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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

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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 ($0.5\,\mu g\,L^{-1}$) 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 gonado-somatic 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.

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

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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 sublethal 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 $1\,\mu g\,L^{-1}$, it can reach $400\,\mu g\,L^{-1}$ 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 $1\,\mu g\,L^{-1}$) in combination with two different water temperatures (16 °C and 21 °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-l 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 12 h/12 h light/dark cycle, and fed on a constant feeding regime (frozen commercial chironomid larvae, 3% of the fish body weight/day,