



# Combined effects of temperature increase and immune challenge in two wild gudgeon populations

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**Abstract** In the context of global changes, aquatic ecosystems are increasingly exposed to multiple stressors that can have unexpected interactive effects on aquatic organisms. Among these stressors, the occurrence of heat waves and pathogens is changing rapidly in freshwater rivers, but their combined effects on fish health are still understudied. In this study, we experimentally tested the crossed effects of increased temperature (mimicking a heat wave) and a standardized immune challenge (mimicking a parasite attack) on wild gudgeon (*Gobio occitaniae*) physiology and behaviour across biological levels from molecules to the whole individual. We also investigated the potential variation of sensitivity among populations by comparing two wild populations from contrasted thermal regimes.

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Combined stressors (i.e. temperature increase and immune challenge) had contrasted effects on fish physiology and behaviour compared to single stressors, but only at the individual level. In particular, the immune challenge inhibited the effect of the temperature on fish behaviour (activity, exploration and foraging) but amplified the negative effect of temperature on fish survival. No interactions were found at other biological levels. This study thus shows that it is essential to consider biotic stressors such as pathogens to better anticipate the effects of global changes on aquatic organisms. In addition, there was a high variability of response between the two gudgeon populations, suggesting that future studies should take into account population variability to better predict the responses of aquatic wildlife to current and future stressors.

**Keywords** Multiple stressors · Climate change · Pathogens · Physiology · Behaviour · Inter-population variability

## Introduction

Freshwater ecosystems are increasingly exposed to numerous stressors which can potentially interact (Hoffmann et al. 2010; Schinegger et al. 2016) and have unexpected effects on fish physiology and behaviour. Among stressors, temperature increase and occurrence of extreme weather are among the most important factors affecting fish physiology (e.g. immune responses, energy reserves and allocations) and behaviour (Brett

1971; Cossins and Bowler 1987). For instance, high temperature can hamper fish immune responses (Dittmar et al. 2014) and enhance fish activity, energy expenditure and oxidative stress (Bartolini et al. 2015; Gandar et al. 2017). Simultaneously, pathogen occurrence is expected to drastically change in the future due to global changes. This could increase the immune costs incurred by fish hosts and strongly affect their physiology and behaviour (Macnab and Barber 2012; Cable et al. 2017). For instance, the reproduction and transmission of ectoparasites such as gyrodactylids are increasing due to climate change, which can trigger a costly immune response and oxidative stress in their fish hosts (Bakke et al. 2007). These molecular effects might interact and have cascading effects on the whole individual, such as reduced fish energy reserves, body condition, reproductive success and survival (Buchmann and Lindenstrøm 2002; Eizaguirre et al. 2009, 2011). However, pathogens and their associated immune challenges are still rarely considered as biotic stressors that could interact with other stressors (Preston et al. 2016), such as increased temperature.

Previous studies outlined the importance of taking into account different levels of biological organisation in stress responses, because different effects might be expressed at the molecular, cellular or individual level (Côté et al. 2016). In this study, we tested the combined effects of stressors (temperature and immune challenge) on fish physiology and behaviour across levels of biological organisation from molecules to the whole individual. We focused on traits related to oxidative stress, DNA damage and immune defences at the molecular and cellular levels, because both temperature and parasites can affect immunity and increase metabolic activity, oxygen consumption and lead to a higher level of oxidative stress (Marcogliese et al. 2005; Stumbo et al. 2012; Halliwell and Gutteridge 2015; Schreck et al. 2016; Birnie-Gauvin et al. 2017). Such molecular effects can have potential cascading effects at the individual level, especially through behavioural adjustment (i.e. plasticity) and effects on fish survival and fitness (Wingfield 2003; Wong and Candolin 2015). We thus focused on fish behaviour and survival because it could have consequences for population dynamics in the wild (Vlez-Espino et al. 2006).

At the molecular level, anti-oxidative molecules are the main defence pathways against reactive oxygen species (ROS) produced by exposure to most environmental stressors. When these anti-oxidative defences are

exceeded by ROS production (i.e. imbalance) damage to lipids, proteins and DNA impacting their structure and functioning are expected (Lushchak 2011). More specifically, temperature increase and immune challenges generally both induce the production of ROS which can increase oxidative damage (Preynat-Seauve et al. 2003; Swindle and Metcalfe 2007; Vinagre et al. 2012). Thus, combined exposure to these multiple stressors could lead to an overproduction of ROS and potentially to high oxidative damage at the molecular level due to imbalance between oxidant and anti-oxidant molecules (Sulmon et al. 2015). This could ultimately lead to primary DNA damage and cause genotoxic effects (Finkel and Holbrook 2000; Birnie-Gauvin et al. 2017). Such genotoxic effects are important to take into account because they can result in cellular dysfunction and apoptosis, with potential cascading deleterious effects at the individual level, particularly on fish survival and thus population dynamic (Anitha et al. 2000; Malev et al. 2010; Cheng et al. 2018).

At the cellular level, immunity is generally strongly affected by environmental stressors. Previous studies showed that temperature strongly affects innate immunity, a central immune trait in fish (Magnadóttir 2006). To measure innate immunity, the local inflammatory immune response is considered as a good indicator of innate immunocompetence (Smits et al. 1999; Martin et al. 2006) and it has been used as a proxy of immune response in various species including fish (Ardia and Clotfelter 2006; Tella et al. 2008; O'Connor et al. 2014). Because temperature could potentially hamper immunity, we expected a lower local immune response in immune challenged fish under high temperature, although immunoactivation or immunodepression can be observed depending on the temperature and species considered (Martins et al. 2011; Dittmar et al. 2014). In addition, depletion of energy reserves is expected under stress exposure, due to reallocation of energy to sustain anti-oxidative and immune defences.

At the individual level, we focused on changes in energy allocation among energy reserves, reproduction or behaviour. Such changes are documented under single stressors, but the effects of multiple stressors on these individual traits remain elusive. Temperature increase and immune challenges both mobilize energy reserves and decrease fish condition and reproduction indices (e.g. Fulton index, Hepatosomatic Index, Gonadosomatic Index) (Chellappa et al. 1995; Marentette and Corkum 2008; Gandar et al. 2017). In

addition, behavioural changes are often the first responses to changes of the environment (Wong and Candolin 2015). For instance, increasing temperature generally increases fish activity, exploration and foraging to sustain energy expenditure (Johansen and Jones 2011; Bartolini et al. 2015; Colchen et al. 2016). Pathogens or immune challenges on the contrary generally trigger a typical sickness behaviour (i.e. a reduction in activity, exploration, foraging and also sociability) which could reduce infection risk and save energy for immunity (Johnson 2002; Bonneaud et al. 2003; Volkoff and Peter 2004). Thus, temperature and immune challenges are expected to have opposite effects (antagonistic) on most behavioural traits, but this hypothesis has been rarely tested. Finally, the immune challenge could potentially amplify the negative effects of temperature on fish survival (synergistic effects) since both stressors are energetically costly. Thus, several types of interaction between stressors could potentially occur (e.g. additive, synergistic or antagonistic) depending on the trait considered (Côté et al. 2016).

Most experimental studies on stressors effects have been performed on animals bred in captivity, which limits our understanding on realistic effects of stressors in the wild. However, wild populations might strongly vary in their responses to stressors depending on their history of exposure. For instance, Dittmar et al. (2014) showed that sticklebacks (*Gasterosteus aculeatus*) populations originating from warmer habitat were more able to cope with a heat wave event. This raises the possibility that populations evolving in stressful environments might develop specific abilities to cope with the same stressor (Crozier and Hutchings 2014). However, empirical studies testing this hypothesis are still rare.

In this study, we aimed at (1) testing how both abiotic (temperature increase) and biotic stressors (an immune challenge mimicking a parasite attack) could interact and shape fish responses across levels of biological organisation. We used multiple markers focusing on key traits that are expected to be affected by both stressors. In addition, we aimed at (2) investigating the inter-population variability in response of wild fish by comparing two wild populations having evolved in thermally contrasted environments.

