



## Effects of chronic exposure to cadmium and temperature, alone or combined, on the threespine stickleback ( *Gasterosteus aculeatus* ): interest of digestive enzymes as biomarkers

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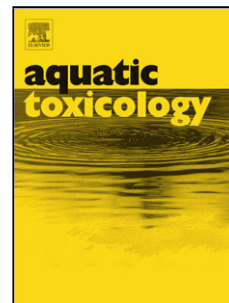
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**Effects of chronic exposure to cadmium and temperature, alone or combined, on the threespine stickleback (*Gasterosteus aculeatus*): interest of digestive enzymes as biomarkers.**

## **Authors**

Younes Mohamed Ismail HANI<sup>1,2</sup>, Cyril TURIES<sup>1</sup>, Olivier PALLUEL<sup>1</sup>, Laurence DELAHAUT<sup>2</sup>, Véronique GAILLET<sup>2</sup>, Anne BADO-NILLES<sup>1</sup>, Jean-Marc PORCHER<sup>1</sup>, Alain GEFFARD<sup>2</sup>, Odile DEDOURGE-GEFFARD<sup>2\*</sup>

<sup>1</sup> *Institut National de l'Environnement Industriel et des Risques (INERIS), UMR-I 02 SEBIO (Stress Environnementaux et Biosurveillance des milieux aquatiques), Verneuil-en-Halatte, France.*

<sup>2</sup> *Université de Reims Champagne-Ardenne (URCA), UMR-I 02 SEBIO (Stress Environnementaux et Biosurveillance des milieux aquatiques), Moulin de la Housse, Reims, France.*

\*Corresponding author:

**Dr. Odile DEDOURGE-GEFFARD**

Université de Reims Champagne-Ardenne (URCA), UMR-I 02 SEBIO (Stress Environnementaux et Biosurveillance des milieux aquatiques)

Moulin de la Housse, B.P. 1039, 51687 Reims, France.

**Tel. : +33 (0) 3 26 91 33 28**

**E-mail : [odile.dedourge@univ-reims.fr](mailto:odile.dedourge@univ-reims.fr)**

## Highlights

- Chronic effects of cadmium and /or temperature were studied.
- Temperature alone impacted antioxidant and energy reserve parameters.
- Digestive enzymes were negatively impacted by the both stress. .
- The negative effect of cadmium was higher and earlier associated with temperature increase.
- Digestive enzymes appear as potential biomarkers of organism's health.

## Abstract

The development of predictive, sensitive and reliable biomarkers is of crucial importance for aquatic biomonitoring to assess the effects of chemical substances on aquatic organisms, especially when it comes to combined effects with other stressors (*e.g.* temperature). The first purpose of the present study was to evaluate the single and combined effects of 90 days of exposure to an environmental cadmium concentration ( $0.5 \mu\text{g. L}^{-1}$ ) and two water temperatures (16 and 21 °C) on different parameters. These parameters are involved in (i) the antioxidant system (superoxide dismutase activity – SOD – and total glutathione levels – GSH –), (ii) the energy metabolism, *i.e.* energy reserves (glycogen, lipids, proteins) and digestive enzymes (trypsin, amylase, intestinal alkaline phosphatase – IAP –), and (iii) biometric parameters (weight, length, Fulton's condition factor, and the gonadosomatic index – GSI –) of threespine stickleback (*Gasterosteus aculeatus*). The second purpose was to determine the interest of the three digestive enzymes as biomarkers in comparison with the other parameters. The higher temperature (21 °C) impacted the anti-oxidant and energy reserve parameters. In liver, GSH levels increased on day 60, while SOD decreased on days 15 and 90, with a significant decrease of protein and lipid energy reserves on day 90. In muscle, the higher temperature decreased SOD activity only on day 90. *G. aculeatus* biometric parameters were also impacted by the higher temperature, which limited stickleback growth after 90 days of exposure. In female sticklebacks, the GSI peaked on day 60 and decreased sharply on day 90, while the highest values were reached at day 90 in the control groups, suggesting impaired reproduction in sticklebacks raised at 21 °C. These results suggest that 21 °C is an upper-limit temperature for

long-term physiological processes in sticklebacks. In contrast, very low-concentration cadmium exposure had no effect on classical biomarkers (energy reserves, antioxidant parameters, biometric parameters). However, digestive enzymes showed an interesting sensitivity to cadmium, which was emphasized by high temperature. The activity of the three digestive enzymes decreased significantly on day 90 when sticklebacks were exposed to cadmium alone, while the decrease was stronger and was recorded earlier (from day 15) when they were exposed to the cadmium-temperature combination. Compared to conventional measurements, digestive enzymes responded rapidly. This could be an important advantage for them to be used as early warning tools to reflect the health status of organisms, particularly for trypsin and IAP activities.

Keywords: antioxidant parameters; digestive enzymes; cadmium stress; energy metabolism; *Gasterosteus aculeatus*; temperature stress.

## 1. Introduction

The water quality of aquatic ecosystems can be assessed using different methods based on chemical and ecological approaches. With over 130 million chemical substances known to date (CAS, 2017), the development of predictive, sensitive and reliable tools to assess the impacts of chemicals is of crucial importance for the biomonitoring of aquatic ecosystems. Among them, biomarkers have occupied an important place in ecotoxicology in the last decades (Forbes et al., 2006). In this context, energy metabolism parameters have emerged as predictable and useful biomarkers to detect the effects of chemicals on different aquatic organisms (De Coen and Janssen, 1997; Charron et al., 2013; Pi et al., 2016). As energy is the most determining biological parameter involved in all the physiological processes of living beings, disturbances of the energy metabolism appear to be a good indicators of the general health status of organisms (De Coen and Janssen, 2003).

Different biological parameters related to the energy metabolism are used in ecotoxicology. They reflect a response to a stress condition, which can be expressed at different levels of living organisms, from macromolecular to individual levels, *e.g.* the adenylate energy charge (AEC), energy reserves (glycogen, lipids, proteins), cellular energy allocation (CEA), serum indicators (glucose, cortisol), condition indices (Fulton's condition factor, the hepatosomatic index). The usefulness of these parameters has been demonstrated in different aquatic organisms, including fish (Dickson et al., 1982; Soengas et al., 1996; Sancho et al., 1998; Almeida et al., 2001; Huntingford et al., 2001; Levesque et al., 2002; Pi et al., 2016). Other parameters representing a crucial step in the energy acquisition stage, *i.e.* digestive

enzymes, have recently been used as health status indicators in aquatic invertebrates (gammarids and daphnia), as well as in fish. In fish, three possible primary sites for pollutant uptake are the body surface, the gills, and the alimentary canal (Perera et al., 2015). Since the latter acts as a secretion site of digestive enzymes, exposure to pollutants could disturb digestive enzyme activity. Hence, several authors have suggested using these parameters as potential biomarkers in ecotoxicology (Dedourge-Geffard et al., 2009; Wang et al., 2015; Caruso et al., 2016). Under stressful situations, energy allocation in fish is disturbed because a large proportion of ingested energy is invested in detoxification, involving the cellular antioxidant defence system: superoxide dismutase (SOD), total glutathione (GSH), glutathione reductase (GR), catalase (CAT), etc. These parameters are also used as classical biomarkers in aquatic biomonitoring, indicating the level of exposure of an organism to chemical substances such as metals or pesticides (Cossu et al., 2000; Sanchez et al., 2007).

Aquatic contamination results from both organic and inorganic pollutants (mainly metals). But in natural ecosystems, living organisms are subjected not only to anthropogenic chemical stresses, but also to multiple stressors, whether natural or introduced into the environment by humans. Temperature is a potent physical stressor and a key factor in the control of the fundamental physiological processes involved in survival, growth, and reproduction of ectotherm organisms (Heugens et al., 2001). The water temperature often fluctuates naturally due to diurnal and seasonal variations that result in extremely low or high temperatures that can be harmful or even fatal to living aquatic organisms (Heugens et al., 2001). Similarly, with the anthropogenic climate change, more extreme high temperature variations can be expected (IPCC, 2013). Although temperature scenarios can be simulated from carbon dioxide emission models (IPCC, 2013), predicting the biological effects of multiple stressors on living beings remains difficult, and even impossible with the current state of knowledge. When it comes to xenobiotics and temperature, some contaminants become more toxic at elevated temperatures and increase the risk of toxic interactions in a global warming context (Hooper et al., 2013). On the other hand, other contaminants become less toxic or remain equally toxic (Weston et al., 2009).

