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Dose- and time-dependent effects of an immune challenge on fish across biological levels

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Abstract

Due to global changes, fish are increasingly exposed to immune challenges associated with disease outbreaks in aquatic ecosystems. Adjustments in physiology and behavior are generally critical to maintaining homeostasis after an immune challenge, but there is limited knowledge on the specific thresholds and dynamics of responses across levels of biological organization in fish. In this study, we tested how different concentrations of an antigens mixture (phytohemagglutinin and lipopolysaccharide) affected innate immunity with potential consequences on oxidative stress, energy reserves, body condition, and behavior across time, using the common gudgeon (*Gobio* sp.) as model species. The immune challenge induced a transitory increase in lytic enzyme activity (i.e., lysozyme) and local immune response (i.e., skin swelling) 2 days after the antigen injection. The available energy stored in muscle was also reduced 4 days after injection, without inducing oxidative stress at the cellular level. Overall, the immune challenge induced limited costs at the molecular and cellular levels but had strong effects at the whole organism level, especially on behavior. Indeed, fish swimming activity and sociability were affected in a dose- and time-dependent manner. These results suggest that immune challenges have dose-dependent effects across levels of biological organization and that behavior is a key response trait to cope with pathogen-induced immune costs in the wild, although fitness consequences remain to be tested.

KEYWORDS

dose–response, ecoimmunology, energy storage, oxidative stress, pathogen, sickness behavior

1 | INTRODUCTION

Global change affects the rate of emerging diseases and disease outbreaks, especially in aquatic ecosystems (Adlard et al., 2015; Okamura et al., 2011). Indeed, anthropogenic activities greatly modify fish–pathogen interactions in freshwater habitats (Dudgeon et al., 2006) and have contributed to several disease outbreaks in fish populations, such as the proliferative kidney disease (PKD; Okamura et al., 2011; Sterud et al., 2007) or the *Gyrodactylus salaris* epidemic (Bakke et al., 2007; Paladini et al., 2014). Consequently, some freshwater fish populations are increasingly exposed to costly immune challenges (Krkošek et al., 2007), but the suite of physiological and behavioral responses against such immune challenges and their associated costs are still poorly known.

When exposed to pathogens, freshwater fish display a cascade of physiological and behavioral responses limiting pathogen proliferation and maintaining fish homeostasis and fitness (Barber et al., 2000; Behringer et al., 2018; Buchmann & Lindenstrøm, 2002). Immunity is a central defense trait, which consists of interconnected innate (nonspecific) and acquired (specific) branches (Lochmiller & Deerenberg, 2000). In fish, the innate immune system is the most critical immune branch, which identifies antigens (Magnadóttir, 2006;

Uribe et al., 2011) and recruits immune cells such as neutrophils, macrophages, and/or lymphocytes locally. This cellular immune response then induces the elimination of pathogens through phagocytosis, the complement system, and several enzymes' secretion. For instance, lytic enzymes such as lysozymes (Alexander & Ingram, 1992; Magnadóttir, 2006) and peroxidase participate in pathogen elimination through the oxidative burst (Ellis, 2001; Quade & Roth, 1997; Rodríguez et al., 2003).

Several studies suggest that these nonspecific immune responses could be extremely costly in birds or mammals (Bonneaud et al., 2003; Lochmiller & Deerenberg, 2000; Sheldon & Verhulst, 1996). However, studies in fish are still scarce, so that the physiological and fitness costs of immune challenges in fish remain debated. During the immune response, metabolic activity increases to support the energy requirements of immune cells (King & Swanson, 2013; Rauw, 2012). Several consequences are expected, such as a depletion of energy reserves in the liver and muscle (Wang et al., 2012), with potentially detrimental effects on body mass and whole-body condition (Bonneaud et al., 2003). For instance, immune challenges can deplete liver glycogen in coho salmon (*Oncorhynchus kisutch*) and rainbow trout (*Oncorhynchus mykiss*) (Wedemeyer et al., 1969) and cause a significant body mass loss in mosquitofish (*Gambusia holbrooki*) (Bonneaud et al., 2016). The immune response itself and the associated increase of metabolic activity can lead to an overproduction of reactive oxygen species (ROS), which may exceed the amount of antioxidant molecules and result in deleterious consequences on cell and tissue integrity (Cherry & Piantadosi, 2015; Costantini & Møller, 2009; Finkel & Holbrook, 2000; Jaeschke et al., 2002; Preynat-Seauve et al., 2003). However, the specific thresholds and timing of these responses are still poorly known.

In addition, immune challenges can affect animal behavior, but the behavioral costs of immune challenges in fish are still elusive (Combes, 2001; Filiano et al., 2016; Johnson, 2002). The so-called "sickness behavior" is a suite of behavioral changes, including a decrease of swimming activity, exploration, and social behavior that can enable hosts to save energy for immunity and/or to limit the exposure to pathogens (Adelman & Martin, 2009; Combes, 2001). For instance, Kirsten et al. (2018) recently showed that immune-challenged zebrafish (*Danio rerio*) displayed reduced swimming activity, sociability, and exploratory behavior, which could benefit individuals by saving energy for immunity, but could also have severe negative consequences for fish fitness by reducing fish ability to forage, find mates, or escape predators (Wong & Candolin, 2015). However, the threshold (e.g., the amount of antigens required) and the timing (i.e., duration) at which immune challenges trigger such behavioral changes are still unclear.

More generally, few studies investigated the cascading effects of immune challenges across levels of biological organization (i.e., from molecules to the whole individual), especially in fish, which limits our ability to predict the impacts of pathogens on fish fitness (Kirsten et al., 2018). Empirical studies are thus needed to test the potential physiological and behavioral changes in immune-challenged fish across time and biological levels.

To address these questions, we used the common gudgeon (*Gobio* sp.) as a model species because it is a common freshwater fish species from European streams, which is widely exposed to immune challenge by numerous pathogens in the wild (Loot et al., 2007). We tested the effects of different concentrations of an antigen mixture (i.e., phytohemagglutinin or PHA and lipopolysaccharide or LPS) on humoral (i.e., lysozyme, hemolytic, and peroxidase activity) and local cellular immune response (i.e., measured by skin swelling). We also measured oxidative stress in fish blood and available energy in muscle cells to test the oxidative and energetic costs of immune challenges. At the organ and individual level, we measured organo-somatic indices, body mass changes, and fish behavioral traits (swimming activity and sociability). To better understand the dose-dependent temporal effects of immune challenges, we tested several antigen doses and monitored the kinetics of fish responses across time.

2 | MATERIALS AND METHODS

2.1 | Model species

The common gudgeon (*Gobio* sp.) was chosen as a model species because it is ubiquitous in Europe's lowland streams (Keith et al., 2011). This benthic fish forages in the substrate for invertebrates and is gregarious, thus displaying complex social behaviors by forming schools (Keith et al., 2011; Pitcher, 1986). Gudgeons are exposed to several parasites in their natural habitats, such as monogenean ectoparasites, copepods, or nematode endoparasites (Loot et al., 2007). The gudgeon is also relatively sedentary and thereby potentially exposed to the same environmental stressors throughout its lifetime (Keith et al., 2011; Stott et al., 1963).