To address these questions, we used an experimental approach on wild gudgeon fish *Gobio occitaniae* because this species is widely distributed and exposed to several environmental conditions in European rivers (Bervoets and Blust 2003; Shinn et al. 2015; Fourtune

et al. 2016). We tested two wild gudgeon populations originating from two contrasted thermal regimes (16 and 24 °C maximum summer temperature). We exposed fish to two environmentally relevant experimental temperatures: a control temperature (17 °C) that is the optimum for this species or a high temperature mimicking a heat wave (24 °C) that is expected to trigger stress responses. Half of the fish were conjointly exposed to an immune challenge mimicking a parasite attack to test the effects of multiple stressors. At the molecular and cellular levels, we expected interactive and potentially synergistic effects of temperature increase and immune challenge on oxidative stress, immunity, and energy reserve, because both stressors involve similar physiological pathways. At the individual level, we expected potentially antagonistic effects of combined stressors on behaviour because opposite effects of temperature and immune challenges are expected on behaviour. Synergistic effects were expected on fish survival because both stressors are energetically costly for organisms. Finally, we expected that the two populations should differ in their responses to experimental stressors, due to different past exposures to environmental conditions in the wild.

## Material and methods

### Model species and sampling sites

We used wild gudgeon (*Gobio occitaniae*) as a model species because it is widely distributed in South-West France and relatively sedentary, thereby exposed to the same environmental stressors throughout their life (Keith et al. 2011). It has a low tolerance to increased temperature and displays a high prevalence of pathogens in the wild (for instance ectoparasites such as Gyrodactylids, Loot et al. 2007). The optimal temperatures of gudgeon range from 16 to 20 °C, and lethal temperature is reached at 27 °C (Tissot and Souchon 2010). Previous studies conducted on this species in the same region showed that gudgeon display contrasted phenotypic responses to physico-chemical conditions, including temperature (Shinn et al. 2015; Fourtune et al. 2016). We used the database from Adour-Garonne Water agency (monthly measures) to select two capture sites (AUSEI and CAUSAL) that differed in mean and maximum summer temperature during 4 years but were relatively similar in other environmental conditions

(Table 1). We chose a time period of 4 years, because it should reflect the temperature experienced by gudgeon during their lifetime before capture (3 to 4 years of longevity; Keith et al. 2011). Sites differed in mean and maximum summer temperature (Table 1) but did not differ in summer temperature variability among years (coefficient of variation, CV, ANOVA,  $F=2.1$ ;  $p=0.19$ ; see Table 1). Sites did not differ in parasite prevalence and intensity. An external and internal parasite inventory was performed on each fish after capture, and only *Gyrodactylus* sp. parasites were detected and had similar prevalence (73% and 61% for AUSEI and CAUSAL respectively) and mean intensity ( $2.0 \pm 2.3$  and  $1.4 \pm 1.7$  for AUSEI and CAUSAL respectively) (difference between sites: glm; prevalence  $\chi^2=1.05$ ,  $p=0.31$ ; intensity  $F=1.15$ ,  $p=0.29$ ).

#### Fish sampling and acclimation

Forty gudgeons (6 to 11 cm of length) were caught by electrofishing (EFKO-FEG 1500) on each study sites in March 2017 and brought to the laboratory. To eliminate potential parasites and standardize fish immune status prior to the experimentation, fish were treated using Praziquantel (Vetofish, France, purity = 1000 mg g<sup>-1</sup>; concentration 3 mg L<sup>-1</sup>). Fish were then acclimated during 30 days in 200 L tanks containing gravel and shelters to reduce stress, equipped with oxygen pumps, mechanical filters and temperature controllers. Temperature was gradually increased by 0.5 °C every 24 h to gradually reach experimental temperatures without triggering stress. During acclimation, physico-chemical parameters were checked every week (pH =  $7.6 \pm 0.7$ ; conductivity =  $314 \pm 17$   $\mu\text{S cm}^{-1}$ ; O<sub>2</sub> dissolved =  $9.5 \pm 0.3$  mg L<sup>-1</sup>) and half of the water was renewed every 2 days. Mortality rate was low (3.75%: 2 individuals from AUSEI and 1 from CAUSAL).

#### Experimental design

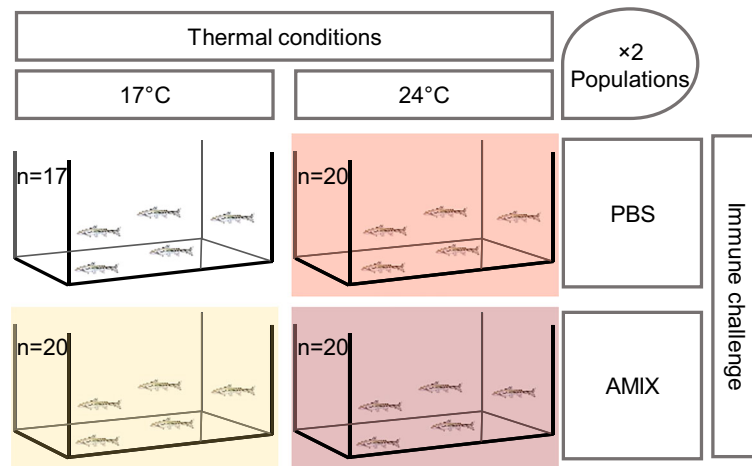
Seventy-seven individuals in total were randomly exposed to 4 different treatments: control without stressor (17 °C PBS  $n=17$ ), temperature increase alone (24 °C PBS  $n=20$ ), immune challenge only (17 °C AMIX  $n=20$ ) and multiple stressors (temperature and immune challenge combined, 24 °C AMIX  $n=20$ ). Within these treatments, 2 populations were tested (AUSEI and CAUSAL) (7 to 10 individuals per treatments and population) (Fig. 1).

We chose two environmentally relevant temperatures: 17 °C, which is the optimal temperature for this species and corresponds to a common temperature found in one of the sites, or 24 °C, which is maximum temperature recorded in the warmest site. In addition, 24 °C is considered as a stressful temperature for gudgeon (lethal temperature 27 °C; Tissot and Souchon 2010). We chose a duration of exposure of 7 days to mimic the average duration of a heat wave in the environment (Beniston et al. 2007).

In order to standardize the immune challenge and mimic an infection by a wide range of pathogens, we injected half of the fish with an antigen mixture (AMIX) of phytohaemagglutinin (PHA) and *Escherichia coli* lipopolysaccharide (LPS) following previous studies in vertebrates, including fish (Tella et al. 2008; Toomey et al. 2010; Otlora-Ardila et al. 2016). The control saline-injected group was injected with a phosphate-buffered saline (PBS group) (Toomey et al. 2010). Fish were injected in the caudal peduncle with 10  $\mu\text{L}$  of PBS or of an antigenic solution mixture (AMIX group) of PHA (PHA, L8754 Sigma-Aldrich, 45  $\mu\text{g}$  10  $\mu\text{L}^{-1}$ ) and LPS (LPS, L2830 Sigma-Aldrich, 45  $\mu\text{g}$  10  $\mu\text{L}^{-1}$ ). Briefly, PHA is a standard lectin inducing a cell-mediated response (e.g. inflammatory response and proliferation of T cells), reflecting a local cellular immune response against a wide range of ectoparasites (Martin et al. 2006; Tella et al.

**Table 1** Environmental characteristics of study sites (Adour-Garonne Water agency database from 2013 to 2016)

Site	Lat.	Long.	Elevation (m)	Water temperature $\pm$ SD (°C)				
				Coefficient of variation	Mean summer	Min summer	Max summer	
AUSEI	43° 41' 48.67" N	1° 21' 29.64" E	116	12.5 $\pm$ 4.7	20.1 $\pm$ 3.8	14.7 $\pm$ 0.5	24.0 $\pm$ 1.8	Warm site
CAUSAL	43° 55' 52.61" N	2° 09' 58.74" E	160	8.6 $\pm$ 2.7	15.5 $\pm$ 1.4	14.3 $\pm$ 0.7	16.1 $\pm$ 1.5	Cold site



**Fig. 1** Experimental design. Fish were exposed either to control temperature 17 °C (optimum) or to high temperature 24 °C corresponding to the highest temperature recorded in the warmest population. Half of the fish was also exposed to an immune challenge caused by an antigen mixture (AMIX) or to a control injection of saline solution (PBS). Seventy-seven individuals in

total were tested in 4 treatment groups: control without stressor (17 °C-PBS, white  $n = 17$ ), thermal stress only (24 °C PBS, orange  $n = 20$ ), immune challenge only (17 °C AMIX, yellow  $n = 20$ ) and multiple stressors (24 °C AMIX, red  $n = 20$ ). Fish were tested in groups of 5 fish, so that 2 replicate tanks per condition and per population were used

2008). The LPS antigen is originating from the membrane of bacteria (*Escherichia coli*) and also triggers a local and systemic innate and adaptive immune response (Swain et al. 2008). The response to the mixture of both antigens is measured through a local skin swelling reflecting local cellular immunity. This skin swelling can easily be measured within 24 to 48 h after injection (Martin et al. 2006; Tella et al. 2008). Antigen injection (AMIX) also triggers a long-lasting circulating immune activation during 4 to 8 days in fish (Le Guernic et al. 2016).