The complex nature of the interactions between toxic substances and temperature has prompted several authors to investigate the issue of combined stresses to improve risk assessment in aquatic environments. Unfortunately, there is a lack of information in the literature on this topic, especially about the effects of chronic low-dose chemical exposure combined to other stresses. In fact, several authors have analysed the effects of xenobiotics, and others have reported the effects of their combination with temperature on fish physiology

(Hallare et al., 2005; Reynaud and Deschaux, 2006; Li et al., 2014; Andersen et al., 2015; Authman et al., 2015; Guardiola et al., 2015; Olsvik et al., 2016). However, most studies have tested short-term exposure at sub-lethal concentrations which do not reflect actual environmental concentrations of toxicants or the real effects of complex interactions with other stressors such as temperature. Furthermore, health effects may be missed in short-term exposure because adverse impacts can take a long time to emerge (Hamilton et al., 2015).

The present study addresses that issue. We determined the joint effects of chronic environmental exposure to temperature (physical stress) and cadmium (chemical stress) on a small teleost fish often used as an animal model in ecotoxicology: the threespine stickleback (*Gasterosteus aculeatus*) (Wootton, 1984; Sanchez et al., 2007).

We selected cadmium as a chemical contaminant model because it is considered as one of the most dangerous inorganic compounds owing to its persistent nature and slow elimination from environmental compartments. This element comes from natural sources and anthropogenic activities such as disposal of industrial, mining, or agricultural wastewater discharges (Sfakianakis et al., 2015). Although the cadmium concentration in fresh aquatic compartments is usually less than  $1 \mu\text{g. L}^{-1}$ , it can reach  $400 \mu\text{g. L}^{-1}$  or more in the most contaminated sites (Foran et al., 2002). That is why it has been classified as a priority pollutant in Europe, in line with the EU Water Framework Directive (WFD). Furthermore, information and recommendations on water quality criteria for cadmium are regularly updated in the US Environmental Protection Agency database (US EPA, 2016). Metabolic alterations related to cadmium exposure in fish species are well documented (Soengas et al., 1996; Almeida et al., 2001; Ferrari et al., 2011; Pretto et al., 2014; Pi et al., 2016).

The first purpose of the present study was to evaluate the effects of single or combined exposure to cadmium/temperature stresses on physiological parameters of the threespine stickleback (*Gasterosteus aculeatus*). Sticklebacks were exposed to an environmental concentration of cadmium (a nominal concentration of  $1 \mu\text{g. L}^{-1}$ ) in combination with two different water temperatures ( $16^\circ\text{C}$  and  $21^\circ\text{C}$ ) for three months. We assessed several parameters throughout the experiment: (i) energy metabolism parameters (glycogen, lipid, protein energy reserves, and trypsin, amylase, intestinal alkaline phosphatase enzyme activities), and (ii) antioxidant parameters (superoxide dismutase activity and total glutathione levels). The second purpose was to determine the interest of the three digestive enzymes as biomarkers in comparison with other parameters considered as classical biomarkers in ecotoxicology.

## **2. Materials and methods**

### **2.1. Ethics Statement**

The experiment was conducted in accordance with the European directive 2010/63/UE for the protection of animals used for scientific purposes. The registration number of the INERIS, where the experiments were conducted, is C60-769-02. The experimental protocols were submitted and reviewed by an ethical committee nationally recognized in France (CREMEAPS, registration number 96).

### **2.2. Fish maintenance conditions**

We used juvenile sticklebacks ( $n=300$ ) of 3 - 3.5 cm standard length, originating from the same population, and born the same year in the INERIS artificial rivers (Verneuil-en-Halatte, France). Three months prior to the experiment, they were transferred from mesocosms to 300-liter laboratory tanks, with a continuous freshwater (0 ppt) circulation system. Water temperature was set at 16 °C, the optimal temperature for sticklebacks reported by Guderley (1994), that was used as a control temperature. Sticklebacks were kept in a thermoregulated room, with a fixed 12h/12h light/dark cycle, and fed on a constant feeding regime (frozen commercial chironomid larvae, 3 % of the fish body weight/day, Europrix, France, *ad libitum*). The feeding level was adjusted at each time-point and classification, and was maintained as a constant ratio of the fish mass, as described by Leloutre et al. (2016).

### **2.3. Experimental design**



Ten days before the onset of the experiment, sticklebacks were randomly distributed in four 90-L laboratory glass tanks (75 *per* tank) as follows: one control group, and three experimental groups (“cadmium” condition, “21 °C” temperature condition, and “cadmium-temperature” condition) (Figure 1). All along the experiment, sticklebacks were kept in a semi-static freshwater (0 ppt) system and fed similarly to maintenance conditions. Each tank was continuously oxygenated and equipped with a multistage water filter (EHEIM Pro III 2073, Germany) and a water conditioner (TANK® TR20, TECO SRL, Italy). The water temperature of the “cadmium-temperature” and “temperature” tanks was gradually increased at a rate of 0.8 °C.day<sup>-1</sup>, from 16 °C (maintenance temperature) to 21 °C (experimental temperature), while the “control” and “cadmium” tanks were maintained at 16 °C. We used 21 °C as a high temperature because it lies within the range of stressful temperatures for sticklebacks (Jordan and Garside, 1972), and slightly exceeds the rise of 1.8 - 4.0°C predicted by global climate change scenarios (IPCC, 2013).

Once the targeted temperature was reached, sticklebacks were kept in the different tanks for 90 days. For the “cadmium” and “cadmium-temperature” conditions, they were exposed to a nominal concentration of 1 µg. L<sup>-1</sup> of cadmium chloride (CdCl<sub>2</sub>, Sigma-Aldrich Chemicals, France). The cadmium concentration was selected in accordance with concentration levels reported in French rivers: 1-10 µg.L<sup>-1</sup> (IRSN, 2004). It was four times as much as the European standard reported in the WFD for class 5 inland surface waters (AA-EQS= 0.25 µg.L<sup>-1</sup>), and slightly exceeded 0.72 µg.L<sup>-1</sup>, the freshwater chronic criterion (CCC) recommended by the US Environmental Protection Agency (US EPA, 2016).

Throughout the experiment, water was renewed every other day, and water parameters were assessed before and after water replacement. Since water parameters (temperature, pH, conductivity, and dissolved oxygen) remained unchanged before and after water renewal, we presented these data as global means (± SD) (Table 1). Concerning cadmium, water concentrations were monitored before and after water renewal, daily during the first month of exposure and weekly for the rest of the study: water samples were acidified with nitric acid 2 %, and total cadmium concentrations were checked by electrothermal atomic absorption spectrophotometry (SpectrAA, GTA110, Varian, Palo Alto, CA, USA). Since the experiment was conducted in semi-static conditions, cadmium concentrations decreased before water replacement. Hence, the actual cadmium concentration to which sticklebacks were exposed was calculated as the time-weighted mean (TW Mean) according to OECD Test Guidelines (OECD,

2012), and was half the targeted nominal concentration (Table 1). No mortality was observed during the acclimation or exposure periods.

