Sensitivity of the Early Development of Ramshorn Snail, *Marisa cornuarietis* (Ampullariidae) to Environmental Chemicals

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Zusammenfassung

In dieser Studie wurde die Eignung des *Marisa cornuarietis* Embryo-Toxizität-Tests (MariETT) für Umweltchemikalien untersucht. Das Ziel der vorliegenden Untersuchungen war es, (1) die Sensitivität des 2006 eingeführten MariETT Assay unter Verwendung ausgewählter Metalle und Pestizide zu bestimmen und diese sowie die Praktikabilität des Tests mit anderen etablierten Biotests zu vergleichen. Folgende Endpunkte wurden untersucht: Die Mortalität, die Ausbildung der Augen und der Fühler, die Herzfrequenz, die Schlupfrate, der Schlupfzeitpunkt, und das Gewicht nach dem Schlupf (Kapitel 1-4). (2) Des weiteren wurde ermittelt, inwiefern histopathologische Änderungen in Organen (Mitteldarmdrüse, Kiemen, Mantelepithel, Epidermis) juveniler *M. cornuarietis* aus subletaler Exposition gegenüber Metallen resultieren (Kapitel 5).

Die Ergebnisse zeigten, dass die embryonale Entwicklung von *M. cornuarietis* sehr empfindlich auf Pestizide und Metalle reagiert. Ein Vergleich von für *M. cornuarietis* ermittelten LOEC Daten mit den entsprechenden Literaturangaben für Zebrabärbling-Embryotoxizitätstests und andere Bioassays mit Embryonen verdeutlicht die hohe Sensitivität des MariETT Assays. Darüber hinaus machen die Ergebnisse der vorliegenden Studie deutlich, dass bei *M. cornuarietis* histopathologische Veränderungen in allen untersuchten Geweben nach Exposition gegenüber Kupfer- und Lithium-Ionen auftreten.

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Summary

1. Research Topic

"Sensitivity of the early development of ramshorn snail, *Marisa cornuarietis* (Ampullariidae) to environmental chemicals".

2. Introduction

2.1 Background of the study

In recent years**,** environmental contamination resulting from industrial use and production of chemicals and their release into the environment, e.g. for agricultural purposes, is one of the main predicaments of the modern world. It is a worldwide problem that needs urgent attention and prevention. Many synthetic chemicals are now in use and most of them eventually reach the aquatic environment. The widespread use of chemicals in urban and rural environments poses a risk of contamination to aquatic ecosystems via various pathways. Aquatic ecosystems are particularly exposed to contaminants that enter the waterway from various sources, and contaminate different compartments of these ecosystems (Lindgaard-Jorgensen and Bender, 1994). The discharge into surface water is also known to be an important mode of entry of pollutants into the environment. Apart from direct discharge, pollutants are sometimes dumped into surface water at considerable distances from the premises where they are produced (Walker et al., 1996). Because of their aquatic distribution, chemicals can affect a wide range of non-target organisms, like aquatic invertebrates and fishes (Faust et al., 1993; Burkepile *et al*., 2000). Whenever non-target organisms are affected, a disruption of the food chain, a modification the food web, or an imbalance in the entire ecosystem, may result from this primary effect (Forget et al., 1998).

The increasing degradation of the aquatic environment by anthropogenic contaminants has been the motivation behind intensive efforts to evaluate the effects of pollutants in numerous biological systems (Herkovits et al., 1997). Attempts to monitor environmental pollution can have one of the two principal objectives - to qualify the distribution of a

contaminant or to measure its impact on the biota of the polluted habitats (Beeby and Richmond, 2002). Various investigations have revealed the presence of a considerable number of chemical pollutants in the water of agricultural and industrial regions (Liszewski and Mann, 1993; Gundacker, 1999; Hamilton et al., 2000; Calamari and Zhang, 2002).

Ecotoxicology has been defined as "the branch of toxicology concerned with the study of toxic effects, caused by natural or synthetic pollutants, to the constituents of ecosystems, animal (including human), vegetable and microbial, in an integral context" (Truhaut, 1977). In this sense, ecotoxicology comprises the integration of ecology and toxicology (Chapman, 1995; Baird et al., 1996; Fig. 1).

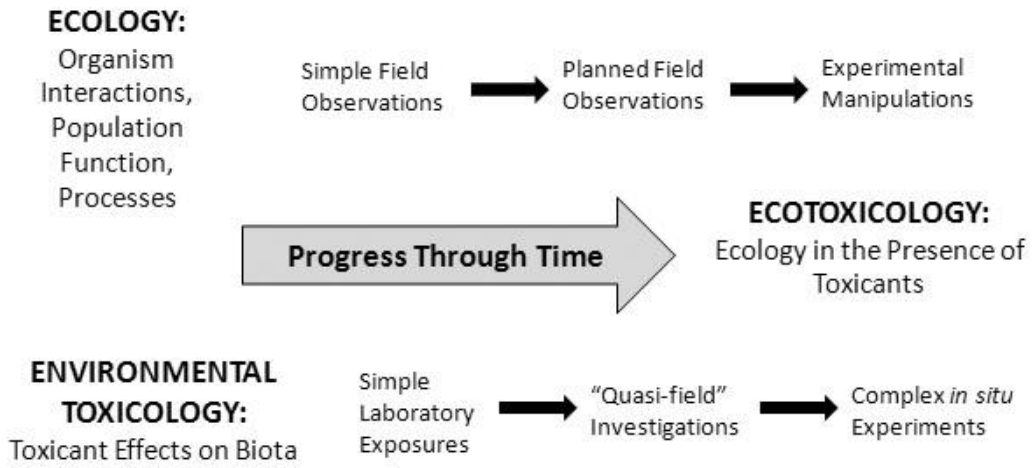

Fig. 1 The development of ecology, environmental toxicology, and ecotoxicology. Initial approaches have not been supplanted but rather have been complemented by subsequent approaches (*modified from Chapman, 2002*).

