# Multistress effects on goldfish (*Carassius auratus*) behavior and metabolism

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**Abstract** Crossed effects between climate change and chemical pollutions were identified on community structure and ecosystem functioning. Temperature rising affects the toxic properties of pollutants and the sensitiveness of organisms to chemicals stress. Inversely, chemical exposure may decrease the capacity of organisms to respond to environmental changes. The aim of our study was to assess the individual and crossed effects of temperature rising and pesticide contamination on fish. Goldfish, *Carassius auratus*, were exposed during 96 h at two temperatures (22 and 32 °C) to a mixture of common pesticides (S-metolachlor, isoproturon, linuron, atrazine-desethyl, aclonifen, pendimethalin, and tebuconazol) at two environmentally relevant concentrations (total concentrations MIX1=8.4 μg $L^{-1}$ and MIX2=42 μg $L^{-1}$). We investigated the sediment reworking behavior, which has a major ecological functional role. We also focused on three physiological traits from the cellular up to the whole individual level showing metabolic status of fish (protein concentration in liver and muscle, hepatosomatic index, and Fulton's condition factor). Individual thermal stress and low concentrations of pesticides decreased the sediment reworking activity of fish and entrained metabolic compensation with global depletion in energy stores. We found that combined chemical and thermal stresses impaired the capacity of fish to set up an efficient adaptive response. Our results strongly suggest that temperature will make fish more sensitive to water contamination by pesticides, raising concerns about wild fish conservation submitted to global changes.

**Keywords** Global change · Temperature warming · Pesticides · Fish · Bioturbation

## Introduction

Agricultural and industrial development has led to a multiplication of pollutants in aquatic and terrestrial ecosystems that may have noxious effects on wildlife and community structures. In field, interactions between contaminants may occur and knowledge lacks about combined effects of these pollutants (Celander 2011). Aquatic ecosystems, which being the final receptacle of many pollutants, are particularly vulnerable to these interactions. France is the fourth largest consumer of pesticides, and chronic contamination of surface water is reported by water quality monitoring programs (CGDD 2011).

At the same time, climate change has been identified as one of the major drivers of ecosystem functioning in the coming decades (Drinkwater et al. 2010). In addition, the modifications in temperature, oxygenation, and acidity patterns may alter the occurrence and the behavior of pollutants (see reviews in Schiedek et al. 2007; Noyes et al. 2009; Holmstrup et al. 2010). They may also alter organism sensitiveness to

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pollutants and even lead to multiple stress effects if they exceed acclimation limits (Noyes et al. 2009; Kennedy and Ross 2012). Inversely, chemical exposures may decrease the resistance of organisms and so their adaptive potential to environmental changes (Noyes et al. 2009). Consequently, importance of crossed effects between climate and pollutions of contaminants has been identified, both on distribution and abundance of species and on ecosystem functioning (Dossena et al. 2012). However, studies on crossed effects between climate change (e.g., temperature) and pesticides on wildlife are often limited (but see Noyes et al. 2009 and citations therein).

Contaminant–temperature interaction is particularly worrying for aquatic ectothermic species (e.g., fish), as the water temperature directly affects their basal and active metabolisms and intervenes in many of biochemical and physiological processes (Cech et al. 1985; López-Olmeda and Sánchez-Vázquez 2011; Manciocco et al. 2014). Pesticides may affect survival, reproduction, and growth of a wide range of aquatic species (Sekine et al. 1996; Graymore et al. 2001; Hayes et al. 2006). In fish, they may induce neurotoxicity, oxidative damages, and genotoxicity and disturb immune system and organ integrity (Polard et al. 2011; Keith et al. 2014). Establishing molecular defense systems (e.g., detoxification, reparation, and protection) has an energetic cost and leads to metabolic compensations (Handy et al. 1999; Marchand et al. 2004). By modifying metabolic rates, the temperature may alter the ability of fish to respond efficiently to chemical stresses (Lemly 1996; Noyes et al. 2009; Kennedy and Ross 2012). Inversely, the metabolic cost of the pollutant exposure may disturb molecular and physiological acclimation process to thermal changes (Gordon 2005; López-Olmeda and Sánchez-Vázquez 2011).

Metabolic, neurological, endocrine, and/or sensory perturbations affect the behavior of fish (Saglio and Trijasse 1998; Cook and Moore 2008). Behavioral changes may, in turn, alter predation avoidance, competitiveness, and ability to feed of fish, with likely consequences on communities and trophic chains (Graymore et al. 2001). Responsive, rapid, linking molecular and physiological responses to highest biologic levels, behavioral bioassays were so used to assess the effect of wide range of stress (Killen et al. 2012; Grassie et al. 2013; Melvin and Wilson 2013).

