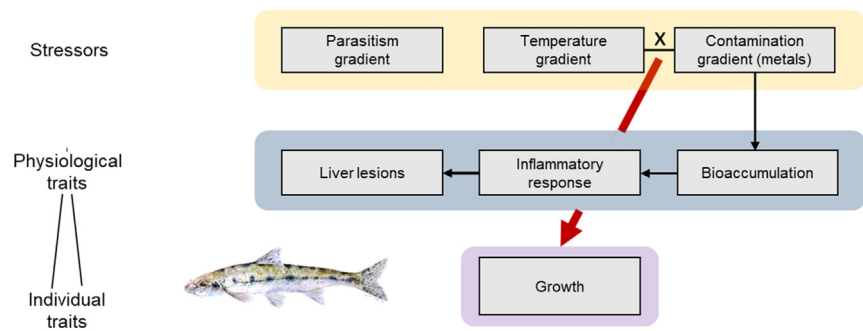
ABSTRACT

Freshwater fish face multiple challenges in human-altered rivers such as trace metal contamination, temperature increase and parasitism. These multiple stressors could have unexpected interactive effects on fish health due to shared physiological pathways, but few studies investigated this question in wild fish populations. In this study, we compared 16 populations of gudgeon (*Gobio occitaniae*) distributed along perturbation gradients in human-altered rivers in the South of France. We tested the effects of single and combined stressors (i.e., metal contamination, temperature, parasitism) on key traits linked to fish health across different biological levels using a Structural Equation Modelling approach. Parasitism and temperature alone had limited deleterious effects on fish health. In contrast, fish living in metal-contaminated sites had higher metal bioaccumulation and higher levels of cellular damage in the liver through the induction of an inflammatory response. In addition, temperature and contamination had interactive negative effects on growth. These results suggest that trace metal contamination has deleterious effects on fish health at environmentally realistic concentrations and that temperature can modulate the effects of trace metals on fish growth. With this study, we hope to encourage integrative approaches in realistic field conditions to better predict the effects of natural and anthropogenic stressors on aquatic organisms.

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1. Introduction

Freshwater ecosystems are increasingly exposed to multiple natural and anthropogenic stressors that could interact and cause unexpected "ecological surprises" (e.g., Christensen et al., 2006; Lange et al., 2018; Schinegger et al., 2016). However, the interactive effects of multiple stressors on fish physiology and health remain largely unknown, which limits our ability to predict the combined effects of environmental stressors on fish populations (Killen et al., 2013; Petitjean et al., 2019; Segner et al., 2014). In human-altered streams, trace metal contamination due to industrial activities and urban wastes, and water temperature increase due to climate change are major stressors affecting the aquatic fauna (Reid et al., 2019; Segner et al., 2014). In addition, parasites are biotic stressors that can affect the physiology (Buchmann and Lindenstrøm, 2002; Slavík et al., 2017; von Gersdorff Jørgensen, 2016) and life-history traits of their hosts (Britton et al., 2011; Šimková et al., 2008). Although the isolated effects of contamination, temperature and parasitism are well documented, their potential interactive effects on fish health remain largely unknown.

These three stressors affect similar physiological pathways, such as immunity, inflammation, and energy management (Dautremepuits et al., 2004; Dittmar et al., 2014; Gandar et al., 2017a), which could result in strong interactive effects. For instance, temperature and contamination can alter fish immunity and affect parasite resistance (i.e., the ability of fish to limit parasite intensity) (Franke et al., 2017; Paul et al., 2014; Sheath et al., 2016). Multiple stressors may also have complex interactive effects on detoxification processes and contaminants bioaccumulation, and trigger inflammatory responses, oxidative stress and cellular damage depending on the combination of stressors. For instance, high temperature can aggravate the effects of contamination by increasing fish metabolism and contaminants uptake (Guinot et al., 2012; Noyes et al., 2009; Sokolova and Lannig, 2008) and may result in higher oxidative stress and cellular lesions (Gandar et al., 2017b; Jacquin et al., 2019). In addition, the activation of costly immune and inflammatory responses under multiple stress is expected to affect energy management and deplete energy reserves (Gimbo et al., 2015; Marteinson et al., 2017; Martin et al., 2003), thereby affecting fish body condition and growth at the individual level (Johnson and Dick, 2001; Jokela and Mutikainen, 1995; Wernicke von Siebenthal et al., 2018).

Recently, environmental stressors have been shown to accelerate the attrition of telomeres, which are conserved tandem repeats of DNA, causing cell senescence when too short (Blackburn, 2005; Monaghan, 2010, 2014; Monaghan and Haussmann, 2006). Several stressors can affect telomere's length, such as contamination (Blévin et al., 2016; Louzon et al., 2019), temperature (Debes et al., 2016; Simide et al., 2016) and parasitism (Stauffer et al., 2017). However, the interactive effects of multiple stressors on telomere shortening are still unknown. More generally, the relative effects of stressors combinations on fish health and their underlying mechanisms are still unclear. Most previous studies investigated the interactive effects of multiple stressors using experimental approaches in controlled laboratory conditions (and usually with laboratory-reared animals) to control environmental variables and test causative links (Petitjean et al., 2019). However, such controlled approaches are sometimes far from realistic conditions (reviewed in Calisi and Bentley, 2009), which can change the outcomes of stress exposure, especially in a multistress framework. There is thus a growing need to investigate the effects of multiple stressors in realistic natural field conditions to better predict the consequences of anthropogenic stressors on wild fish populations.

In this study, we tested the effects of realistic gradients of trace metal contamination, temperature, and parasitism on fish physiological traits (i.e., metals bioaccumulation, available energy, immunity, cellular damage, telomere length), and their potential cascading effect on fish health (i.e., body condition, parasite intensity, growth) in 16 populations of gudgeon (*Gobio occitaniae*) from the Garonne watershed, South of France. We used Structural Equation Modelling (SEM) analyses to test hypotheses on direct and indirect effects of stressors by comparing several causal scenarios linking environmental factors and response traits at different biological levels (e.g., Blanchet et al., 2009; Fourtune et al., 2016), to decipher the multiple effects of combined anthropogenic stressors on wild fish populations.

2. Material and methods

2.1. Model species

The gudgeon (*Gobio occitaniae*) was chosen as a model species because it is widely distributed in South-West France and is exposed to several stress factors in its natural environment. This species is relatively sedentary and thereby exposed to the same environmental stressors during its lifetime (Keith et al., 2011; Stott et al., 1963). The gudgeon is a benthic species and is thus exposed to contaminants through sediments, water and food. Moreover, this species is exposed to a wide range of temperatures and parasites in the wild, especially gyrodactylids (Loot et al., 2007). Gyrodactylids are monogenean ectoparasites feeding on fish skin and fins with important effects on fish immunity and life-history traits (Bakke et al., 2007; Buchmann and Lindenstrøm, 2002). Previous studies showed a relatively high allelic richness and low genetic differentiation (Fst) between sites within our study area (Côte et al., unpublished; Fourtune et al., 2016), suggesting high gene flow among our study populations. Interestingly, gudgeon generally displays contrasted phenotypic responses to environmental variations among populations (Knapen et al., 2004; Shinn et al., 2015).