The scope of ecotoxicology in recent years has been broadened to include organisms, populations and whole ecosystems. Its basic principle lies on the detection, monitoring and prediction of the effects of environmental contaminants on ecosystems (Forbes and Forbes, 1994).

The effects of stressors in the environment extend to various biological levels of organization, from molecules, cells, organs and individual organisms to populations,

biological communities and ecosystems. To quantify these effects on organisms bioassays are carried out. Bioassays are typically conducted to measure the effects of a substance on a living organism and are essential in the development of new drugs and in monitoring environmental pollutants. Up to the present, the biological effects of environmental contaminants have been subject to many studies. There are numerous biological tests designed to determine toxicity in aquatic ecosystem, and these fall mainly into three categories, i.e. toxicity test with fish, *Daphnia*, and algae. For practical reasons, the goal always is a test which would be fast, simple, reproducible, precise, accurate, standardized, cost effective, convenient, and sensitive (Isenberg, 1993). A wide range of biological indicators has been developed to detect and assess exposure and effects of contaminants at sublethal levels, so-called biomarkers. Biomarkers are now routinely used in biomonitoring programs (Moore et al., 2004; Hyne and Maher, 2003; Bonacci et al., 2007; De la Torre et al., 2007; Kopecka and Pempkowiak, 2008). The use of biomarkers and bioassays has led to the generation of numerous comparative toxicity data which provide important information on variation in responses of aquatic species to chemicals e.g. pesticides and are useful for determining margins of safety for aquatic biota, either prospectively (before manufacture and use) or retrospectively (after manufacture and use) (Adams 1995; Graney et al., 1994).

Fish are largely used for the assessment of the quality of the aquatic environment and as such can serve as bioindicator species of environmental pollution (Lopes et al., 2001; Whitefield and Elliott, 2002; Dautremepuits et al., 2004). Among invertebrates, considerable differences in sensitivity to environmental contaminants also have been reported (Roast et al., 1999; Lund et al., 2000; Gagnaire et al., 2008; Pablo et al., 2008). While fishes have been considered very suitable organisms for pollution monitoring in aquatic ecosystem (Van der Oost et al., 2003), a comparatively low number of studies on invertebrates are available. Consequently, the toxicity of contaminants has been tested mainly in fish and some adult invertebrates (e.g. *Daphnia*) but, in most cases, acute toxicity (mortality, immobility) has been determined. However, to understand the sublethal effects of environmental contaminants on animal health, their action should be studied during the main life cycle phases of an organism, especially during the first stages of development, which are generally considered to be particularly sensitive to the toxic action of chemicals.

It is now widely accepted that the early life stages of many aquatic species are highly sensitive to both inorganic and organic toxicants (Borthwick et al., 1985; Fridman et al., 2004; Schirling et al., 2006; Beiras and Bellas, 2008; Scheil and Köhler, 2009). Embryo tests or early life stage tests have become important tools for environmental risk assessment, and have been shown to be especially sensitive indicators of a number of types of aquatic pollution (Devlin and Mottet, 1992; Pickering and Lazorchak, 1995). Measures of toxicity derived from these early life stage tests can provide a strong indication of the potential range of biological effects of toxicant action (U.S. EPA, 2002). These tests are also able to provide data of high biological relevance at the interface between the individual and the population levels (Schirling et al., 2006).

Following several studies on endocrine disruption in *Marisa cornuarietis* (e.g. Oehlmann et al., 2000; Schulte-Oehlmann et al., 2000; Schirling et al., 2006) and, consequently, the establishment of a novel embryo toxicity test using this species (Schirling et al., 2006), the ontogenetic development of *M. cornuarietis* was especially selected for this study.

In order to understand interactions of contaminants with molecular or cellular targets, however, effects should be studied at the sub-organs levels administering sublethal concentrations. Histopathological responses include changes in cell and tissue structure in response to toxic pollutants (Meyers and Hendricks, 1982). Histopathological changes in aquatic animals serve to act as biomarkers in assessing the integrity of the surrounding environment (Lajtner et al., 1996), and a powerful tool for monitoring anthropogenic contamination (Stentiford et al., 2003). It is imperative that histological biomarkers are indicative of pollutant action to the overall health of monitor species in the ecosystem (Velkova-Jordanoska and Kostoski, 2005). In this context, studies of the hepatopancreas of molluscs are especially important because it is the central metabolic organ of these animals. The peroxisomes and lysosomes of mollusc hepatopancreatic cells can suffer morphological and functionalchanges caused by pollutants; and there changes may be valuable as bioindicators of pollution (Moore, 1985; Cajaraville et al., 1989; Fahimi and Cajaraville, 1995). Histopathological effects of chemicals on the great ramshorn snail, *Planorbarius corneus* were studied, and this species gives more sensitive response to chemicals than those of some other aquatic organisms (Klobucar et al., 1997; Klobucar et al., 2001; Otludil et al., 2004). The investigation of histopathological changes in organs of snail is an accurate way to assess the effects of xenobiotic compounds in experimental studies (Triebskorn,

1989; Triebskorn and Köhler, 1992). Hence, this study was undertaken to examine the effect of different metals at sublethal concentrations on histopathology of *M. cornuarietis.*

2.2 Aims of the study

The purpose of this study was to determine the sensitivity of the recently introduced *M. cornuarietis* embryo toxicity test (MariETT) using selected metals (inorganic pollutants) and pesticides (organic pollutants), and to compare its sensitivity and practicability to other established bioassay e.g. *Dar*T or AMPHITOX (Nagel, 2002; Fridman et al., 2004) (Chapters 1-4). Furthermore, this study aimed at assessing the sublethal effects of metals in juveniles of *M. cornuarietis* under laboratory conditions, by means of histopathology (Chapter 5).