2.2 | Animal care

Three hundred forty gudgeons were purchased from the fish farming Les Viviers de Haute Corrèze (Courteix, France) in size range 10–14 cm (13.3 ± 2.7 g). At the fish farm, fish were raised in natural ponds without selective breeding programs nor vaccination treatment. Fish were brought to the laboratory and were immediately deparasitized using Praziquantel (Prescription no. 2529; purity = 1000 mg g^{-1}; concentration: 3 mg L^{-1}; VetoFish) to eliminate potential parasites and standardize their immune status before the experimentation. Fish were then acclimated for 30 days in four 200 L tanks (85 fish per tank) containing oxygen pumps, mechanical filters, gravel, and shelters to reduce stress. During acclimation, temperature and light regime were 14°C and 12:12 h, respectively. Physico-chemical parameters were monitored every week (pH 7.9 ± 0.2; conductivity = 324 ± 29 µS cm^{-1}; $O_{2\,dissolved}$ = 11.4 ± 1.1 mg L^{-1}). Half of the water was renewed every week to ensure proper conditions. Fish were fed daily (about 1% of the total biomass per tank) with commercial fish food (JBL Propond Sterlet S) to ensure a sufficient

daily food supply (Flammarion et al., 1998; Kestemont et al., 1991). No mortality occurred during the acclimation period.

2.3 | Antigens

To standardize the immune challenge among individuals and mimick an infection by a wide range of pathogens, we used a mixture of two antigens: LPS (i.e., *Escherichia coli* lipopolysaccharide, serotype: O111:B4, L2830; Sigma-Aldrich) and PHA (i.e., *Phaseolus vulgaris* phytohemagglutinin-P, L8754; Sigma-Aldrich). Both antigens are commonly used in ecoimmunological studies to trigger inflammatory, and more broadly, innate immune response in various vertebrates, including fish (Ardia & Clotfelter, 2006; Bonneaud et al., 2003; Otálora-Ardila et al., 2016). The LPS antigen is an endotoxin from the membrane of Gram-negative bacteria, which triggers a substantial and costly local and humoral innate immune response (Swain et al., 2008). The PHA is a plant protein 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., 2008). Because the gudgeon's immune system is poorly known, we injected both antigens conjointly in an antigen mixture to maximize our chance to trigger a significant immune response.

2.4 | Experimental design

Before the experiment, 20 fish were randomly selected and used as a reference untreated (i.e., uninjected) group. They were sampled immediately after acclimation (untreated fish sampled at $T = 0$; Figure 1). The remaining fish were marked with visible implant elastomers (Northwest Marine Technologies, Inc.) to identify each individual for behavioral assays.

Fish were then randomly assigned to one out of four experimental treatments (Figure 1) and maintained in the same conditions as for acclimation (i.e., temperature 14°C, light regime 12:12 h, half of the water renewed every week). There were $n = 80$ fish per treatment, distributed in four replicate tanks ($n = 20 \times 4$ replicate tanks per treatment). Fish involved in the experiment were anesthetized using benzocaine at 50 mg L^{-1} and subcutaneously injected in the caudal peduncle with 10 µl of phosphate-buffered saline solution (control-saline group named: PBS, $n = 80$ control fish) or to different concentrations of each antigen at 4.5, 9.0, or 18.0 g L^{-1} (treatment names: low, medium, and high, respectively) using Hamilton syringe (10 µl, 26-gauge). Hence, fish from low, medium, and high-dose groups were injected with 10 µl of PBS containing 45 µg of PHA and 45 µg of LPS, 90 µg of PHA and 90 µg of LPS, or 180 µg of PHA and 180 µg of LPS, respectively. We chose these concentrations based on previous studies using a constant ratio of antigens concentration for fish biomass corresponding approximately to 4.5, 9, and 18 mg kg^{-1} of each antigen (see Ardia & Clotfelter, 2006; Johansen et al., 2006; Jolly et al., 2014; Le Guernic et al., 2016).

We then monitored fish responses across time (0, 2, 4, 8, and 16 days) by sampling 20 fish per time point in each treatment group (Figure 1). These time durations were chosen based on the literature showing significant changes in immune activity at 2 and 4 days postinjection in Atlantic salmon (*Salmo salar*) and three-spined stickleback (*Gasterosteus aculeatus*), respectively (Langston et al., 2001; Le Guernic et al., 2016), while behavioral changes can be detected at 2, 4, and 8 days in mice (Zhao et al., 2019). In addition, we sampled fish 16 days after the immune challenge to observe potential lasting consequences on fish energy mobilization and behavior.

At each time point, videos were recorded to measure fish behavior (details below). Fish were then euthanized using an anesthetic overdose (benzocaine, 150 mg L^{-1}), weighed (±0.1 g) and measured (±0.1 cm). Blood samples were collected from the caudal vein in heparinized syringes (1 ml Terumo syringe, 0.45 × 13 mm needle). The collected blood samples were centrifuged (4°C, 2000*g*) for 10 min to retrieve plasma. Plasma samples were aliquoted in two parts and kept at −20°C for subsequent immune and oxidative damage and antioxidant capacity assays (see below). Fish were then dissected for sex determination and further analyses on tissues. Spleen and liver

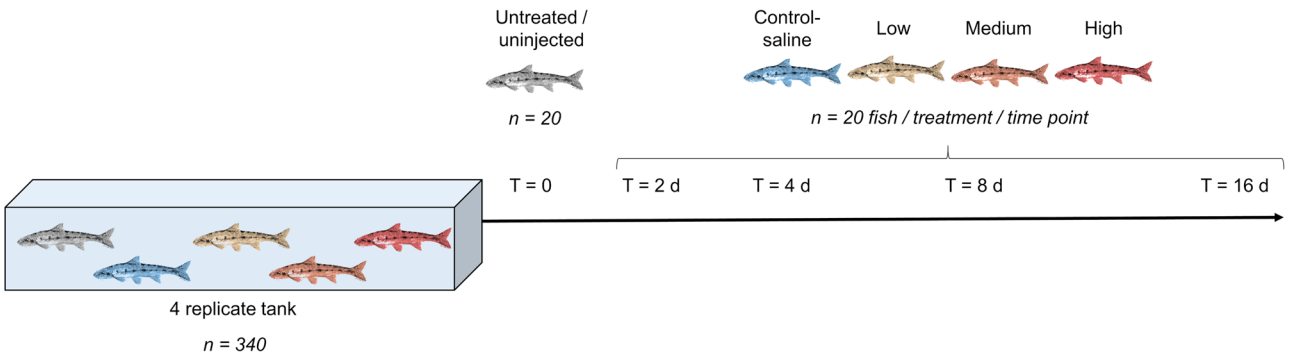

FIGURE 1 Experimental design: at the end of the acclimation, 20 fish were sampled and considered as untreated (uninjected) fish. The remaining fish were randomly assigned to four treatments ($n = 80$ fish per treatment): control-saline (injected only with a control-saline solution of PBS, in blue), low-dose (injected with 4.5 g L^{-1} of each antigen, in yellow), medium-dose (injected with 9.0 g L^{-1} of each antigen, in orange), and high-dose group (injected with 18.0 g L^{-1} of each antigen, in red). At each time point (2, 4, 8, and 16 days), we collected 20 fish from each experimental group. PBS, phosphate-buffered saline solution [Color figure can be viewed at wileyonlinelibrary.com]

were weighed (±1 mg) to calculate condition indices while the white muscle of fish was immediately frozen in liquid nitrogen and stored at −80°C to assess fish energy reserves (see below).