2.2. Sampling sites

We selected 16 study sites in the Garonne watershed (Fig. 1) along three environmental gradients (i.e., trace metal contamination, water temperature and parasite). Contamination levels and temperatures were calculated based on a dataset from the Adour-Garonne Water Agency (Table 1) from summer 2012 to summer 2016 (i.e., during 4 years, which corresponds to the lifetime duration of the gudgeon) (SIE, 2016). We first computed a contamination gradient using a Toxic Unit (TU) for trace metals elements based on previous studies (Kuzmanović et al., 2016; Sprague, 1970). For each study site, a TU was computed for 9 trace metal elements (i.e., aluminium-Al, arsenic-As, cobalt-Co, lead-Pb, zinc-Zn, nickel-Ni, cadmium-Cd, chromium-Cr, and copper-Cu) depending on their concentration and toxicity:

$$TU\,(\textbf{\textit{Toxic Unit}})_{site} = \log \sum_{i=1}^{n} \frac{C_i}{LC_{50}}$$

C_i corresponds to the average concentration of an element in the water column measured by the Water Agency between 2012 and 2016. LC_{50} corresponds to the lethal concentration for 50% of *Pimephales promelas* fish in an ecotoxicity bioassay (data from the ECOTOX database, USEPA, 2008). A higher TU thus reflects a higher metal toxic risk for fish. We completed this Water Agency dataset with direct measurements of trace metal concentrations in the water collected during fish sampling (see Table S1 for concentrations). Direct measurements of trace metals concentrations were indeed consistent with the Water Agency database. We also computed a TU for organic pollutants (i.e., pesticides, polycyclic aromatic hydrocarbons), but few organic pollutants were reliably detected. Also, most sites had similar and low TU values for organic pollutants. Consequently, the TU gradient of organic contamination was not included in the final analysis, and only trace metal contamination was taken into account in this study.

Temperature was recorded by the French water agency each month. We computed a gradient of water temperature using a PCA analysis with seasonal mean water temperature (mean of 3 months for each
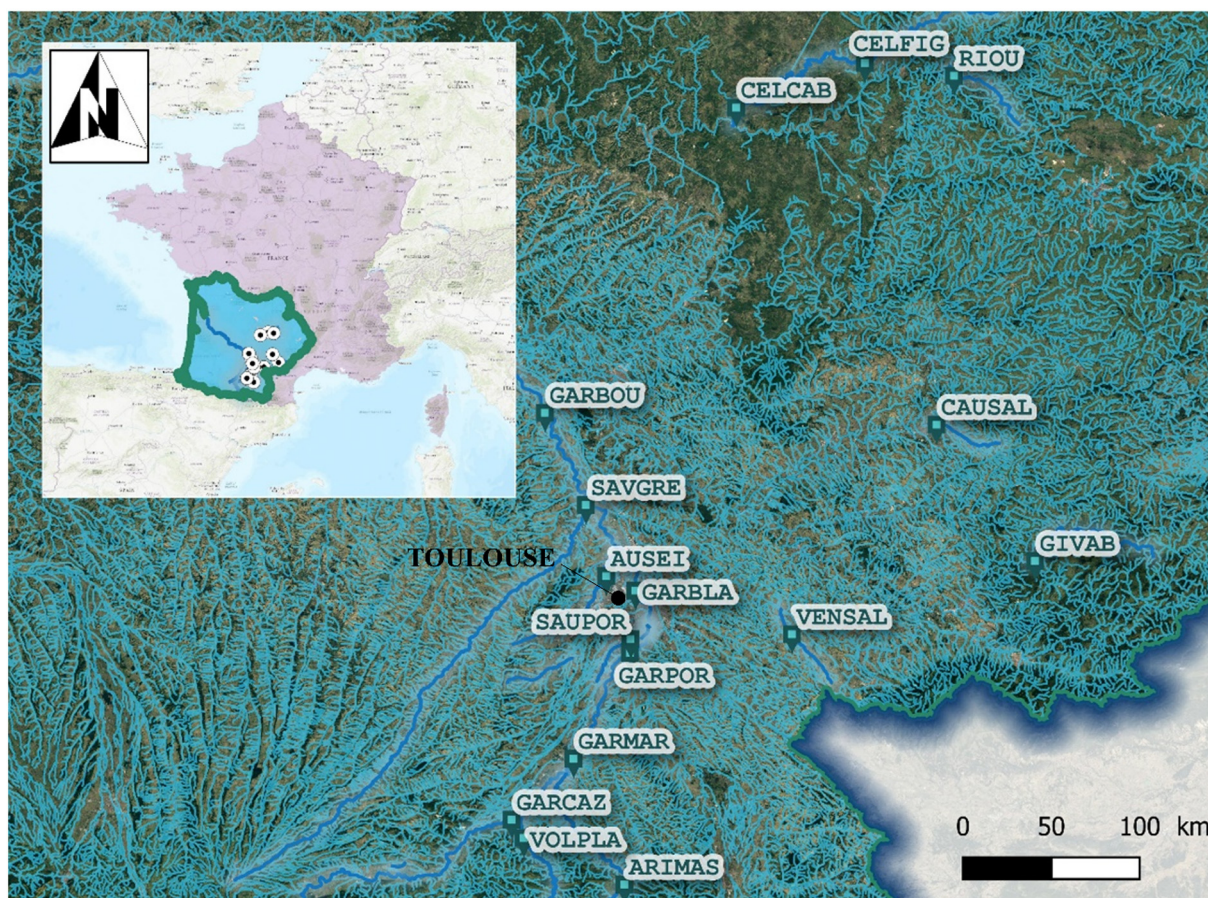
Fig. 1. Location of the 16 study sites in the Garonne watershed (South-West France).

season) and variation coefficients for each study site (see Table S2), to take into account both mean and variations of temperatures among sites. While the first PCA axis was mostly explained by the mean temperature across seasons (35.7% of explained variance), the second axis was mostly explained by the variations coefficient of temperature (30.1% of explained variance) (Fig. S1). Because the gradient of temperature variation (i.e., second PCA axis) did not have any effect on the traits measured in this study, we only reported the effects of the mean temperature gradient (i.e., first PCA axis) (Table 1) (e.g., min/max mean summer temperature: 14 °C/21 °C; min/max mean winter temperature: 6 °C/11 °C, see Petitjean, 2019 and Table S2 for more details).

We scanned all fish for ecto- and *endo*-parasites (skin, fin, gill, and gut), and the most prevalent and abundant parasite was *Gyrodactylus*

Table 1

Characteristics of study sites: location (Longitude and Latitude in Lambert 93 coordinates), number of fish used in statistical analysis and summary of the three environmental gradients. The contamination gradient corresponds to an integrative index of the toxic risk for fish (Toxic unit - TU) based on dissolved trace metals concentrations in the water. The temperature gradient corresponds to the mean water temperature (first PCA axis, see Fig. S1). The parasite prevalence was calculated as the mean Gyrodactylus prevalence per site (% of infected fish per site), reflecting the encounter probability between this socially transmitted parasite and its host in each site (i.e., risk of being infected). The Variance Inflation Factor (VIF) of the three predictors was below the threshold of 3, showing low collinearity among the three gradients (Zuur et al., 2010).

Sites	Stream name	Longitude	Latitude	Number of fish	Contamination gradient	Temperature gradient	Parasite prevalence
GIVAB	Le Gijou	652,983	6,287,090	16	−1.0	−3.5	52
VOLPLA	Le Volp	546,845	6,231,930	16	−0.9	−1.2	36.7
GARMAR	La Garonne	557,517	6,248,020	15	−0.9	−1.2	48.3
CELFIG	le Célé	619,393	6,388,240	19	−0.9	−1.7	46.7
ARIMAS	L'Arize	567,492	6,221,890	18	−0.9	−1.3	70.8
CELCAB	le Célé	593,305	6,379,640	15	−0.8	0.4	10
GARCAZ	La Garonne	544,488	6,235,740	27	−0.8	−1.7	10
GARPOR	La Garonne	569,591	6,269,450	3	−0.8	0.2	12.5
CAUSAL	Le Caussels	633,072	6,315,030	19	−0.7	−0.4	60.7
SAVGRE	La Save	561,198	6,299,830	7	−0.6	2.3	14.3
VENSAL	La Vendinelle	602,916	6,272,670	11	−0.6	−1.1	30
GARBLA	La Garonne	570,981	6,282,179	17	−0.5	0.2	40
SAUPOR	La Saudrune	569,799	6,272,239	5	−0.3	3.7	66.7
GARBOU	La Garonne	553,270	6,318,800	7	−0.3	0.6	20
AUSEI	L'Aussonnelle	565,033	6,285,220	8	−0.2	2.3	73.3
RIOU	Le Riou Mort	637,385	6,385,460	15	1.3	−0.3	86.7

sp., which was consistent with previous studies (Loot et al., 2007). We thus focused our study on this parasite. The mean *Gyrodactylus* prevalence per site (i.e., percentage of infected fish in a population) and parasite intensity (i.e., number of the parasite on each infected fish) were assessed by thoroughly scanning fins under a dissecting microscope (x10) (e.g., Jacquin et al., 2016).

