Comparative Biochemistry and Physiology, Part A xxx (2016) xxx–xxx

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part A

journal homepage: www.elsevier.com/locate/cbpa

Development of functional trait biomarkers for trace metal exposure in freshwater clams (*Musculium* spp.)☆

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ARTICLE INFO

Article history:
Received 23 November 2015
Received in revised form 27 March 2016
Accepted 9 April 2016
Available online xxxx

Keywords:
Biomarker
Bivalve
Locomotion
Pollution
Reproduction
Metal

ABSTRACT

Exposure to trace metals typically causes oxidative stress; these consequences are better-characterized in estuarine and marine species than in freshwater species. How cellular-level responses to metal pollution influence whole-organism and population-level traits is poorly understood. We tested whether exposure to single metals (zinc and cadmium) and to metal mixtures (water in equilibrium with sediment from a highly polluted lake) alters two ecologically-relevant traits in freshwater clams, locomotion and reproduction. Fingernail clams (*Musculium* spp.) from unimpacted habitats were exposed to single metals and the metal mixture for up to 49 days. The single metal doses (≤5 mg/L Zn and ≤20 μg/L Cd) were not toxicologically meaningful as clam survival, burial, and climbing activity did not differ across treatments. Water in equilibrium with the lake sediment contained cadmium, copper, lead, and zinc. Clams exposed to this metal mixture had decreased climbing activity but no change in burial activity. Metal-exposed clams had lower fecundity (number of shelled juveniles extruded by adult clams) and patterns in metal accumulation corresponded with lake sediment dose and clam activity. In contrast to the functional traits, stress protein expression and whole-clam glycogen content did not vary across treatment groups. These results indicate that fingernail clams of the genus *Musculium* are appropriate for development as sentinel species for metal pollution and can serve as a model for determining how metal pollution alters metabolic allocation patterns in freshwater organisms.

1. Introduction

Trace metal pollution is a worldwide problem that arises from mining and smelting activities, atmospheric deposition, industrial effluent, sewage outfalls, and oil and chemical spills (Bryan, 1971; Pacyna and Pacyna, 2001; Callender, 2003). Metals accumulate in aquatic habitats, particularly in sediments (e.g., Bryan and Langston, 1992). Metal movement through habitats and consequences for algae, macrophytes and animals are best characterized in coastal habitats (Anderson, 1977; Collier et al., 2013). Metal accumulation patterns and biomarkers of damage and defense are routinely monitored in coastal crustaceans and molluscs, especially those that are aquacultured (for review, Bryan, 1971; Luoma, 1983; Boening, 1999; Rainbow, 2002; Amiard-Triquet et al., 2013) and have been described in freshwater crayfish (Kouba et al., 2010), mussels (e.g., Johns, 2012) and clams (e.g., Doherty, 1990; Andrès et al., 1999; Gunther et al., 1999).

Bivalves are useful models for understanding the consequences of metal pollution at the cellular, organismal, and population levels (Boening, 1999; Berthet, 2013). Most bivalves are sedentary and will interact with the sediment and water column. Many species are widely distributed, allowing comparisons of pollution across broad geographic ranges. Bivalves have large water filtration capacities (for review, Vaughn and Hakenkamp, 2001), readily accumulate metals in their tissues (e.g., Wallace and Luoma, 2003; Johns, 2012) and are an important route for trophic transfer from algae to fish, waterfowl and humans (e.g., Rainbow et al., 2007; Cui et al., 2011).

The consequences of metal exposure in bivalves fall into several general categories. Given the pro-oxidant nature of metals (Regoli, 2012), bivalves exposed to metals may exhibit lipid peroxidation (Franco et al., 2006), altered antioxidant expression and/or activity (Li et al., 2012; Borković-Mitić et al., 2013), and DNA damage (Black et al., 1996). Additional consequences include alterations in lysosome stability (Domouhtsidou et al., 2004), mitochondrial function (Ivanina et al., 2011) and ion transport (Rocha and Souza, 2012). Biomarkers of defense include metallothioneins and stress proteins (Amiard-Triquet et al., 2013). However, even with sources of variation (e.g., seasonality) taken into account, cellular-level responses are not consistent across studies. Recent reviews have highlighted this issue and the importance of

☆ This is a submission from the Second International Conference on Oxidative Stress in Aquatic Habitats, which was held in La Paz, BCS, from November 11–14, 2015.