In total, 20 fish per treatment and per time point were used, but due to difficulties in collecting blood or tissue on some small individuals, sample sizes can vary across groups (from $n = 7$ to $n = 20$ depending on the considered trait, treatment, and time point). We checked the injection of PBS itself was not stressful for fish by comparing saline-injected fish (PBS) with untreated fish for all traits (see results in Table 1 below). There was no difference in sex ratio (Kruskal–Wallis test $\chi^2 = 19.54$, $p = .24$) nor fish mass (Kruskal–Wallis test $\chi^2 = 14.13$, $p = .59$); and size (Kruskal–Wallis test $\chi^2 = 12.87$, $p = .68$) among treatments. Survival rate was 98.75% and was not significantly different among treatments (binomial LM, $\chi^2 = 18.0$, $p = .32$), with a total of four dead fish (i.e., one from low- and three from high-dose treatment groups), showing that the experimental treatments resulted in very low mortality.

2.5 | Compliance with ethical standards

Experimental procedures were conducted under the establishment approval for vertebrate experimentation No. A3113002 and were endorsed by the ethical committee No. 073 (authorization No. 8538) as stated in the French and European legislation for animal experimentation (European directive 2010/63/UE).

2.6 | Molecular level: Oxidative stress index

We measured the nonenzymatic antioxidant capacity of the plasma barrier (Isaksson, 2013) using the OXY-adsorbent test (expressed in mMeq HClO; Diacron International, Grosseto, Italy) and both active oxidant action and oxidative damage (Alberti et al., 2000) using d-ROM test (expressed in mMeq H_2O_2; Diacron International). Both assays were performed according to the manufacturer instructions with some modifications for fish plasma sample, as suggested in previous studies (Bagni et al., 2007; Hoogenboom et al., 2012; Petitjean et al., 2020). Intra- and inter-plate repeatability was 88.2 ± 8.9% and 89.1 ± 2.1%, respectively for OXY-adsorbent test and 95.4 ± 4.0% and 95.9 ± 2.3% for d-ROM test. We then used the ratio of oxidative damage divided by the antioxidant capacity multiplied by 1000 to calculate an oxidative stress index (Costantini et al., 2006; Herborn et al., 2011), reflecting the oxidative status of each fish.

2.7 | Molecular level: Immune response

We measured the humoral immune response through three parameters: the lysozyme activity, the hemolytic (complement) activity, and the peroxidase activity in fish plasma.

Lysozymes are essential antimicrobial proteins of the innate immune system that alter the cell wall of Gram-positive and Gram-negative bacteria (Magnadóttir, 2006). We measured lysozyme activity using a turbidometric assay according to previous studies (Cha et al., 2008; Kumari & Sahoo, 2005). Briefly, lyophilized *Micrococcus lysodeikticus* cells (Gram-positive bacteria, ATCC No. 4698; Sigma-Aldrich) were suspended in a sodium citrate buffer (0.05 M, pH 5.5) at a concentration of 0.2 mg ml^{-1}. One hundred fifty microliters of cell suspension was added to fish plasma (15–20 µl) in 96-well microtiter plates. Lysozyme activity was then measured through the decrease of absorbance (at 450 nm) along a 40 min kinetics of 5 min intervals and corrected according to the volume of plasma used in the assay. A unit of lysozyme activity (U) was defined as the quantity of sample required to induce a reduction in absorbance of 0.001 min^{-1}.

The hemolytic activity is a nonspecific measure of the innate immune system, activated by numerous micro-organisms, which measures the activity of the complement immune system of fish (Magnadóttir 2000, 2006). We measured the hemolytic activity of fish plasma according to previous methods (Magnadóttir, 2000; Sakai, 1992). Briefly, red blood cells of sheep (bioMérieux) were diluted in an Hanks' balanced salt solution (HBSS) buffer containing Mg^{2+} to reach a concentration of 3%. Serial dilutions of 20 µl of fish plasma sample (six dilutions per samples: $1/10^e$, $1/20^e$, $1/40^e$, $1/80^e$, $1/160^e$, $1/320^e$) were made within 96-well microtiter plates and 100 µl of the 3% blood cell suspension was added in each well. After 60 min of incubation at 21°C, the microplate was centrifuged at 800*g* for 5 min, and 100 µl of the supernatant was transferred to another microplate to read sample absorbance at 450 nm. Within each plate, maximum and spontaneous hemolysis (i.e., positive and negative controls) were made by adding 100 µl of distilled water or HBSS buffer to the blood cell suspension, respectively. Hemolytic activity was then measured as the concentration of plasma necessary to lyse 50% of red blood suspension (U ml^{-1}).

The peroxidase activity is a measure of enzymes' activity (i.e., myeloperoxidase and eosinophil peroxidase) released during the oxidative burst by leukocytes to fight pathogens (Guardiola et al., 2013; Rodríguez et al., 2003). We measured the peroxidase activity of fish plasma according to previously described methods (Guardiola et al., 2013; Quade & Roth, 1997). Briefly, 5 µl of plasma was diluted in 500 µl of HBSS buffer without Ca^{2+} or Mg^{2+}. We then placed 60 µl of diluted plasma in 96-well microtiter plates and added 100 µl of enzyme substrate composed of 1 ml of tetramethylbenzidine and dimethylsulfoxide (TMB and DMSO; 1:1 m/v; Sigma-Aldrich), 20 µl of 3% hydrogen peroxide (30% H_2O_2; Sigma-Aldrich), and 9 ml of citrate phosphate buffer (0.05 M, pH 5.0) in each well. After an incubation of 2 min, we added 50 µl of sulfuric acid (2 M, H_2SO_4; Sigma-Aldrich) to stop the reaction and read sample absorbance at 450 nm. A unit of peroxidase activity (U) was defined as quantity of enzyme per milliliter of plasma necessary to oxidize 1 mole of H_2O_2 per minute.

2.8 | Cellular level: Local immune response

We assessed the local immune response by measuring the local skin swelling (i.e., thickness measurement) of the caudal peduncle after

immune challenge or control injection following previous studies in fish (Ardia & Clotfelter, 2006; O'Connor et al., 2014). The local immune response reflects the intensity of the local inflammation and the proliferation of T cells (Ardia & Clotfelter, 2006; O'Connor et al., 2014; Tella et al., 2008). The thickness of the caudal peduncle was measured three times by the same operator and averaged before and after the injection of the antigen mixture or PBS using a thickness gauge (Elcometer® 124) to calculate the intensity of the local immune response as the difference of peduncle thickness before and after injection divided by the thickness before injection × 100. The intra-individual repeatability was 97.0 ± 1.2% before injection and 96.2 ± 1.0% after injection.