We used the mean parasite prevalence (i.e., percentage of infected fish per site) to assess a gradient of parasitism within each study site (i.e., risk of being infected) (Table 1). Indeed, gyrodactylids are socially transmitted parasites (Bakke et al., 2007) that are transmitted through fish to fish contacts, so that the risk of being infected depends partly on the proportion of infected fish in the population (i.e., the mean parasite prevalence in each site) (Patterson and Ruckstuhl, 2013; Poulin, 2006; Sol et al., 2003). We also counted the number of parasites on each infected fish to assess parasite resistance (i.e., the ability of the host to limit parasite proliferation), which partly depends on fish immunity (Cable and van Oosterhout, 2007; López, 1998; van der Oost et al., 2003).

We checked that the mean parasite prevalence per site was stable across years (2016 and 2017) in a subset of four study sites (ANOVA: effect of the year: F = 1.60, p = 0.21), as shown in previous studies (Gotanda et al., 2013), showing that mean gyrodactylids prevalence is generally consistent across years and could be a reliable relative measure of risk of being infected among sites, although seasonal variations probably occur. To check the multicollinearity among the three selected gradients (i.e., contamination, temperature, parasitism), we computed a Variance Inflation Factor (VIF) as recommended by Zuur et al. (2010). The VIF was below the threshold of 3 for each stressor, suggesting that collinearity was low and that the estimate for each environmental gradient could be reliably assessed (Zuur et al., 2010). Because other environmental variables could also affect fish health, we computed an index with potential physico-chemical confounding variables in a PCA gathering pH, dissolved O_2 concentrations, suspended organic matter, conductivity, total organic carbon, total phosphorus, nitrates, nitrites and ammonium. The first axis, explained 63.2% of the variance (see Fig. S2), and was included as a physico-chemical covariate in all statistical models, and removed when non-significant.

2.3. Sample collection

A total of 440 Fish were sampled by electrofishing (EFKO-FEG 1500). All fish were weighed ($\pm$0.1 g) and measured ($\pm$0.1 cm). Physiological assays were conducted on a subset of 3 to 27 fish per study sites depending on the trait considered (i.e., total sampling size $n = 218$, see Table 1) after euthanasia using anesthetic overdose (Eugenol, 1 mL.L^{-1}) during spring (May–June). These fish were dissected for sex determination and their liver was weighed ($\pm$1 mg) to calculate hepatosomatic index. For some individuals, organs were too small to measure some traits, so that sample size can vary depending on the physiological trait considered. The fish muscle was aliquoted in two parts, which were kept at -20 °C for subsequent measurement of trace metal bioaccumulation and available energy. Fish scales were collected to determine the age and growth of each individual during its first year of life. Pelvic fins were collected and stored at -20 °C to measure telomere length in somatic tissues. Blood samples were collected from the caudal vein in heparinized syringes (1 mL Terumo syringe, 0.45 × 13 mm needle) to measure immunity.

2.4. Physiological trait: bioaccumulation of trace metals

The first part of fish 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)

using hot plates. We assessed the reliability of the analyses using TORT-3 (lobster hepatopancreas) certified reference material. Concentrations recovery for the 9 metals considered (i.e., As, Al, Co, Pb, Zn, Ni, Cd, Cr, Cu) reached an average of 94 $\pm$ 6% with a minimum value of 85 $\pm$ 12% and a maximum value of 104 $\pm$ 8% for As and Cr respectively (for more details see Table S3). Concentrations of the 9 trace metal elements in fish muscle (see Table S3) were centered, scaled and summed. Finally, the summed concentrations were log-transformed to calculate a bioaccumulation index.

2.5. Physiological trait: available energy

The second part of fish muscle was used to assess the available energy as the sum of total carbohydrate, lipid and protein contents in fish muscle following previous studies (Gandar et al., 2016, 2017a; Smolders et al., 2004). The amount of available energy in muscle cells is a useful marker of energy status, because it is critical for the maintenance of biological functions of fish, especially under stress (Gandar, 2015). We used the protocol from De Coen and Janssen (1997) modified by (Gandar et al., 2017a). 25 mg of fish muscle were homogenized in 1 mL of ultrapure water using Fastprep®-24 homogenizer. Muscle suspension was divided into 2 aliquots. The first aliquot was mixed with trichloroacetic acid (TCA) and incubated -20 °C. Then, the sample was centrifuged to split up the supernatant and the pellets. 50 µL of the supernatant was read at 492 nm against glucose in TCA using CLARIOstar® (BMG LABTECH) for the determination of total carbohydrate content (µg.mg^{-1} muscle wet weight). The pellet was resuspended in a sodium hydroxide solution (NaOH, 1 M) and mixed with Bradford reagent before reading the absorbance in triplicate at 595 nm against bovine serum albumin in 0,2 M NaOH for total protein concentration determination (µg.mg^{-1} muscle wet weight). The second aliquot was mixed with chloroform (CHCl$_3$), methanol (CH$_3$OH) and sulfuric acid (H$_2$SO$_4$) and centrifuged. H$_2$SO$_4$ was added to the bottom phase, and vanillin-phosphoric acid reagent was added before reading the absorbance in triplicate at 525 nm against tripalmitin in chloroform for total lipid concentration determination (µg.mg^{-1} muscle wet weight). Concentrations of carbohydrates, proteins and lipids were then transformed to 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.

2.6. Physiological trait: immunity

The Neutrophils/Lymphocytes ratio (N/L inflammatory immune ratio) was used as a marker of inflammatory immune response: the number of neutrophils increases and lymphocytes decreases under both stress and infection (reviewed in Davis et al., 2008). Blood smears were made immediately after blood collection and stained with May-Grunwald (Sigma-Aldrich, 1:2 v:v) and Giemsa (Sigma-Aldrich, 1:20 v:v) stains. 100 leucocytes (monocytes, neutrophils and lymphocytes) cells were then numbered after identification (see Table S4 for identification criteria) under an Axioskop microscope (Zeiss, Germany, x40 magnification) to assess the relative abundance of each cell type. We then calculated a neutrophils/lymphocytes (N/L) ratio as a marker of inflammatory immune response.