**Table 1. Average water parameters and measured cadmium concentrations recorded during the experiment.**

Conditions	Water parameters				Measured cadmium concentrations ( $\mu\text{g. L}^{-1}$ )
	Temperature ( $^{\circ}\text{C}$ )	pH	Conductivity ( $\mu\text{S}$ )	Dissolved Oxygen ( $\text{mg/L}$ )	
<b>Control</b>	$16.10 \pm 0.38$	$8.33 \pm 0.22$	$464.60 \pm 64.19$	$9.40 \pm 0.48$	$0.01 \pm 0.002$
<b>Cadmium</b>	$16.45 \pm 1.10$	$8.24 \pm 0.20$	$484.31 \pm 71.93$	$9.41 \pm 0.46$	$0.56 \pm 0.27$
<b>Temperature</b>	$21.13 \pm 0.44$	$8.14 \pm 0.23$	$488.46 \pm 59.02$	$8.51 \pm 0.54$	$0.01 \pm 0.001$
<b>Cadmium-Temperature</b>	$21.10 \pm 0.50$	$8.09 \pm 0.18$	$524.40 \pm 77.52$	$8.38 \pm 0.54$	$0.53 \pm 0.21$

#### 2.4. Dissection and sample preparation

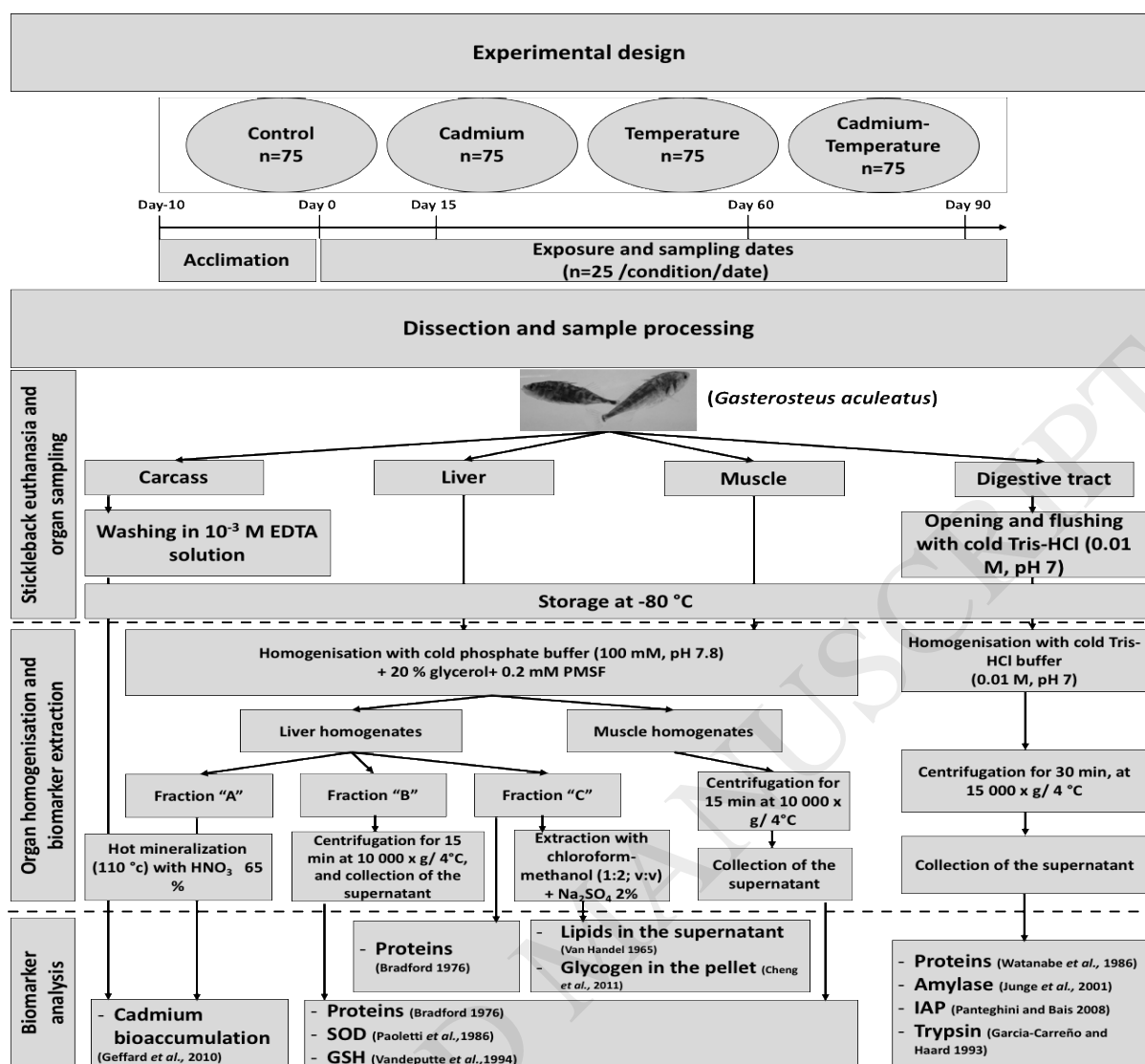
Sticklebacks ( $n=25$  per condition) were sampled on days 15, 60, and 90, after a 24h starvation period recommended by Langeland et al. (2013) for digestive enzyme assays. At each sampling date, sticklebacks were anaesthetized with  $70 \text{ mg. L}^{-1}$  of MS 222, CAS No: 886-86-2, and then killed by cervical dislocation (section of the cranial-vertebral junction). Then they were weighed, standard length was measured, and organs (liver, muscle, and the whole digestive tract) were sampled and frozen at  $-80^{\circ}\text{C}$ , until homogenisation and analysis (Figure 1).

Liver and muscle samples (n=10/condition/sampling date) underwent a common homogenisation step, using ice-cold phosphate buffer (100 mM, pH 7.8) with 20 % glycerol and 0.2 mM PMSF. Liver homogenates were divided into three fractions: A, B, and C. Fraction A was used for cadmium bioaccumulation assays (see paragraph 2.5.), fraction B for anti-oxidant parameters, and fraction C for energy reserve parameters (Figure 1).

Fraction B and muscle homogenates were centrifuged for 15 min (10,000 x g, 4°C), and the supernatants were used to assess antioxidant parameters (Figure 1). Fraction C was used to assess protein reserves, and then an extraction was performed with chloroform/methanol (1:2; v:v) and sodium sulphate (Na<sub>2</sub>SO<sub>4</sub>) 2 % to assess lipid (in the supernatant) and glycogen (in the pellet), respectively, after a 15-min centrifugation step (1,000 x g, 4 °C).

The whole digestive tracts of all sticklebacks (n=25/condition/sampling date) were rinsed on ice using cold Tris-HCl buffer (0.01 M, pH7), and then homogenised in the same buffer. Digestive homogenates were then centrifuged at 15,000 x g (30 min, 4°C), and the supernatants were collected and used for enzymatic activity analysis (Figure 1).

Tissue homogenisation (except for carcass) was performed using a PRECELLYS24<sup>®</sup> homogeniser (BERTIN TECHNOLOGIES, France).



**Figure 1. Experimental design and schematic diagram of sample processing and biomarkers analysis.** Fish were sacrificed on days 15, 60 and 90, and organs were sampled to assess cadmium bioaccumulation, antioxidant parameters (superoxide dismutase activity– SOD – and total glutathione levels– GSH –), and energy metabolism parameters: energy reserves (glycogen, lipids, proteins) and digestive enzyme activities (trypsin, amylase, and intestinal alkaline phosphatase – IAP –).

## 2.5. Metal analysis

Cadmium bioaccumulation was assessed in stickleback liver (fraction A) and carcass (Figure 1). Fraction A underwent hot solubilisation with nitric acid at  $110^{\circ}\text{C}$  (1 mL of  $\text{HNO}_3$  for 1 mL of homogenate), and samples were diluted with ultrapure water before cadmium analysis. Stickleback carcasses (after the kidney, spleen, and gonads were removed), were washed in a solution of  $10^{-3}$  M EDTA to remove cadmium from the fish surface, and frozen at  $-80^{\circ}\text{C}$  until mineralisation and cadmium measurements (Figure 1). Carcass tissues were

digested individually at 110 °C by adding 5 mL of concentrated nitric acid (HNO<sub>3</sub> 65 %). Carcass digestates were then adjusted to 10 mL with ultrapure H<sub>2</sub>O before cadmium analysis. After the mineralisation step, cadmium was assayed in all samples by electrothermal atomic absorption spectrophotometry (SpectrAA, GTA110, Varian, Palo Alto, CA, USA), as described by Geffard et al. (2010). Results were expressed as µg. g<sup>-1</sup> wet wt.