Because these gudgeon fish are gregarious, they were placed and tested in groups of 5 fish per tank, so that 2 replicate tanks per condition and per population were used. Experimental tanks were 50 × 30 × 30 cm with opaque glass containing 37 L of water previously filtered at 0.2 μm, treated on activated carbon, UV lamp and dechlorinated water. Each tank was equipped with temperature controller and mechanical water filtration. Oxygen supply was kept constant over every treatment using air pump in order to avoid the decrease of dissolved oxygen level in warmer tank (17 °C:  $17.4 \pm 0.3$  °C,  $O_2$  (dissolved) =  $10.2 \pm 0.2$  mg L<sup>-1</sup>,  $n = 25$ ; 24 °C:  $24.0 \pm 0.2$  °C,  $O_2$  (dissolved) =  $9.7 \pm 0.3$  mg L<sup>-1</sup>,  $n = 25$ ). During the exposure fish were sustained under a day/night cycle photoperiod of 12/12 h. Fish were fed daily using thawed midges (*Chironomus* sp.). There was no difference of sex ratio ( $\chi^2 = 0.44$ ,  $p = 0.93$ ) and fish mass ( $6.5 \pm 0.2$  g); and size ( $8.6 \pm 0.1$  cm) among treatments (ANOVA mass  $F = 0.27$ ,  $p = 0.85$ ; ANOVA size  $F = 0.49$ ,  $p = 0.69$ ).

#### Sample collection

At the end of the experiment, fish were euthanized using anaesthetic overdose (benzocaine, 150 mg L<sup>-1</sup>). Fish were then weighed, measured and dissected for sex determination and further analyses on tissues. Blood samples were collected from the caudal vein in heparinised syringes (1 mL Terumo syringe, 0.45 × 13 mm needle) and 2 μL of total blood fraction was collected, diluted at 1:100 in a cryopreservative buffer (250 mM sucrose, 40 mM trisodium citrate, 5% dimethylsulphoxide, pH 7.6 adjusted with 1 M citric acid), deep-frozen in liquid nitrogen and conserved at -80 °C until measurement of primary DNA damage (genotoxicity assessment) in erythrocytes using the comet assay. The remaining blood was centrifuged (4 °C, 2000×g) for 10 min, and plasma was kept at -20 °C for subsequent oxidative damage and anti-oxidant capacity assays. After dissection, the spleen, liver and gonad were weighed ( $\pm 1$  mg) for body condition index calculation while fish muscles were immediately frozen in liquid nitrogen and stored at -80 °C to assess fish energy reserves.

#### Molecular level: oxidative stress index

Following previous studies, we measured anti-oxidant capacity and oxidative damage in the plasma to calculate an oxidative stress index considered as the ratio of



oxidative damage divided by the anti-oxidant capacity multiplied by 1000 (Costantini and Dell’Omo 2006; Herborn et al. 2011). Anti-oxidant capacity represents the anti-oxidant power of the plasma barrier composed of exogenous (e.g. tocopherols, carotenoids and flavonoids) and endogenous (e.g. proteins, bilirubin, uric acid, cholesterol and GSH) compounds (Isaksson 2013). Specifically, OXY-adsorbent test (Diacron International, Grosseto, Italy) measure the anti-oxidant capacity of the plasma by quantifying the overall ability of compounds present in a plasmatic solution to cope with the *in vitro* oxidant action of hypochlorous acid (HOCl; an endogenously produced oxidant) (Hoogenboom et al. 2012). In accordance with method from Bagni et al. (2007) and Hoogenboom et al. (2012), plasma was previously diluted (1:100) with ultra-pure water. Then, 5  $\mu\text{L}$  of the diluted plasma and 200  $\mu\text{L}$  of HOCl solution were incubated in 96-well microplate for 10 min at 37 °C. The same relative volumes were used for certified standards and blanks. After incubation time, 5  $\mu\text{L}$  of chromogen *N,N*-diethyl-*p*-phenylenediamine was added to each well and absorbance was read at 490 nm using CLARIOstar (BMG LABTECH). For anti-oxidant capacity assessment, intra- and inter-plate repeatabilities were  $86.5 \pm 10.6\%$  and  $86.1 \pm 7.8\%$  respectively.

Oxidative damage was analysed using d-ROM test (Diacron International, Grosseto, Italy). The d-ROM test allows to indirectly measure plasmatic hydroperoxides (ROOH), which is both considered as active oxidant and a reactive oxygen metabolite (ROM) resulting from the effect of ROS on organic substrates (carbohydrates, lipids, amino acids, proteins, nucleotides) (Beaulieu et al. 2010), and act as precursors of end-products of lipid peroxidation (Hoogenboom et al. 2012). This marker is thus considered to assess both active oxidants capacity and oxidative damage (Alberti et al. 2000). We used the method implemented for fish by Bagni et al. (2007) and Hoogenboom et al. (2012). Eight microlitres of undiluted plasma, 200  $\mu\text{L}$  of 0.01 M acetic acid/sodium acetate buffer (pH 4.8) solution and 2  $\mu\text{L}$  of chromogen (*N,N*-diethyl-*p*-phenylenediamine) were incubated in 96-well microplate for 75 min at 37 °C. The same relative volumes were used for certified standards and blanks. Incubation was made in 96-well microplate and centrifuged at 3500 rpm for 2 min. Clarified supernatant was then transferred in 96-well microplate for absorbance reading at 490 nm using CLARIOstar (BMG LABTECH). For ROM assessment, intra- and inter-plate repeatabilities were  $95.9 \pm 3.9\%$  and  $93.7 \pm 2.5\%$  respectively.

Finally, we calculated an oxidative stress index as the ratio of oxidative damage divided by the anti-oxidant capacity multiplied by 1000 (Costantini and Dell’Omo 2006; Herborn et al. 2011). Due to the difficulties of taking a large enough quantity of blood to carry out all our measurements, sample size among treatment was as follows: control (17 °C PBS)  $n = 8$ , immune challenge only (17 °C AMIX)  $n = 10$ , thermal stress only (24 °C PBS)  $n = 13$  and combined stressors (24 °C LPS)  $n = 10$ .

#### Molecular level: DNA damage

Oxidative stress can cause DNA disruption and genotoxicity. In order to assess genotoxicity, we used the alkaline comet assay which is one of the most used approaches (Santos et al. 2016). It allows to detect a wide array of primary DNA damage such as single and double strand breaks, alkali-labile, incomplete repair sites and DNA cross-links (Tice et al. 2000). Here, primary DNA damage was assessed using comet assay performed on cryopreserved erythrocytes in accordance with the method described by Singh et al. (1988) modified by Santos et al. (2014). Microscope slides were covered with melted normal agarose in PBS (0.8%) and dried overnight at 20 °C. Erythrocyte suspension was mixed with 1% low melting agarose made in HBSS (37 °C), and 100  $\mu\text{L}$  of the mix was scattered on the slides and placed at 4 °C for 10 min allowing the agarose polymerization. Slides were then incubated in a lysing solution for 1 h at 4 °C in a dark room (2.5 M NaCl, 100 mM Na<sub>2</sub>EDTA, 10 mM Tris-HCl, 1% Triton X-100, and 10% DMSO, pH 10) and put under dim yellow light in a horizontal electrophoresis tank filled with electrophoresis buffer (300 mM NaOH, 1 mM EDTA, pH > 13) for 40 min at 4 °C allowing the unfolding of DNA before electrophoresis step (0.66 V.cm<sup>-1</sup>, 24 min). Slides were then washed 3 times for 5 min with a neutralization buffer (0.4 M Tris-HCl, pH 7.5) and dried in absolute ethanol prior their staining using 0.02% ethidium bromide solution. Primary DNA damage of 100 randomly selected cells per slide was randomly analysed using an Axioskop epi-fluorescence microscope (Zeiss, Germany) and an image analysis system (Comet IV software, Perceptive Instruments, UK). We then recorded tail intensity (percentage in tail DNA) as a proxy of DNA damage rate (Collins 2004). For this assay, sample size among treatment was as follows: control (17 °C PBS)  $n = 16$ , immune challenge

only (17 °C AMIX)  $n = 18$ , thermal stress only (24 °C PBS)  $n = 17$  and combined stressors (24 °C LPS)  $n = 13$ .