2.7. Physiological trait: liver lesions

Liver lesions were quantified by standard histological techniques following previous studies in fish (Cuevas et al., 2016; Jacquin et al., 2019; van Dyk et al., 2007). Briefly, the liver was dissected, immediately fixed in 4% formalin, and then paraffin-embedded. 4 µm thick sections were stained with hematoxylin, eosin saffron and observed under a Nikon microscope (Eclipse Ni) equipped with a digital camera (DS-Ri1).
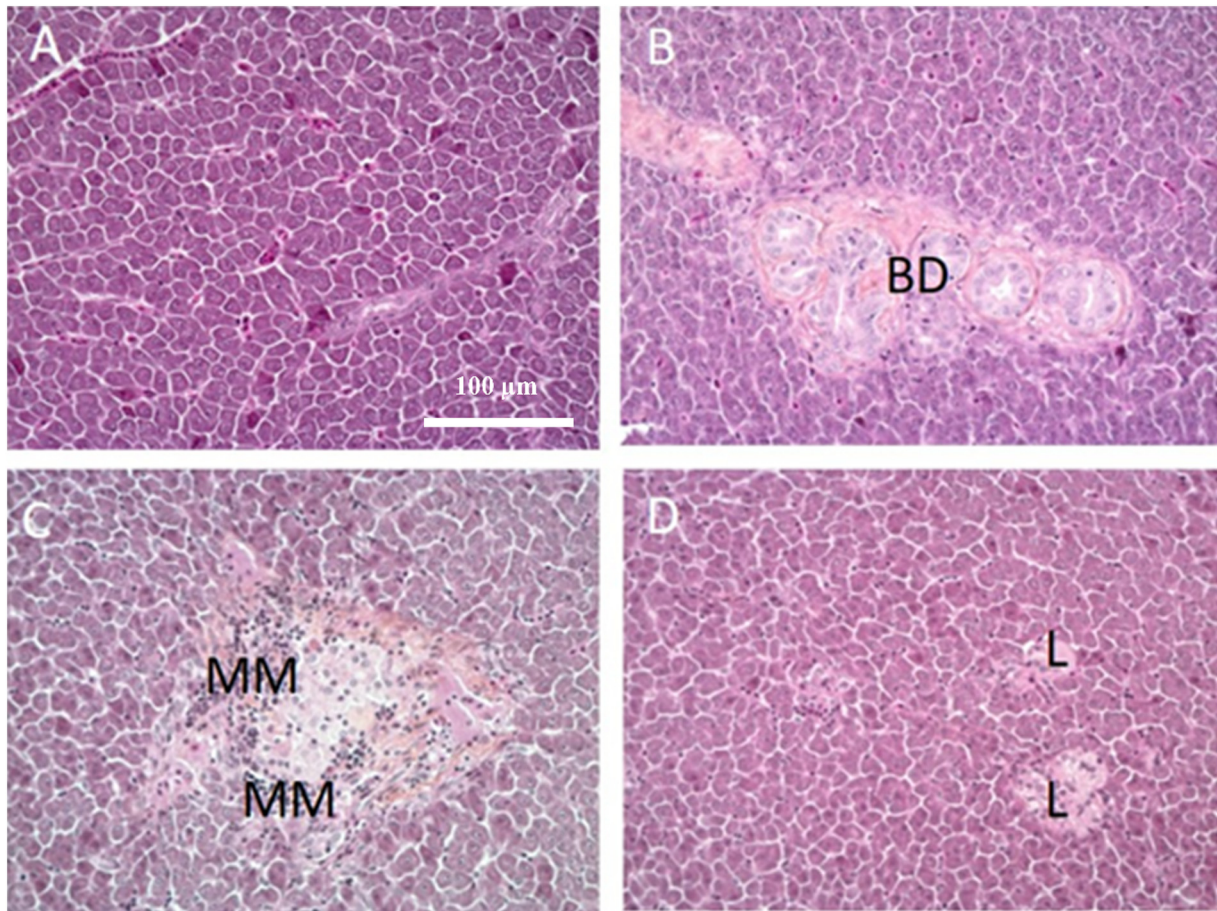
Fig. 2. Histopathological observations of gudgeon livers. A. Normal liver parenchyma with bile ducts. B. Proliferating bile ducts with surrounding fibrosis (BD). C. Focal MelanoMacrophage hyperplasia reflecting an over-inflammation (MM). D. Focal hepatocellular necrosis with altered nuclear morphology and clear cytoplasm reflecting cell apoptosis (L). For each liver elementary lesion, a score between 0 and 3 was determined according to the severity of the lesion. A composite global histopathological score was then calculated as the mean value of the different scores as already published (Jacquin et al., 2019). The scale bar represents 100 μm.

Three main hepatic lesions were recorded in liver sections: the proliferation of bile ducts and fibrosis reflecting liver hyperplasia (Fig. 2B), melano-macrophage aggregates reflecting an over-inflammation of hepatocytes (Fig. 2C), lysis and nuclear alterations reflecting cell apoptosis (Fig. 2D) (see Wolf and Wheeler, 2018). For each hepatic lesion, a score between 0 and 3 was associated according to the severity of the lesion. A global histopathological score reflecting the severity of the lesions was then calculated as the mean value of the different scores following previous studies (Jacquin et al., 2019).

2.8. Individual trait: parasite intensity

Gyrodactylus sp. parasite intensity (i.e., number of parasites on each infected fish) were assessed by thoroughly scanning fins under a dissecting microscope (x10) (e.g., Jacquin et al., 2016). Infection by the socially transmitted *Gyrodactylus* sp. ectoparasite mostly occurs through fish to fish contacts (Bakke et al., 2007; Richards et al., 2010). We thus used the mean parasite prevalence per site (i.e., the percentage of infected fish in each study site) as a proxy of the risk of being infected. In contrast, we used the parasite intensity in infected fish (i.e., the number of parasites per infected fish) as an index of parasite resistance (i.e., fish ability to control the infection through the immune system) (Cable and Van Oosterhout, 2007; López, 1998; van der Oost et al., 2003).

2.9. Individual trait: growth

We measured fish growth rate during their first year of life from scales using the back-calculation method reflecting a scale-proportional hypothesis described by Hile (1941):

$$\text{Growth rate} = -\left(\frac{a}{b}\right) + \left\langle L + \left(\frac{a}{b}\right) \right\rangle \times \left(\frac{R1}{Rtot}\right)$$

with a: the slope and b: the intercept of the linear regression between the size of the fish (L) and the radius of the whole scale (Rtot). R1 corresponds to the length of the radius between the nucleus and the first annuli of the scale.

2.10. Individual trait: condition indices

Two condition indices were calculated to measure the general condition of fish. First, we calculated the Fulton Condition Factor (FCF, fish mass corrected by its cubic length) as an index of general body condition that reflects the general energy reserves of the fish (Smolders et al., 2002). Second, the hepatosomatic index (HSI, liver mass corrected by the body mass x100) was measured as a proxy of energy content in the liver (Chellappa et al., 1995).

2.11. Individual trait: telomere length

Telomere length is increasingly used as an integrative marker of stress exposure in wild animals (Angelier et al., 2018; Bize et al., 2009; Haussmann and Marchetto, 2010; Monaghan, 2014). Briefly, telomere length was determined by quantitative PCR using a BioRad CFX 96 (Bio-Rad Laboratories, Hercules, California, USA) according to Cawthon (2002) with minor modifications. We measured telomere length in pelvic fins since this somatic tissue is the main target of gyrodactylids ectoparasites, which are known to feed on it (Bakke et al., 2007). Briefly, pelvic fin samples were digested with proteinase K and DNA was extracted from pelvic fins using the DNeasy Blood and tissue kit (Qiagen). The universal telomere primers designed by Cawthon (2002) were used for amplification of the telomere repeats. The control single-copy gene Recombination Activating Gene 1 (RAG1) was selected and amplified using specific primers (McLennan et al., 2019) designed for the gudgeon (RAG1-F: 5′-AGAGAGAGGGG GCTAGATGA-3′ and RAG1-R: 5′-CCATGCTTCTCGCTGACATCAGCAGC AGCCTTCA-3′). qPCR was then performed using 2.5 ng of DNA per reaction. The universal telomere primers designed by Cawthon (2002) were used at a concentration of 800 nM, and primers RAG1-F/RAG1-R at 300 nM. All samples were randomly distributed across the PCR plates. Amplification efficiencies reached Mean $\pm$ SE: RAG1, 96.97 $\pm$ 0.78; TEL, 99.60 $\pm$ 2.05, and the average inter-plate variation of the Ct values was 1.63 for the telomere assay and 0.74 for the RAG-1 assay, respectively. Telomere length (T/S ratio) was expressed relative to the internal single gene control (RAG1), according to Cawthon (2002).