## **2.6. Biological analyses**

### **2.6.1. Fish biometric parameters**

Stickleback growth was assessed by monitoring the evolution of their weight and length throughout the experiment. Sex was determined, and the gonado-somatic index (GSI = gonad mass (g) x [body mass (g)]<sup>-1</sup> × 100) and Fulton's condition factor (K= Weight (g) x [Length (mm)<sup>3</sup>]<sup>-1</sup> × 100) were measured.

### **2.6.2. Oxidative stress parameters**

Before GSH and SOD activity assays, cytosolic protein concentrations were determined according to Bradford's (1976) method using bovine serum albumin (Sigma-Aldrich Chemicals, France) as a standard. Antioxidant biomarkers were assayed according to the methods of Vandeputte et al. (1994) and Paoletti et al. (1986), respectively, adapted to microplates and optimised for threespine stickleback as described by Sanchez et al. (2007). Results were expressed as U. g<sup>-1</sup> of protein for SOD activity and µmol. g<sup>-1</sup> of protein for GSH.

### **2.6.3. Energy metabolism parameters**

#### **a) Energy reserves**

Protein contents were measured according to Bradford's (1976) method using bovine serum albumin (Sigma-Aldrich Chemicals, France) as a standard. Lipid and glycogen were measured according to protocols adapted from Van Handel (1965) and Cheng et al. (2011), respectively, using triolein (Sigma-Aldrich Chemicals, France) and glucose solutions (Sigma-Aldrich Chemicals, France) as standards.

#### **b) Digestive enzymes activities**

Amylase, intestinal alkaline phosphatase (IAP) activities and cytosolic gut protein contents were measured according to the methods of Junge et al. (2001), Panteghini and Bais (2008), and Watanabe et al. (1986), respectively, using Thermo-Scientific Gallery ready-to-use

reagents. Trypsin activity was measured according to Garcia-Carreño and Haard (1993), using N-benzoyl-DL-arginine 4-nitroanilide hydrochloride (BAPNA, 3 mM) as a substrate. All enzymatic techniques were conducted at 37 °C by kinetic colorimetric assay at 405 nm, adapted on an automated photometric analyser (Gallery™, Thermo Fisher Scientific Oy) and optimised for threespine stickleback. Each enzyme activity was expressed as U. g<sup>-1</sup> of protein.

## 2.7. Statistical analyses

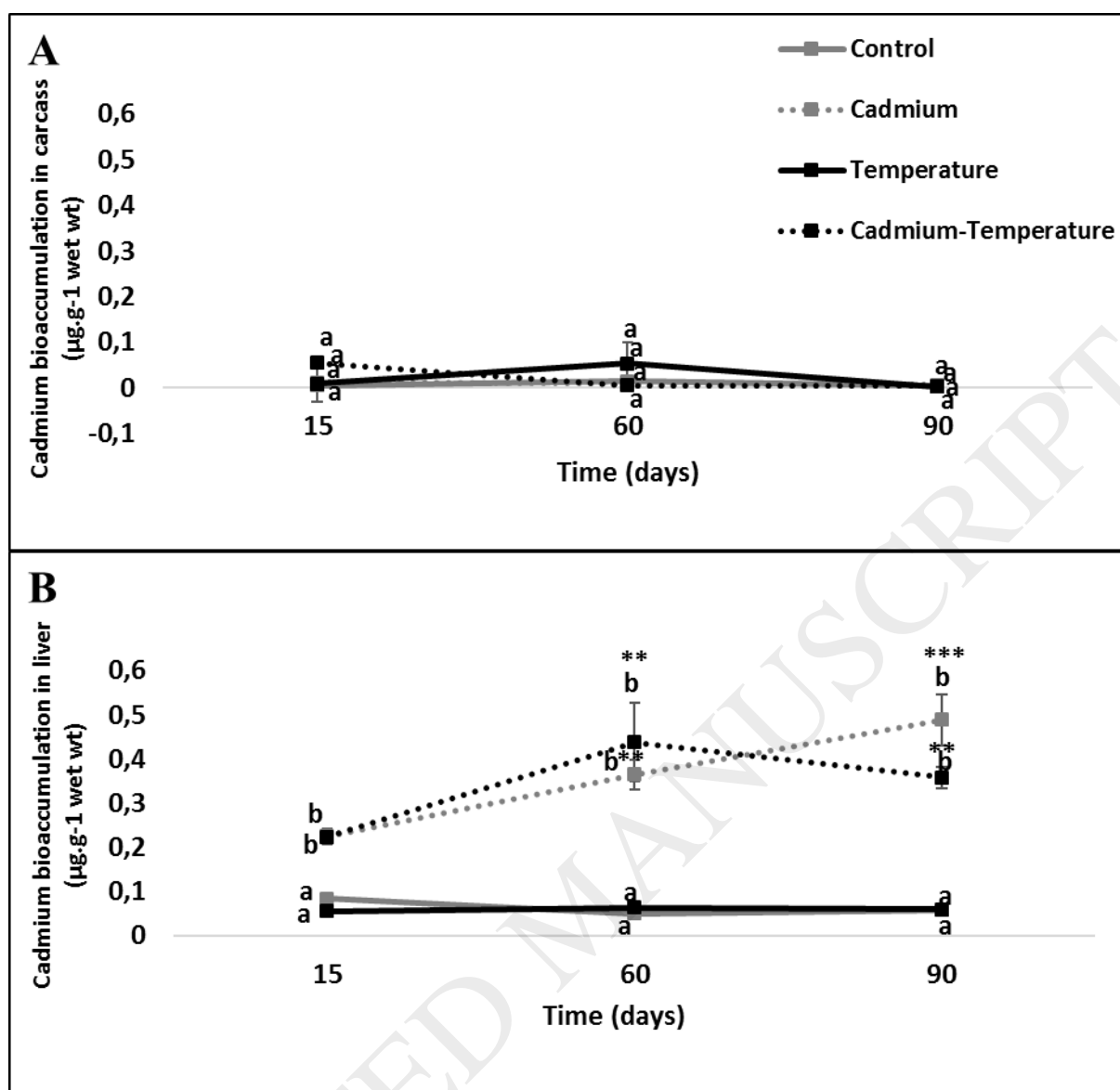
Statistical analyses were performed using R statistical software (v3.3.1) with  $\alpha = 0.05$ . Before statistical treatment, normality and homogeneity tests (Shapiro's and Levene's tests, respectively) were performed, followed by a parametric test (two-factor ANOVA), with time point and treatment group as factors. When data were not normally distributed, log, Box-Cox or square-root transformation were used. Tukey's HSD post-hoc test was performed to compare conditions at each time-point. Results were expressed as means  $\pm$  SEs.

## 3. Results

### 3.1. Cadmium bioaccumulation

We assessed cadmium bioaccumulation in *G. aculeatus* carcass (Figure 2 A) and liver (Figure 2 B) after 15, 60, and 90 days of exposure. No bioaccumulation was observed in carcass whatever the experimental condition (Figure 2 A). However, in liver, cadmium bioaccumulated in both "cadmium" and "cadmium-temperature" conditions. Although cadmium concentrations did not significantly differ between the two groups at each sampling date, the trends of the bioaccumulation kinetics differed, but nonsignificantly (Figure 2 B).

Compared to the initial values of day 15, cadmium bioaccumulation was significantly higher (1.5- to 1.9-fold) on days 60 and 90 in the "cadmium" and "cadmium-temperature" groups ( $p < 0.01$ ), whereas no bioaccumulation was observed in the control and "temperature" groups.



**Figure 2.** Cadmium bioaccumulation in *G. aculeatus* carcass (A) and liver (B) after 15, 60, and 90 days. Sticklebacks nonexposed to cadmium and kept at 16 °C were considered as the control group. For a same time point, different letters indicate significant differences between groups (mean  $\pm$  S.E; n=10,  $p<0.05$ ). For a same group, asterisks indicate significant temporal differences with respect to initial values on day 15 (\* $p<0.05$ ; \*\* $p<0.01$ ; \*\*\* $p<0.001$ ).