Sediment reworking by fish is the consequence of foraging activity, reproduction, and/or predator avoidance behavior (Reise 2002; De Vries 2012; Shirakawa et al. 2013) or simply results of swimming movements near the ground (Montgomery et al. 1996). Temperature is considered as the "abiotic master factor" influencing the behavior of fish (Brett 1971), and a significant effect of the temperature warming on the sediment reworking by fish was demonstrated in several freshwater species (Canal et al. 2015). In addition, contaminations at sub-lethal levels may cause "ecological death," which occurs when disruption on complex behaviors compromises individual survival and performance (Scott and Sloman 2004). The sediment bioturbation by aquatic species has substantial impacts on the streambed physicochemistry parameters and plays a critical role in aquatic system functioning (Shirakawa et al. 2013). Fish are important actors, although generally underestimated, of this sediment bioturbation (Shirakawa et al. 2013; Peoples et al. 2014), so any changes in their sediment reworking behavior can have deleterious effects on aquatic system functioning (Ieno et al. 2006).

To our knowledge, no study has yet investigated the effects of the water contamination by realistic pesticide cocktails on the sediment reworking by fish, despite its major ecosystemic role. The aim of this study was so to assess the effects of pesticide exposure and temperature rising on fish sediment reworking behavior. We also tested the hypothesis that metabolic perturbations can partly explain the behavioral changes observed.

The goldfish (*Carassius auratus*, Linnaeus 1758, Cyprinidae family) is an Asiatic species introduced in French lentic water areas during the twentieth century (e.g., ponds, backwaters of rivers, floodplain waterbody) (Keith et al. 2011). Moreover, its use in ecotoxicological tests is growing fast (Bretaud et al. 2000; Cavas and Konen 2007; Feng et al. 2013). Fish were exposed to a mixture of pesticides found in the rivers of the south-west France at two different environmental relevant concentrations (S-metolachlor, isoproturon, linuron, tebuconazol, aclonifen, atrazine-desethyl, and pendimethalin for total concentrations of 8.4 and 42 μg $L^{-1}$). Experiments were conducted at two realistic water temperatures which occur in this area (22 and 32 °C). Sediment reworking behavior was followed during 96 h, and physiological endpoints were assessed at the end (Fulton's condition factor (FCF), hepatosomatic index (HSI), and protein concentrations in liver and white muscle).

## Material and methods

### Fish species and acclimation

Fish were purchased from the fish farming Carpio (Consac, France) in the size range 10–12 cm. They were first acclimatized for 2 weeks in opaque tanks under controlled conditions (18 °C with a 12:12-h light regime). Water was aerated and dechlorinated prior to fish introduction. Half of the water was renewed every day, and fish were fed daily with commercial pellets. They were gradually acclimatized to experimental temperatures during 15 days. The temperature was increased by 0.5 °C every 12 h until reaching the experimental temperature. Three days before the experiment, fishes were starved to optimize their foraging behavior during the experimental period. No mortality occurred during the acclimation period.

## Mixtures of pesticides

The mixtures were developed on the basis of the pesticide contamination of the Save River (France), assessed from March 2008 to November 2009 (Polard 2011). The Save River watershed, located in the Gascogne area, is mainly used for intensive agriculture (corn, wheat, and sunflower). High contamination levels of surface waters and sediment by triazines (atrazine, DEA, cyanazine), ureas (isoproturon, linuron, chlorotoluron), and anilides (metolachlor, metazachlor) have been reported in the Gascogne area during spring flood (Devault et al. 2009; Polard et al. 2011). According to the analysis conducted by Pollard (2011), we selected six herbicides and one fungicide using three criteria: the frequency of detection, the concentration, and the representation of the different families of molecules detected. Selected molecules are S-metolachlor, isoproturon, linuron, tebuconazol, aclonifen, atrazine-desethyl, and pendimethalin (cf. Table 1). Pesticide standards were diluted in acetone (0.3 mL $L^{-1}$ of water) and demineralized water to obtain two mixtures: MIX1 and MIX2 for total concentrations of 8.4 and 42 μg $L^{-1}$, respectively. The proportions between the molecules, calculated from analysis conducted by Polard (2011), are preserved in the two mixtures. The MIX1 concentrations correspond to the highest level of contamination observed not only in the Save river after spring flood (Polard 2011), but also in other French rivers (Garnouma et al. 1998; Debenest 2007; IFEN 2007; Taghavi et al. 2010). The MIX2 is more concentrate to reflect situations of highest levels of contamination measured in Europe and USA (Kreuger 1998; Battaglin et al. 2000; Graymore et al. 2001).

Pesticides were obtained from Sigma-Aldrich (St. Louis, MO, USA): S-metolachlor (CAS-No: 87392-12-9, PESTANAL®, analytical standard), isoproturon (CAS-No: 34123-59-6, PESTANAL®, analytical standard), linuron (CAS-No: 330-55-2, PESTANAL®, analytical standard), Atrazine-desethyl (CAS-No: 6190-65-4, PESTANAL®, analytical standard), aclonifen (CAS-No: 74070-46-5, PESTANAL®, analytical standard), pendimethalin (CAS-No: 40487-42-1, PROWL®, analytical standard), and tebuconazol (CAS-No:107534-96-3, PESTANAL®, analytical standard). Acetone (CAS: 67-64-1, Fisher Chemical, HPLC solvent) was purchased from Fisher Scientific (Illkirch, France).