#### Cellular level: local immune response

The local immune response caused by mixture of antigen (AMIX) or control saline (PBS) injection was measured as the skin swelling (thickness) of the caudal peduncle 48 h after injection using a calliper following previous studies in fish (Ardia and Clotfelter 2006; O'Connor et al. 2014). The thickness of the caudal peduncle was measured 3 times and averaged before and after the antigenic

injection using a thickness gauge (Elcometer 124). A previous pilot study on gudgeon showed that this time lag was optimal to measure skin swelling in this species (unpublished data). Although antigen injection (AMIX) can reflect several components of the immune response, skin swelling has been validated as a reliable measure of local immune capacity across a variety of taxa, including fish. An important skin swelling response reflects a strong innate immune system and a high investment in immune defence (Ardia and Clotfelter 2006; Tella et al. 2008; O'Connor et al. 2014). The intensity of the local immune response was calculated as follows:

$$\text{Local Immune Response} = \frac{\text{Mean Caudal peduncle thickness}_{(t=48h)} - \text{Mean Caudal peduncle thickness}_{(t=0)}}{\text{Mean Caudal peduncle thickness}_{(t=0)}} \times 100$$

Intra-individual repeatability of the local immune response measurements (three repeated measures on the same individual) was very high ( $99.4 \pm 0.3\%$  after injection). Sample size for immune local response measurement among treatment was as follows: control (17 °C PBS)  $n = 16$ , immune challenge only (17 °C AMIX)  $n = 19$ , thermal stress only (24 °C PBS)  $n = 18$  and combined stressors (24 °C LPS)  $n = 16$ .

#### Cellular level: available energy

The available energy in muscles was calculated as the sum of total carbohydrates, lipid and protein contents. The amount of available energy in muscle cells is a good marker of energy status, because it is critical for the maintenance of biological functions of fish, especially under stress (Gomes et al. 2015; Gandar et al. 2016). We used protocol from De Coen and Janssen (1997) modified by Gandar et al. (2017). Twenty-five milligrams of fish muscles was homogenized in 1 mL of ultrapure water using Fastprep-24 homogenizer. Muscle suspension was divided in 2 aliquots. The first aliquot was mixed with trichloroacetic acid (TCA) and incubated – 20 °C. Then, sample was centrifuged to split up the supernatant and the pellets. Fifty microlitres of the supernatant was read at 492 nm against glucose in TCA using CLARIOstar (BMG LABTECH) for determination of total carbohydrate content ( $\mu\text{g mg}^{-1}$  muscles wet weight). The pellet was resuspended in sodium hydroxide (NaOH, 1 M). Then, suspension was mixed with

Bradford reagent before reading absorbance in triplicate at 595 nm against bovine serum albumin in 0.2 M NaOH for total protein concentration determination ( $\mu\text{g mg}^{-1}$  muscles wet weight). The second aliquot was mixed with chloroform ( $\text{CHCl}_3$ ), methanol ( $\text{CH}_3\text{OH}$ ) and sulphuric acid ( $\text{H}_2\text{SO}_4$ ) and centrifuged. The  $\text{H}_2\text{SO}_4$  was added to the bottom phase, and vanillin-phosphoric acid reagent was added before reading absorbance in triplicate at 525 nm against tripalmitin in chloroform for total lipid concentration determination ( $\mu\text{g mg}^{-1}$  muscles wet weight). Concentrations of carbohydrate, protein and lipid were then transformed to energetic values using their enthalpy of combustion (17, 24 and  $39.5 \text{ kJ g}^{-1}$  respectively) and summed to obtain the available energy in muscles. Sample size for energy reserve among treatment was as follows: control (17 °C PBS)  $n = 16$ , immune challenge only (17 °C AMIX)  $n = 19$ , thermal stress only (24 °C PBS)  $n = 18$  and combined stressors (24 °C LPS)  $n = 13$ .

#### Individual level: condition indices

Four condition indices were calculated to reflect the general condition of the individual based on organ and whole body mass of fish. First, we measured the daily body mass change before and after the experiment (4 days before injection, and 7 days after injection) to measure changes in whole body condition. Then, the splenosomatic index (SSI, spleen mass corrected by the body mass  $\times 100$ ) was calculated as a proxy of spleen

contraction. The spleen contraction results in the release of erythrocytes and lymphocytes in the blood and thus plays an important role in hematopoiesis and immune responses and is representative of the stress response (Nilsson 1983; Maule and Schreck 1990; Pearson and Stevens 1991). The hepatosomatic index (HSI, liver mass corrected by the body mass  $\times 100$ ) is considered as a proxy of energy content in the liver (Chellappa et al. 1995). And finally, the gonadosomatic index (GSI, gonad mass corrected by the body mass  $\times 100$ ) is a proxy of the reproductive status (Marentette and Corkum 2008).

Because condition indices were partly correlated (Online Resource 1), we used a PCA to extract a global condition index from the first and the second axis. The first axis representing fish global body condition index composed of GSI, HSI and daily body mass change while the second axis is represented mostly by SSI. The sum of the variance explained by both axes of the PCA was 70.8%. Hence, fish with a higher global body condition index had thus a lower body mass loss and a higher liver and gonad mass related to their body mass. Sample size for global condition index among treatment was as follows: control (17 °C PBS)  $n = 16$ , immune challenge only (17 °C AMIX)  $n = 18$ , thermal stress only (24 °C PBS)  $n = 18$  and combined stressors (24 °C LPS)  $n = 12$ .

#### Individual level: behaviour

At the individual level, fish behaviour was analysed at day 4 after the AMIX injection in their own experimental tank to avoid handling stress. For this purpose, fish were marked using a visible implant elastomer (Northwest Marine Technologies, Inc., Shaw Island, Washington) before the experiments to monitor behaviour. Videos were recorded by Sony HandyCam HD camera (50 Hz; 1920  $\times$  1080p) and analysed using JWatcher software (Blumstein et al. 2001) for 10 min based on previous studies (Jacquin et al. 2017; Lopez-Luna et al. 2017).

Four behavioural traits were chosen because they are commonly affected by temperature and pathogens: activity, exploration, foraging and sociability. Activity was measured as the time spent swimming (Calfee et al. 2016). In order to measure fish exploration, the tank was divided in two main areas, i.e. a central area and a margin area corresponding to the border of the tank (8 first cm from the tank glass). Then, exploration was measured as the time spent in a central area of the tank (thigmotaxis)

following previous studies (Rosemberg et al. 2011; Benhaïm et al. 2016). Foraging was measured (Nowicki et al. 2012) as the number of foraging events (fish digs the substrate) and the latency to forage after distributing chironomids larvae (Winandy and Denoël 2015). Sociability was assessed using the number of encounters between individuals (number of times an individual touch another individual with the head) and the average nearest neighbour distance (ANND). The ANND was calculated as the distance between the nearest neighbour and the focal individual (mean of 10 measures taken every 30 s during 5 min). Lower ANND reflects lower distances between individuals and thus higher sociability (Bartolini et al. 2015; Colchen et al. 2016).

Because these behavioural traits were correlated (Online Resource 2), we used PCA to extract two synthetic variables named “general activity” (PCA1, 38.7% of variance explained) and “sociability” (PCA2, 19% of variance explained) for a total of 57.7% of the explained variance. Fish with a higher general activity index on the first PCA axis swam for a longer time and foraged more frequently (number of foraging events) with a lower latency to forage. Fish with a lower index on the second axis were more sociable, i.e. had more social contacts and stayed closer to their conspecifics (smaller ANND).