### 3.2. Stickleback growth and biometric parameters

As regards weight and length at any given time point, no difference was noted between the treated and control groups ( $p<0.05$ ), except on day 90, when weight (1.3-fold,  $p<0.01$ ) and length (1.15-fold,  $p<0.01$ ) significantly decreased in sticklebacks from the “temperature” and “cadmium-temperature” groups as compared to the control group. However, a significant

temporal increase in weight and length was noted in all groups on days 60 and 90 as compared to initial values of day 15 (Table 2).

As regards Fulton's condition factor at any given time point, no significant difference was noted between the treated and control groups, except on day 90 when Fulton's condition factor was significantly higher in the treated groups than in the control group (1.2-fold,  $p < 0.01$ ). Whatever the condition, no temporal effect was observed on Fulton's index when compared to the initial values of day 15, except a significant 1.27-fold decrease on day 90 ( $p = 0.0001$ ) in the control group.

As for the GSI, no significant difference was observed between exposed and control groups in males. Whatever the condition, no temporal effect was observed, except in males of the cadmium group, in which a significant 1.8-fold decrease was recorded on days 60 and 90 as compared to the initial value of day 15 ( $p < 0.05$ ). In females, GSI values were negatively impacted by the elevated temperature and its combination with cadmium on day 90 as compared to the control and "cadmium" groups ( $p < 0.05$ ). A temporal effect was observed in all female groups when compared to initial values of day 15. In fact, in the control and "cadmium" conditions, GSI values increased significantly from day 60, to reach the highest values on day 90 (5-fold higher than values on day 15,  $p < 0.0001$ ). However, in female sticklebacks from the "temperature" and "cadmium-temperature" groups, the highest GSI values were observed on day 60 (5.7- to 6-fold higher than on day 15,  $p < 0.001$ ), followed by a sharp decrease on day 90 to reach similar values to those of day 15.



**Table 2. Evolution of fish biometric parameters, anti-oxidant parameters, and energy reserves under the effect of temperature and/or cadmium exposure, after 15, 60, and 90 days.** For a same time point, different letters indicate significant differences between exposed groups (mean  $\pm$  S.E; n=10, p<0.05). For a same exposed group, asterisks indicate significant temporal differences as compared to initial values of day 15 (\*p<0.05; \*\*p<0.01; \*\*\*p<0.001).

Conditions		Fish biometric parameters				Oxidative stress				Energy reserves (J)			
		Weight (mg)	Length (mm)	Fulton's factor (K)	GSI		Liver GSH (μmol. g <sup>-1</sup> of prot)	Muscle GSH (μmol. g <sup>-1</sup> of prot)	Liver SOD (U. g <sup>-1</sup> of prot)	Muscle SOD (U. g <sup>-1</sup> of prot)	Glycogen	Lipids	Proteins
					Males	Females							
Day 15	Control	618.60 ± 51.83a	35.20 ± 0.91a	1.38 ± 0.02a	1.34 ± 0.49ab	2.31 ± 1.74a	9.77 ± 0.59a	4.66 ± 0.39a	1304.78 ± 98.71a	479.43 ± 54.53a	11.59 ± 2.03a	88.56 ± 13.89a	95.58 ± 11.71a
	Cadmium	659.50 ± 43.95a	36.9 ± 0.69a	1.29 ± 0.04a	1.79 ± 0.70a	2.22 ± 0.96a	8.47 ± 0.66a	4.07 ± 0.49a	1726.37 ± 116.33ab	524.82 ± 131.04a	19.18 ± 2.66a	104.06 ± 13.37a	118.09 ± 12.31a
	Temperature	573.20 ± 56.59a	34.5 ± 1.18a	1.36 ± 0.03a	0.83 ± 0.74b	1.80 ± 0.57a	7.98 ± 0.61a	4.52 ± 0.30a	1666.71 ± 176.46ab	601.66 ± 34.41a	16.18 ± 1.83a	102.25 ± 8.49a	92.65 ± 10.07a
	Cadmium-Temperature	535.40 ± 49.36a	34.10 ± 1.12a	1.31 ± 0.02a	0.87 ± 0.54b	1.92 ± 0.86a	8.54 ± 1.39a	4.19 ± 0.46a	1987.34 ± 56.60b	566.13 ± 63.05a	12.61 ± 2.43a	86.82 ± 10.97a	83.14 ± 10.13a
Day 60	Control	1015.10 ± 89.55a**	41.30 ± 0.90a*	1.40 ± 0.03a	1.16 ± 0.50a	7.35 ± 4.72a***	9.94 ± 0.99a	3.81 ± 0.31a	1622.01 ± 201.77a	886.22 ± 170.65a	40.24 ± 7.97a***	160.88 ± 14.98a**	197.07 ± 20.60a*
	Cadmium	1098.8 ± 71.57a**	42.40 ± 0.76a**	1.42 ± 0.03a	0.97 ± 0.34a**	9.79 ± 6.19a***	9.98 ± 0.84a	4.08 ± 0.39ac	1788.74 ± 204.30a	818.56 ± 215.89a	53.42 ± 8.08a***	182.86 ± 15.41a**	263.56 ± 24.16b**
	Temperature	1009.5 ± 51.39a***	40.80 ± 0.46a***	1.47 ± 0.03a	0.87 ± 0.45a	11.89 ± 7.36a***	10.64 ± 1.26a	5.88 ± 0.55b	2039.11 ± 208.22a	1062.84 ± 148.67a*	47.27 ± 8.15a**	147.06 ± 10.78a*	195.40 ± 11.30a***
	Cadmium-Temperature	939.00 ± 47.72a***	40.90 ± 0.88a***	1.37 ± 0.03a	0.99 ± 0.57a	11.07 ± 5.13a***	10.95 ± 1.17a	5.15 ± 0.53bc	1850.28 ± 108.17a	944.58 ± 199.60a	28.59 ± 4.39a*	163.62 ± 11.29a***	204.03 ± 15.19ab***
Day 90	Control	1134.80 ± 111.02a***	47.10 ± 2.19a***	1.08 ± 0.06a***	0.90 ± 0.50a	12.36 ± 7.12a***	5.45 ± 0.63a*	4.55 ± 0.36a	737.76 ± 83.88a	279.98 ± 9.73a	25.62 ± 2.18a**	206.55 ± 17.64a***	291.10 ± 40.12a***
	Cadmium	1123.50 ± 115.01a**	43.10 ± 1.44a***	1.36 ± 0.03b	0.92 ± 0.47a*	12.94 ± 7.09a***	6.49 ± 0.83a	4.36 ± 0.45a	1054.15 ± 170.73ab	289.64 ± 12.55a	29.66 ± 5.31a	224.38 ± 21.71a***	290.38 ± 42.77a***
	Temperature	873.9 ± 51.71b**	40.80 ± 1.37b***	1.29 ± 0.06b	0.40 ± 0.29a	2.05 ± 1.48b	5.18 ± 0.83a	5.78 ± 0.41a	990.07 ± 100.65ab*	570.77 ± 61.26b	25.84 ± 5.64a	168.25 ± 10.62b***	172.49 ± 23.67b**
	Cadmium-Temperature	813.00 ± 56.81b**	39.30 ± 1.18b**	1.32 ± 0.03b	0.54 ± 0.29a	4.63 ± 4.41b	8.23 ± 0.28a	4.87 ± 0.42a	1401.87 ± 102.62b**	483.32 ± 65.38b	36.91 ± 5.83a**	168.41 ± 10.45b***	172.76 ± 19.56b***

### 3.3. Oxidative stress parameters

There was no significant difference in GSH activity (Table 2) between treated groups all along the experiment, except in muscle, in which a significant 1.3-fold increase was noted on day 60 ( $p<0.05$ ) in the sticklebacks of the “temperature” and “cadmium-temperature” conditions as compared to the control group. Furthermore, no temporal effect was observed on liver or muscle GSH levels in any group.