Final concentrations of pesticides in water were quantified by HPLC-DAD for aclonifen and HPLC-MS/MS for other pesticides. Analyses were performed by the Laboratoire Départemental de l'Eau de la Haute-Garonne (county laboratory water, Saint Alban, France). At the end of the experience, observed concentrations were closed from expected concentrations, except for the MIX2 at 32 °C with a 19 and 64 % decrease in S-metolachlor and pendimethalin concentrations, respectively.

## Experimental design

The experimental design is shown in Fig. 1a, b. Fish were placed individually in 30-L opaque aquaria in temperature-controlled rooms (22 and 32 °C, 12 fish for each combination pesticides-temperature). They were exposed during 96 h to three treatments of pesticides (CONTROL, MIX1, and MIX2). CONTROL aquaria received only acetone. Prior to fish introduction, aquaria and all equipment were presaturated with the mixture of pesticides that they will receive during 1 day. Air pumps were placed in each aquarium to supply water with oxygen. Water and pesticides solutions were renewed in half every day to limit concentration variations. No mortality occurs during the exposure.

Temperature, pH, conductivity, and oxygen concentration were assessed every day (Fig. 1c).

**Table 1** Composition and characteristics of the two mixtures of pesticides: MIX1 and MIX2 for total concentrations of 8.4 and 42 μg $L^{-1}$, respectively

| Chemicals | Family | Use | LC50-96 h fish (mg/L) (min-max) | Molecular weight (g/mol) | CLP classification (acute/chronic) | Mixture concentrations (μg $L^{-1}$) | |
|---|---|---|---|---|---|---|---|
| | | | | | | MIX1 | MIX2 |
| S-metolachlor | Chloroacetanilide | Herbicide | 1.23–12 | 283.79 | C1/C1 | 2.4 | 12.0 |
| Linuron | Urea | Herbicide | 3.15–31.1 | 249.11 | C1/C1 | 2.0 | 10.0 |
| Isoproturon | Substituted urea | Herbicide | 18–54.41 | 206.28 | C1/C1 | 1.2 | 6.0 |
| Tebuconazole | Triazole | Fungicide | 4.4 | 307.81 | NC/C2 | 1.2 | 6.0 |
| Aclonifen | Diphenyl ether | Herbicide | 0.67 | 264.66 | C1/C1 | 0.8 | 4.0 |
| Atrazine-desethyl | Triazine | Herbicide | ND | 187.63 | ND | 0.4 | 3.0 |
| Pendimethalin | Dinitroaniline | Herbicide | 0.138–0.418 | 281.31 | C1/C1 | 0.4 | 2.0 |
| Total concentrations | | | | | | 8.4 | 42.0 |

*LC50-96 h* concentration which causes 50 % mortality at 96 h of exposure, *CLP* classification of chemical risk in aquatic system (CE 1272/2008): *C1* very toxic and *C2* toxic, *ND* no data, *NC* not concerned

### Assessment of the sediment reworking behavior

The method proposed by De Nadaï-Monoury et al. (2013) was used to experimentally quantify sediment reworking by fish. All aquaria were filled with a 5-cm layer of commercial white quartz sand (1–2.5 mm in diameter). A thin layer of dark blue sand (same diameter as the white sand) was used as tracers and sprinkled on the white sand surface. Tap water was added in the aquaria, paying attention not to disturb the sediment layer. Aquaria were placed on polystyrene plates (20 mm thick) to isolate them from vibrations and lighted by an indirect light source (12:12-h light regime) to reduce fish stress.

Pictures of the sediment layer were taken with a digital camera Canon EOS 20D before fish introduction (T0) and then every 24 h to follow sediment reworking (T24 to T96). Automatic pixel count was performed by Image-Pro Plus software (Media Cybernetics) (see details in De Nadaï-Monoury et al. 2013 and Canal et al. 2015). The number of white pixels was reported to the total surface of the aquarium, standardized with respect to the initial picture (T0) and to the weight of individuals to obtain the percentage of surface reworked per gram of fish (% $g^{-1}$).

### Measures of physiological endpoints

At the end of the experiment, fish were weighted (to the nearest 10 mg) and measured (fork length to the nearest mm). Fish were euthanized by concussion, and livers and fillets (white muscles) were collected, immediately frozen in liquid nitrogen, and conserved at −80 °C. FCF, HSI, and protein concentrations were calculated as follows.