#### Statistics

To test the effects of treatments on fish traits across biological levels, we used linear mixed-effects models (lme4 package; Bates et al. 2015) on each trait: oxidative stress index (log-transformed), DNA damage, local immune response, available energy, general body condition index (first condition PCA axis; see Online Resource 3), Splenic Index (second condition PCA axis; see Online Resource 3), general activity (first behavioural PCA axis, log-transformed) and sociability (second behavioural PCA axis). The replicate tank was included as a random effect to take into account possible shared conditions in the same tank. Temperature treatment (17 vs 24 °C), injection treatment (PBS vs AMIX injection), population of origin and their interactions were included as fixed factors. Survival was analysed using generalized linear model with a binomial distribution. Best models were then selected by stepwise model selection approach by eliminating non-significant variables starting with interactions (see Table 1 for summary of the best final models). Triple interactions were not significant and removed from final models. Fish size and sex were added as covariates in all models but removed



from final models when non-significant. When interactions were significant, we used contrast post-hoc analyses (emmeans package; Lenth et al. 2017) with false discovery rate adjustment to analyse differences between groups. Five percent was used as a significance threshold.

To compare the magnitude of stressor effects (single or multiple), we computed standardized effect sizes (compute.es package) Hedges'  $g$  and 95% confident interval (CI) from GLMM model outputs (e.g.  $z$  values or  $t$  value) following methods described in Hedges et al. (1999) and Del Re (2013). When 95% CI overlapped 0, treatment effect was considered as not significant. When effect size and its 95% CI was lower or higher than 0, treatment effect was significantly as negative or positive, respectively (Hedges et al. 1999). When the effect of multiple stressors was significant, we used an interactive effect analysis. We compared the magnitude of interactive vs additive effects of multiple stressors, following methods described in Côté et al. (2016) and Lange et al. (2018). The effect of multiple stressors (here 24 °C-AMIX treatment group) was compared to the expected additive effects of both single stressors alone (i.e. the sum of the individual stressor effects) using standardized effect sizes Hedges'  $g$  and their 95% confident interval (CI) as follows:  $g = \frac{(Y_{12}-Y_2)-(Y_1-Y_{ct})}{2s} \times J(m)$  where  $Y_{ct}$ ,  $Y_1$ ,  $Y_2$  and  $Y_{12}$  correspond to the mean effect of control (no stressor), stressor 1 (temperature), stressor 2 (immune challenge) and multiple stressors (temperature and immune challenge), respectively.  $s$  corresponds to the pooled sampling variance and  $J(m)$  is the correction for small sampling bias across all treatments groups following Gurevitch et al. (2000) and Lange et al. (2018). When the interactive effect of multiple stressors was significantly lower (no overlap of CI) than the expected additive effect of each stressor, it was considered as antagonistic. When the interactive effect was significantly higher, it was considered as synergistic (Gurevitch et al. 2000; Lange et al. 2018). All analyses were performed with R, version 3.3.3 (R Development Core Team 2013).

## Results

### Molecular level: oxidative index and DNA damage

Oxidative stress index and DNA damage rate did not significantly differ among treatments (Fig. 2A, B).

However, fish from CAUSAL population had a higher oxidative stress index ( $1.8 \pm 0.1$  and  $1.3 \pm 0.2$  respectively;  $F = 5.11$ ,  $p = 0.03$ ) and DNA damage rate ( $19.4 \pm 1.1$  and  $15.1 \pm 1.0\%$ , respectively,  $F = 8.66$ ,  $p < 0.01$ ) compared to fish from AUSEI population (Table 2).

### Cell level: local immune response

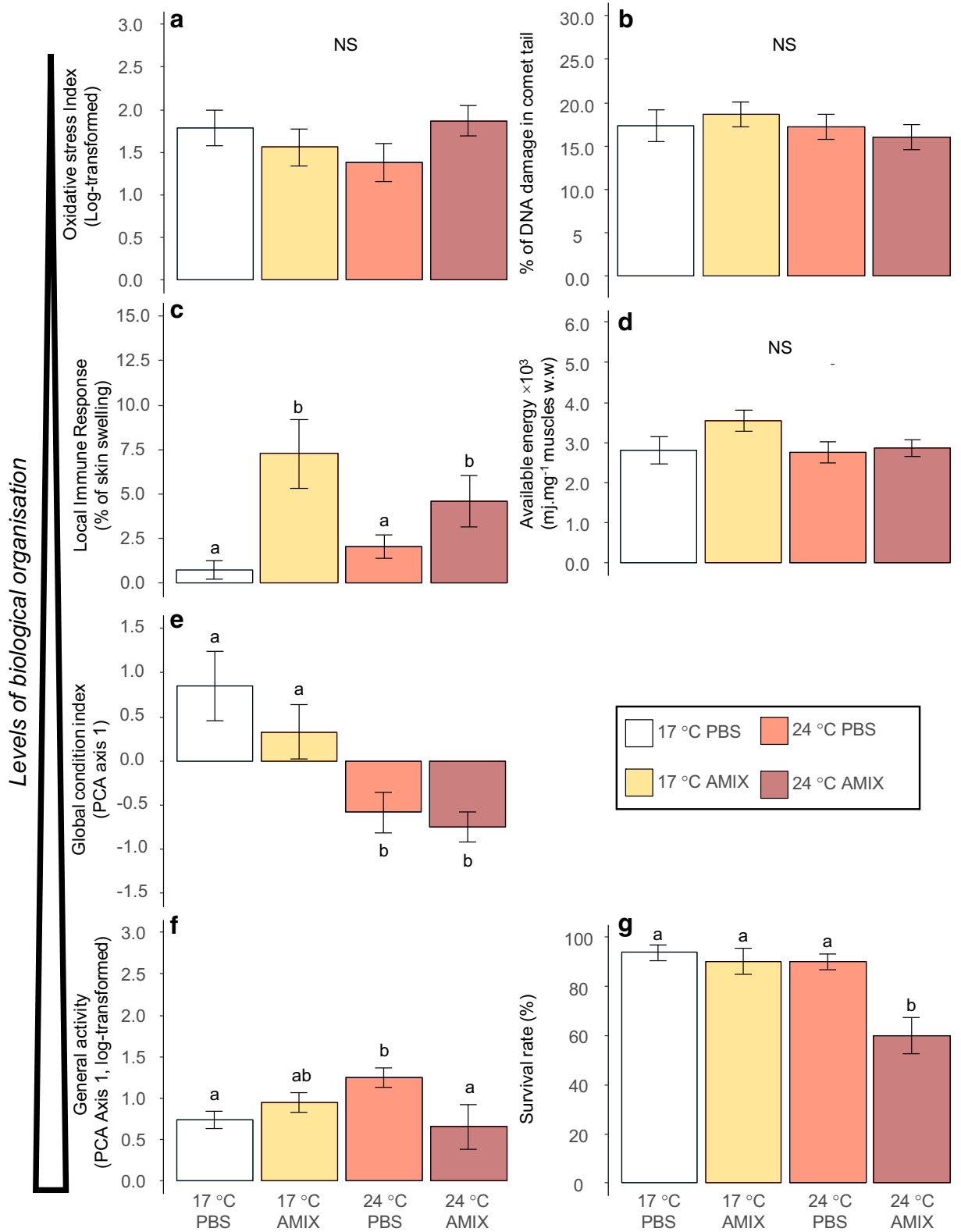
The local immune response was not significantly affected by temperature increase (Table 2). However, antigen-injected fish (AMIX) exhibited a significantly higher local immune response compared to saline-injected fish (PBS) (Table 2; Fig. 2C). The interaction between stressors and the population was not significant (Table 2). The effect size of the immune challenge on local immune response was large ( $1.23$  [ $0.49$ ;  $1.97$ ]; Fig. 3A).

### Cell level: available energy

The available energy in fish muscles was not significantly different among treatments (Fig. 2D). However, CAUSAL fish had higher available energy in their muscles compared to AUSEI fish ( $3.5 \times 10^3 \pm 0.2 \times 10^3$  and  $2.5 \times 10^3 \pm 0.2 \times 10^3$  mj  $\text{mg}^{-1}$  of tissue respectively, effect of population  $F = 12.25$ ,  $p < 0.001$ ) (Table 2).