As regards SOD activity, a consistent trend was recorded in both liver and muscle all along the experiment. In fact, SOD activity was higher in the treated groups than in the control groups. But the difference was significant in liver only on days 15 (1.5-fold,  $p=0.04$ ) and 90 (1.9-fold,  $p=0.004$ ) in the “cadmium-temperature” group, and in muscle only on day 90 (1.7-fold,  $p=0.01$ ), in the “temperature” and “cadmium-temperature” groups. A temporal effect was observed only on day 90 in liver and on day 60 in muscle. In liver, a significant decrease of SOD activity was observed in the “temperature” (1.6-fold,  $p=0.04$ ) and “cadmium-temperature” (1.4-fold,  $p=0.006$ ) groups as compared to initial values of day 15. However, in muscle, a significant increase of SOD activity occurred in the “temperature” group (1.7-fold,  $p=0.04$ ) only on day 60 as compared to initial values of day 15.

### 3.4. Energy metabolism parameters

#### 3.4.1. Energy reserves

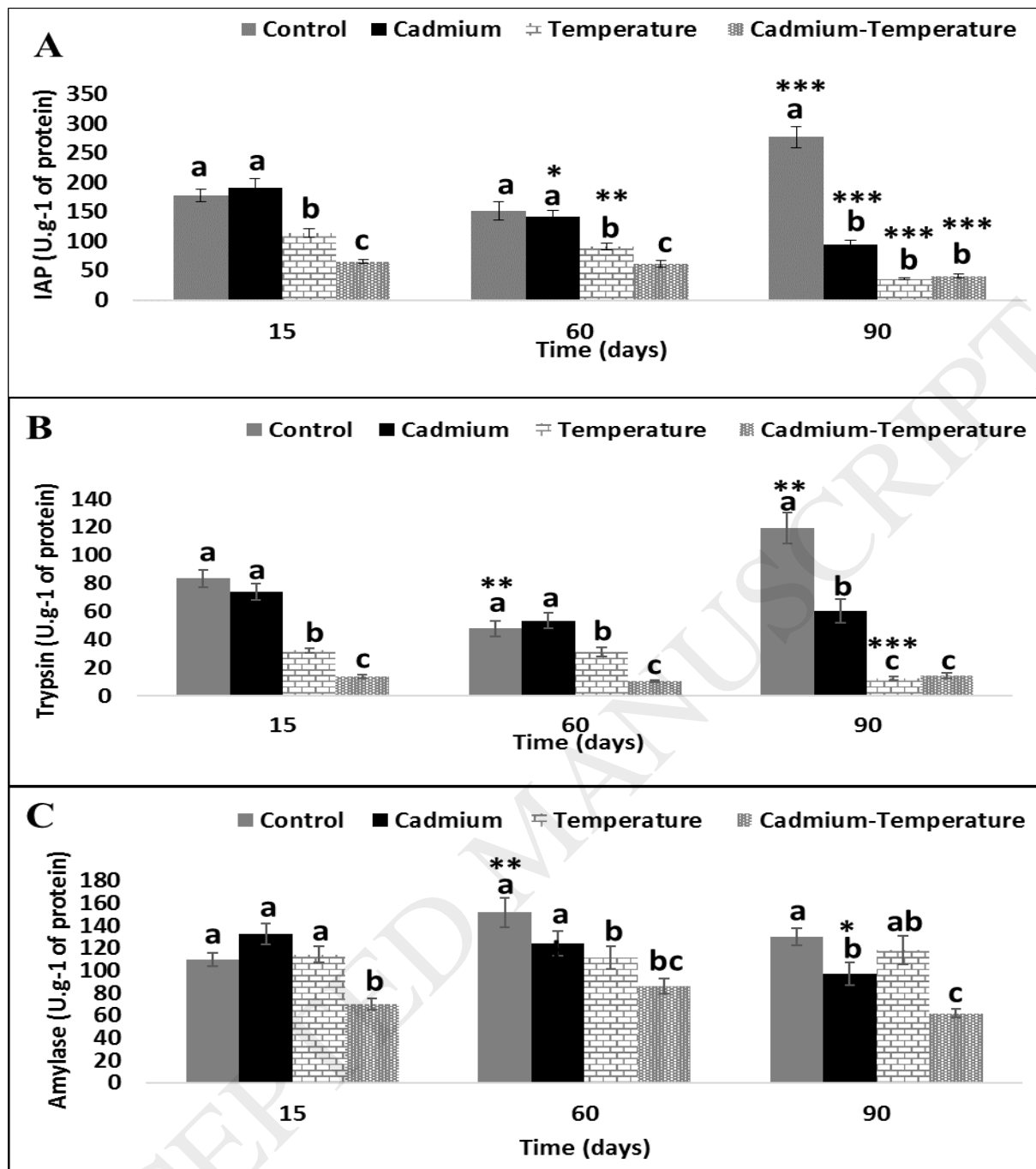
For a same date, neither cadmium nor temperature, nor again their combination had any significant effect on glycogen throughout the experiment (Table 2). However, when compared to initial values of day 15, a significant 2- to 3-fold increase in glycogen values was observed in all groups on day 60 ( $p<0.05$ ), but only in the control and “cadmium-temperature” groups on day 90 (2-fold increase,  $p<0.01$ ).

Lipid and protein quantities exhibited the same patterns throughout the experiment. In fact, no significant difference was noted between the treated groups on days 15 and 60 ( $p>0.05$ ). However, as compared to the control and “cadmium” groups, a significant decrease of protein and lipid quantities was recorded on day 90 (1.6-fold,  $p=0.009$ ) in the “temperature” and “cadmium-temperature” groups. A significant temporal increase of lipid and protein quantities was observed all along the experiment in all groups (1.5 to 3-fold increase,  $p<0.05$ ).

### 3.4.2. Digestive enzyme activities

Throughout the experiment, IAP and trypsin activities were highly modulated in response to cadmium, temperature, and their combination, and followed a similar trend in all groups (Figures 3 A and 3 B). In the control groups, when compared to initial values of day 15, a significant increase of IAP activity was noted on day 90 ( $p < 0.0001$ ), whereas a significant decrease of trypsin activity ( $p = 0.006$ ) was observed on day 60, followed by a significant increase ( $p < 0.0001$ ) on day 90. Compared to the control groups, cadmium had a significant effect only on day 90, and was responsible for *ca.* 2.9-fold and 1.9-fold reductions of IAP and trypsin activities, respectively ( $p < 0.0001$ ). Temperature caused a significant decrease in IAP and trypsin activities from day 15 until day 90 (7-fold decrease,  $p < 0.0001$ ). The effect of cadmium on the activity of the two digestive enzymes was aggravated in the presence of temperature stress, and was observed earlier after day 15. In fact, IAP and trypsin activity levels significantly decreased by 2.5 to 4.5-fold on days 15 and 60 ( $p < 0.00001$ ), and 7-fold on day 90 ( $p < 0.00001$ ) in the “cadmium-temperature” groups as compared to the control groups.

Amylase activity (Figure 3 C) changed less than trypsin and IAP activities when sticklebacks were exposed to cadmium or temperature, with a significant 1.3-fold decrease from day 60 ( $p < 0.05$ ) in the “temperature” group, and 2.1-fold only on day 90 ( $p = 0.01$ ) in the “cadmium” group. Exposure to the higher temperature enhanced the effects of cadmium on amylase activity: in the “cadmium-temperature” groups as compared to the control groups, amylase activity decreased significantly earlier, similarly to IAP and trypsin activities, from day 15 (1.5-fold decrease,  $p < 0.001$ ), and remained reduced until day 90 (2.1-fold decrease,  $p = 0.01$ ). Compared to initial values on day 15, amylase activity remained unchanged in all groups throughout the experiment, except in the control group on day 60 (1.4-fold increase,  $p < 0.01$ ) and in the cadmium group on day 90 (1.3-fold decrease,  $p < 0.05$ ).