3. Materials and methods

3.1 Experimental settings

The *Marisa* Embryo Toxicity Test (*MariETT*) was conducted under laboratory settings using selected chemicals. Preliminary experiments were conducted to validate pesticide and metal sensitivity of the embryonic development of the ramshorn snail, *Marisa cornuarietis* (Chapter 1). In the following chapters, the experiments emphasized on the sensitivity of the *MariETT* incomparison to other biotests focusing on selected pesticides (Chapter 2) and selected metals (Chapters 3 and 4). Since the embryonic development of *M. cornuarietis* revealed to be highly sensitive to metals, the histopathological study was conducted in Chapter 5 to describe and qualify sublethal effects of copper and lithium in early juveniles of *M. cornuarietis.*

The *Marisa cornuarietis* strain used for egg production originated from a breeding stock of the Zoological Institute in Frankfurt/Main University, Germany (gratefully donated by J. Oehlmann). Adult snails were maintained in 120 L glass aquaria containing water with a conductivity of ≈ 800 µS/cm (based on tap water, conductivity adjusted with NaCl), a pH of ≈ 7.5, and a temperature of 24 ± 1 ^{o}C. The photoperiod was 12h:12h (light:dark). Aquaria were part of a circulation system with aerated and filtered water. Snails were fed daily with commercial dry food flakes (TetraMin, Tetra GmbH, Germany) or carrots.

3.2 Test Organisms

The ramshorn snail, *Marisa cornuarietis* (Linnaeus, 1758) (Fig. 2a) belongs to the class Gastropoda, subclass Prosobranchia, family Ampullariidae (apple snails) and genus *Marisa*. They are a common inhabitant of stagnant and slowly running freshwater ecosystems in south and Central America. The snail can reach a diameter of 40 to 60 mm and feeds on both freshwater plants and eggs from other snails. Like other ampullariid species, it possesses a lung in addition to a monopectinate gill, but in contrast to other species of this family, it does not occasionally leave the water but deposits its egg masses below the water line. The egg clutches consist of 20-80 eggs which are embedded in a gelatinous mass sticking to submerge objects or, in captivity, to aquaria walls (Fig. 2b). The gelatinous material has several purposes, besides keeping the eggs together and protecting them, it serves as food material for the growing young snail. Depending on the temperature, the development of the embryo until hatch takes between 8 and 20 days (about 12 days at 26 ^{o}C). The ontogeny was described in detail by Demian and Yousif (1973).

M. cornuarietis is easy to be maintained and bred in aquaria under laboratory conditions, has a short generation interval, a short spawning interval, and transparent eggs, enabling the monitoring of developmental features using stereomicroscopy, which collectively make this species suitable as an organism for toxicological studies.

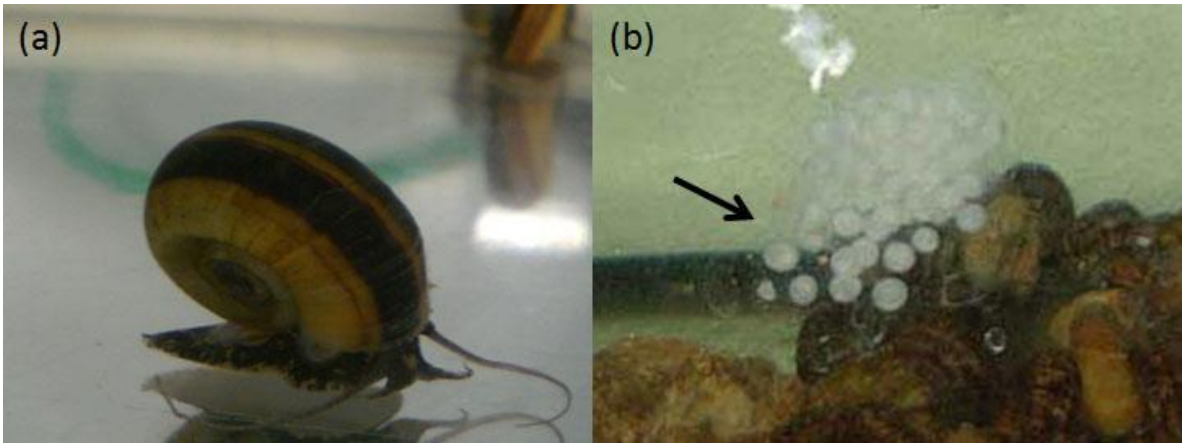

Fig. 2 Adult ramshorn snail, *Marisa cornuarietis* (a) and an egg clutch (arrow) sticking to the aquaria wall (b).

3.3 Exposure Conditions

The chemicals used in this study were metals (inorganic toxicants) and pesticides (organic toxicants). As a representative metal group, copper, lead, lithium, nickel, palladium and zinc were selected. Atrazine, chlorpyrifos, flubendiamide, imidacloprid, methiocarb and Mesurol® snail pellets were used as a representative pesticide group.

Pesticides and metals were applied as pure chemicals (metals as chlorides) using stock solutions, except palladium was applied as a commercially available $PdCl_2$ standard solution, and Mesurol® snail pellets (Bayer CropScience, Germany) were crushed and dissolved with the same water as used for animal stock maintainance to the final nominal concentrations of one time application rate (0.5 g/m^2, ≅ 1270 µg/L methiocarb), three times application rate (1.5 g/m^2, ≅ 3810 µg/L methiocarb), and 10 times application rate (5 g/m^2, ≅ 12 700 µg/L methiocarb).

