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Secondary vitellogenesis persists despite disrupted fecundity in amphipods maintained on metal-contaminated sediment: X-ray fluorescence assessment of oocyte metal content

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ABSTRACT

Melita plumulosa is an epibenthic, detritivorous amphipod found in estuaries along the eastern coast of Australia. It has been utilized as a test organism in rapid ten to thirteen days reproduction toxicity tests for sediment quality assessment. The fecundity of females in the toxicity test has been found to be inhibited by exposure of the amphipods to contaminated sediments enriched with zinc and other metals. This study investigated the proposal that interference in vitellogenesis is the cause of reproductive toxicity of metals in crustaceans. Inspection of the ovaries from amphipods on day 6 of the test either from control or Zn/Pb/Cd/Cu-spiked sediment, that were nearing completion of vitellogenesis, showed that the females in all treatments were producing similar numbers of oocytes undergoing secondary vitellogenesis. The distribution of the Zn, Cu and Pb in the oocytes and ventral caeca of females was examined by X-ray fluorescence microscopy. Elemental mapping revealed a dense accumulation of Zn in primary oocytes and a uniform distribution of Zn and Cu in the secondary oocytes in all treatments. Zn and Cu were also observed to be uniformly distributed in the ventral caeca. Pb was not detected in either of these tissues. The apparent normal morphology and the typical number of oocytes undergoing secondary vitellogenesis suggest that vitellogenesis was not being disrupted by Pb displacing Zn in the metal-binding domain of vitellogenin in amphipods exposed to the contaminated sediment during the test. Alternative mechanisms for the reproductive toxicity of amphipods exposed for six days to metal-contaminated sediment are discussed.

1. Introduction

Melita plumulosa is an epibenthic, detritivorous amphipod found in estuaries along the eastern coast of Australia. This species has been demonstrated to be more sensitive to metal-contaminated sediments than other endemic amphipods from Australia and New Zealand (King et al., 2006), can be cultured under laboratory conditions (Hyne et al., 2005), and has been effectively employed as a test organism in acute and chronic toxicity tests (Gale et al., 2006; Mann et al., 2009; Simpson and Spadaro, 2011). Exposure of *M. plumulosa* to metal-spiked sediments in a 13-day reproductive toxicity test resulted in a significant reduction in fecundity. It was also demonstrated that a mixture of metals (40:5:3:1 for Zn, Pb, Cu, Cd on a molar basis) at concentrations approaching the sediment quality guideline trigger values (ANZECC/ARMCANZ, 2000) rather than individual metals caused the reduced fecundity (Mann et al., 2009). When *M. plumulosa* were exposed in the laboratory to sediment obtained from the field that was contaminated with these metals, the amphipods accumulated significant amounts of Zn, Pb, Cu and Cd after 42 days (Gale et al., 2006; see also Supplementary data). However, the tissue distribution of the metals following exposure of *M. plumulosa* to metal-contaminated sediment is unknown. Previous studies in other gammarid amphipods have indicated that Zn and Cu are distributed primarily in the ventral caeca (also referred to as hepatopancreatic caeca) (Icely and Nott, 1980; Nassiri et al., 2000). Zn and Cu remained approximately equally distributed between cytosolic and insoluble fractions in the amphipods collected from either clean or contaminated sites and experimentally exposed to the metals with a small percentage loosely adsorbed onto the exoskeleton (Mouneyrac et al., 2002). The ventral caeca of the amphipod *Orchestia gammarellus* also was found to store a significant proportion of accumulated cadmium, with the majority of the accumulated cadmium present in the

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cytosol, probably bound to metallothioneins (Nassiri et al., 2000). In a study of the distribution of trace metals amongst various body tissues of the freshwater amphipod *Gammarus fasciatus*, Pb was more concentrated in the exoskeleton than in any other body parts. However, Zn and Pb were also present in relatively high concentrations in the eggs and ovaries of *G. fasciatus* (Amyot et al., 1996).