2.12. Statistics

We used a three-step approach to test the effects of multiple stressors on fish health. First, we used preliminary linear mixed-effects models (LMM) to select the main stressor combinations affecting fish health, i.e., select the significant double interactions between contamination, temperature and mean parasite prevalence on each fish trait (i.e., bioaccumulation, available energy, N/L inflammatory immune ratio, liver lesions, telomere length, parasite intensity, condition indices, growth). All response variables were log-transformed and centered and scaled to meet Gaussian distribution. The sampling site was included as a random effect. Fish size (a proxy of fish age: correlation size/age: $R^2 = 0.76$, $p < 0.001$) was added as a covariate because it can strongly affect physiological responses and bioaccumulation in fish. The physico-chemical gradient (first PCA axis) and sex were also added as covariates. Best models were selected by top-down model selection according to the Akaike Information Criterion (AIC) (see Table 2). When ΔAIC was below or equal to 2, we retained the simplest model. Before model fitting, we checked the collinearity of the predictors using the VIFs and a threshold value of 3 and found low collinearity between gradients (Zuur et al., 2010). When interactions between paired stressors were significant, we made posthoc analyses by splitting the dataset into two parts based on the median value of one of the predictors involved in the interaction and running LMM on both separate datasets.

Second, we investigated the direct and indirect effects of these selected stressor combinations on fish health using Structural Equation Modelling (SEM). SEM are probabilistic models that unite multiple predictors and response variables in a single complex causal network to test different scenarios involving causal relationships (Bollen, 1989; Lefcheck, 2016; Shipley, 2016). This property of SEM makes them more adequate to describe a system (i.e., direct and indirect effects) and predict causal relationships compared to conventional univariate analyses (Grace, 2008). We constructed SEM models from the R package PiecewiseSEM, which implements models with random effects (Lefcheck, 2016). We built SEM networks based a priori on existing literature and knowledge about the effects of stressors (i.e., predictors) on each trait. We also included all significant double interactions between stressors retained in the first statistical LMM approach (see above). Best SEM models were then selected according to AIC. When ΔAIC was below or equal to 2, we applied the parsimony principle and retained

Table 2
Best final linear mixed models (preliminary step) explaining fish traits along stressor gradients according to the Akaike Information Criterion (AIC). When ΔAIC was below or equal to 2, we retained the simplest model. For the effect of the contamination gradient metal on metal bioaccumulation, the reported effect corresponds to the interaction between the contamination gradient and fish size.

Trait	Statistics	Variables					
		Contamination gradient	Temperature gradient	Parasite prevalence	Size	Sex	Contamination: temperature gradient
Bioaccumulation	Estimate $\pm$ SE	0.188 $\pm$ 0.077	–	–	−0.110 $\pm$ 0.093	–	–
$n = 197$	t	2.43	–	–	−1.18	–	–
	p	**0.015**	–	–	0.238	–	–
Available energy	Estimate $\pm$ SE	0.434 $\pm$ 0.117	–	–	−0.268 $\pm$ 0.073	–	–
$n = 197$	t	3.71	–	–	−3.69	–	–
	p	**<0.001**	–	–	**<0.001**	–	–
N/L immune ratio	Estimate $\pm$ SE	–	–	0.213 $\pm$ 0.072	0.174 $\pm$ 0.071	−0.350 $\pm$ 0.105	–
$n = 161$	t	–	–	2.97	2.43	−3.34	–
	P	–	–	**0.003**	**0.015**	**<0.001**	–
Liver lesions	Estimate $\pm$ SE	–	–	–	0.248 $\pm$ 0.011	–	–
$n = 114$	t	–	–	–	2.35	–	–
	p	–	–	–	**0.019**	–	–
Infection intensity	Estimate $\pm$ SE	–	–	0.163 $\pm$ 0.061	–	–	–
$n = 82$	t	–	–	2.66	–	–	–
	p	–	–	**0.008**	–	–	–
Growth	Estimate $\pm$ SE	−0.252 $\pm$ 0.155	−0.116 $\pm$ 0.209	0.367 $\pm$ 0.159	0.372 $\pm$ 0.053	–	−0.770 $\pm$ 0.301
$n = 197$	t	−1.63	−0.56	2.31	7.08	–	−2.56
	p	0.103	0.578	**0.021**	**<0.001**	–	**0.010**
Body condition	Estimate $\pm$ SE	–	0.270 $\pm$ 0.128	–	–	−0.405 $\pm$ 0.130	–
$n = 168$	t	–	2.10	–	–	−3.12	–
	p	–	**0.035**	–	–	**0.002**	–
Hepato-somatic Index	Estimate $\pm$ SE	–	–	–	−0.197 $\pm$ 0.122	−0.827 $\pm$ 0.180	–
$n = 123$	t	–	–	–	1.62	−4.59	–
	p	–	–	–	0.106	**<0.001**	–
Telomere length	Estimate $\pm$ SE	–	–	–	–	–	–
$n = 115$	t	–	–	–	–	–	–
	p	–	–	–	–	–	–

the most straightforward model. Because models were qualitatively similar with or without sex and physicochemical variables, we present only the simplest models without these variables in the results. Because sample sizes vary for each trait, we conducted separate SEM models for each response trait at the individual level to obtain the most reliable estimate for each relationship. We thus present a different SEM model per response trait where each link is characterized by an unstandardized estimate, which reflects the expected (linear) change in the response with each unit change in the predictor and p-value. Sample sizes are smaller for liver samples ($n = 113$) because we screened cellular lesions in the liver of a subset of individuals so that a separate SEM is presented on the liver (liver lesions). The accuracy of models was finally assessed using the goodness of fit, which is a test of directed separation available with the summary function of the R package PiecewiseSEM. Briefly, when a p-value > 0.05, the fit was considered as correct.

Third, we computed total, direct and indirect effects from the unstandardized estimates given by the SEM models on traits at the individual level. The indirect effects of each stressor on individual traits were computed through each physiological traits (i.e., indirect pathway in the full model) according to the Sobel product of coefficients approach (Sobel, 1982). To test the significance of the effects (direct and indirect), we used the error propagation method and computed the 95% confidence interval of indirect and total effects from the standard errors of each path given in the summary statistics of the considered SEM.

3. Results

3.1. Effects of stressors on parasite intensity

First, preliminary LMM shows that parasite intensity in infected fish (i.e., number of parasites per infected fish, reflecting fish ability to limit parasite proliferation) was not linked to any stressor gradient, excepted the mean parasite prevalence (i.e., the proportion of infected fish in the study site) (Table 2).

SEM analyses (goodness of fit: 0.414, CFI = 18.652, df = 18, n = 82) were consistent and showed a higher number of parasites per infected fish in sites with higher mean parasite prevalence (estimate: 0.33, p = 0.023) (see Fig. S3). However, we did not find any significant relationship between immunity or any physiological traits and parasite