The test solutions were prepared by diluting the stock solutions with the same water as used for adult snail maintainance. The final nominal concentrations of the test solutions for embryo toxicity testing were shown in Table 1. Regarding the histopathological study, the final nominal concentrations of 0, 5, 10, 25, 50 and 75 µg Cu^{2+}/L or 0, 50, 100, 200, 1000 and 5000 µg Li^+/L, respectively, were used.

Table 1 Nominal concentrations of the test solutions for embryo toxicity testing.

Test chemicals		Nominal concentrations (µg/L) tested
Pesticides:	Atrazine (Sigma-Aldrich, Germany)	0, 100, 1000, 10 000, 30 000
	Chlorpyrifos (Sigma-Aldrich, Germany)	0, 150, 200, 350, 500
	Flubendiamide (Sigma-Aldrich, Germany)	0, 100, 200
	Imidacloprid (Sigma-Aldrich, Germany)	0, 10 000, 25 000, 50 000
	Methiocarb (Sigma-Aldrich, Germany)	0, 100, 250, 500
Metals:	Cu^{2+} ($CuCl_2$, 99%, Acros organics, Great Britain)	0, 50, 100, 250
	Pb^{2+} ($PbCl_2$, ≥ 98%, Fluka, Switzerland)	0, 5000, 10 000, 15 000
	Li^+ (LiCl, ≥ 99%, Fluka, USA)	0, 1000, 2500, 3000
	Ni^{2+} ($NiCl_2$, ≥ 98% p.a., Roth, Germany)	0, 0.1, 1, 10, 100
	Pd^{2+} ($PdCl_2$, standard solution, Sigma-Aldrich, Germany)	0, 50, 100, 500
	Zn^{2+} ($ZnCl_2$, ≥ 98% p.a., Merck, Germany)	0, 100, 200, 500, 1000, 2000, 5000

3.4 Embryo Toxicity Test

M. cornuarietis deposits its eggs during the night in relatively large, soft, gelatinous egg masses which are usually attached to the side wall of the aquaria. Freshly laid egg masses were removed from the aquaria walls and divided with a razor blade. To exclude possible effect of genetic differences, 20 eggs (5 each from 4 randomly chosen egg masses deposited during the same night) were distributed into either control groups (with aquarium water) or exposure groups, and incubated at 27 ^{o}C for further investigations. For each treatment plus control, 9 replicate Petri dishes were used (n=9). The control water and the test solutions were renewed daily. The number of hatched individuals often differed from the number of eggs due to twin, triplet, or quad hatchlings. The development of the snail embryos from the day of egg laying until hatching was observed using a stereomicroscope.

The following endpoints were observed: mortality (%), formation of tentacles and eyes (%), heart rate (min^{-1}), and hatching success (%). Weight after hatching (mg wet wt.) was also measured. The endpoints were recorded daily, at the suggested days of development as shown in Table 2. For the tests, mortality < 10% in the control was accepted as valid. Mortality was defined by coagulation of the embryo or cessation of the heart beat; this was recorded daily during the experiment. To measure snail fresh weight after hatching, five hatchlings were chosen randomly, and transferred to the surface of soft paper tissue. When the adhesive water was removed completely from the hatchling, the five individuals were pooled and weighed on an analytical balance (Kern, type 770). Possible malformations of the embryos also were recorded. The experiments with pesticides were performed using glass Petri dishes, the stock and test solutions were kept in glass bottles as well. Polystyrene Petri dishes and polyethylene bottles were use for the metal test solutions.

Table 2 Schedule to investigate different endpoints of embryonic development of *Marisa cornuarietis* (based from the original protocol by Schirling et al., 2006)

Endpoints	1d	2d	3d	4d	5d	6d	7d	8d	9d	10d	11d	12d	13d	14d
Formation of tentacles				+	+	+								
Formation of eyes					+	+	+							
Heart rate									+					
Hatching										+	+	+	+	+
Mortality				+	+	+	+	+	+	+	+	+	+	+
	Eggs opaque			Eggs clear up						Eggs transparent				

3.5 Histopathological Study

3.5.2 Slide Preparation

Seven-days-old juvenile snails from copper (0, 5, 10, 25, 50 and 75 µg Cu^{2+}/L), lithium (0, 50, 100, 200, 1000 and 5000 µg Li^{+}/L), and control groups (shell diameter of about 2 mm) were fixed in Bouin's solution (150 ml picric acid, 50 ml formalin, and 10 ml acetic acid) for 3 days. Samples were washed for 3 x 10 min with 70% ethanol containing one drop of 32% NH_3, dehydrated in a graded series of ethanol, and embedded in Techno-vit (Heraeus Kulzer, Germany). Samples were cut into 5 µm sections using an automatic rotary microtome (2050 Supercut, Reichert-Jung, Germany) and spread on microscope slides. Finally, the sections were stained with hematoxylin-eosin (HE) or periodic acid Schiff reagent (PAS) and alcian blue, and covered with Eukitt (Roth, Germany).

3.5.3 Histopathological Investigations

Histopathological changes in hepatopancreas, epidermis, and gills of the snails were studied under a light microscope (Axioskop 2, Zeiss, Germany). Organs of 10 animals per treatment were semi-quantitatively evaluated. Three different sections per organ were qualitatively described, and semi-quantitatively assessed by calculation of mean assessment values (MAV) which were calculated for n=10 animals per treatment. For semi-quantitative assessment of the reactions, histopathological symptoms were classified according to the state of cellular pathology: control status, status of reaction and status of destruction.

4. Results and Discussion

Chapter 1: Embryo toxicity of pesticides and heavy metals to the ramshorn snail, *Marisa cornuarietis* (Prosobranchia). *Chemosphere* 75: 1539-1547 (2009).