2.9 | Cellular level: Available energy

The amount of available energy in white muscle cells is a good marker of energy status because it is critical for the maintenance of biological functions of fish, especially under stress (Gandar et al., 2016; Gomes et al., 2015). We thus used previously validated protocols (De Coen & Janssen, 1997; Gandar et al., 2017) to measure the total available energy in fish muscle by summing energetic values of carbohydrates, proteins, and lipids content expressed in mJ mg^{-1} of muscle (see Gandar et al., 2017; Petitjean et al., 2020).

2.10 | Organ level: Conditions indices

Three condition indices were calculated to reflect the general condition of fish based on organ mass. Firstly, we measured an averaged daily body mass change by calculating body mass change before and after the experiment. We then standardized this value by the number of experimental days fish experienced to measure an averaged daily changes in whole-body condition. Secondly, we measured the SplenoSomatic Index (SSI, spleen mass corrected by the body mass × 100) as a proxy of spleen state resulting from the production of erythrocytes and lymphocytes and their release in the blood during the immune and stress responses (Maule & Schreck, 1990; Nilsson, 1983; Pearson & Stevens, 1991). Finally, we measured the HepatoSomatic Index (HSI; liver mass corrected by the body mass × 100) as a proxy of energy content in the liver (Chellappa et al., 1995).

2.11 | Individual level: Behavior

To measure fish behavior, fish were placed by groups of five individuals (from the same tank and the same treatment) in a rectangular arena (75 × 50 cm) containing 30 L of water. During behavioral measurements, fish were kept in groups of five individuals to reduce stress and record social behaviors. After 5 min of acclimation, videos were recorded using Webcam Logitech C922 Pro Stream (30 fps, 1080p) and blindly analyzed using Boris software (Friard & Gamba, 2016) for 10 min based on previous studies (Jacquin et al., 2017; Lopez-Luna et al., 2017; Petitjean et al., 2020). Three behavioral traits were chosen because they are commonly affected by pathogens and immune challenges: swimming activity, exploration, and sociability (Barber et al., 2000). To measure fish swimming activity and exploration, the tank was virtually divided in six areas (25 × 25 cm). The swimming activity was measured as the time swimming and the number of crossed lines (area limits) for 10 min (Jacquin et al., 2017; Lopez-Luna et al., 2017; Petitjean et al., 2020), while the exploration was measured as the number of visited areas following previous studies (Jacquin et al., 2017). Sociability was assessed as the number of encounters between individuals (number of times an individual touches another individual with the head; Geffroy et al., 2014).

Because behavioral traits related to swimming activity and exploration were partly correlated (see Figure A1), we used a principal component analysis (PCA) to extract one synthetic variable representing the "general activity" of fish (PCA axis 1, 52.9% of variance explained). Fish with a higher general activity index on the first PCA axis swam for a longer time and explored more areas. The number of contacts was used as a proxy of sociability (Colchen et al., 2016; Geffroy et al., 2014).

2.12 | Statistics

To test whether time and antigen doses affect fish responses, we used linear mixed-effects models (LMM; lme4 package; Bates et al., 2015) on each trait: oxidative stress ratio (log-transformed), lysozyme (root-square transformation), hemolytic activity (root-square transformation), peroxidase activity (log-transformed), local immune response (skin swelling), total available energy in muscle (log-transformed), daily mass change (log-transformed), SSI (log-transformed), HSI (log-transformed), general activity (first behavioral PCA axis), and sociability (number of contacts, log-transformed). The experimental replicate tank was included as a random effect to take into account possible shared conditions in the same tank. The fixed explanatory variable was the combination of the treatment group (PBS, low dose, medium dose, or high dose of antigens) and time postinjection (0, 2, 4, 8, and 16 days). Fish size and sex were added as covariates in all models but removed from final models when non-significant. When the effect of the fixed explanatory variable (i.e., treatment group combined with the time postinjection) was significant, we used contrast post hoc analyses (emmeans package; Lenth et al., 2017) with False Discovery Rate adjustment (Benjamini & Hochberg, 1995) to analyze differences between each treatment (i.e., dose and time) and the control-saline group within a given time point. To control the potential effect of captivity and elastomers injection, we compared fish involved in the experiment (including control-saline PBS fish) and untreated fish sampled at $T = 0$ day. Statistical analyses were made using the open-source software R (V 3.5.2).

3 | RESULTS

3.1 | Molecular level: Oxidative stress

We found no difference in oxidative stress between untreated fish and fish involved in the experiment. In addition, the oxidative stress index was not affected by any treatment (LMM; $\chi^2 = 22.2$, $p = .14$; Table 1). More specifically, oxidative damage and antioxidant capacity (LMM; $\chi^2 = 20.1$, $p = .22$ and $\chi^2 = 18.3$, $p = .31$, respectively) were not significantly affected by antigen injection, whatever the time point.

3.2 | Molecular level: Humoral immune response

Fish from the control-saline PBS group had a marginally higher lysozyme activity compared to untreated fish, but only after 16 days of exposure (LMM post hoc test, estimate = 1.57 ± 0.65, $p = .05$; see Table 1), suggesting the injection itself, even of the PBS saline solution triggered a response compared to untreated (i.e., noninjected) fish but only at long time scales. Fish injected with a medium and high dose of antigens (9.0 and 18.0 g L^{-1}, respectively) had increased lysozyme activity compared to the control-saline PBS group 2 days after injection only (LMM post hoc test, estimate = 1.98 ± 0.65, $p = .02$; estimate = 1.51 ± 0.63, $p = .05$, respectively; Table 1 and Figure 2).

In contrast, we did not find any significant effect of the antigen injection on hemolytic and peroxidase activities of fish plasma (LMM, $\chi^2 = 24.4$, $p = .082$ and $\chi^2 = 26.6$, $p = .046$, respectively), whatever the time (Table 1).

3.3 | Cellular level: Local immune response

Since untreated fish were not injected, we did not measure their local immune response before the experiment. We found that all injected fish displayed a significant local immune response (i.e., skin swelling) compared to the control-saline PBS group, whatever the concentration of antigen injected and the time (LMM, $\chi^2 = 88.2$, $p < .001$; Table 1) except for fish injected with the low dose of antigens (4.5 g L^{-1}) after 8 days of exposure (LMM post hoc test, estimate = 3.16 ± 2.70, $p = .24$).

3.4 | Cellular level: Available energy

The amount of available energy (i.e., sum of lipids, proteins, and carbohydrates) in fish muscle increased significantly in all treatment groups compared to the untreated fish (LMM, $\chi^2 = 46.6$, $p < .001$; see Table 1) suggesting that the experiment itself increased fish reserves, except in fish injected with medium and high dose of antigens 4 days after antigen injection (LMM post hoc test, estimate = 835.8 ± 383.1, $p = .079$; estimate = 664 ± 360.9, $p = .14$, respectively; Table 1). This difference between fish groups treated with medium and high dose of antigens 4 days after injection and other experimental groups was mainly due to a reduced amount of lipids stored in fish muscle (LMM post hoc test on lipids separately: medium dose: estimate = 835.8 ± 383.1, $p = .08$; high dose: estimate = 664 ± 360.9, $p = .14$, respectively).