**Figure 3. Intestinal alkaline phosphatase (IAP) (A), trypsin (B) and amylase (C) activities under the effect of temperature and/or cadmium after 15, 60, and 90 days. Sticklebacks maintained at 16 °C and non-exposed to cadmium were considered as the control group.**

For a same time point, different letters indicate significant differences between exposed groups (mean  $\pm$  S.E; n=25, p<0.05).

For a same exposed group, asterisks indicate significant temporal differences with respect to initial values of day 15 (\*p<0.05;

\*\*p<0.01; \*\*\*p<0.001).

## 4. Discussion

### 4.1. Cadmium exposure and bioaccumulation

We conducted our experiment in semi-static conditions, and water was renewed every other day to keep the cadmium concentration constant. Although a nominal concentration of  $1 \mu\text{g.L}^{-1}$  of cadmium was targeted, the real water cadmium concentration calculated at the end of the experiment was 2-fold lower, *i.e.*  $0.5 \mu\text{g.L}^{-1}$ . Therefore sticklebacks were exposed chronically to a very low cadmium concentration, below the freshwater chronic criterion (CCC) recommended by the US Environmental Protection Agency:  $0.72 \mu\text{g.L}^{-1}$  (US EPA, 2016), and 2-fold higher than the annual average value (AA-EQS=  $0.25 \mu\text{g.L}^{-1}$ ) reported by the European Directive 2013/39/EU for inland surface waters (Class 5). Despite this very low concentration (supported by our bioaccumulation results), we noted adverse effects on certain parameters throughout the experiment. In fact, cadmium accumulated in liver but not in carcass. These results are not surprising considering the very low concentration to which sticklebacks were exposed. The literature reports that even at high concentrations, cadmium accumulates at higher rates in liver than in carcass. Moreover, the liver is the main xenobiotic-detoxifying organ (Cazenave et al., 2006; Vergauwen et al., 2013). Contrary to our expectations, cadmium accumulation in liver was not influenced by the elevated temperature, and no correlation was observed between these two parameters. This goes against literature results, since increased metal accumulation along with increasing temperature has been widely reported in many species as one of the mechanisms whereby temperature influences metal toxicity (Vergauwen et al., 2013; Perera et al., 2015).

### 4.2. Effects on biometric parameters

Temperature is a potent physical stressor for fish, with major effects on the metabolism, growth, and fundamental biochemical processes (Hovel et al., 2015). It had a major effect on stickleback biometric parameters: at  $21^\circ\text{C}$ , sticklebacks (exposed to cadmium or not) weighed less than those maintained at  $16^\circ\text{C}$  (with or without cadmium exposure) after 90 days of exposure. These results confirm that sticklebacks, as a cold-water species, prefer cool temperatures ( $<18^\circ\text{C}$ ) (Guderley, 1994). They also suggest that  $21^\circ\text{C}$  is an upper tolerance limit for their long-term growth, even if they are fed *ad libitum*, a rare occurrence in the natural habitats of this species (Wootton, 1984). Similarly, the growth capacity of the coral reef fish *Acanthochromis polyacanthus* was severely limited, even with high food rations, after 3 months of exposure to a high water temperature (Munday et al., 2008).

Concerning Fulton's condition factor, neither temperature nor cadmium had an effect on sticklebacks. However, a decrease was observed in the control fish on day 90 and could be

explained by the increase of fish length, since the K-factor is a crude parameter that takes into account fish weight and the cube of fish length. The GSI was not impacted by cadmium exposure in females, but was highly influenced by temperature conditions (alone or in combination with cadmium): at 21 °C (with or without cadmium exposure), the GSI peaked on day 60, whereas at 16 °C (with or without cadmium exposure), the peak was reached only after 90 days. These results suggest that oocyte formation was promoted earlier in females raised at 21 °C. High temperature has been reported to augment gonadal development in teleost fish, including sticklebacks (Guderley, 1994; Acharia et al., 2014). However, degeneration of gonad morphology following exposure to a constant high temperature has been reported too (Acharia et al., 2014). This may explain why the GSI sharply decreased on day 90 in sticklebacks raised at 21 °C (exposed to cadmium or not), suggesting impaired reproduction.

### **4.3. Effects on core biomarkers**

#### **4.3.1. Antioxidant parameters**

Despite a nonsignificant increasing trend of SOD activity in the cadmium-treated groups as compared to the control groups, we detected no sign of oxidative stress. Heavy metals are known to accumulate in fish tissues and catalyse reactions that generate reactive oxygen species (ROS) and lead to oxidative stress. In fish, antioxidant parameters can be induced or inhibited by heavy metals depending on the dose, the species, and the organ (Sanchez et al., 2005; Farombi et al., 2007; Zheng et al., 2016). In our study, the absence of oxidative stress at this concentration could be due to the protective role of other organs (*i.e.* the gut) that may have sequestered cadmium more than the liver and muscle did, resulting in effects other than those of oxidative stress. Cadmium compartmentalisation and sequestration in the digestive tract has been reported in fish exposed to waterborne heavy metals (Andres et al., 2000; Vergauwen et al., 2013). Unfortunately, we did not study cadmium bioaccumulation in the digestive tract to support this hypothesis.

Although cadmium had no impact on oxidative stress, temperature (alone or in combination with cadmium) influenced the two antioxidant parameters. GSH levels significantly increased in muscle on day 60, and SOD activity also significantly increased in liver on days 15 and 90, and in muscle only on day 90. Several studies have shown that oxidative stress biomarkers are highly sensitive to temperature, but their response depends on fish species (Figueiredo-Fernandes et al., 2006; Vinagre et al., 2012; Madeira et al., 2013). In Nile tilapia (*Oreochromis niloticus*) raised at 17 and 27 °C for 45 days, no difference was observed in SOD or GST activity (Figueiredo-Fernandes et al., 2006). However, in juvenile seabass (*Dicentrarchus labrax*), catalase activity was higher at 18 °C and 28 °C than at 24 °C, their

thermal optimum. The authors concluded that fish experience thermal stress at temperatures beyond their thermal optimum (Vinagre et al., 2012). Sticklebacks are typically present in waters where temperatures range from 4 to 20°C (Wootton, 1984). Even if a temperature of 21 °C slightly exceeds their optimal thermal range, they may be exposed to this temperature in natural habitats now and then. Long-term exposure to this temperature seems to be stressful, and these results confirm that 21 °C can be considered as an upper limit temperature for long-term physiological processes in sticklebacks.

#### 4.3.2. Energy reserves

As for antioxidant parameters, cadmium at 0.50 µg. L<sup>-1</sup> had no effect on *G. aculeatus* energy reserves, unlike temperature. In fact, no significant difference in carbohydrate quantities was recorded during the experiment in any treated group as compared to the controls. However, a significant decrease in lipid and protein quantities was observed on day 90 in groups exposed to 21 °C (exposed to cadmium or not), with a concomitant decrease in stickleback weight and size. Environmental temperature has major effects on fish physiology, and high temperatures accelerate metabolic rates (Wootton, 1984). The peculiarity of ectothermic animals lies in their ability to acclimate to different temperatures in their environment, with maximum and minimum tolerance limits (i.e. their tolerance range). When environmental temperatures are beyond the range of their tolerance limits, they can lead to physiological disturbances and decreased fitness. This situation is called “stress condition”. Bioenergetics plays a determining role in living organisms when it comes to stress tolerance (Klepsatel et al., 2016). A balance between energy input and expenditure is required to allow for a sufficient energy supply (aerobic scope of an organism) to cover the cost of basal maintenance as well as other functions (growth, reproduction) (Sokolova, 2013). Under stressful conditions, optimal energy allocation is affected: the aerobic scope is reduced, maintenance and survival take priority while growth and reproduction are stopped, and energy reserves are depleted (Klepsatel et al., 2016). In our study, glycogen levels did not differ between the different groups, while protein and lipid contents decreased after 90 days of exposure. Glycogen stores were not affected by cadmium exposure. This result was not surprising since other parameters (i.e. lipids, proteins) were not affected either, considering the very low cadmium concentration to which sticklebacks were exposed. However, in the “temperature” and “cadmium-temperature” groups, sticklebacks may have kept their glycogen stores for a possible episode of hypoxia in the case of a temperature increase. In some circumstances (e.g. warm vegetated or ice- and snow-covered waters), natural habitats of sticklebacks may become deoxygenated (Wootton, 1994). In addition, it is well known that oxygen consumption in sticklebacks, as in other fish species, rises along with