Marisa cornuarietis has recently become the subject to a number of ecotoxicological studies which have revealed sensitivity of this species to endocrine disruptive chemicals (Oehlmann et al., 2000; Schulte-Oehlmann et al., 2000) and resulted in the description of a highly reproducible embryo toxicity test (Schirling et al., 2006). Therefore, the purpose of the study was to investigate the sensitivity of the recently introduced *M. cornuarietis* embryo toxicity test (MariETT) using the mentioned selected pesticides (atrazine and imidacloprid) and heavy metals (nickel and zinc), and to compare its sensitivity and practicability to other biotests, particularly the *D. rerio* embryo toxicity test (*Dar*T). In the present study, metals and pesticides were selected as the test chemicals because of the following reasons: (1) metals are usually considered as inorganic pollutants, (2) most dangerous and persistent organic chemicals are pesticides, their uses raises a number of environmental concerns. Endpoints used in this study included mortality, formation of tentacles and eyes, heart rate, hatching and weight after hatching. Results showed a significant delay in the hatching rate with increasing concentrations of atrazine, nickel and zinc. Imidacloprid and zinc resulted in a significant decrease of the heart rate. Furthermore, a significant delay in the formation of tentacles and eyes were observed only for metal treatments (nickel and zinc). All treatments did not revealed any significant changes regarding mortality, and weight after hatching. No significant malformations were noted among all developing embryos throughout the exposure periods. Comparing lowest observed effect concentrations (LOECs) of the tested chemicals in our study with available from established biotests data, it become obvious that LOECs for *M. cornuarietis* for all treatments were lower than those recorded for embryo of *D. rerio* (Görge and Nagel, 1990; Senger et al., 2006; Kienle et al., 2008; Langer, 2007, unpublished). Also other test species were reported to display a rather limited sensitivity to the chemicals tested here (Herkovits et al., 2000; Carr et al., 2003; Bringolf et al., 2004; Gheorghiu et al., 2006; Nadelle et al., 2009). The comparison of the *M. cornuarietis* LOECs

data with corresponding literature data on other bioassays gives evidence for the remarkably high sensitivity of this novel MariETT assay.

Chapter 2: The sensitivity of ramshorn snail, *Marisa cornuarietis*, embryos to the pesticides chlorpyrifos, flubendiamide, methiocarb, and Mesurol® snail pellets. *Bulletin of Environmental Contamination and Toxicology* (submitted)

The purpose of this study was to quantify the toxicity of insecticides/molluscicides on the embryonic development of *Marisa cornuarietis* using pesticides with three different modes of action, and, again, to assess the degree of sensitivity of the *Marisa cornuarietis* embryo toxicity test (MariETT) comparing to other established biotests. The following pesticides were used: chlorpyrifos, an organophosphate insecticide; flubendiamide, a new class of insecticides; methiocarb and Mesurol® snail pellets, a carbamate pesticide. The same method and endpoints were used previously (Chapter 1). The study showed that chlorpyrifos dramatically increased mortality rates and affected embryonic development in *M. cornuarietis*, as the formation of tentacles and eyes, hatching success, and weight after hatching. The differences observed between the current study and previous reports indicated a particular sensitivity of *M. cornuarietis* embryos to chlorpyrifos in comparison to other established test organisms (Varó et al., 2000; Humphrey et al, 2004; Sparling and Fellers, 2007; Kienle et al., 2009; Robles-Mendoza et al., 2009). Regarding flubendiamide, no effects were observed in the *Marisa* embryo toxicity test on any of the endpoints up to concentrations at the limits of water solubility. This is consistent with previous work showing no significant changes in the early life stages of fish (Hall, 2007). Flubendiamide exerts extremely potent and broad spectrum activity within the insect order Lepidoptera, its mechanism of action is ryanodine receptor (RyR) activation, but no significant activity has been reported for insects outside this clade (Tohnishi et al., 2005; Ebbinghaus et al., 2007; Lahm et al., 2009). Methiocarb at the environmentally relevant concentration (500 µg/L) showed significant changes regarding mortality, and formation of tentacles. Altinok et al. (2006) also reported concentrations of 4.82 to 5.43 mg/L methiocarb to kill 50% of juvenile rainbow trout (*Oncorhynchus mykiss*). These data indicate the *M. cornuarietis* embryos to be about one order of magnitude more sensitive to methiocarb. In contrast to sole

methiocarb, dissolved in water, Mesurol® snail pellets at 1 time application rate (equivalent to 1270 µg/L methiocarb) did not reveal any effects on *M. cornuarietis* embryos. This might indicate that the methiocarb pellet formulation is less effective as a contact poison than it is after ingestion. Since the data did not indicate any significant embryotoxicity induced by flubendiamide and Mesurol® snail pellets at application rate, the present study has demonstrated that that the MariETT assay is particular sensitive to the active ingredient of selected pesticides (chlorpyrifos and methiocarb).

Chapter 3: Metal sensitivity of the embryonic development of the ramshorn snail *Marisa cornuarietis* (Prosobranchia). *Ecotoxicology* 19: 1487-1495 (2010).

and

Chapter 4: Turning snails into slugs: induced body plan changes and formation of an internal shell. *Evol Dev* 12(5): 474-483 (2010).*