intensity (Fig. S3). Thus, this effect was mainly direct and no underlying physiological mechanism could be detected in our study (Fig. 5A).
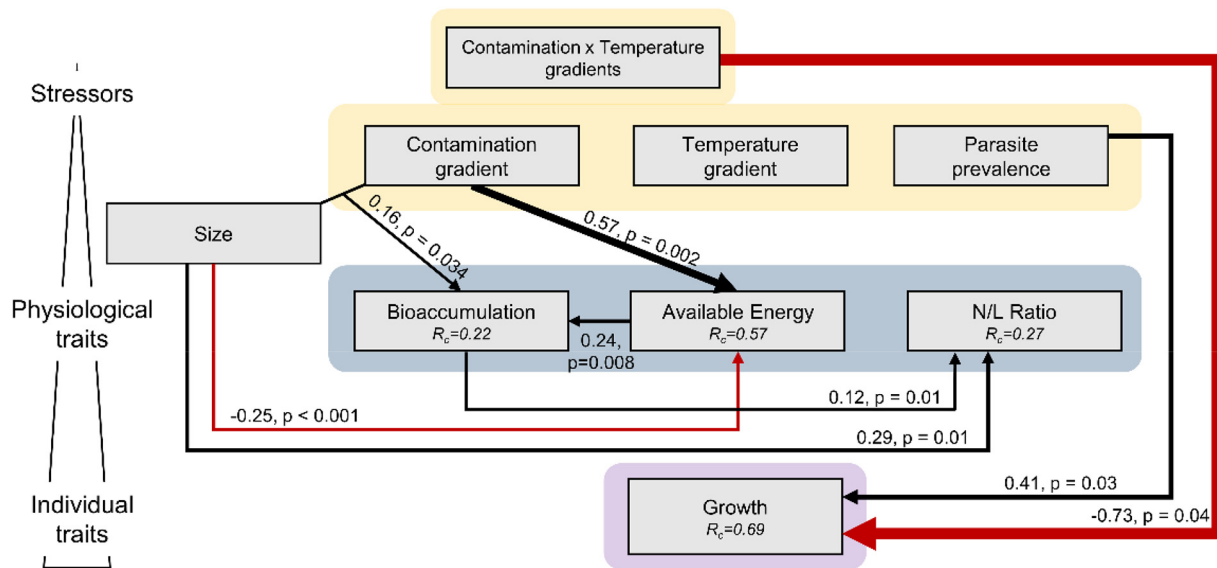
3.2. Effects of stressors on growth

Preliminary LMM shows that growth was significantly affected by fish size, mean parasite prevalence and the interaction between temperature and contamination (Table 2). Indeed, fish growth was higher in locations with higher mean parasite prevalence (estimate: 0.41, p = 0.03, Fig. 3) but was not affected by parasite intensity (LMM: estimate = 0.04, $t = 0.66$, p = 0.81). In addition, posthoc analyses show that fish growth decreased significantly with contamination in warmer sites (LMM estimate: -0.41, F = 4.5, p = 0.03) but not in colder sites (LMM estimate: 3.54, F = 1.4, p = 0.23).

SEM analyses (goodness of fit: p = 0.907, CFI = 2.136, df = 6, $n = 197$) were consistent, we above described results but we did not detect any significant relationships between growth and any physiological traits (Fig. 3). Thus, we found only direct effects of stressors on growth related to the mean parasite prevalence and to the contamination: temperature interaction (Fig. 5B).

However, here some physiological traits were directly affected by stressors, especially contamination (Fig. 3). First, fish from more contaminated sites (i.e., higher TU) had more available energy in their muscle (estimate: 0.57, p = 0.002, Fig. 3), and small fish had less available energy in their muscle (effect of size: estimate −0.25, p < 0.001, Fig. 3).

Second, the bioaccumulation of metals in muscle was positively affected by the interaction between fish size and the contamination gradient (estimate: 0.16, p = 0.034, Fig. 3): in more contaminated sites bigger fish bioaccumulated more metals in their muscle (LMM estimate: 0.17, F = 8.9, p = 0.004) compared to smaller fish (LMM estimate: -0.17, F = 1.0, p = 0.332). In addition, the amount of available energy had a positive effect on metal bioaccumulation (estimate: 0.24, p = 0.008, Fig. 3): fish having more available energy in their muscle also bioaccumulated more metals in their muscle. More particularly, by fitting the same SEM structure on lipid, protein and carbohydrate content independently, we found that lipid content explained this pattern (lipid: estimate: 0.27, p = 0.004, protein: estimate: −0.10, p = 0.163, carbohydrate: estimate: 0.07, p = 0.446). In other words, fish with more lipids in their muscles also bioaccumulated more metals.

Fig. 3. Structural Equation Models (SEM) testing the effects of stressor gradients on growth and underlying physiological traits (goodness of fit: p = 0.907, CFI = 2.136, df = 6, n = 197). Significant links are represented by solid arrows (red: negative relationship, black: positive relationship). Unstandardized coefficient and p-values are given alongside arrows. Line width represents the strength of the relationship (unstandardized coefficient). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
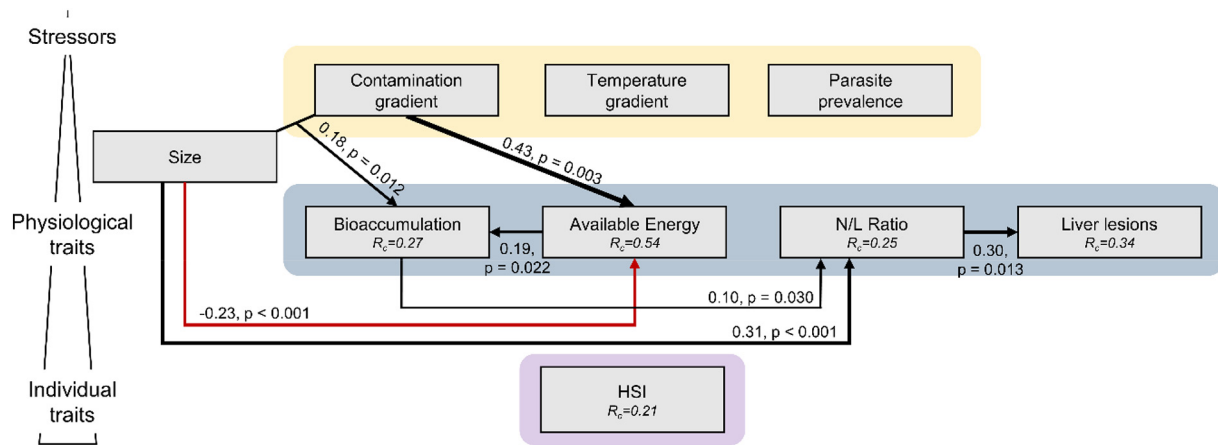
Fig. 4. Structural Equation Models (SEM) testing the effects of stressors on the liver HepatoSomatic Index (HSI) and underlying physiological traits (goodness of fit: p = 0.690, CFI = 14.592, df = 18, n = 113). The sample size for this analysis is $n = 113$ because histopathological analyses on the liver were conducted on a subset of individuals. We thuspresent here a separate SEM model on liver variables. Significant links are represented by solid arrows (red: negative relationship, black: positive relationship). Unstandardized coefficient and p-values are given alongside arrows. Line width represents the strength of the relationship (unstandardized coefficient). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Third, bigger fish (estimate: 0.29, p = 0.01, Fig. 3) and fish with more metals in their muscle (estimate: 0.12, p = 0.01, Fig. 3) had a higher N/L inflammatory immune ratio. More particularly, by fitting the same SEM structure on neutrophils and lymphocytes counts independently, we found that metal bioaccumulation significantly increased neutrophils counts (estimate: 0.73, p = 0.01) while it did not significantly affect lymphocytes count (estimate: −0.72, p = 0.09).

3.3. Effects of stressors on body condition

Preliminary LMM shows that body condition was significantly affected by the temperature gradient only (Table 2). SEM analyses (goodness of fit: p = 0.888, CFI = 1.142, df = 4, $n = 218$) show that fish from warmer sites had a higher body condition (estimate: 0.37, p = 0.04). However, we did not find any significant relationships between body condition and any physiological traits. Thus, temperature had only direct and positive effects on fish body condition (Fig. 5C).

3.4. Effects of stressors on the liver

Preliminary Mixed Models show that the Hepatosomatic Index (i.e., HSI, relative liver mass) was not affected by any stressor (Table 2). Since we screened cellular lesions in the liver of a subset of individuals, we represented a separate SEM on this subset of individuals to infer potential indirect relationships between stressors and the liver HSI through physiological changes. SEM analyses (goodness of fit: p = 0.690, CFI = 14.592, df = 18, $n = 113$) show that the HSI was not significantly affected by stressor gradients nor by physiological traits (Fig. 4) neither directly nor indirectly (Fig. 5D).