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http://dx.doi.org/10.1016/j.cbpa.2016.04.012
1095-6433/© 2016 Published by Elsevier Inc.

integrating across levels of biological organization (Amiard-Triquet and Amiard, 2013; Amiard-Triquet et al., 2013; Roméo and Giambérini, 2013). Those reviews, and others (Mouneyrac et al., 2013; Ivanina and Sokolova, 2015), emphasize ecologically-relevant traits like metabolism, performance and reproduction.

Many studies of metal pollution have subjected the bivalves to acute (≤96 h) exposures (but see Black et al., 1996; Ivanina et al., 2011) or have assessed cellular-level and functional traits in field caging assays or in populations from polluted habitats (Bodin et al., 2004; Perceval et al., 2004; Tlili et al., 2013). Here we took a different approach by conducting laboratory assays of 42–49 days in which we measured performance traits, reproduction and survival weekly. We measured stress protein expression and glycogen content only at assay midpoints or endpoints to test for sustained changes in protein expression or use of energy reserves. This array of metrics encompassed most of the categories into which metabolic resources are allocated (Sokolova et al., 2012a). This design allowed us to explore the consequences of chronic metal exposure on traits that integrate multiple physiological systems and that likely influence population persistence (locomotion, reproduction, survival).

We worked with freshwater “fingernail” clams of the genus *Musculium* (Corbiculacea; Mackie, 2007). While their utility for biomonitoring was acknowledged more than 50 years ago (Ingram et al., 1953), clams in the genera *Musculium*, *Sphaerium* and *Pisidium* are rarely used as sentinel species. This is surprising given their abundance in all freshwater habitat types (>3000 per square meter, Mackie, 2007) and their close evolutionary relationship to a widely-studied model (*Corbicula*). Fingernail clams are hermaphroditic and exhibit facultative vivipary, internally brooding shelled larvae and releasing juveniles (Mackie, 1978a). Calculating fecundity is straightforward because many stages of larvae and juveniles are visible at low magnification or to the naked eye. Brood size varies with water temperature and organic pollution (Mackie, 1978b), aquaculture waste (Kullman et al., 2007), and dissolved O_2 (Joyner-Matos et al., 2011).

Clams were exposed to dissolved metals (zinc or cadmium) or to sediment from a lake that is associated with the Bunker Hill Superfund site in northern Idaho (Supplemental Fig. 2). A century of mining in the Coeur d'Alene (CDA) region of Idaho deposited >110 million tons of tailings into the CDA drainage (LeJeune et al., 2000), some of which has accumulated in “chain lakes” in the CDA River floodplain (Sprenke et al., 2000). Clams are sparsely distributed in the CDA drainage but are abundant in a neighboring water body that is used as a reference site (e.g., Shea et al., 2012). Doses in the single metal assays were representative of the CDA drainage and “multi-metal” assays used sediment from a heavily impacted chain lake. To evaluate performance, we measured clam burial. As these clams are found attached to vegetation in the water column (unlike *Corbicula*, which lives at the sediment/water interface), we created an assay for clam climbing behavior. In select assays we measured antioxidant and stress protein expression and glycogen content. Finally, in assays in which clam survival or performance differed across treatments, we measured whole-body metal concentrations and two aspects of reproduction, the number of extruded juveniles and the number of brooded larvae.

2. Materials and methods

2.1. Collection of clams

Clams were collected from Turnbull National Wildlife Refuge (TNWR), which is unimpacted by metal pollution (avoids the possibility of using clams that are tolerant of metals Sloss et al., 1998; Phillips and Hickey, 2010). Clams were collected from two sites on the refuge. Long Lake, a large permanent wetland, is located at 47° 25′ north latitude and 177° 33′ west longitude. Turnbull Laboratory for Ecological Studies (TLES) pond, a permanent to semi-permanent wetland, is located at 47° 25′ north latitude and 177° 33′ west longitude. Our collections included two species, *Musculium lacustre* and *Musculium securis* (T. Lee, pers. Comm.), which often co-occur and can be difficult to distinguish (Mackie, 2007).