In this study, I investigated the effects of metal ions on the embryonic development of the ramshorn snail, *Marisa cornuarietis,* by exposing embryos to varying concentrations of copper, lead, lithium, and palladium. Own results showed that copper concentration of 50 µg Cu^{2+}/L caused earlier hatching and reduced the weights of newly hatched snails. Copper at a concentration of 100 µg Cu^{2+}/L, resulted in 100% mortality immediately after the snails had hatched, and a copper concentration of 250 µg Cu^{2+}/L caused extreme effects in snail embryo development. *M. cornuarietis* with a LOEC of 50 µg Cu^{2+}/L, is slightly more sensitive than many other aquatic species (Krishnakumar et al., 1990; Milam et al., 2005; Prato et al., 2006). Regarding palladium, only highest palladium concentration (500 µg Pd^{2+}/L) had a significant delay in the formation of tentacles and eyes, also significantly affected heart rate and hatching success. In response to lithium treatment, *M. cornuarietis* embryos with high concentration of Li^{+}: 2.5 mg/L caused 10.0 ± 6.0 % shell-less snails and 3 mg/L caused 20.0 ± 9.5 % shell-less snails. After hatching, the shell-less snails individual reached a maximum age of 5 months. During their lifetime, the animal steadily grew and gained mass but did not change their outer appearance. In addition, the mortality, the formation of tentacles and eyes and the hatching success showed significant differences for embryos in control and

treatment groups at concentrations of 2.5 mg Li^+/L and 3 mg Li^+/L. Furthermore, 3 mg Li^+/L was found to induce significant changes on the wet weight of newly hatched snails. In contrast to developmental biology, which has used Li^+ as an agent to experimentally disrupt embryonic development in older studies not much attention has been paid to the effects of lithium in an ecotoxicological context, and very few published data are available with which to compare own results. No effect of lead was found on the embryonic development of *M. cornuarietis* regarding heart rate or the weight of newly hatched snails. However, in embryos exposed to 15 mg Pb^{2+}/L, the mortality was shown to increase significantly, and also a significant delay in the formation of tentacles and eyes, and a significantly earlier hatching were found. The present study indicates that the MariETT assay is sensitive to copper and lithium compared to data reported for fish species (Rougier et al., 1996; Long et al., 1998; WHO, 2002; Osman et al., 2007). The toxicity of metals (in this study and results appeared in Chapter 1) on the embryonic development of *M. cornuarietis* was in the order: Ni > Cu > Zn > Pd > Li > Pb.

*Banthita Sawasdee contributed to the $PdCl_2$ and LiCl experiments only here.

Chapter 5: Histopathological effects of copper and lithium in the ramshorn snail,

***Marisa cornuarietis* (Gastropoda, Prosobranchia). Chemosphere (in press).**

Although several researchers have studied toxic effects of metals in snails and bivalves, up to now, there are not many data on metal-induced histopathological changes in gastropods which might serve as basic indicators to assess hazards in molluscs. Own previous studies (Chapter 1, Chapter 3, and Chapter 4) revealed embryonic development of *Marisa cornuarietis* to be highly sensitive to metals. Therefore, the purpose of the study was to describe and quantify sublethal effects of metals in newly hatched snails by means of histopathology. To investigate histopathological alterations induced by effects of copper and lithium in *M. cornuarietis*, the hepatopancreas, epidermis, and gill of young snails were studied and described. The results showed that both copper and lithium caused significant histopathological changes in the hepatopancreas, epidermis and mantle tissues, and gills of

the exposed snails. However, the most severe reactions became evident in the hepatopancreas, which is the main metabolic organ in snails and also involved in detoxification and accumulation of heavy metals (Dallinger and Wieser, 1984; Tanhan et al., 2005). From this study, it can be concluded that the toxicity of copper on histopathological changes in *M. cornuarietis* is about 10 times higher than the toxicity of lithium. This observation is in agreement with own results obtained in previous experiments (Chapter 3).

5. Concluding statements

Although the relevance of early life stages for the toxicity assessment of chemiclas has gained increasing attention in the past years, the knowledge of effects on invertebrate embryos still remains very limited. The particular ecophysiological characteristics of the various species of freshwater snails that can be easily reared in the laboratory should shortly enable a wide range of ecotoxicity tests to be set up in the laboratory for valuable monitoring programs in freshwater ecosystems. In this context, Schirling et al. (2006) have established a protocol for an embryo test with *Marisa cornuarietis*, and have demonstrated that toxicity effects were not only found in adult animals, but occur very early in ontogeny. On the basis of this paper, the entirety of my own studies has shown that the use of *M. cornuarietis* embryos with a focus on both developmental endpoints and histopathology should be a suitable and powerful invertebrate test system in assessing the effects of toxic substances in aquatic ecosystem. For almost all substances, own results revealed the exceptional sensitivity of the MariETT test and, therefore, its potential to replace or supplement established biotests used in aquatic ecotoxicology.

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Eigenanteil an den durchgeführten Arbeiten in den zur Dissertation eingereichten Publikationen und Manuskripten

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Chapter 1: Embryo toxicity of pesticides and heavy metals to the ramshorn snail, *Marisa cornuarietis* (Prosobranchia)*

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Abstract

An invertebrate embryo toxicity test with the ampullariid snail, *Marisa cornuarietis*, to assess the toxicity of pesticides and heavy metals recently was established. Snail embryos were treated with atrazine (100, 1000, 10,000 and 30,000 µg/L), imidacloprid (10,000, 25,000, and 50,000 µg/L), Ni^{2+} (0.1, 1, 10, and 100 µg/L) or Zn^{2+} (100, 200, 500, 1000, 2000, and 5000 µg/L). The effect of these substances were examined by monitoring the following endpoints: mortality, formation of tentacles and eyes, heart rate, hatching, and weight after hatching. Effects in term of a significant delay on the formation of both tentacles and eyes were found after treatment with 100 µg/L Ni^{2+} or 200 µg/L Zn^{2+}. The heart rate was shown to significantly decrease at 25,000 µg/L imidacloprid or 1000 µg/L Zn^{2+}. At 100 µg/L atrazine, 10 µg/L Ni^{2+}, or 1000 µg/L Zn^{2+} a significant delay in hatching became visible. No significant mortality was observed for the tested concentrations of atrazine, imidacloprid, or Ni^{2+}, while 5000 µg/L Zn^{2+} resulted in 100% mortality after 10 days. The weight of freshly hatched individuals remained unaffected in all treatments. On the basis of the lowest observed effect concentrations (LOECs) recorded, we could show the *Marisa cornuarietis* embryo toxicity test (MariETT) to react up to three orders of magnitude more sensitive (for metals) and at least one order of magnitude more sensitive (for the tested organics) than the established *Danio rerio* embryo test.