In contrast, we found that the histopathological index (i.e., index of cellular lesions in the liver) was positively linked to the N/L inflammatory immune ratio (estimate: 0.30, p = 0.013, Fig. 4). We also fitted the same SEM statistical models on each lesion separately, i.e., proliferating bile ducts with surrounding fibrosis (BD on Fig. 2), MelanoMacrophage hyperplasia (MM on Fig. 2), and cell apoptosis (L on Fig. 2). We found that biliary alterations (i.e., proliferating bile ducts) and surrounding hepatic fibrosis mostly contributed to the observed pattern (BD: estimate: 0.26, p = 0.026, MM: estimate: 0.02, p = 0.886, L: estimate: 0.02, p = 0.886). In other words, fish from more contaminated sites had higher metal bioaccumulation in their muscle (estimate: 0.18, p = 0.012, Fig. 4), higher N/L inflammatory immune ratio (estimate: 0.10, p = 0.030, Fig. 4), and more severe cellular lesions in their liver, especially a proliferation of bile duct and fibrosis (Fig. 4).

3.5. Effects of stressors on telomere length

LMM (Table 2) and SEM analyses show that telomeres length was not affected, either directly or indirectly, by any stressor gradient (goodness of fit: p = 0.525, CFI = 11.05, df = 12, $n = 107$) (Fig. 5E).

4. Discussion

Different environmental stressors affected different traits related to fish physiology and health. First, parasitism risk had limited deleterious effects and temperature had positive effects on fish body condition, while trace metal contamination appeared to trigger a cascade of physiological changes, which seems to contribute to liver lesions. In addition, the combination of contamination and elevated temperature reduced fish growth. These results highlight the importance of studying the effects of multiple stressors across biological levels.

4.1. Effects of parasitism

The mean parasite prevalence (i.e., the proportion of infected fish in each site) had limited effects on fish health. In addition, contamination and temperature gradients did not affect mean parasite prevalence nor parasite intensity, contrary to previous studies (Marcogliese, 2016; Poulin, 1992; Sures et al., 2017). This suggests that the ranges of our perturbation gradients were limited and did not affect *Gyrodactylus* parasites or that we could not detect any effect with our sampling design, but further studies are needed to confirm these hypotheses.

We found that infected fish living in sites with higher mean prevalence of *Gyrodactylus* (i.e., sites with a higher infection risk) also had higher parasite intensity (i.e., a higher number of parasites per infected individual) (Poulin and Guégan, 2000). In other words, The higher the proportion of infected hosts, the higher the parasite intensity (i.e., the number of parasites per infected host) suggesting either that these sites harbor more virulent parasites and/or that fish sampled here have a weaker immune system, and thus a lower ability to limit the proliferation of parasites. However, we found no relationship between immune or physiological parameters and parasite intensity, so that the underlying physiological mechanisms remain unknown. Several explanations could explain this result. First, parasite intensity was relatively low (i.e., 0 to 14 parasites per fish) compared to other studies (up to 100 parasites per fish, Van Oosterhout et al., 2006), which could explain the absence of link with physiological changes. Alternatively, parasite intensity could vary across seasons, and/or heavily infected fish
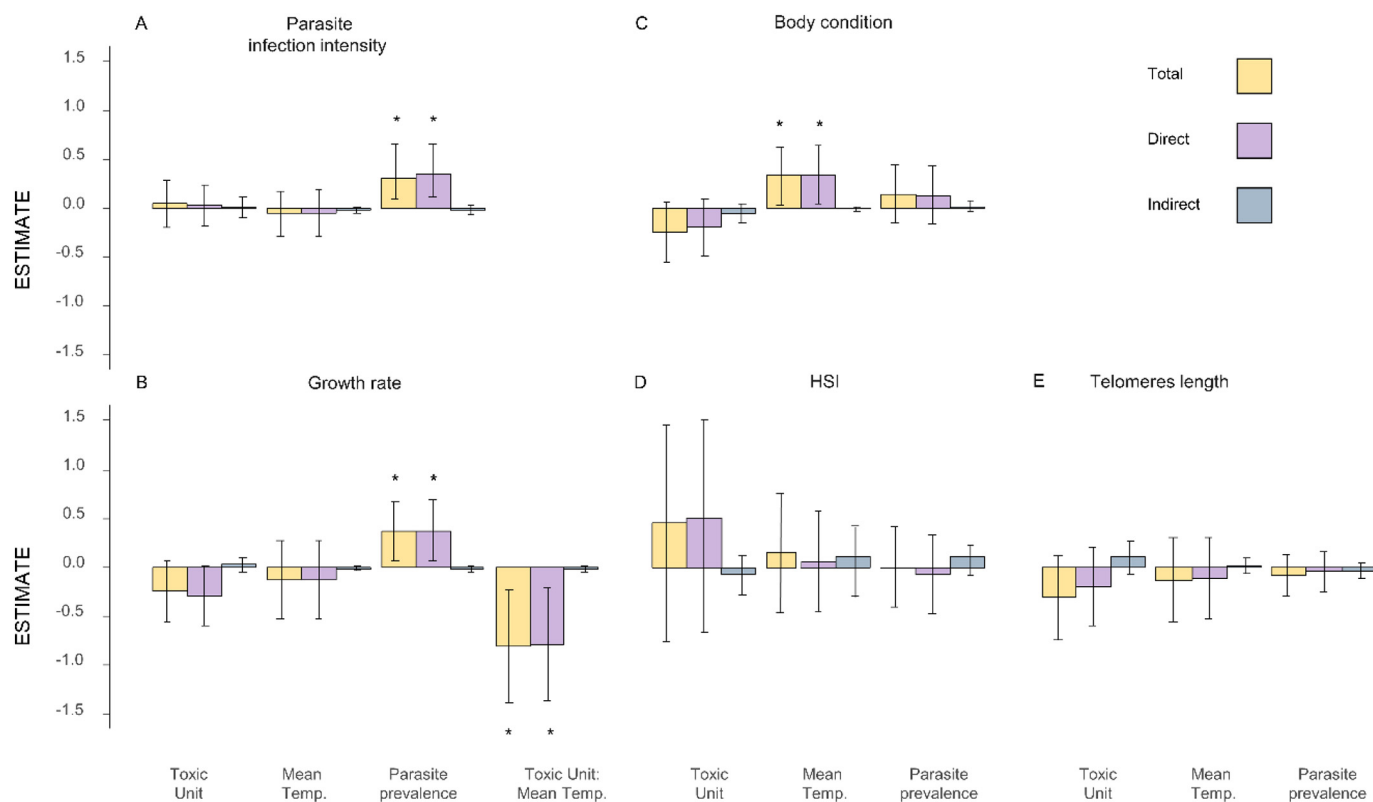
Fig. 5. Total, direct and indirect effects of stressors on, infection intensity ($n = 82$, A), growth rate ($n = 197$, B), body condition index ($n = 218$, C), HSI ($n = 113$, D) and telomere length ($n = 107$, E). Total effects are represented by a yellow bar (sum of direct and indirect effects). Direct effects are represented by a pink bar (estimate of the relationship between the stressor and measured trait at the individual level). Indirect effects through physiological traits are represented by a blue bar (sum of the products of path coefficients along all indirect physiological pathways). Error bars correspond to the 95% confidence interval computed by the standard error propagation method. Stars indicate effects that are significantly different from zero. Here, we did not find any indirect effects of stressors on individual levels across fish physiology. Indeed, when stressors had significant (total) effects on traits at the individual level, it was exclusively due to direct effects. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

could have been counter-selected in the wild and not sampled in this study (Cable and van Oosterhout, 2007; van Oosterhout et al., 2007). Also, in our study, the mean parasite prevalence (i.e., risk of being infected) was assessed through the mean parasite prevalence per site (i.e., percentage of infected fish in each site) because *Gyrodactylus* are socially transmitted parasites. However, some other unmeasured parameters such as parasite density in the water column/sediment, fish behavior, habitat structure might also affect infection risk, so that further studies might be needed to assess more precisely parasitism risk (McCallum et al., 2017).