Interference with vitellogenesis has been proposed as a mechanism for reproductive toxicity of metals in crustaceans (Hook and Fisher, 2001). Vitellogenin and lipovitellin 1 (the latter being a yolk protein derived from the N-terminal moiety of vitellogenin) have been shown to be Zn- and Zn/Cd-binding proteins, respectively, in the oocytes of the frog *Xenopus laevis* (Montorzi et al., 1994, Sunderman et al., 1995). Displacement of zinc in the metal-binding domain of these proteins by Pb and/or Cd is a possible mechanism causing the reproductive toxicity of these metals to *M. plumulosa*. Adipose tissue (or body fat) has been shown to be the site of vitellogenin synthesis in the amphipod *O. gammarella*, and it releases vitellogenin into the hemolymph only when the ovaries are in secondary vitellogenesis (Meusy et al., 1983). Vitellogenin is transported by the hemolymph to the ovary where it is accumulated in the form of yolk protein (vitellin) in oocytes undergoing secondary vitellogenesis to form the mature egg cells or ova (Charniaux-Cotton, 1985). A prominent feature of amphipod oocytes undergoing secondary vitellogenesis is the uptake by endocytosis of vitellogenin which gives the oocytes a bright color due to the presence of carotenoids associated with vitellin (Zerbib, 1980).

The ovary of mature female amphipods is a paired organ with an elongated flattened tubular shape that lies dorsolaterally to the mid-gut (Ford et al., 2005). It possesses a thin germarium zone at the dorsal part of the ovary next to the basal lamina of the ovarian membrane. Some oogonia cells in the germination zone that develop by mitosis leave this zone and undergo meiotic prophase and differentiate into primary oocytes (Zerbib, 1980). During the reproductive season, or at the beginning of a new intermolt period of a continuously breeding amphipod, a cohort of primary oocytes synchronously go into secondary vitellogenesis, and start to grow at the same rate to form a linear row of mature ova that ovulate at the end of the molt cycle (Hyne, 2011).

X-ray fluorescence microscopy (XFM) imaging allows the determination of the distribution and relative concentration of trace elements within biological samples. The technique directly detects the emitted characteristic X-rays of the sample's elements without the need for radioactive labeling or fluorescent tagging (Dillon, 2012). This technique facilitates determination of the distribution of elements in tissues, while still maintaining cellular integrity. It has been employed in numerous studies of the cellular distribution of exogenous metals (Bertsch and Hunter, 2001; Dillon et al., 2002; Munro et al., 2008; Dillon, 2012).

In the present study, the ovaries together with the ventral caeca of female amphipods were isolated following their maintenance on metal-spiked sediment for the first molt cycle of the reproductive toxicity test to assess whether secondary vitellogenesis was occurring. The distribution of the metals, Zn, Cu and Pb, in the isolated ovarian and ventral cecal tissue was then examined using XFM imaging.

2. Materials and methods

2.1. Test organism

Cultures of *M. plumulosa* were established with animals collected from the northern shores of the Hawkesbury River near Brooklyn (33°32′3″S, 151°11′51″E) NSW, Australia (salinity, 27.0–33.8‰). The cultures were maintained under the conditions recommended by Hyne et al. (2005) on silty sediments collected from Bonnet Bay, in the lower Woronora River, Sydney, Australia, (34°0′24″S, 151°3′27″E).

2.2. Control sediment and metal-spiked sediment

The properties and contaminant concentrations in the control sediment from Bonnet Bay have been well characterized (Simpson et al., 2004) and had previously been demonstrated to support high fecundity in *M. plumulosa* (Mann and Hyne, 2008; Mann et al., 2009, 2010). The sediment was collected from the surface layer (top 2–4 cm) using a hand shovel, press-sieved through 1.1 mm mesh to remove large debris and stored in the dark in plastic bags at 4 °C for a maximum of two months. The metal-spiked sediment was prepared with sediment collected from Durras Lake, a saline coastal lagoon 275 km south of Sydney (35°39′18″S, 150°16′33″E). The sediment was spiked with a mixture of Zn/Pb/Cd/Cu (500/250/20/30 mg/kg) as described in detail previously (Mann et al., 2009) according to the procedures outlined in Simpson et al. (2004).