Clams were collected with a 1 mm mesh D-net (40 cm diameter) that was held vertically, lowered until it touched the sediment and then gently moved up and down over a short distance, agitating the vegetation on which the clams are found. The net was rinsed and clams were separated from the debris through the use of a 500 μm sieve. Clam shell length (adductor–adductor) was measured as an indicator of reproductive status (Kilgour and Mackie, 1990). Adult clams (≥5 mm length) were placed in 1-l collection vessels filled with lake water and kept cool. Clams were acclimated with feeding at 15 ± 1 °C for 3 days. In the 2012 assays the clams were fed an equal mix of *Chlorella* and *Chlamydomonas* that were cultured in Bolds Basal media (Carolina Biological Supply). In the 2014 assays the clams were fed only *Chlorella*. While no clam mortality was detected during the acclimation, many juveniles were extruded during every acclimation period.

2.2. Assay design

Assays were conducted in 2 L glass jars which were placed in random order into a Jewel Oceanic 55 gal aquarium or a 100 gal Rubbermaid stock tank that was cooled with a Julabi chiller. Both systems were maintained at 15 ± 1 °C. The jars were continuously aerated and they were kept in a room that had a 12 h light/12 h dark cycle. Five replicate jars were run for each treatment. Each jar contained either five (2012) or seven (2014) clams that were housed individually in cages constructed from 7.6 cm of PVC pipe (1.9 cm inner diameter, thin wall). In the single metal assays and the first sediment assay, the cages had 1 mm nylon mesh secured over each end. In the remaining assays, we used a 0.5 mm nylon mesh. The cages were placed on end in the jars; clams could not bury in the sediment. The cages were randomly numbered and clams were handled in random numerical order and blind to treatment ID in all measurements. At the start of each assay, the clam shell length was measured to ensure that clams of all size classes were present in each treatment. Clams were fed daily.

In the single metal assays (2012), the jars were filled with 1500 mL of dechlorinated water and were dosed with stock solutions of either zinc sulfate (heptahydrate) or cadmium sulfate (anhydrous) that were made with nanopure water. The jar water was replaced halfway through each assay. In the Low Zinc assay (started 7/13/2012, 45-day assay), doses ranged from 0 to 1000 μg/L Zn. In the High Zinc assay (8/28/2012, 42-day assay), doses ranged from 0 to 5000 μg/L Zn. In the Cadmium assay (8/16/2012, 45-day assay), doses ranged from 0 to 20 μg/L Cd. Metal concentrations in the Low Zinc and Cadmium assays were consistent with dissolved metal concentrations in water bodies in the CDA drainage (Sprenke et al., 2000; USGS, 2011). Surface water pH in the chain lakes in the CDA drainage ranges from 6.8–7.4 and the interstitial water pH ranges from 6.5–7.5 (Sprenke et al., 2000; Balistrieri et al., 2003); the dechlorinated water used in our assays was pH 7.6. In the High Zinc and Cadmium assays, dissolved O_2 levels were 6.89 ± 0.2 mg/L in all jars.

In the sediment assays, clams were exposed to sediment that was collected from Killarney Lake, Idaho (47° 31′ north latitude and 116° 33′ west longitude), which is connected to the CDA River via a dredged channel and is used as a settling pond to collect contaminated sediment (Sprenke et al., 2000). The sediment from Killarney Lake typically has some of the highest metal concentrations in the drainage, with ranges of 0.02–146 mg/kg Cd, 48–12,800 mg/kg Pb, and 100–34,150 mg/kg Zn (LeJeune et al., 2000). In 2012 and 2014 we collected sediment from the same site using an Ekman dredge (water depth of 4.5 m). The sediment was autoclaved before being mixed with autoclaved uncontaminated sand (playground sand) to achieve mixtures of 0%, 12.5%, 25%, 50% and 100% sediment. The 2 L (exposure) jars contained 1350 ml of dechlorinated water and 350 ml of the sediment/sand mix; the jars were allowed 24–48 h for settling. The jar water was replaced

Please cite this article as: Schoonover, C.M., et al., Development of functional trait biomarkers for trace metal exposure in freshwater clams (*Musculium* spp.), Comp. Biochem. Physiol., A (2016), http://dx.doi.org/10.1016/j.cbpa.2016.04.012