### Individual level: global body condition index

The global body condition (from PCA axis 1; see Online Resource 3) was negatively affected by temperature alone, but not by the immune challenge alone nor the multiple stressors or population (Fig. 2E). More specifically, detailed analyses on each trait showed that fish lost more body mass per day ( $17$  °C =  $-0.50 \pm 0.08\%$ ,  $24$  °C =  $-0.98 \pm 0.14$ ,  $F = 9.1$ ,  $p < 0.01$ ) and had a lower HSI ( $17$  °C =  $0.91 \pm 0.06$ ,  $24$  °C =  $0.66 \pm 0.05$ ,  $F = 10.2$ ,  $p < 0.01$ ) at  $24$  °C compared to  $17$  °C. Female GSI also decreased with temperature increase ( $17$  °C =  $5.27 \pm 0.08$ ,  $24$  °C =  $2.46 \pm 0.04$ ,  $F = 19.2$ ,  $p < 0.001$ ). The effect of temperature on the global body condition was large ( $-1.16$  [ $-1.9$ ;  $-0.42$ ]). The Splenic Index (PCA axis 2; see Online Resource 3) was not significantly affected by treatment nor population (temperature  $F = 0.3$ ,  $p = 0.6$ ; immune challenge  $F = 0.003$ ,  $p = 1.0$ ; population  $F = 0.7$ ,  $p = 0.4$ ).



**Fig. 2** (A) Oxidative stress index among treatments (control, white 17 °C PBS; thermal stress only, orange 24 °C PBS; immune challenge only, yellow 17 °C AMIX; combined stressors, red 24 °C AMIX). (B) DNA damage (percentage of DNA in comet tail). (C) Local immune response measured as the percentage of skin swelling at injection. (D) Available energy measured in white muscles. (E) Global condition index (PCA axis 1, reflecting mostly HSI, GSI, daily mass change). (F) General activity (PCA axis 1, reflecting mostly time in central area, time in water column, time swimming, latency to forage and foraging events). (G) Survival rate (%). NS indicate non-significant differences among treatments while different letters above bars indicate significant differences after post-hoc contrast analyses

### Individual level: Behaviour

General activity (PCA axis 1) was significantly affected by the interaction between temperature and immune challenge (Table 2). In control saline-injected fish, general activity increased with temperature (PCA axis 1, 17 °C PBS vs 24 °C PBS  $p = 0.015$ ) (Fig. 2F). On the contrary, in AMIX immune-challenged fish, temperature did not

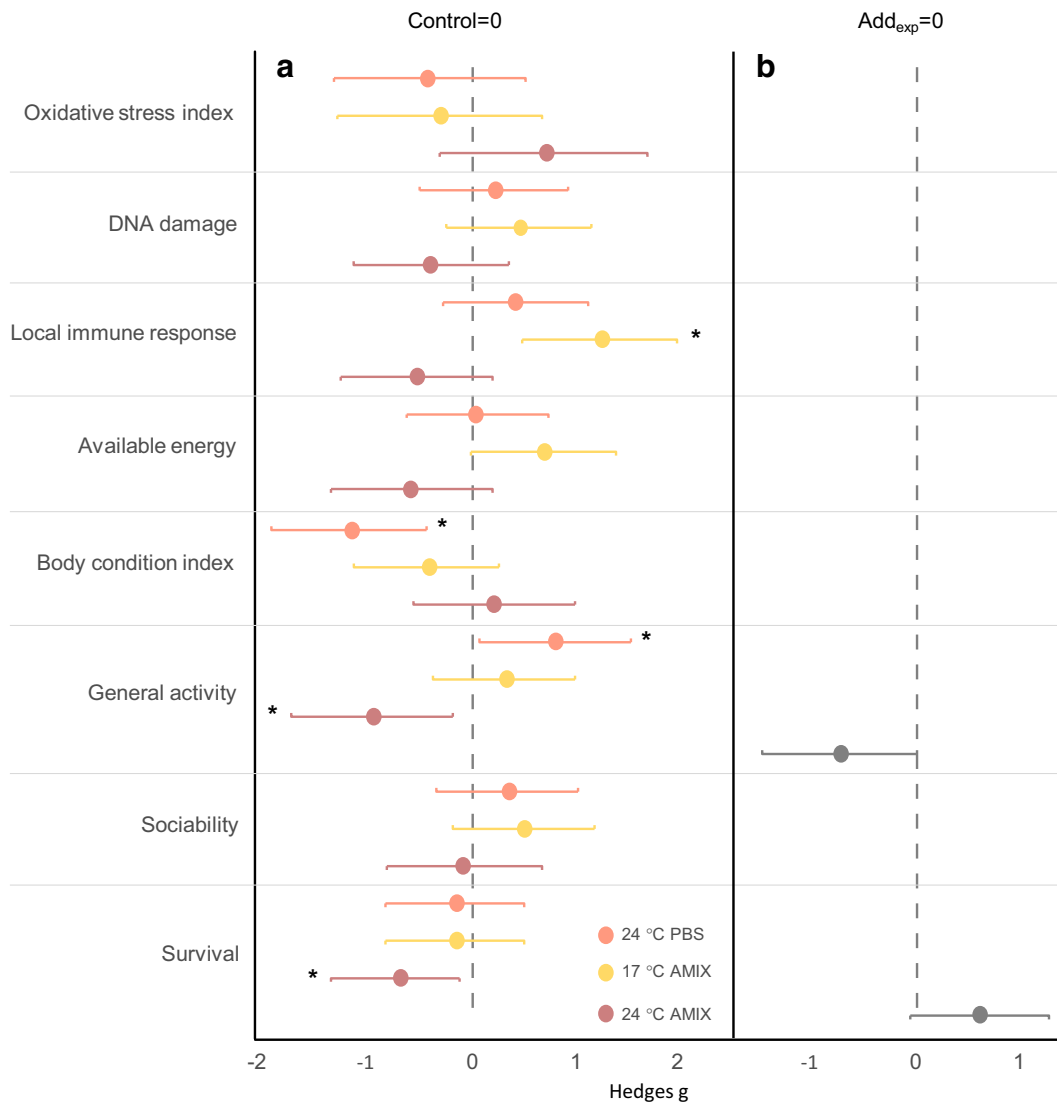
significantly affect the general activity (PCA axis 1, 17 °C AMIX vs 24 °C AMIX  $p = 0.207$ ). Accordingly, the effect size of multiple stressors was significantly negative and large ( $g = -0.93 [-1.69; -0.16]$ ; Fig. 3A). Temperature and immune challenge had marginally antagonistic effects on the general activity (i.e. the immune challenge inhibited the effect of temperature) compared to additive effects as shown by the interactive effect analysis ( $g = -0.73 [-1.47; 0.01]$ ; Fig. 3B).

In addition, populations had different responses to the immune challenge as shown by the significant population-by-immune challenge interaction on general activity and sociability (Table 2). More specifically, immune challenge significantly decreased activity in CAUSAL fish (PBS CAUSAL vs AMIX CAUSAL:  $t = 3.4, p = 0.002$ ), but not in AUSEI fish (PBS AUSEI vs AMIX AUSEI:  $t = -1.8, p = 0.94$ ) (Fig. 4). On the contrary, immune challenge decreased sociability in AUSEI fish ( $t = -2.9, p = 0.021$ ) but not in CAUSAL fish ( $t = 0.4, p = 0.828$ ) as shown in Fig. 4.