The Fulton's condition factor (FCF) presents the global body condition and the "general well-being" of fish (Smolders et al. 2002). It was calculated from weight and length parameters of fish:

$$\mathrm{FCF} = \frac{\text{weight (g)}}{\text{lenght (cm)}^3} \quad (1)$$

The hepatosomatic index (HSI) represents a global measurement of the liver growth status and is correlated with energy contents (Chellappa et al. 1995). It was calculated as the relation between the liver and the total weights:

$$\mathrm{HSI} = \frac{\text{liver weight(g)}}{\text{total weight (g)-liver weight(g)}} \times 100 \quad (2)$$

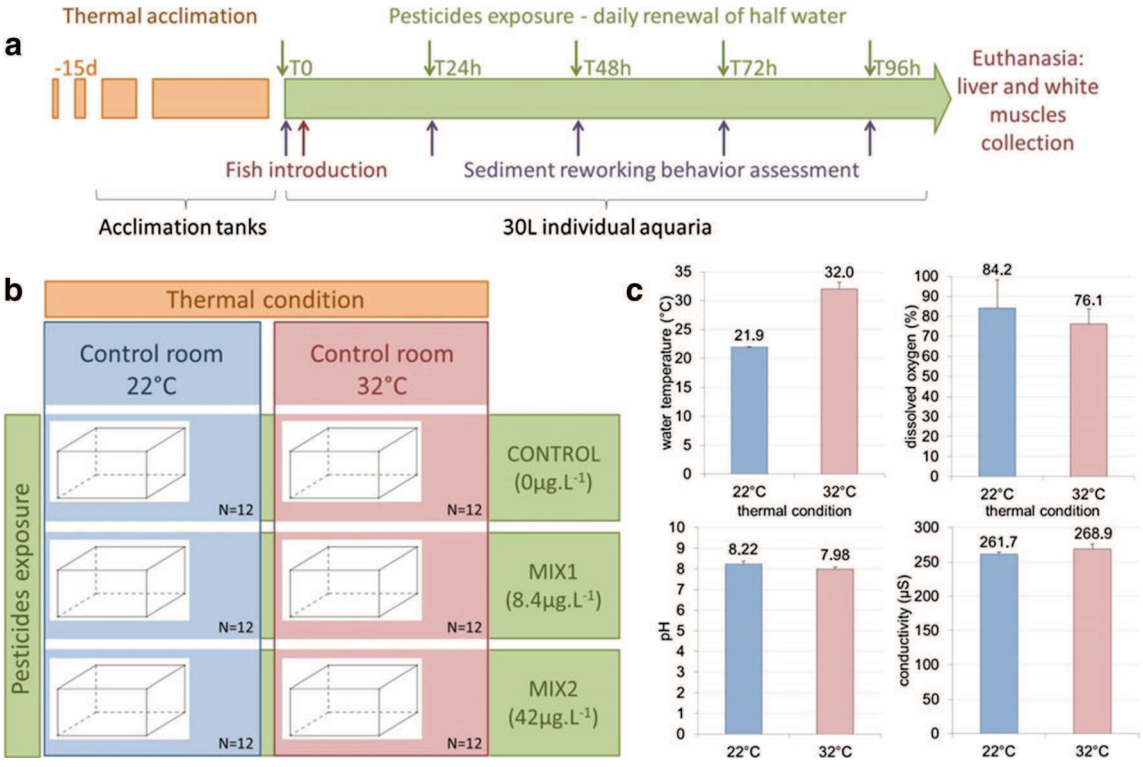


**Fig. 1** Effects of pesticide exposure and temperature rising on behavior and physiological parameters of an aquatic fish species, *Carassius auratus*: experimental design. **a** Timeline of the experience: fish were acclimated during 15 days to experimental temperature in collective tanks and then exposed to pesticides during 96 h in 30 L individual aquaria. Sediment reworking behavior was measured daily, and physiological responses were assessed at the end of the experiment. **b** Experimental design: fish were exposed at two temperatures (22 and 32 °C) to a mixture of seven common pesticides at different concentrations: CONTROL (0 µg $L^{-1}$), MIX1 (total concentration= 8.4 µg $L^{-1}$), and MIX2 (total concentration=42 µg $L^{-1}$). *N*=12 fish for each thermal × exposure condition. **c** Measures of some water physicochemical parameters in each thermal condition: water temperature (°C), dissolved oxygen (%), pH, and conductivity (µS). Measures were realized daily in each aquarium. Mean±SD. *N*=12

Proteins are one of the major energy reserves in aquatic species. To obtain the protein concentration, samples of liver and muscles were homogenized in 4 vol (*v*/*w*) of 40 mM Tris-HCl (pH 8.8), 2 mM EDTA, and protease inhibitors (1 μL/1 mL of solution) at 4 °C using a FastPrep® homogenizer. Homogenates were centrifuged at 10,000*g* for 10 min at 4 °C. Process was repeated once, and supernatants were stored at −80 °C. Protein concentrations were quantified using a microplate spectrophotometer (FLUOstar OMEGA, BMG LABTECH), according to the principle of protein–dye binding (Bradford 1976).