Keywords: atrazine, embryo toxicity test, imidacloprid, *Marisa cornuarietis*, nickel, zinc

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Introduction

The ramshorn snail *Marisa cornuarietis* Linneaus (Ampullariidae) is a common inhabitant of stagnant and slowly running freshwater ecosystems in south and central America. The snail can reach a diameter of 40 to 60 mm and feeds on both freshwater plants and eggs from other snails. Like other ampullariid species, it possesses a lung in addition to a monopectinate gill, but in contrast to other species of this family, it does not occasionally leave the water but deposits its egg masses below the water line. The egg clutches consist of 20-80 eggs which are embedded in a gelatinous mass sticking to submerge objects or, in captivity, to aquaria walls. The gelatinous material has several purposes, besides keeping the eggs together and protecting them, it serves as food material for the growing young snail. Depending on the temperature, the development of the embryo until hatch takes between 8 and 20 days (about 12 days at 26 ^{o}C). The ontogeny was described in detail by Demian and Yousif (1973).

M. cornuarietis has recently become the subject to a number of ecotoxicological studies which have revealed sensitivity of this species to endocrine disruptive chemicals (Oehlmann et al., 2000; Schulte-Oehlmann et al., 2000; Schirling et al., 2006). With its largely transparent eggs, *M. cornuarietis* provides the opportunity to follow the development of the embryo from outside three days after fertilization until the time of hatching. This allows the investigator to record sublethal parameters which are affected by treatment with different chemical compounds and has therefore resulted in the description of a highly reproducible embryo toxicity test (Schirling et al., 2006).

Atrazine (2-chloro-4-ethylamino-6-isopropylamine-S-triazine) is a widely used selective triazine herbicide for the control of broadleaf weeds and grasses in agriculture. It is a photosynthetic electron transport inhibitor; it impairs the Hill reaction by binding the polyphenoloxidase of complex B of photosystem II (Schulz et al., 1990). Atrazine is mobile in soils under conditions of high rainfall and this can result in contamination of surface and ground waters (WHO, 1990). In Central European surface waters, atrazine concentrations of more than 400 µg/L have been detected (Umweltbundesamt, Germany, 1997). Atrazine has been banned in some industrialized countries since the early 1990's, however, due to its persistance, remains among the most significant water pollutants (Elezovic et al., 1994). It is

still present in river sediments and surface waters, and deethylatrazine, its major metabolite, has been detected in animal tissues, soil, and water (De Lorenzo et al., 2001). The ecotoxicological relevance of atrazine, especially in aquatic ecosystems, has been reviewed by Huber (1993) and Solomon et al. (1996). In zebrafish, *Danio rerio*, atrazine at 1.3 µg/L decreased the survival of juvenile fish and also increased the number of deformations and edema in early life stage (Görge and Nagel, 1990). Furthermore, atrazine at 0.4 mg/L disturbed the normal development to long-pec stage in zebrafish and, at concentrations between 10 and 20 mg/L, it caused retardations in organogenesis, a slowdown of movement, and functional disturbances of the heart and circulatory system (Wiegand et al., 2001). It was also found to change the behavior of zebrafish at an environmentally relevant concentration of 5 µg/L (Steinberg et al., 1995). Atrazine causes many physiological disturbances including increased respiration, decreased reflexes, inhibition of acetylcholine esterase activity, and derangements of osmoregulation in freshwater fish (Hussein et al., 1996). Because of its lipophilicity, atrazine bioconcentrates in organisms. A bioaccumulation factor (BCF) of 19 was found in zebrafish embryos, whereby the chorions absorbed only a small amount of atrazine but provided no protection against this pesticide (Wiegand et al., 2000). Atrazine is toxic to different amphibian species (Clements et al., 1997; Fort et al., 2004; Freeman and Rayburn, 2004), and a number of recent studies indicate that atrazine is an endocrine disrupter (Sanderson et al., 2000; Thomas and Doughty, 2004; Bringolf et al. 2007). In the freshwater molluscs, *Physa acuta* and *Ancylus fluviatilis*, atrazine induced significant changes in grazing behaviour, and different movement patterns and velocity (Rosés et al., 1999).

The rather new insecticide, imidacloprid (1- (6-chloro-3-pyridinylmethyl)-N-nitro imidazolidin-2-ylideneamine) is a systemic compound with a novel mode of action (Moriya et al., 1992; MacDonald & Meyer, 1998). Imidacloprid was the first member of a new pesticide family called neonicotinoids acting as a blocker of the nicotinergic acetylcholine receptor (Bai et al., 1991). This insecticide is effective for controlling aphids, white flies, thrips, scales, psyllids, plant bugs, and various other pest species. Imidacloprid degrades in soil and plants into a number of different derivatives. The hydrolytic half-life of imidacloprid in groundwater is greater than 30 d at pH 7 and 25 °C, and the half-life of imidacloprid in soil was found to range from 28.7- 48.7 days in three different soil types (Sarkar et al., 1999;

Sarkar et al., 2001). Cox (2001) reported that imidacloprid is persistent in soil, as the concentration of tested soil samples had not decreased 1 year after application. Imidacloprid has been classified by the US Environmental Protection Agency as both a toxic class II and class III agent. The US EPA (1998), has set the acute reference dose (RfD) to 0.42 mg/kg/d, and the chronic RfD to 0.057 mg/kg/d. Several studies have provided evidence that imidacloprid has toxic effects to aquatic invertebrates, birds, and fishes (Cox, 2001; Eissa, 2004; Philippe et al., 1999). In fish, imidacloprid caused stress symptoms in juvenile medaka (Sánchez-Bayo and Goka, 2005), but its acute toxicity was limited. The LC_{50} of imidacloprid for the earthworms *Aporrectodea nocturna* and *Allolobophora icterica* was between 2 and 4 mg/kg dry soil, whereas sublethal effects revealed by significant decreases in weight were already observed at concentrations of 0.5 and 1 mg/kg dry soil (Capowiez et al., 2005).

