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Investigating heritability of cadmium tolerance in *Chironomus riparius* natural populations: A physiological approach

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HIGHLIGHTS

- Physiological responses to cadmium were assessed in midge populations with heritably different Cd-tolerance.
- Protein profiles varied with Cd exposure and Cd-tolerance of populations.
- Cd-tolerant populations had higher baseline levels of metallothioneins and glutathione and higher energy consumption with Cd.
- Cd exposure increased glutathione and lipid peroxidation in Cd-sensitive populations.
- Cd-tolerance is related with different constitutive levels and plasticity of defence mechanisms.

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ABSTRACT

Physiological responses allow populations to cope with metal contamination and can be involved in the evolution of tolerance under historical metal contamination scenarios. Here we investigate physiological aspects that might be underlying the heritable high tolerance to cadmium (Cd) in two *Chironomus riparius* populations collected from historically metal contaminated sites in comparison to two populations from reference sites.

To evaluate differences in the physiological response to short-term Cd exposure, protein expression profiles, metallothioneins [MTs] and several antioxidant defences such as total glutathione (GSH_t), catalase (CAT) and glutathione-*S*-transferases [GSTs], were measured in all four populations reared for at least 8 generations under laboratory clean conditions. Cd-induced oxidative damage in lipids and energy related parameters (energy consumption and energy reserves) were also assessed.

Results showed two major gradients of protein profiles according to Cd concentration and population tolerance. Furthermore, Cd-tolerant populations showed higher baseline levels of MTs and GSH_t while Cd-sensitive populations, collected from reference sites, showed significant induction of GSH_t levels with Cd exposure that were nonetheless insufficient to avoid increased oxidative damage to lipids. Cd exposure had no clear effects on the antioxidant enzymes or energy reserves but triggered a general increase in energy consumption. Finally, energy consumption was higher in Cd-tolerant populations across experimental conditions.

Altogether, results demonstrate that inherited Cd-tolerance in these midge populations is related, at least in part, with different constitutive levels and plasticity of different defence mechanisms confirming the validity of using multiple physiological traits when studying evolution of tolerance.

1. Introduction

Plasticity in organisms' physiology together with genetic adaptation can play an important role in how natural populations can cope with environmental change (Bonduriansky and Day, 2008; Gienapp et al., 2008; Day and Bonduriansky, 2011; Schlichting

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and Wund, 2014; Stoks et al., 2014). This is not only because stress-induced physiological responses can facilitate short-term persistence of populations (i.e. before genetic adaptation can occur) (Pigliucci, 2005; Latta et al., 2007; Pestana et al., 2016), but also because variation in the direction and magnitude of the plastic responses is indicative of adaptive evolution to environmental stress, including chemical contamination (Sturmbauer et al., 1999; Haap et al., 2016; Oexle et al., 2016).

Metal contamination is widespread in many ecosystems and some populations are able to reduce their adverse effects through a suite of biochemical and physiological adjustments that allow them to live under such harsh conditions (Janssens et al., 2009; Sandbichler and Höckner, 2016). As such, evolution of tolerance and genetic adaptation to metals has been demonstrated in invertebrate populations under historical metal contamination (Agra et al., 2011; Ribeiro et al., 2012).

Yet, studies demonstrating the evolution of physiological responses to metals or altered patterns of the induced responses as the outcome of microevolution are rare. This is somewhat surprising given the considerable variation in the magnitude, direction and number of metal-induced physiological traits that have been demonstrated in a variety of organisms (Leiniö and Lehtonen, 2005; Xie et al., 2008). Moreover, variation in these responses has been also observed for conspecific populations (Gillis et al., 2014; Pain-Devin et al., 2014) highlighting the potential evolutionary importance of these physiological plastic traits. Nevertheless, it is always difficult to conclusively distinguish whether responses observed in different populations are genetically determined or are the result of plasticity in physiological traits and thus to disentangle the relative contribution of genetic adaptation and of phenotypic plasticity in the evolution of tolerance (Ghalambor et al., 2007; Merilä and Hendry, 2014).

In the present study we investigate whether evolution of cadmium (Cd) tolerance in the non-biting midge *Chironomus riparius* (Meigen, 1804) is associated to different physiological defence mechanisms that are usually triggered by metal exposure. *C. riparius* is a model species in ecotoxicology and several physiological responses have been previously demonstrated to confer tolerance to metals including Cd.