2.3. Test water

Seawater was collected from Port Hacking (NSW, Australia) and stored in a fiberglass tank outside the laboratory until required. The seawater was filtered through a series of three filters (Stefani Australasia, Welshpool, Australia) consisting of a 5 μm particle cartridge, a 2 μm dual phase particle/organic adsorption cartridge and a 0.5 μm ion-exchange cartridge to maintain low Zn concentrations. The salinity was adjusted to 25‰ using Sydney tap-water that had been passed through a mixed bed filter and activated carbon filter, after which it was allowed to stand for six weeks for dechlorination. The water used for culturing, toxicity and exposure tests was stored in a plastic tank in the laboratory to allow temperature acclimation.

2.4. Analytical chemistry

All glass and plastic-ware for analyses were usually new and were cleaned by soaking in ten percent (v/v) HNO_3 (BDH, Analytical Reagent grade) for a minimum of 24 h, followed by thorough rinsing with deionized water (Milli-Q, Millipore). All chemicals were analytical reagent grade or equivalent analytical purity. Measurement of pH, salinity, temperature and dissolved oxygen were made with electrodes from WTW (Weilheim, Germany), each calibrated according to manufacturer instructions. The method for porewater extraction (centrifugation at 800g for 5 min) has been described previously (Mann et al., 2009). The porewater and overlying water (OW) samples were membrane filtered (0.45 μm) immediately following collection and acidified with concentrated HNO_3 (two percent HNO_3 (v/v), Tracepure, Merck). Dissolved metal concentrations in digested sediments were determined by inductively coupled plasma atomic emission spectrometry (ICP-AES, Spectroflame EOP, Spectro Analytical Instruments) calibrated with matrix-matched standards (QCD Analysts). Dissolved metal concentrations in seawater samples were determined by inductively coupled plasma mass spectrometry (ICP-MS). The limit of reporting (LOR) for the metals in seawater was 1 μg/l. As a check on analytical quality, a blank seawater sample fortified with the metals of interest was analyzed and recoveries for Cd, Cu, Pb and Zn of 99–104 percent were obtained.

2.5. Pretest amphipod preparations

At 24 °C, embryos are retained within the marsupium for seven days before hatching and release (Mann and Hyne, 2008). For the reproduction test, male and female amphipods were segregated for seven days to allow females to release pre-existing broods as described previously (Mann et al., 2009). All amphipods destined for testing were kept on Bonnet Bay sediment at 24 ± 1 °C with a normal feeding regime (twice per week) with Sera® micron (Sera, Heinsberg, Germany), the final feed occurring one day before commencing the test.

2.6. Synchronous ovarian cycle of reproduction toxicity test

A reproductive test was performed essentially as described in detail previously in Mann et al. (2009), except the overlying water was renewed every third day. Five gravid females and seven males were added to each of the five replicate 250 ml beakers containing either 20 g of the control Bonnet Bay sediment or the Zn/Pb/Cd/Cu spiked sediment with 200 ml of overlying 25‰ seawater. One of these replicates was dedicated to the collection of water samples and for physicochemical analyses. The beakers were covered with cling-wrap plastic to reduce evaporation and placed in a temperature controlled room at 24 °C with a 12/12 h light/dark cycle. Gentle aeration was supplied to each beaker through a Pasteur pipette connected to an aquarium air-pump. While embryos carried by the gravid females were undergoing development in the external marsupial pouch during the test, maturation of the primary oocytes was occurring concurrently within their ovaries in preparation for the next spawning during day seven of the reproduction test. At day six of the