3.5 | Organ level: Conditions indices

There was no effect of the injection itself (untreated vs. control-saline PBS group) nor treatments on the daily mass change (LMM, $\chi^2 = 24.2$, $p = .09$) and the SSI of fish ($\chi^2 = 35.7$, $p = .003$, but see nonsignificant multiple comparisons among treatment groups in Table 1), whatever the concentration of antigens and time (Table 1). Regarding the HSI, there was no significant difference between fish groups injected with antigens and control-saline PBS group (Table 1). However, fish injected with control-saline solution and with high doses of antigens had a higher HSI than untreated fish 16 days after injection reflecting a higher amount of energy in the liver (LMM post hoc test, estimate = 0.53 ± 0.13, $p = .015$; estimate = 0.57 ± 0.14, $p = .015$, respectively).

3.6 | Individual level: Behavior

We did not measure behavioral response before the experiment (untreated fish). In treated fish, we found that antigen injection significantly affected fish general activity (LMM, $\chi^2 = 52.9$, $p < .001$; Table 1). Indeed, at 4 days, fish injected with medium and high dose of antigens decreased their general activity compared to saline-injected fish (LMM post hoc test, estimate = −1.18 ± 0.43, $p = .027$; estimate = −1.73 ± 0.43, $p < .001$, respectively; Table 1 and Figure 3). Detailed analyses on each separate behavioral trait confirmed that fish injected with high dose had a lower swimming time and explored fewer areas than control-saline fish at 4 days (LMM post hoc test, estimate = −21.7 ± 6.57, $p = .01$ and estimate = −0.45 ± 0.18, $p = .05$, respectively).

However, fish then increased their swimming activity. Indeed, fish from the low and medium dose groups displayed a higher general activity than control-saline injected fish at 16 days (LMM post hoc test, estimate = 1.09 ± 0.44, $p = .04$; estimate = 1.43 ± 0.43, $p = .006$, respectively; Table 1 and Figure 3).

Fish sociability (number of encounters between individuals) was also significantly affected by the immune challenges. Indeed, the number of contacts was significantly lower in fish injected with the high dose of antigens 4 days after injection compared to saline-injected fish, respectively (LMM post hoc test, estimate = −0.50 ± 0.17, $p = .045$; Table 1).

4 | DISCUSSION

Altogether, our results indicate that different antigen mixtures induce differents effects in fish and that responses differed depending

TABLE 1 Linear mixed models (LMM) explaining fish responses to treatments across time (2, 4, 8, or 16 days): untreated $T = 0$, injection of control-saline solution (PBS), injection of a low (4.5 g L^{-1}), medium (9.0 g L^{-1}), or high (18.0 g L^{-1}) dose of LPS and PHA antigens

Biological levels	Response traits unit	Time (days)	Treatments							
			Untreated, $T = 0$ Mean ± *SE*	Control PBS Mean ± *SE*	Low Mean ± *SE*	Medium Mean ± *SE*	High Mean ± *SE*	*n*	χ^2	*P*
Molecular	Oxidative stress index (mMeq H_2O_2/mMeq HClO)	2	4.5 ± 1	2.6 ± 0.6	3.8 ± 0.8	4.4 ± 1.2	4.5 ± 1.6	302	22.2	.14
		4		1.7 ± 0.4	2.4 ± 0.5	2.7 ± 0.6	3.6 ± 0.9			
		8		3.6 ± 0.8	6.3 ± 3.8	3.7 ± 1.9	1.7 ± 0.3			
		16		1.7 ± 0.4	1.9 ± 0.6	4.9 ± 1.2	2.4 ± 0.8			
	Lysozyme activity (U ml^{-1})	2	4.7 ± 2.6 A	4.9 ± 2.1 A a	6.3 ± 2.2 A a	18.3 ± 3.8 B b	16 ± 4.2 B b	279	36.7	.002
		4		8.8 ± 2.1 A a	12 ± 3.4 A a	11.8 ± 3.2 A a	9.9 ± 4.3 A a			
		8		10.8 ± 3.5 A a	18.3 ± 3.9 B a	14.9 ± 3.5 B a	11.9 ± 3.1 A a			
		16		14 ± 3.1 B a	14.4 ± 3 B a	5.9 ± 2.2 A a	18.9 ± 4 B a			
	Hemolytic activity (U ml^{-1})	2	12.9 ± 3.7	4.3 ± 1.6	11 ± 2.2	9.7 ± 3.5	7.7 ± 1.4	260	24.4	.082
		4		5.9 ± 2.1	6.6 ± 2	13.3 ± 5	9.5 ± 3.1			
		8		3.4 ± 1.6	5.2 ± 1.9	6.2 ± 4.6	5.4 ± 1.7			
		16		6.4 ± 2.2	4.3 ± 1.2	16.2 ± 5	12.4 ± 5.6			
	Peroxidase activity (U ml^{-1})	2	4.3 ± 2.6 A	23.9 ± 14 A a	69.7 ± 55.7 A a	25.6 ± 10.6 A a	41.5 ± 21.3 A a	221	26.6	.046
		4		68.9 ± 41.5 A a	40.4 ± 20.9 A a	90.3 ± 36.5 A a	132.4 ± 56.7 A a			
		8		46.1 ± 24.3 A a	96.5 ± 49.3 A a	104.5 ± 37.3 A a	97.5 ± 35.6 A a			
		16		115.9 ± 67.7 A a	47.2 ± 27.6 A a	124 ± 65 A a	171.1 ± 57.3 B a			
Cellular	Local immune response (swelling percentage)	2	NA	2.7 ± 1.7 a	9.5 ± 1.8 b	10.1 ± 2.1 b	12.2 ± 2 b	316	88.2	<.001
		4		4.8 ± 1.4 a	13.4 ± 1.5 b	12.6 ± 2.3 b	11.6 ± 2.1 b			
		8		2.7 ± 1.8 a	5.9 ± 2.2 a	11.6 ± 1.7 b	13.1 ± 2 b			
		16		−2.2 ± 2.2 a	5.1 ± 1.8 b	5.6 ± 2.4 b	7 ± 2.2 b			
	Available energy (mJ. mg_{muscle}^{-1})	2	3192.2 ± 152 A	4762.7 ± 401 B a	4924.4 ± 362.6 B a	5013.1 ± 349.8 B a	4837.5 ± 286.7 B a	323	46.6	<.001
		4		4857.6 ± 347.3 B a	4576.9 ± 291.8 B a	4386.9 ± 285.4 A a	4195.2 ± 254.8 A a			
		8		5355.9 ± 382.1 B a	5305.7 ± 314.2 B a	4803.6 ± 308.9 B a	4756.1 ± 300 B a			
		16		4889.7 ± 440 B a	4716.6 ± 311.1 B a	5527.3 ± 434.9 B a	5020.3 ± 418.3 B a			