Interestingly, we found that fish living in sites with a higher mean parasite prevalence had a faster growth during the first year of their life. One potential explanation is that habitats that are favorable for parasites are also beneficial for fish growth, for instance linked to higher food availability (Atalabi et al., 2018). Alternatively, high mean parasite prevalence (i.e., higher risk of being infected) could select for faster life-history strategies and a faster growth rate to cope with stressful environments (Kortet et al., 2010), but further longitudinal studies across seasons and life stages are now needed to test this hypothesis.

4.2. Effects of temperature gradient

The temperature gradient alone did not negatively affect any physiological nor individual trait in our study. On the contrary, fish living in warmer sites had a higher body condition. This might be explained by the moderate range of the temperature gradient found in our study sites (min/max mean summer temperature: 14 °C/21 °C) that was within the optimum temperature of gudgeon (i.e., critical limit temperature: 27 °C, Tissot and Souchon, 2010). In addition, increased mean

water temperature could enhance secondary production (Benke, 1998; Reynolds and Benke, 2005), which could in turn increase food availability for gudgeon. But, temperature may also sharply rise above the optimal temperature, for instance during summer heatwaves and could adversely affect fish health through physiological changes (Kassahn et al., 2009; Pörtner, 2010; Pörtner and Farrell, 2008). However, we could not test this hypothesis in our study because the resolution of the temperature records was too low to assess the effects of rapid temperature changes on fish physiology and health and further field studies using temperature dataloggers are now necessary.

4.3. Effects of contamination gradient

The levels of water metal contamination found in our study sites were above the Annual Average-Quality Standard defined by the European Parliament (Directive n°2013/39/UE) for the preservation of aquatic habitats and wildlife (see Table S1). Accordingly, several effects were observed at the physiological level.

First, metals were significantly bioaccumulated in fish muscle in a size-dependent manner (i.e., age-dependent) (Bird et al., 2008; Farkas et al., 2003; Pyle et al., 2005). Muscle generally bioaccumulate less metals than other organs such as the liver and spleen (Bervoets et al., 2009; Cretì et al., 2010; Rajotte and Couture, 2002). This suggests that along the metal contamination gradient, fish accumulated a fairly high amount of metals in all their organs. Besides, water contamination also increased the available energy in muscle, especially lipids, which could be due to pollution-induced disturbances in lipid metabolism (Bano and Hasan, 1989; Melvin et al., 2019) and/or due to increased food uptake in contaminated sites (Yang et al., 2013). Interestingly, in

our study, this pollution-induced increase in available energy increased in turn the bioaccumulation of metals in muscle (Schäfer et al., 2015). This suggests that contamination by metals could result in positive feedback loops amplifying the adverse effects of contamination on fish physiology. However, this hypothesis remains to be empirically tested in controlled laboratory conditions.

In addition, SEM models showed that metal bioaccumulation was related to the increased N/L inflammatory immune ratio, partly due to increased neutrophils number, which suggests that trace metals triggered a significant over-inflammation (Dethloff et al., 1999; Don Xavier et al., 2019), which in turn may increase liver cellular lesions. Indeed, histopathological analyses reveal an increase of biliary alteration and hepatic fibrosis, which are likely to reflect chronic cytotoxicity, over-inflammation and more globally liver alteration (Lukin et al., 2011; Wolf and Wolfe, 2005). This suggests that metal contamination has deleterious consequences for cell integrity and liver function through both direct toxicity and over-inflammation (Jaeschke et al., 2002; Rikans and Yamano, 2000; Xu et al., 2018). These results raise concerns about the long term effects of realistic levels of pollution on fish health and population persistence. However, we did not find any effects of these physiological changes on individual traits such as growth or body condition contrary to previous studies (Bervoets and Blust, 2003; MacKenzie et al., 1995; Pietrock and Marcogliese, 2003; Pyle et al., 2005), perhaps because fish with lower fitness were counter-selected in the wild and not sampled in our field study. Further longitudinal studies across time and life stages are now needed to better estimate the effects of metal contamination on fish fitness.

4.4. Multiple stressor effects

Interestingly, we found an interactive effect of temperature and contamination gradients on fish growth. Contamination decreased fish growth, but only in warmer sites. This suggests that elevated temperature aggravates the effects of pollution, as shown in previous studies (Noyes et al., 2009; Gandar et al., 2017a; Jacquin et al., 2019). Here, growth was affected during the first year of life of gudgeon, but no other deleterious effects were observed, which suggests that multiple stressors have more severe consequences during early life stage, a critical period of fish development. Stressful conditions are known to inhibit growth in fish, which is partly due to the inhibition of growth hormones (Barton, 2002; McCormick et al., 1998). In addition, high temperature increases metabolism and accelerates contaminants uptake (Noyes et al., 2009; Sokolova and Lannig, 2008), which can increase the costs of detoxification and defenses and decrease the energy invested in somatic growth (Petitjean et al., 2019; Sokolova et al., 2012; Sokolova, 2013). However, in this study, the interaction between contamination and temperature gradients had no effects on physiological traits nor energy reserves, so that the underlying physiological mechanisms remain to be determined.

We found no effect of stressors on telomere length, a proxy of whole-life stress exposure. The literature reports contrasting results on the relationship between telomere shortening and stress exposure in wild animals, depending on the stressor, species, sex, and tissue collected (Angelier et al., 2018; Chatelain et al., 2020; Louzon et al., 2019). In our study, we only measured telomere length in somatic tissues (i.e., pelvic fin). Thus further studies in other organs such as the liver and immune organs (Giraudeau et al., 2019) are now necessary to better understand the links between stress exposure and telomere shortening in wild animals.

5. Conclusion

To conclude, this study shows that parasitism and temperature alone had limited deleterious effects on fish health in human-altered rivers. In contrast, trace metal contamination increased metal bioaccumulation and lipid storage in muscle, likely through a positive feedback

loop, which led to cellular damage in the liver, although few effects were detected at the individual level. In addition, trace metal contamination decreased fish growth when combined with elevated temperature, which suggests that multiple stressors could affect fish life-history traits, although further studies are needed to assess the consequences of multiple stressors on fish fitness and, ultimately on population dynamics (e.g., structure and abundance). In addition, further experimental studies under controlled conditions and/or in semi-natural setting using cross-transplantations would also be useful to test the underlying mechanisms and potential local adaptation of populations to environmental stressors. With this study, we hope to encourage future studies to take into account multiple stressors in environmentally realistic conditions to better understand and anticipate the effects of anthropogenic stressors on fish populations.

Compliance with ethical standards

Fish sampling was conducted under the authorization of local authorities (N° 2016-995, 2016-15av, 2016-21av, PE-2016-014, E-2016-130) and in compliance with French and European legislation (European directive 2010/63/UE).

CRediT authorship contribution statement

Quentin Petitjean: Conceptualization, Investigation, Formal analysis, Writing - original draft, Writing - review & editing. **Séverine Jean:** Conceptualization, Investigation, Writing - review & editing. **Jessica Côte:** Methodology, Conceptualization, Investigation, Writing - review & editing. **Thibaut Larcher:** Investigation, Writing - review & editing. **Fréderic Angelier:** Investigation, Writing - review & editing. **Cécile Ribout:** Investigation, Writing - review & editing. **Annie Perrault:** Investigation, Writing - review & editing. **Pascal Laffaille:** Investigation, Writing - review & editing. **Lisa Jacquin:** Methodology, Conceptualization, Investigation.

Declaration of competing interest

The authors declare that they have no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2020.140657.

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