### Statistical analyses

Due to the lack of homogeneity of variances between some groups, comparisons between groups were performed using non-parametric statistics: the effect of pesticide exposure at each temperature was analyzed by Kruskal–Wallis (KW) tests and Dunn's post-tests, while comparisons between temperature conditions were performed by Mann–Whitney (MW) tests. To evaluate the interaction effect between temperature and pesticide exposure, we used factorial ANOVA for physiological responses and multivariate analysis of variance (MANOVA) with repeated measures for the sediment reworking behavior, assuming that the ANOVA test is quite robust against violations of the homogeneity of variance assumption (Lindman 1974).

## Results

### Sediment reworking activity

We showed a significant crossed effect between temperature, pesticide concentrations, and time on the percentage of surface reworked by fish (MANOVA analysis, Table 2). The percentage of surface reworked steadily increased over time whatever the condition of temperature or pesticide concentrations (Fig. 2), although a slight slowdown was observed from 48 h. Significant individual effects of pesticides and temperature were observed at 24, 48, 72, and 96 h of exposure (Table 2), while crossed effect between pesticides and temperature was significant at 48, 72, and 96 h of exposure. At 22 °C, the pesticide exposure significantly decreased the percentage of surface reworked by fish at each time step (Fig. 2a, Kruskal–Wallis (KW) test, $p<0.01$). This effect increased over time and was significant after 24 h of exposure for MIX1 and 48 h for MIX2 (Dunn post-tests, $p=0.001$ and 0.038, respectively). After 96 h of exposure, the percentage of surface reworked was reduced by 54 and 41 % in fish exposed, respectively, to MIX1 and MIX2 compared to CONTROL (Dunn post-tests, $p=0.000$ and 0.006, respectively). In CONTROL fish, temperature rising decreased by 21 % the percentage of surface reworked after 96 h (Mann–Whitney (MW) test, $p=0.017$). On the contrary, pesticide exposure at 32 °C had no significant effect on the percentage of surface reworked regard to 22 and 32 °C controls (Fig. 2b, KW test: $p>0.05$ at each time step).

### Physiological endpoints

Interaction between pesticides and temperature had no significant effects on the FCF (factorial ANOVA, $p=0.15$). Temperature significantly increased the FCF (MW, $p<0.000$ for all pesticide conditions, Fig. 3a). At 22 °C, pesticide exposure tended to decrease the FCF, but changes were not significant (Kruskal–Wallis (KW) tests, $p=0.37$). At 32 °C, pesticide exposure had no effect on FCF (KW test, $p=0.92$).

Concerning the hepatosomatic index (HSI), crossed effect between pesticide concentrations and temperature was significant (factorial ANOVA, $p=0.022$, Fig. 3b). Pesticide exposure altered significantly the HSI at 22 °C but not at 32 °C

**Table 2** Factorial MANOVA/ANOVA analyses of the effect of pesticide exposure, temperature warming, and time on the percentage of surface reworked by *Carassius auratus* (% $g^{-1}$)

| Analysis | *p* values | | | |
|---|---|---|---|---|
| | Temperature | Pesticides | Temperature × pesticides | Time effect |
| Multivariate analysis | 0.002 | 0.001 | 0.000 | 0.000 |
| Interaction with time | 0.406 | 0.182 | 0.003 | – |
| Univariate analyses | | | | |
| Percentage of surface reworked at T 24 h | 0.005 | 0.030 | 0.072 | – |
| Percentage of surface reworked at T 48 h | 0.020 | 0.002 | 0.001 | – |
| Percentage of surface reworked at T 72 h | 0.002 | 0.005 | 0.000 | – |
| Percentage of surface reworked at T 96 h | 0.012 | 0.002 | 0.000 | – |

Fish were exposed during 96 h to a mixture of seven pesticides at different concentrations (CONTROL, MIX1 8.4 μg $L^{-1}$, and MIX2 42 μg $L^{-1}$) and two temperatures (22 and 32 °C). The percentage of surface reworked was assessed every 24 h. $N=12$