(Continues)

TABLE 1 (Continued)

Biological levels	Response traits unit	Time (days)	Treatments							
			Untreated, $T = 0$ Mean ± *SE*	Control PBS Mean ± *SE*	Low Mean ± *SE*	Medium Mean ± *SE*	High Mean ± *SE*	*n*	χ^2	*P*
Organ	Daily mass change (Percentage)	2	NA	0.94 ± 0.45	−0.87 ± 0.18	−0.54 ± 0.17	−0.51 ± 0.36	316	21.7	.12
		4		−0.26 ± 0.23	0.11 ± 0.20	0.38 ± 0.33	0.01 ± 0.22			
		8		−0.41 ± 0.05	−0.41 ± 0.06	−0.54 ± 0.21	−0.42 ± 0.05			
		16		−0.30 ± 0.06	−0.34 ± 0.06	−0.31 ± 0.03	−0.35 ± 0.07			
	SSI (Relative spleen mass percentage)	2	0.48 ± 0.06 A	0.35 ± 0.05 A a	0.32 ± 0.04 A a	0.31 ± 0.04 A a	0.41 ± 0.06 A a	336	35.7	.003
		4		0.38 ± 0.05 A a	0.42 ± 0.05 A.a	0.41 ± 0.1 A a	0.45 ± 0.06 A a			
		8		0.58 ± 0.07 A a	0.44 ± 0.05 A a	0.35 ± 0.04 A a	0.59 ± 0.1 A a			
		16		0.58 ± 0.09 A a	0.44 ± 0.07 A a	0.43 ± 0.07 A a	0.54 ± 0.08 A a			
	HSI (relative liver mass percentage)	2	0.7 ± 0.1 A	0.6 ± 0.1 A a	0.8 ± 0.1 A a	0.8 ± 0.1 A a	0.8 ± 0.1 A a	336	65.9	<.001
		4		0.8 ± 0.1 A a	0.9 ± 0.1 A a	0.9 ± 0.1 A a	0.9 ± 0.1 A a			
		8		1 ± 0.1 A a	0.8 ± 0.1 A a	0.8 ± 0.1 A a	0.8 ± 0.1 A a			
		16		1.4 ± 0.2 B a	1.1 ± 0.1 A a	1 ± 0.1 A a	1.3 ± 0.1 B a			
Individual	General activity (PCA axis)	2	NA	−0.1 ± 0.2 a	0.5 ± 0.2 a	−0.3 ± 0.2 a	−0.5 ± 0.6 a	316	52.9	<.001
		4		0.9 ± 0.3 a	0.3 ± 0.2 a	−0.3 ± 0.2 b	−0.8 ± 0.5 b			
		8		0.3 ± 0.2 a	0.3 ± 0.2 a	0.7 ± 0.3 a	0.1 ± 0.1 a			
		16		−0.9 ± 0.5 a	0.2 ± 0.2 b	0.5 ± 0.2 b	−0.9 ± 0.4 a			
	Sociability (contacts counts)	2	NA	64.7 ± 6.2 a	64 ± 7.1 a	65.3 ± 6.8 a	60.6 ± 8.8 a	316	32.1	<.01
		4		65.3 ± 6.7 a	61.3 ± 6.8 a	45.1 ± 4.1 a	46.7 ± 8.6 b			
		8		46.3 ± 6 a	44.4 ± 6 a	67.9 ± 7.8 a	56.8 ± 6.3 a			
		16		53.3 ± 6.5 a	51.3 ± 7.2 a	60.1 ± 5 a	54.8 ± 4.2 a			

Note: The χ^2 and *p* value on the right correspond to the ANOVA tests testing the effect of the treatment group combined with the time postinjection. Different upper case letters indicate significant differences due to the experiment itself (i.e., differences between the experimental group and untreated fish euthanized at $T = 0$). Different lower case letters indicate significant differences between control-saline fish (PBS) and antigens-injected fish (low, medium, and high dose) at each time point (2, 4, 8, and 16 days) from post hoc tests.

Abbreviations: ANOVA, analysis of variance; LPS, lipopolysaccharide; PBS, phosphate-buffered saline solution; PHA, phytohemagglutinin.

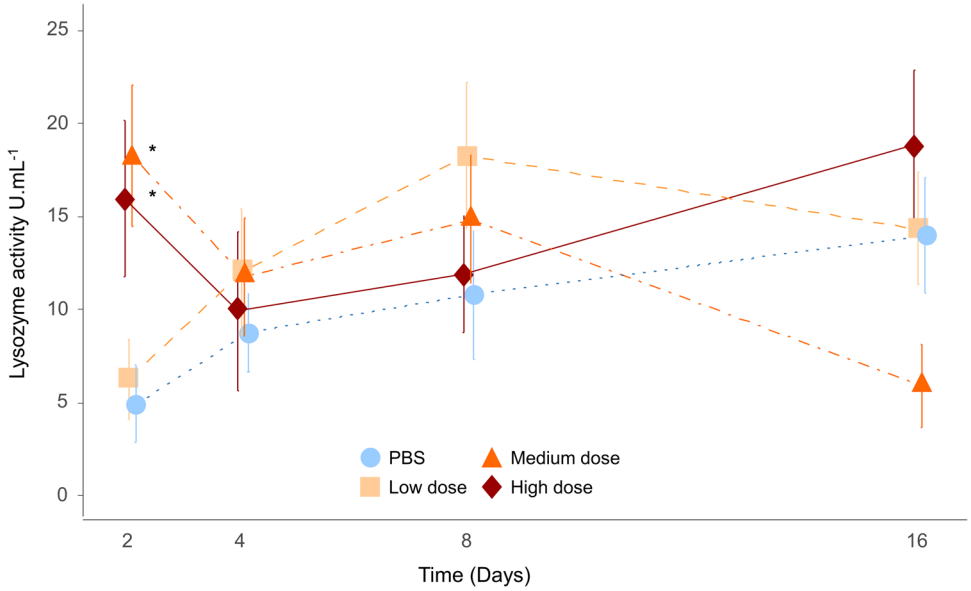

FIGURE 2 Mean lysozyme activity in the plasma ± *SE* (in U ml^{-1}) of fish in different treatment groups across time (blue circles and dotted line: control-saline group; yellow squares and dashed line: low dose of antigens 4.5 g L^{-1}; orange triangles and dot-dashed line: medium dose of antigens 9.0 g L^{-1}; red diamonds and solid line: high dose of antigens 18.0 g L^{-1}). Stars indicate significant differences between antigen-injected fish and the control-saline group (PBS injected fish, in blue) within each time point. PBS, phosphate-buffered saline solution [Color figure can be viewed at wileyonlinelibrary.com]

on the biological level considered. Medium and high doses of antigens had the highest effects, notably on immunity, energy reserves, and behavior (Table 2), and appeared to be more costly than a low dose of antigens. Hence, this suggests that the immune response and the associated costs vary according to the intensity of the immune challenge in a dose-dependent manner. However, these costs were limited since we did not find any significant difference in available energy and fish condition indices (i.e., body and organ mass) between antigen-injected and control-saline fish. In addition, oxidative stress indices were not affected.