**Table 2** Best final mixed models explaining fish responses to stressors at different biological levels. Temperature (17 vs 24 °C), immune challenge (PBS vs AMIX injection), population and their interactions were included as fixed factors. Sex and size were included as covariates but removed from most final models. Triple interactions were not retained in final models. Best models were

selected by stepwise model selection. For each significant fixed factor reported, the summary of the statistical output of the best model is given as follows: Est. and SE correspond to the estimated fixed effect and its standard error respectively; the  $F$  and  $p$  value correspond to the Fisher  $F$  statistic (type III Wald  $F$  test) and to the  $p$  value of the fixed factor respectively

Biological level	Response trait	Fixed factor	Est.	SE	$F$	$p$
Molecular	DNA damage	Population	4.32	1.47	8.66	0.005
	Oxidative stress index	Population	0.47	0.21	5.11	0.030
Cellular	Immune response	Immune challenge	0.20	0.05	13.78	<0.001
	Available energy	Population	0.93	0.27	12.25	<0.001
Individual	Global body condition (PCA axis 1)	Temperature	-1.87	0.43	18.28	<0.001
		Sex	-1.35	0.38	11.93	<0.001
		Temperature:sex	1.17	0.57	4.08	0.048
	General activity (PCA axis 1)	Temperature	0.50	0.17	8.81	0.004
		Immune challenge	0.72	0.21	11.66	<0.001
		Population	-0.14	0.17	0.74	0.394
		Immune challenge:population	-0.98	0.24	16.62	<0.001
	Sociability (PCA axis 2)	Temperature:immune challenge	-0.74	0.24	9.39	0.003
		Immune challenge	1.13	0.39	8.58	0.005
		Population	1.05	0.37	7.79	0.007
Survival rate	Immune challenge:population	-1.28	0.53	5.67	0.020	
	Temperature	0.58	1.27	0.21	0.65	
	Immune challenge	0.58	1.27	0.21	0.65	
	Temperature:immune challenge	2.37	1.27	6.17	0.04	



**Fig. 3** (A) Effect size (hedges'  $g$ ) and 95% confidence intervals (CI) of treatment effects (thermal stress only, orange 24 °C PBS; immune challenge only, yellow 17 °C AMIX; combined stressors, red 24 °C AMIX) relative to control at different biological levels. Dashed line represent control treatment. When 95% CI did not overlap 0 (control), the effect of the treatment was significant. Asterisks indicate significant effect of treatment. (B) Interactive

effects of multiple stressors compared to additive effects of single stressors (only on traits where multiple stressors effects were significant). When the interactive effect was significantly lower than the expected additive effect (95% CI did not overlap  $Add_{exp} = 0$ ), it was considered as antagonistic. When it was significantly higher, it was considered as synergistic (see “Material and methods”)

#### Individual level: survival

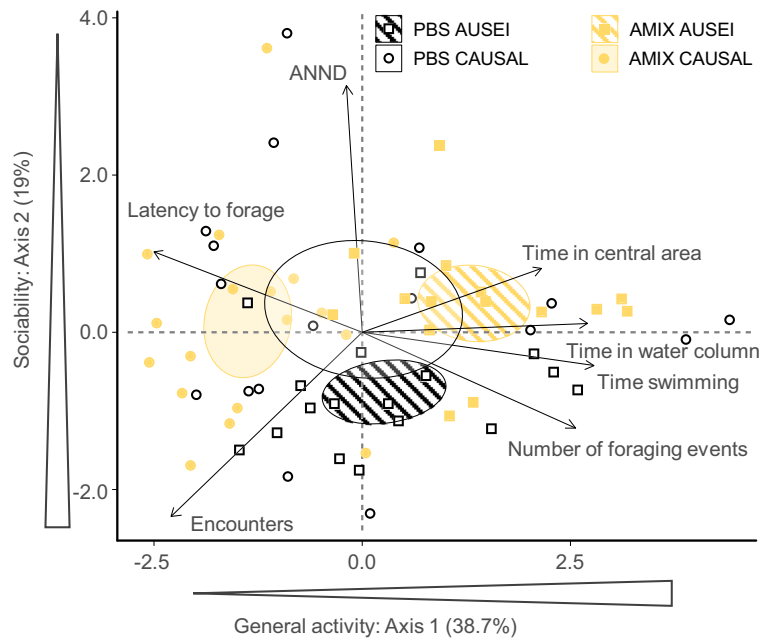
Survival was significantly lower in individuals exposed to combined stressors ( $60 \pm 14\%$ ) compared to fish exposed to control ( $94 \pm 6\%$ ) and single stressors (17°CAMIX  $90 \pm 10\%$ , 24°C PBS  $90 \pm 6\%$ ) (Fig. 2G). Accordingly, the effect size of combined stressors on survival rate was significantly negative ( $-0.68 [-1.35; -0.12]$ ) (Fig. 3A). The interactive effect analysis shows

that combined stressors had a marginally synergistic effect compared to the expected additive effects of both stressors ( $g = 0.59 [-0.07; 1.25]$ ) (Fig. 3B).

#### Discussion

In this study, we tested the effects of multiple stressors (temperature and immune challenge) on gudgeon across

**Fig. 4** PCA of behaviour of fish exposed to multiple stressors (each dot represents an individual). Graphical representation shows the immune treatment and populations groups (temperatures are pooled). Open squares and dots represent control individuals (PBS) from AUSEI and CAUSAL population respectively, while yellow squares and dots represent immune challenge individual (AMIX) from AUSEI and CAUSAL population respectively. White and yellow ellipses represent the 95% confidence interval of immune treatment (control: PBS and immune challenge: AMIX, respectively) while open and hatched ellipses represent the population (CAUSAL and AUSEI population, respectively)



biological levels and the inter-population variability in fish responses. Combined stressors had interactive effects, but only at high levels of biological organisation (behaviour and survival) (summary in Table 3). More specifically, immune challenge inhibited the effects of temperature on activity, and fish exposed to multiple stressors had a decreased survival rate. In addition, populations differed strongly in their responses to stressors, showing that it is important to take into account inter-population variability of sensitivity in wild fish.

#### Single stressor effects

Increased temperature alone had limited effects at the molecular and cellular level, but strong consequences at the individual level. More specifically, temperature alone did affect neither oxidative stress, DNA damage, immune response nor available energy in muscles, contrary to our expectations (Vinagre et al. 2012; Madeira et al. 2013) (Dittmar et al. 2014; Gandar et al. 2017). It is possible that the experimental temperature alone and/or the duration of treatment were not high enough to trigger significant physiological responses because 24 °C is slightly under the upper limit of the gudgeon (27 °C). Alternatively, it is possible that captivity induced stress that would mask the effects of temperature, which is consistent with the high basal levels of oxidative stress and DNA damage observed (17% of DNA damage rate

compared to 5% usually found) (Anitha et al. 2000; Santos et al. 2014; Cheng et al. 2018). At the individual level, high temperature decreased body condition and increased activity as expected. This suggests that high temperature caused energy reallocation from the liver and gonads (decreased HSI and GSI) to maintain energy reserves in muscles (Anacleto et al. 2018) and sustain locomotion, foraging and survival (Gerry and Ellerby 2014; Bonneaud et al. 2016; Sacristán et al. 2017). Accordingly, in our study, survival rate did not decrease under high temperature alone.

The immune challenge alone caused a significant local immune response as expected (Ellis 2001). Although this immune challenge does not entirely reflect the pathogenic effects of parasites, it triggered a costly immune response that is likely to affect other traits across biological levels. However, the immune challenge did influence neither oxidative stress index, available energy in muscles nor condition indices at the molecular and cellular levels. It is possible that the chosen immune challenge was not strong enough to cause significant physiological adjustments and/or that the timing of measure did not enable us to detect it. Whatever the underlying mechanism, this is consistent with the absence of increased DNA damage measured by the comet assay. This suggests either that the energetic cost of the immune challenge was limited and/or that individuals could compensate the increased energy



**Table 3** Summary of results across biological levels. Interactive effects of multiple stressors were observed at the individual level only. In addition, populations (CAUSAL and AUSEI) differed depending on the trait considered. NS indicate non-significant effect

Biological Level	Response trait	Treatment effect	Conclusion multiple stressors	Population variability	Conclusion on population variability	General conclusion
Molecular	Oxidative stress	NS	Basal damage was high and not affected by treatments	CAUSAL > AUSEI	CAUSAL fish had higher damage than AUSEI, suggesting a higher sensitivity to captivity	No effect of treatment. Basal damage were high and different between populations
	DNA damage	NS	Immune challenged fish had a higher immune response	NS	No variability	Immune stressor triggered a significant immune response
Cellular	Local immune response	Immune challenge alone	No treatment effect	CAUSAL > AUSEI	CAUSAL population had higher amount of available energy than AUSEI	No effect of treatments. Populations had different available energy
	Available energy	NS	High temperature decreased condition	NS	No variability	High temperature decreased condition but populations did not vary in their response to temperature
Individual	General body condition index	Temperature alone	Multiple stressors effect: immune challenge inhibited the effect of temperature	Immune challenge × population CAUSAL < AUSEI	Immune challenge decreased activity in CAUSAL but not in AUSEI fish	Marginally antagonistic effects of multiple stressors on activity and inter-population variability
	General activity (PCA axis 1)	Temperature × immune challenge	Immune challenge decreased sociability (in AUSEI)	Immune challenge × population CAUSAL > AUSEI	Immune challenge decreased sociability in AUSEI but not in CAUSAL fish	Immune stressor alone decreased sociability but high inter-population variability
	Sociability (PCA axis 2)	Immune challenge alone	Multiple stressors effect only	NS	No variability	Multiple stressors had marginally synergistic effects on survival
	Survival	Temperature × immune challenge				

demand by changing their behaviour (Wingfield 2003). Accordingly, at the individual level, the immune challenge triggered a decrease of general activity in fish from the CAUSAL population and decreased sociability in fish from the AUSEI population. This is consistent with a sickness behaviour, which usually allows saving energy for immunity (Volkoff and Peter 2004; Bonneaud et al. 2016; Kirsten et al. 2018) and avoid pathogen spread (i.e. “behavioural resistance”) (Barber et al. 2000). Survival was not affected by the immune challenge alone.