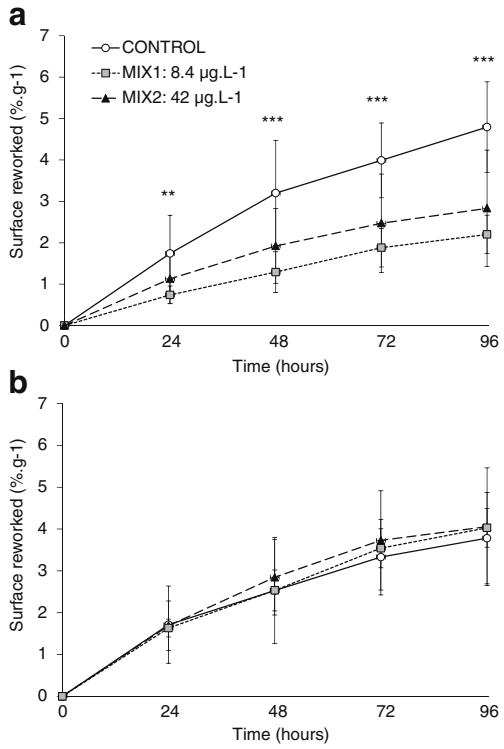


**Fig. 2** Effects of pesticide exposure and temperature rising on sediment reworking activity of an aquatic fish species, *Carassius auratus*. Evolution of the mean surface reworked (% $g^{-1}$) by fish, exposed during 96 h to a mixture of seven pesticides at different concentrations (CONTROL, MIX1/4.8 µg $L^{-1}$, and MIX2/42 µg $L^{-1}$) and at two temperatures **a** 22 °C and **b** 32 °C. The percentage of surface reworked was assessed every 24 h. *Bars* show standard deviations. $N=12$. *Asterisks*: Kruskal–Wallis test of the pesticide effect at 22 and 32 °C with *$p<0.05$, **$p<0.01$, and ***$p<0.001$

(KW tests, $p=0.004$ and 0.278, respectively). At 22 °C, MIX1 exposure decreased the HSI compared to CONTROL (Dunn post-tests, $p=0.009$). In contrast, MIX2 exposure had no significant effect. At 32 °C, pesticide exposure increased the HSI, but not significantly (KW tests, $p=0.27$). A substantial but non-significant decrease of the HSI was observed with temperature rising in CONTROL fish (MW tests, $p=0.065$).

Pesticide concentrations and temperature had significant effects on protein concentrations in liver and muscle (Fig. 3c, d), but crossed effect between temperature and pesticide concentrations was significant only for the protein concentration in muscle (factorial ANOVA, $p=0.002$ for muscle and 0.073 for liver). At 22 °C, pesticide exposure decreased the protein concentration both in liver and in muscle (KW test,

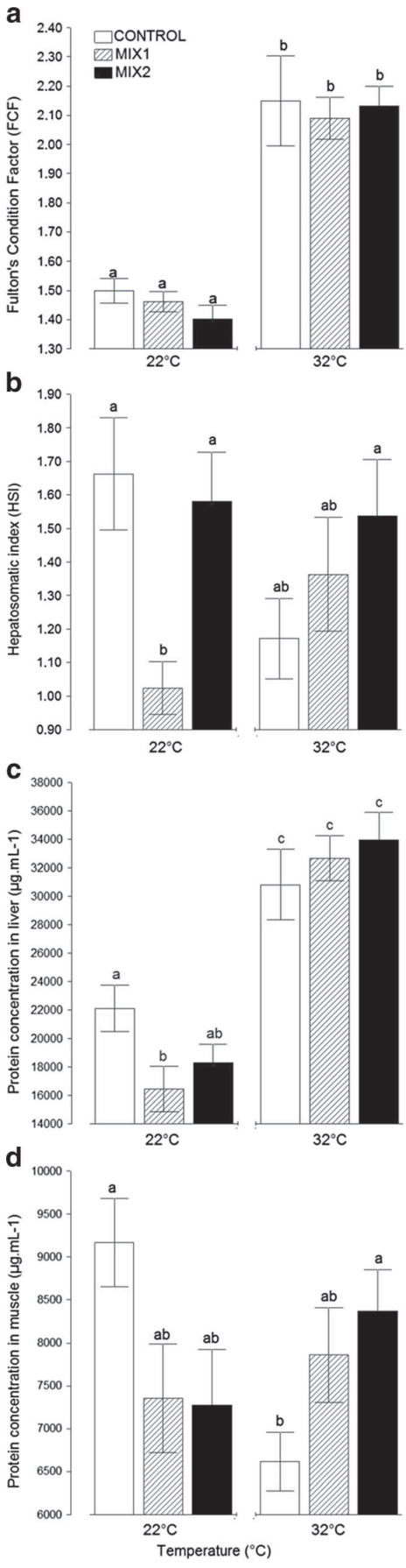


**Fig. 3** Effects of pesticide exposure and temperature rising on physiological parameters of an aquatic fish species, *Carassius auratus*. **a** Fulton's condition factor, **b** hepatosomatic index, **c** protein concentration in liver (µg $mL^{-1}$), and **d** protein concentration in muscle (µg $mL^{-1}$). Goldfish were exposed during 96 h to a mixture of seven pesticides at different concentrations (CONTROL, MIX1/4.8 µg $L^{-1}$, and MIX2/42 µg $L^{-1}$) and at two different temperatures (22 and 32 °C). *Letters* indicate homogenous groups. Mean±SEM. $N=12$

$p$=0.050 and 0.059 in liver and muscle, respectively), but this decrease was only significant in liver for MIX1 compared to the CONTROL (Dunn's post-test, $p$=0.048). In the opposite, pesticide exposure at 32 °C increased protein concentration in muscle, with MIX2 significantly different from the CONTROL (Dunn's post-test, $p$=0.038). Temperature significantly increased protein concentration in liver (Dunn's post-test, $p$<0.05 for all conditions of exposure) and decreased protein concentration in muscle in CONTROL group (Dunn's post-test, $p$=0.000).