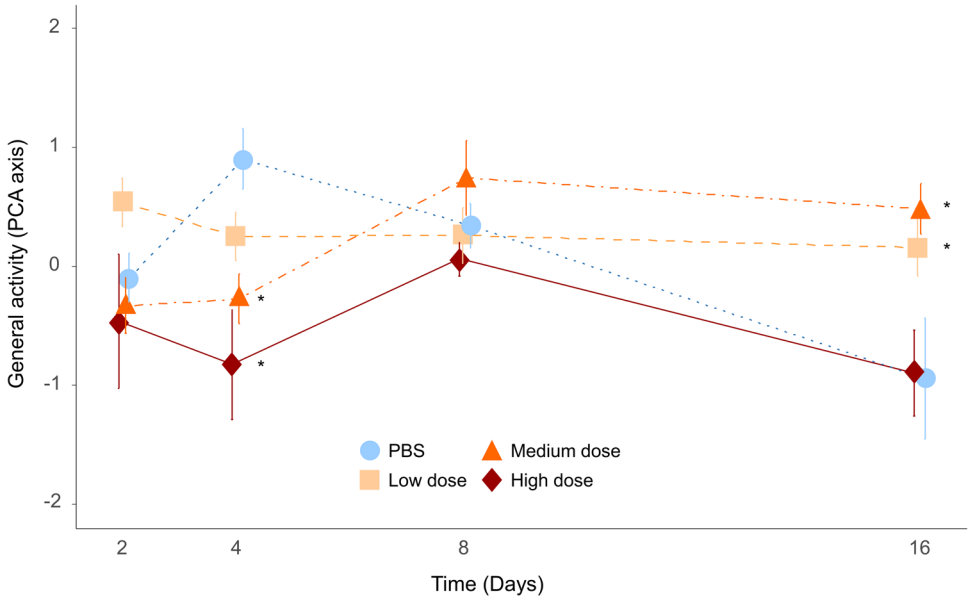

FIGURE 3 Mean general activity (first PCA axis scores, see Figure A1) ± *SE* of fish in different treatment groups across time (blue circles and dotted line: control-saline group (PBS); yellow squares and dashed line: low dose of antigens 4.5 g L^{-1}; orange triangles and dot-dashed line: medium dose of antigens 9.0 g L^{-1}; red diamonds and solid line: high dose of antigens 18.0 g L^{-1}). Stars indicate significant differences between the control-saline group and immune-challenged groups within each time point. PBS, phosphate-buffered saline solution; PCA, principal component analysis [Color figure can be viewed at wileyonlinelibrary.com]

TABLE 2 Summary of the main results

	Antigens concentration		
Response traits	**Low dose (4.5 g L^{-1})**	**Medium dose (9.0 g L^{-1})**	**High dose (18.0 g L^{-1})**
Immunity	Significant local immune response at the cellular level but no humoral response at the molecular level	Significant local (cellular level) and humoral (molecular level: lysozyme) immune response at 2 days	
Energy reserves	At the cellular level, the amount of available energy in fish muscle increases significantly in all treatment groups compared to untreated fish, except in fish injected with a medium and high dose of antigens at 4 days. At higher biological levels, few effects were detected on condition indices		
Behavior	Fish swimming activity increases at 16 days	Fish swimming activity first decreases at 4 days and then increases at 16 days	Fish swimming activity decreases at 4 days Fish sociability (contacts) decreases at 4 days

Moreover, the type of response varied according to the time course of the immune challenge (Table 2). At the molecular level, the humoral immune response was triggered 2 days after the immune challenge, while effects at higher biological levels on energy reserves and behavior did not appear until 4 days post-injection. This suggests that an immune challenge rapidly triggers a transitory immune response with potential cascading effects on energy management and behavior without affecting fish body condition nor survival (only four dead fish from the low- and high-dose treatment groups). Physiological and behavioral changes triggered by the immune challenge might thus enable fish to maintain fish body condition and fitness to cope with pathogen attacks in the wild, although further studies using live pathogens would be needed to test this hypothesis.

4.1 | Effects of immune challenge on fish immunity

The immune challenge had different effects on fish immune traits depending on the concentration of antigens injected. At the molecular level, results were contrasted. We did not find any effect of antigen injection on hemolytic and peroxidase activities. This suggests that the immune challenge did not trigger the expected systemic immune response and/or that the hemolytic activity (i.e., activation of the complement system) was altered by coagulation in fish plasma (Bexborn et al., 2009; Markiewski et al., 2007). Accordingly, lysozyme activity rapidly increased in fish injected with the medium and high doses of antigens but the response did not last longer than 2 days, which contrasts with previous studies reporting longer responses (Bich Hang et al., 2013; Nayak et al., 2008; Paulsen et al., 2003). These discrepancies might be explained by interspecific variability in antiparasitic defenses among fish species as shown in previous studies (Fänge et al., 1976; Grinde et al., 1988; Saurabh & Sahoo, 2008). Accordingly, in our study, gudgeons fish exhibited lower lysozyme activity than other freshwater fish species such as the common carp (*Cyprinus carpio*) (Xu et al., 2019) and the striped catfish (*Pangasianodon hypophthalmus*) (Bich Hang et al., 2013).

On the contrary, at the cellular level, the local immune response (i.e., skin swelling) increased significantly in all injected fish, whatever the antigen concentration. This result is consistent with the effects of both LPS and PHA on local immune response (Ardia & Clotfelter, 2006; O'Connor et al., 2014; Otálora-Ardila et al., 2016; Tella et al., 2008), which is due to the local infiltration of neutrophils, macrophages, and lymphocytes and more broadly local inflammation (Ardia & Clotfelter, 2006). The type of PHA used in our study may explain the long-lasting effect (i.e., until 16 days) of the antigens mixture on swelling measurement because PHA-P may elicit higher cells agglutination and tissue damage at the injection point than other types of PHA (Tylan & Langkilde, 2017). Whatever the mechanisms, our results show that the immune challenge was sufficient to trigger a significant immune response and that skin swelling might be a reliable and rapid measure of immune activation in fish.

In summary, our results show that the experimental immune challenge was efficient in triggering a significant immune response. Indeed, the immune challenge was mainly detected in the short term (i.e., only at 2 days) in the circulating plasma and for a more long-lasting period at the local cellular level (i.e., skin swelling). These results suggest the set up of a nonspecific inflammatory response, involving the local recruitment of neutrophils and macrophages at the location of antigen injection (Afonso et al., 1998; von Gersdorff Jørgensen, 2016). Interestingly, such short-term inflammation is usually costly and could potentially affect other traits related to energy management in fish, as investigated below.