Taken together, these results suggest that single stressors (increased temperature or immune challenge alone) had limited detrimental effects at the molecular and cellular levels but triggered changes in energy allocation between traits and organs that could allow fish to maintain homeostasis and limit short-term mortality. This suggests that fish are able to set up adequate physiological responses to face environmentally realistic single stressors with limited effects on fitness. However, such physiological processes could be altered when facing multiple stressors.

#### Multiple stressors

Combined thermal and immune stressors caused complex interactive effects, but mostly at the individual level. At the molecular level, multiple stressors had neither significant combined effects on oxidative stress nor DNA damage. In our study, the integrative oxidative stress index represents the ratio between damage caused by ROS and the non-enzymatic anti-oxidant capacity of the plasma. The level of both damage caused by ROS (i.e. ROM) and non-enzymatic anti-oxidant capacity was not significantly different among treatments. This suggests that in our study, fish exposed to multiple stressors appear capable to maintain the balance between anti-oxidant defences and ROS production. Nevertheless, measuring more precisely others traits related to the enzymatic anti-oxidant capacity of the plasma (such as superoxide dismutase, catalase or glutathione peroxidase activity) could reflect more accurately the anti-oxidant defences against ROS production during the stress response and highlight potential physiological adjustment at the molecular level (e.g. Gandar et al. 2017).

At the cellular level, multiple stressors had no significant combined effects on the local immune response. This could be due to a low effect of each single stressor on the local immune response or to the chosen immune

trait that is not sensitive enough. In both cases, it would thus be interesting to measure other immune traits such as leukocyte counts, oxidative burst or immune gene expression that could be more sensitive to stress exposure (Dittmar et al. 2014; Jolly et al. 2014).

Interestingly, we found significant combined effect of stressors at high level of organisation. Behaviour was particularly sensitive: general activity (i.e. activity, exploration and foraging) decreased in fish exposed to combined stressors. More specifically, temperature strongly increased fish general activity in control saline-injected fish as expected, but this effect of temperature was not significant in immune-challenged fish. This suggests an antagonistic effect of temperature and immune challenge, which could be explained by opposite effects of both stressors on behaviour. Indeed, temperature generally increases activity, sociability and foraging due to increased metabolism (Johansen and Jones 2011; Bartolini et al. 2015; Colchen et al. 2016). On the contrary, pathogens and immune challenges generally decrease these traits due to sickness behaviour, allowing to save energy for immunity (Johnson 2002; Bonneaud et al. 2003; Volkoff and Peter 2004). In the wild, such antagonistic effects of multiple stressors could have important consequences on population persistence. For instance, increased general activity caused by temperature increase allows fish to find more food to sustain the energetic demand of accelerated metabolism (Brett 1971; Nowicki et al. 2012). Our results suggest that concomitant exposure to pathogens and/or immune challenges could inhibit this behavioural response and potentially modify behavioural strategies set up to cope with single stressor exposure.

In this context, such antagonistic effects of multiple stressors could thus limit the ability of fish to sustain their energy expenditure and significantly reduce their fitness. Accordingly, in our study, we observed strong decrease in survival of fish exposed to combined stressors (30% decrease). Although further studies are needed to understand the underpinning mechanisms, our results suggest that multiple stressor effects could thus have far-reaching consequences for individual fitness and population dynamics in the wild. For these reasons, combined effects of stressors at individual level, and especially on behaviour, should be considered in future studies.

In our study, fish were not exposed to pathogens in order to standardize immune challenges and avoid potential pathogenic effects of parasites (Seppala and Jokela 2011). It is likely that pathogen effects would

even be higher than immune challenge effects. In addition, high temperature can increase pathogen prevalence and virulence, which could also amplify detrimental effects of multiple stressors. Taken together, these results also underline the importance of taking into account biotic stressors (here pathogens), because they could strongly affect the response to other abiotic stressors (here temperature). This could help better predicting population responses to human-altered environments.

### Variability between populations

Interestingly, the two chosen populations exhibited strong differences in basal physiological parameters and in physiological responses. More precisely, CAUSAL fish exhibited a higher level of oxidative stress and DNA damage compared to AUSEI fish. This suggests that CAUSAL fish could be more sensitive to captivity and/or have been exposed to higher levels of stressors in the wild before they were captured, but further studies on F1 and F2 fish are now necessary to explore the underlying mechanisms.

In addition, populations had different responses to immune challenge but not to temperature, contrary to our expectations. Thermal regime of capture sites could be too similar to induce differences, although we chose sites with similar contrasts as previous studies (Dittmar et al. 2014). Alternatively, other environmental parameters could differ between sites, and further studies on a higher number of sites are now necessary. Interestingly, populations strongly differed in their response to the immune challenge. These differences could be due to different exposure or costs of infection in the wild (Scharsack et al. 2016), but sites had similar macroparasite prevalence and mean infection intensity which does not support this hypothesis. It is however possible that other parasites were not detected (microparasites).

Interestingly, populations differed in the behavioural traits responding to the immune challenge: CAUSAL fish decreased their general activity, while AUSEI fish reduced their sociability. Hence, our results suggest that different populations could have evolved different behavioural strategies and adjust different behavioural traits (activity/exploration or sociability) to face pathogens. Similarly, Dingemanse et al. (2007) and Bell et al. (2010) found that biotic stressors (predators) could shape behavioural

response and syndrome among populations of three-spined stickleback (*Gasterosteus aculeatus*). However, in our case, the costs and benefits of such combinations of behavioural traits (i.e. behavioural syndromes) on fitness remain to be formally tested.

Whatever the underlying mechanisms, our results highlight the importance of taking into account the inter-population variability to better predict stressor effects in the wild. However, in our study, we only tested two populations and many other environmental factors besides temperature could differ between sites. Further studies on a higher number of replicate populations are now in progress to disentangle the effects of multiple environmental stressors.

### Conclusion

We found significant interactive effects of multiple stressors (combined temperature and immune challenge) but only at high levels of biological organisation (i.e. on behaviour and survival). This suggests that multiple stressors could have unexpected antagonistic effects on behaviour and potential synergistic detrimental effects on fish survival and fitness. However, the underlying mechanisms remain to be elucidated, because no interaction effect was found at the molecular and cellular levels on the studied traits. This highlights the importance of taking into account biotic stressors such as pathogens to understand complex effect of multiple stressors on aquatic organisms. In addition, populations varied in their response to the immune challenge, suggesting alternative behavioural strategies to face pathogens. This highlights the importance of taking into account inter-population variability to better understand the effect of current and future stressors in the wild.

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**Compliance with ethical standards** All experimental procedures were conducted in compliance with French and European legislation for animal experimentation (European directive 2010/63/UE). Fish capture was conducted under the authorization PE 2017-001 and fish were treated for parasites according to the prescription 2529, VetoFish. Experimental procedures were conducted under the French animal handler's certificate (no. 31-103), the establishment approval for vertebrate experimentation no. A3113002, and were approved by the ethical committee no. 073 (authorization no. 8538).

**Conflict of interest** The authors declare that they have no conflict of interest.

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