## Discussion

### Behavioral response to pesticide exposure and temperature rising

We found that pesticide exposure decreased the sediment reworking activity of goldfish, which is consistent with other studies. Shinn (2010) showed that a mixture of atrazine, linuron, and S-metolachlor (with respective concentrations of 10, 15, and 45 μg $L^{-1}$) inhibits swimming behavior in *Oncorhynchus mykiss*. Atrazine alone affects burst swimming reactions of the goldfish after 24-h exposure at 0.5 μg $L^{-1}$ and alters grouping behavior, surfacing activity, and sheltering at 5 μg $L^{-1}$ (Saglio and Trijasse 1998). Behavioral perturbations may follow neurological, endocrine, sensory, or metabolic disruptions (see review in Scott and Sloman 2004). Beyond the metabolic adjustment related to stress response, atrazine and S-metolachlor are known to entrain endocrine perturbations and sensory disruptions in fish (Bisson 2002; Wolf and Moore 2002; Becker et al. 2009). So, the behavioral response to pesticide exposure could be (1) an adaptive strategy to decrease the energy consumed by the general activity or (2) a toxic effect of the pesticides themselves.

Unexpectedly, temperature rising also decreased the sediment reworking activity of fish. Indeed, Reynolds & Casterlin (1979) measured the locomotor activity of the goldfish exposed to a gradient of temperatures and found a two-fold increase in locomotion at 32 °C compared to 22 °C. Temperature rising often leads to increase the basal and active metabolism of fish and so to increase their general locomotor activity (López-Olmeda and Sánchez-Vázquez 2011; Canal et al. 2015). But, behavioral adjustments to temperature are not linear and depend on the severity and the duration of the stress exposure. Sullivan et al. (2000) suggested that, in salmon species, general activity increases with temperature rising—parallel to the metabolic rate—and then decreases beyond a certain threshold which marks the boundary between the zones of thermal tolerance and resistance. Here, both experimental temperatures were on either side of the optimal temperature of the goldfish (28 °C), within the theoretic tolerance range (Bret 1946). But, intraspecific genetic diversity and/ or difference in thermal life history between populations may impact the thermal sensitiveness of fish (see a review in Pörtner 2002). Anyway, our results suggest that in our study, goldfish placed at 32 °C were outside their tolerance range. Particularly, at high temperatures, the cellular demand in oxygen increases, leading to an oxygen insufficiency (Pörtner 2002). Survival to thermal stress then depends on metabolic adjustments and cellular defense and reparation systems. The subsequent drop in the aerobic scope and the metabolic compensation to defense system induction can cause a decrease in global activity (Sullivan et al. 2000; Sokolova 2013).

### Physiological responses to pesticide exposure and temperature rising

We found that pesticide exposure and temperature rising significantly disrupt the metabolic balance of juvenile's goldfish, from the cellular to the individual level. Pesticides exposed fish had decreased protein concentration in liver and HSI and tended to have a lower FCF. Except for the FCF, the metabolic response was stronger at the lowest concentrations. Thermal-exposed fish had decreased protein concentration in white muscle but increased FCF and hepatic protein concentration.

Glycogen, lipids, and proteins are the three major energetic reserves in aquatic species. The HSI permits to estimate the energy status of the liver; insofar, it is correlated with lipids and glycogen reserves (Chellappa et al. 1995; Zheng et al. 2013). Under stress, hepatic glycogen rapidly provides glucose, via glycogenolysis pathway, which supplies other organs readily usable energy (Moon and Foster 1995). Moreover, a decrease in lipid stores and the disruption of lipid metabolism were shown for several pollutants and fish species (Smolders et al. 2003; Zheng et al. 2013; Castelli et al. 2014). Modification in the protein concentration results from a perturbation in the protein turnover, with an imbalance between the synthesis and the degradation rate of proteins (Smolders et al. 2003). In the white muscle, proteins are the main source of energy. Inversely, the liver metabolic response primarily involves lipid and glycogen reserves, and proteins are used ultimately when other reserves are depleted (Smolders et al. 2003). If not, the hepatic protein synthesis may be increased to produce defense systems, including heat shock proteins (Viant et al. 2003).