temperature (Wootton, 1994; Sollid and Nilsson, 2006). We hypothesised that sticklebacks were genetically programmed to survive in poorly oxygenated habitats. Hence, a rising temperature (21 °C) led to the onset of acclimation mechanisms to prepare sticklebacks to cope with a possible hypoxic period that did not happen because all experimental tanks were continuously oxygenated. As a consequence, glycogen stores were not affected, but protein and lipid reserves were used for gluconeogenesis. Hypoxic periods are characterized by the onset of adaptation mechanisms that allow certain fish species such as carp to be more hypoxia- or anoxia-tolerant (Sollid and Nilsson, 2006). In such conditions, besides gill remodelling to improve oxygen uptake from water, fish increase their liver glycogen reserves (Sollid and Nilsson, 2006; Ballantyne, 2013), and the activity of several gluconeogenic enzymes (De Boeck et al., 1997). Hence, in case of a long hypoxic or anoxic period, the longer the utilization of the glycogen store can be delayed, the greater the chances for fish to survive (Sollid and Nilsson, 2006).

#### 4.4. Effect on digestive enzymes

Unlike antioxidant and energy reserve parameters, digestive enzymes showed an interesting sensitivity to cadmium and its combination with temperature. Cadmium alone progressively decreased the activity of the three digestive enzymes significantly only on day 90. The digestive tract is one of the main routes of pollutant absorption, and several authors have reported inhibiting or stimulating effects of organic and inorganic contaminants on the digestive enzymes of aquatic organisms depending on the exposure route and/or the dose (Dedourge-Geffard et al., 2012; Filippov et al., 2013). In our study, the impact on digestive enzymes indicates that cadmium at 0.50 µg. L<sup>-1</sup> passed through or accumulated in the digestive tract. Zebrafish exposed to cadmium 5 µM in water accumulated more cadmium in the gut than in the liver (Vergauwen et al., 2013). Unfortunately, we have no data on cadmium bioaccumulation in the digestive tract to support this information.

Contrary to what most studies on digestive enzyme activities report in the literature, sticklebacks were chronically exposed to a very low cadmium concentration representative of the concentrations found in aquatic environments (IRSN, 2004). *In vivo* exposure of rosy barb fish (*Puntius conchonius*) to 48 to 181 µg. L<sup>-1</sup> of mercuric chloride decreased intestinal acid phosphatase (ACP) activity, but increased IAP and lactate dehydrogenase activities (Gill et al., 1990). When exposed to a sublethal concentration of cadmium (6.8 mg. L<sup>-1</sup>) for 30 days, fossil cat (*Heteropneustes fossilis*) showed inhibition of three intestinal enzymes, *i.e.* trypsin, IAP, and ACP, while pepsin activity increased in stomach (Sastry and Gupta, 1979). Moreover, amylase and protease activities were inhibited after 60 min of *in vitro* exposure to 5, 25, and



50 mg.L<sup>-1</sup> of cadmium, and carbohydrase activity was reduced after 60 days of *in vivo* exposure to 5 mg.L<sup>-1</sup> of cadmium in Mozambique tilapias (*Oreochromis mossambicus*) (Golovanova et al., 1994). The inhibitory effect of heavy metals on enzymes can operate through two mechanisms: (i) a direct effect, *i.e.* a modification of the spatial conformation of the enzyme through the interaction of the pollutant with essential elements such as Ca<sup>2+</sup> ions (in the case of cadmium for example), resulting in altered enzyme activity, and (ii) an indirect effect *via* interactions with synthesis and/or secretion mechanisms (Dedourge-Geffard et al., 2012).

Temperature also impacted *G. aculeatus* digestive enzymes (trypsin, IAP, amylase), and aggravated the effect of cadmium when the two stresses were combined. The effect of temperature (with or without cadmium exposure) was recorded earlier, *i.e.* from day 15, as compared to the single effect of cadmium. Fish are heavily influenced by environmental temperature because they are heterothermic animals. When we exposed sticklebacks to an elevated temperature (21 °C) in the absence of cadmium, trypsin and IAP activities decreased from day 15, while amylase activity remained stable throughout the experiment. These results are not consistent with the literature. Numerous authors have indeed investigated the effect of temperature on fish digestive enzymes. In general, when temperature increases, digestive enzyme activities increase too (Hofer, 1979a, 1979b; Kuz'mina et al., 2002; Bowyer et al., 2014).

Two hypotheses can be proposed to explain why the inhibiting effect of cadmium on digestive enzymes was promoted at 21 °C. The first hypothesis is that the elevated temperature was responsible for a high rate of cadmium accumulation in the gut (contrary to what was observed in liver), leading to enhanced effects of cadmium on digestive enzymes. The second hypothesis is that sticklebacks raised at 21°C produced new IAP, trypsin and amylase isoenzymes, as reported in other fish species as an acclimation mechanism to temperature (Gelman et al., 2008). These new isoenzymes were more vulnerable to the effects of cadmium than those produced at 16 °C.

Despite the very low cadmium concentration to which sticklebacks were exposed, and compared to classical biomarkers of metal exposure, *G. aculeatus* digestive enzymes responded rapidly. Therefore, they appear as an interesting early warning tool to reflect the health status of organisms and the chemical quality of the surrounding water. The highest sensitivity of digestive enzyme responses suggests that these measurements are relevant for assessing the effects of water quality on the digestive capacity of *G. aculeatus*. The interest of these enzymes as potential biomarkers of the health status of organisms and of the chemical quality of aquatic

ecosystems is particularly true for trypsin and IAP activities. These results are in agreement with Lemieux et al. (1999) and Rungruangsak-Torrissen et al. (2006), who reported that these enzymes are limiting factors for fish growth. However, further studies are necessary to more precisely characterise the potential effects of other contaminant families (e.g. PAHs, pesticides) and abiotic and biotic parameters on the above-described enzyme activities before using them as biomarkers because they can be confounding factors.

## 5. Conclusions

This study aimed to (i) evaluate the combined effects of 90 days of exposure to an environmental dose of cadmium and an elevated water temperature (21 °C) on threespine stickleback physiology, and (ii) to evaluate the interest of stickleback digestive enzymes (IAP, trypsin, amylase) as new biomarkers. Sticklebacks were exposed to a very low cadmium concentration (0.50 µg. L<sup>-1</sup>), which is below the freshwater chronic criterion (CCC) of 0.72 µg. L<sup>-1</sup> recommended by the US Environmental Protection Agency (EPA).

On the one hand, a temperature of 21 °C, which is cogent with a predicted global temperature rise of 1.8-4.0 °C, impacted *G. aculeatus* antioxidant parameters and protein and lipid energy reserves after a long exposure period. In the same way, stickleback growth was limited under the effect of the high temperature, and reproduction also seemed to be impacted. This could have rather serious consequences on stickleback populations in a long-term climate warming context. These results suggest that 21 °C is an upper tolerance limit for long-term physiology processes in threespine sticklebacks.

On the other hand, cadmium alone at the very low concentration of 0.50 µg. L<sup>-1</sup> had no effect on any parameters, except digestive enzymes that were negatively impacted after 90 days of exposure. This effect on digestive enzymes was aggravated and recorded earlier under the higher temperature. Compared to conventional measurements of the effects of metals on fish physiology (*i.e.* biometric parameters, antioxidant biomarkers, energy reserves), *G. aculeatus* digestive enzymes responded rapidly and therefore appear as interesting early warning tools for reflecting the health status of organisms.

Reproduction is one of the most important biological functions in living organisms. It would therefore be of great interest to investigate the relationship between early impairment of *G. aculeatus* digestive enzyme activities and the reproductive function.

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