Firstly, it is well established that metallothioneins (MTs) are specifically implied in the cellular protection against metal toxicity and consequent oxidative stress (Kumari et al., 1998; Klaassen et al., 1999; Sandbichler and Höckner, 2016) and, thus, upregulation of the levels of MTs has been postulated to constitute adaptive responses of natural populations to metal exposure in aquatic environments (Gillis et al., 2014; Weng and Wang, 2014). These low molecular weight cysteine-rich proteins play a key role in the regulation of the homeostasis of essential metals and in the detoxification of non-essential metals such as Cd (Amiard et al., 2006; Janssens et al., 2009; Isani and Carpenè, 2014; Sandbichler and Höckner, 2016). Although other proteins such as heat shock proteins and phytochelatins have been implied in the physiological responses to metals in invertebrates (Planelló et al., 2010; Gonçalves et al., 2016; Sandbichler and Höckner, 2016), upregulation of the levels of MTs has been shown to be induced by Cd exposure in *C. riparius* (Fabrik et al., 2008; Toušová et al., 2016).

It has been also shown that metals, and Cd in particular, can induce oxidative stress in aquatic invertebrates (Valavanidis et al., 2006; Connon et al., 2008; Lushchak, 2011; Chandurvelan et al., 2013). In an attempt to protect the cellular integrity of organisms, several enzymatic and non-enzymatic antioxidant cellular defences are upregulated to cope with the increase of reactive oxygen species (ROS) caused by metal exposure (Valavanidis et al., 2006; Lushchak, 2011; Sandbichler and Höckner, 2016). Among these, relationships have been established between metal stress and the content of reduced glutathione (GSH) or the enzymatic activities of glutathione-*S*-transferases (GSTs) and catalase (CAT) (Barata et al., 2005; Gravato et al., 2006). GSH, the most abundant cellular thiol, is a radical scavenger that is used as a first line of defence to prevent the interactions of metals with main cellular structures. GSTs are a large family of enzymes that mostly catalyse the conjugation of various toxic compounds with the thiol group of GSH for subsequent elimination as less toxic and more water soluble and extractable compounds. Finally, CAT is an antioxidant enzyme that degrades hydrogen peroxide into water and oxygen (Valavanidis et al., 2006; Lushchak, 2011). Nonetheless, the excessive production of ROS caused by metal exposure may overwhelm defence responses and disrupt the cellular redox balance, causing oxidative damage to susceptible biological macromolecules including lipid, protein and DNA oxidation that can trigger serious cellular injuries (Livingstone, 2001; Bertin and Averbeck, 2006). Due to this, variations in the levels of lipid peroxidation (LPO) have been extensively used as indicators of the inability of antioxidant defence systems to adequately protect membrane integrity from free radical attacks (Boudet et al., 2013; Wu et al., 2013; Gillis et al., 2014).

The activation of cellular defence mechanisms, however, is a highly energy-demanding process and thus enhanced defence systems that improve the ability to cope with chemical stress are expected to have high metabolic requirements (Sibly and Calow, 1989; Sokolova et al., 2012). For this reason, additional analysis of the patterns of energy consumption and energy available for metabolism may provide important clues to better understand and predict population-level consequences of chemical stress (De Coen and Janssen, 2003; Rodrigues et al., 2015a; Campos et al., 2016) given the critical role of energy budget in stress adaptation and tolerance (Sibly and Calow, 1989).

The main goal of the present study was to demonstrate if these physiological responses were related to tolerance. For that, we used two *C. riparius* populations collected from historically metal-contaminated sites and for which higher tolerance towards acute Cd exposure was observed in comparison to two *C. riparius* populations collected from reference sites (Pedrosa et al. submitted for publication). We hypothesize that heritable differences in the physiological defence mechanisms against Cd exposure between Cd-tolerant and Cd-sensitive populations have to exist. Because all populations were cultured for several generations under common garden laboratory clean conditions before these ecotoxicological tests (i.e. before acute tests and the measurement of physiological responses to Cd exposure) we can exclude major acclimation or carry-over effects and assure that responses were the result of microevolution (Uusi-Heikkilä et al., 2015). By investigating the effects of short-term Cd exposure on protein expression profiles, levels of MTs, biomarkers of oxidative stress and energy related parameters, it is expected to shed light on the physiological processes mediating the differential Cd tolerance among these *C. riparius* natural populations.

2. Material and methods

2.1. Source populations

The four *C. riparius* populations used in all our experiments were collected from the Ave and Vouga river basins, Northern of Portugal. Population CEL was collected from a metal-impacted site located in the village of Celeirós, in the Este River, downstream the city of Braga. The river received direct urban and industrial discharges, without any previous treatment, during decades which resulted in the accumulation of great amounts of metals including Cd that can reach concentrations of 5.96–144.00 mg/kg in the local sediments (Soares et al., 1999). Population EST was collected from S.