Fish exposed to the lowest pesticide concentrations (MIX1) at 22 °C showed a significant decrease in the HSI and the protein concentrations in the white muscles (and to a lesser extent in liver). This global depletion in energy stores may indicate a drastic increase in energy requirement, which cannot be compensated by alimentation, fish being starved. This is consistent with the intermediary stress response describe by Selye (1950). Energetic stores were mobilized to furnish the energy necessary for the molecular and physiological defense system induction. In the opposite, fish exposed to the highest

concentration of pesticides (MIX2) have shown an HSI closed to the CONTROL value and a non-significant decrease in protein concentrations in organs. Unexpectedly, metabolic disruptions seemed to be more important at the lowest concentration. At highest concentrations, the metabolic response to pesticide exposure could be hidden by other adaptive responses or direct toxic effects of pesticides. Biagianti-Risbourg and Bastide (1995) have shown an increase in HSI due to the sequestration of fat-soluble pesticides in liver lipid droplets. Other authors suggested that liver may swell with pesticide exposure in order to increase its detoxification capacity (Arnold et al. 1995; Bacchetta et al. 2014) or in consequences of degenerative changes in the liver tissue (Arnold et al. 1995; Guardiola et al. 2014). The absence of classic dose/response relationship may have so three reasons: (1) different modes of action of pesticides at different concentrations, including a low-dose effect for the endocrine active substances (i.e., atrazine or S-metolachlor) (EFSA 2010), (2) different physiological responses—or strategies—depending on the concentration of toxic substances, or (3) the passage of a metabolic compensation state to a non-compensation state when stress becomes too severe (Sokolova 2013).

Thermal-exposed fish (CONTROL/32 °C) exhibited a significant decrease in the muscular protein concentrations in muscle while the protein concentration in liver increased. The dichotomy between the liver and the muscle protein responses to temperature rising was observed to in *O. mykiss* (Viant et al. 2003). These authors suggested that muscle proteins were used to compensate the metabolic cost of thermal acclimation, while the liver protein synthesis was enhanced, probably to produce thermal defenses. The decreased HSI, although not significant, may also indicate a growing energy requirement due to thermal acclimation.

### Energetic strategy and multistress effects

Thermal and chemical stress required the induction of a whole range of defense systems, including detoxification, protection, and/or reparation systems, to maintain homeostasis (Iwama 1998; Martínez-Álvarez et al. 2005). Temperature warming induces heat shock proteins among other defense systems (Iwama et al. 1998). Atrazine and isoproturon are known to induce in the goldfish the hepatic expression of proteins involved in defense against oxidative stress (Fatima et al. 2007; Meng et al. 2011) and in general process of detoxification (Meng et al. 2011). Thermal- or low-pesticide-dose-exposed fish had decreased energy reserves, indicating a growing energy demand related to cost of defense system induction. It is a classic adaptive response to stress. The decrease in the sediment reworking behavior could then result energetic trade-offs between activity, reproduction, growth, and maintenance (see among others Handy et al. 1999; Marchand et al. 2004; Roze et al. 2013).

The energy balance is a key factor in the stress response (Selye 1950; Barton 2002). Ultimately, the ability of organisms to mobilize energy for defense systems determines, in large part, their ability to cope with a stressor (Sokolova 2013). When the organism cannot—or no longer—counteract for the effects of stress, harmful health effects appear. When defense systems are insufficient to combat the damage caused by stress, the metabolic responses disappeared and organism enters in "metabolic arrest" (Sokolova 2013). This metabolic arrest increases the survival time of stressed organisms. But, this strategy is unsustainable over the long term (Sokolova 2013). In this study, goldfish exposed to the highest concentration of pesticides presented behavioral changes but no metabolic responses. Moreover, when fish were exposed to combined thermal and pesticides stresses, their behavioral and metabolic responses to individual stressor decreased or disappeared. Most studies showed an increase in the toxicity of pesticides on fish with temperature. We can therefore hypothesize that in both cases, goldfish entered in metabolic arrest state. Pesticide exposure can so potentially compromise the adaptation of fish to temperature changes and vice versa.

In this study, we show for the first time that realistic pesticide contaminations in our experimental condition may alter a major fish ecosystemic function: the sediment reworking. Our results show behavioral and metabolic perturbations caused by each stressor separately and crossed effects between these two factors. Our findings suggest that (1) low environmental relevant concentrations of pesticides lead to behavioral and metabolic adaptive response in the goldfish, (2) fish may elicit different adaptive responses in function of the kind and the intensity of stress (metabolic compensation or metabolic arrest), and (3) fish may be unable to set up efficient adaptive responses when they are exposed to combined thermal and pesticide challenges, with potential dramatic consequences. Further analyses are however needed, including longer studies and molecular approaches, to deepen these results. Ultimately, this study confirms the difficulty to predict adverse outcomes of environmental pollutions on fish and so implications for aquatic ecosystem functioning, without taking into account physical parameters like temperature warming. It also strongly suggests that temperature will make fish more sensitive to water contamination by pesticides and, inversely, raising concerns about wild fish conservation submitted to global changes.

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