4.2 | Effects of immune challenge on fish energy reserves

Contrary to expectations, the available energy in muscle did not decrease in fish injected with antigens mixture compared to the control-saline group. Indeed, energy reserves (mostly lipids) deposited in fish muscle increased in all fish involved in the experiment compared to untreated ($T = 0$) fish, except in fish injected with medium and high doses of antigens at 4 days. This suggests that

artificial feeding in captivity induces rapid storage of energy reserves in muscle, but that fish injected with the highest antigens concentrations had lower storage of energy (particularly lipids) in their muscle. This result is consistent with previous studies indicating that immune challenges increase metabolic activity and immunity at the expense of energy reserves in various vertebrate species (Bonneaud et al., 2003; Cabrera-Martínez et al., 2018; King & Swanson, 2013). Indeed, the inflammatory response is particularly costly because it triggers the secretion of several cytokines (e.g., TNFa) involved in the remobilization of fatty acids to produce lipoproteins to fight pathogens and to fuel the immune response (Grunfeld & Feingold, 1992; Johansen et al., 2006).

However, we likely underestimated these costs in captivity because captive fish likely increased food consumption to cover the costs of immunity, resulting in limited changes in body condition indices and survival. Accordingly, in our study, the HSI and the body mass were not significantly affected by the immune challenge, contrary to other studies (Bonneaud et al., 2016; Johansen et al., 2006). In addition, the immune challenge had no effects on the oxidative stress index measured in our study, whatever the concentration of antigens injected. This confirms that the potential increase of metabolic activity and inflammatory response had no visible deleterious effects on oxidative stress and thus limited expected cellular damage when food is abundant (Cherry & Piantadosi, 2015; Costantini & Dell'Omo, 2006; Costantini & Møller, 2009), although detailed studies on more specific oxidative markers would be necessary to confirm this hypothesis.

In summary, our results suggest that fish have the ability to manage the short-term costs of the immune challenges without strong depletion of energy reserves nor oxidative damage, likely due to the increased food availability and consumption in captivity that could enable fish to cover the costs of immunity. However, this hypothesis needs to be tested by controlling the food supply individually during exposure. In the wild, immune challenges might have more deleterious consequences for fish if food accessibility is limited, especially if the behavior is also affected by immune challenges, with potential consequences for fish ability to forage (Dantzer, 2004; Exton, 1997; Volkoff & Peter, 2004).

4.3 | Effects of immune challenge on fish behavior

Fish injected with the medium and high antigen doses decreased their swimming activity after 4 days. This is consistent with the expected increase in energetic demand for the immune response (Kirsten et al., 2018). However, at the lowest antigen concentration, fish swimming activity was not affected by the immune challenge, which suggests a dose-dependent effect of antigens on fish behavior. This supports that immune challenge with medium and high doses of antigens increases immunity at the expense of swimming activity, which is a classical sickness behavior allowing fish to save energy for immunity (Dantzer, 2004; Johnson, 2002; Kirsten et al., 2018). However, a potential side effect is that less active fish could be less able to find food (Dantzer, 2004; Johnson, 2002; Volkoff & Peter, 2004), which could explain the transient reduction of available energy stored (i.e., at 4 days) in the muscle of antigen-injected fish (Volkoff & Peter, 2004). Interestingly, the reduction of swimming activity and exploration was then followed by an enhanced activity at 16 days, which could help to offset the cost of the immune response by increasing foraging and food consumption in the long term, but this hypothesis remains to be formally tested.

We also found a decreased sociability (i.e., contact between individuals) in fish injected with the high dose of antigen at 4 days according to previous studies (Dugatkin et al., 1994; Kirsten et al., 2018). Such behavioral alterations could be part of the sickness behavior and could reduce pathogen spread (i.e., "behavioral resistance") among conspecifics in the wild (Barber et al., 2000), but could also have side effects on the ability of fish to find food efficiently (Pitcher et al., 1982) or escape predator in the wild (Ward et al., 2011).

In summary, our results suggest that in the short term, behavioral adjustments involved in the sickness behavior might allow energy reallocation from behavior (e.g., swimming activity, exploration, sociability) to immunity (e.g., macrophages, natural killer, and T cells; Dantzer, 2004; Exton, 1997). In addition, we showed that a low dose of antigens had limited adverse effects on behavior, while high concentrations of antigens decreased swimming activity and sociability, probably due to the high-energy demand for immunity. Such energy reallocation could have detrimental effects on fish's ability to find food and hence amplify the energetic costs of immune challenges, but this remains to be formally tested. Although behavioral assay under laboratory condition may not reflect real behavior in the wild (Calisi & Bentley, 2009), our study shows a dose-dependent effect of immune challenges which could result in contrasting effects on fish ability to acquire resources but also to escape from predators, with consequences for fish fitness. Further studies investigating physiological and behavioral changes induced by an immune challenge in a more realistic context are thus needed (e.g., infection by parasites under different environmental conditions) to infer potential cascading effects on fish fitness in the wild.

5 | CONCLUSION

Immune challenges triggered a significant nonspecific immune response at the local cellular level and, to a lesser extent, on circulating immunity (i.e., lysozyme activity) but with high differences depending on the immune challenge's intensity. Contrary to our expectations, we found limited evidence for oxidative stress or depletion in energy reserves in different organs, likely due to increased feeding in captivity. Interestingly, the immune challenge triggered behavioral changes such as a transient reduction in swimming activity and sociability depending on the dose injected, which likely saved energy for immunity but might have detrimental consequences for fish foraging ability, mating, or vulnerability to predators. Further research in a more realistic biotic context with parasites and predators

is now needed to better understand the consequences of immune challenges on fish fitness in human-altered rivers.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in figshare at https://doi.org/10.6084/m9.figshare.13182665.v1

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APPENDIX

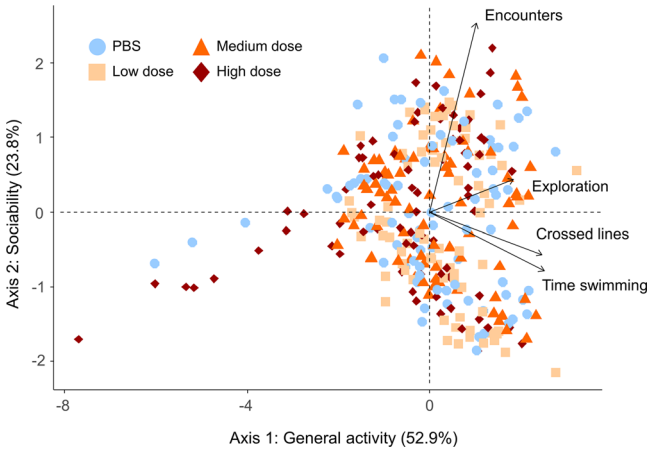

Figure A1. Principal component analysis (PCA) of the behavior of fish exposed to three doses of antigen mixture (Control: blue circles, low: yellow squares, medium: orange triangles, and high: red diamonds), each point represents an individual. Axis 1 mostly represents the swimming activity of fish (time swimming, crossed lines, and exploration). Axis 2 mostly represents the sociability of fish (number of encounter or contacts between individuals) [Color figure can be viewed at wileyonlinelibrary.com]