The heavy metal nickel (Ni) is an essential element, required by various organisms for a range of structural organizations and metabolic activities. This metal is a component of several enzyme systems, mainly dehydrogenases and transaminases. Nickel is an ubiquitous element and occurs in soil, water, air, and in the biosphere. Most of nickel is used for the production of stainless steel and other nickel alloys. Nickel compounds are also used as catalysts, pigments, and in batteries. Nickel is introduced into the hydrosphere by removal from the atmosphere, by surface run-off, by discharge of industrial and municipal waste, and also following natural erosion of soils and rocks. Levels in natural fresh waters have been found to range from 2 to 10 µg/L (IPCS, 1991). In Europe, nickel is listed on the European Commission List II (Dangerous Substances Directive) and regulated through the Council of European Communities because of its toxicity, persistence, and affinity for bioaccumulation (Bubb and Lester, 1996). Nickel toxicity in aquatic invertebrates varies considerably according to the species and abiotic factors. In oligochaetes, nickel salts caused a toxic effect by decreasing the hemoglonin concentration (Tabche et al., 1999). A 96-h LC_{50} of 0.5 mg Ni^{2+}/L has been reported for *Daphnia* spp., while, in mollusks, 96-h LC_{50} values were around 0.2 mg/L in two freshwater snail species and 11 µg/L in a bivalve (IPCS, 1991). In fish, 96-h LC_{50} values generally fall within the range of 4-20 mg Ni^{2+}/L (IPCS, 1991). However, nickel toxicity to fish is modulated by other environmental factors and, thus, variation in water hardness was found to change the 48-h LC_{50} in rainbow trout

(*Oncorhynchus mykiss*) from about 80 to 20 mg/L (Brown, 1968). Nickel was also reported to affect the development of sea urchin (*Anthocidaris crassispina*) embryo: at the highest concentration tested, 480 µg Ni^{2+}/L completely inhibited embryo development (Kobayashi and Okamura, 2004).

Zinc is the second most important trace metal in the human body after iron. Zinc ions are now considered neurotransmitters. Cells in the salivary gland, prostate, immune system and intestine are other types that use zinc signalling (Hershfinkel et al., 2007). Total brain zinc concentrations range at a micromolar range level, whereby the zinc is stored together with glutamate in synaptic vesicles and may serve as an endogenous neuromodulator in synaptic neurotransmission (Barañano et al., 2001; Takeda, 2001). As Smart (2004) described, zinc plays important roles in excitatory glutamate receptors activation and inhibitory GABA signal transduction. Even though zinc is an essential requirement for a healthy body, too much zinc can be harmful. Excessive absorption of zinc can suppress copper and iron absorption. The free zinc ion in solution is highly toxic to plants, invertebrates, and fish. A recent example showed 6 mM Zn^{2+} to kill 93% of adult *Daphnia magna* (Muyssen et al., 2006). In monogenean ectoparasites (*Gyrodactylus turnbulli*), Zn^{2+} at concentrations of up to 240 µg/L caused a significant decrease of reproduction and survival (Gheorghiu et al., 2006). Zinc was also suggested to change acetylcholinesterase and ectonucleotidase activities in zebrafish (*Danio rerio*) brain (Senger et al., 2006). Existing literature on accumulated zinc concentrations in biota was recently reviewed by Rainbow (2006).

As exemplarily reported here, the toxicity of pesticides and heavy metals has been tested mainly in fish and adult invertebrates. However, toxicity data for developing invertebrate embryos are largely lacking. The main aim of this work therefore was to check out the sensitivity of the recently introduced *M. cornuarietis* embryo toxicity test (MariETT) using the mentioned selected pesticides and heavy metals, and to compare its sensitivity and practicability to other biotests, particularly the *D. rerio* embryo toxicity test (DarT).

Methods

Experimental animals

The *Marisa cornuarietis* (Mesogastropoda : Ampullariidae) strain used for egg production originated from a breeding stock of the Zoological Institute in Frankfurt/Main, Germany

(gratefully donated by J. Oehlmann). Adult snails were maintained in 120 L glass aquaria filled with tap water to which seasalt (hobby-marin, Dohse Aquaristik, Germany) was added up to a water conductivity of about 820 µS/cm. The water temperature was adjusted to 24 ± 1 ^{o}C, the light - dark regime was 12h/12h. Stock animals were fed fish flake food (Hagen, Germany) and fresh carrots once a day.

Exposure conditions

Effects of pesticides were investigated by exposing developing *M. cornuarietis* embryos inside their egg to a range of atrazine (Sigma-Aldrich, Germany) (0, 100, 1000, 10,000 and 30,000 µg/L) and imidacloprid (Sigma-Aldrich, Germany) concentrations (0, 10,000, 25,000 and 50,000 µg/L). Solutions were prepared with the same water as used for the animal stock culture. For the test with atrazine and imidacloprid, glass Petri dishes were used and the stock solutions were kept in glass bottles.

The heavy metals, nickel and zinc were applied as $NiCl_2$ x 6 H_2O (≥ 98% p.a., Roth, Germany) and $ZnCl_2$ (≥ 98% p.a., Merck, Germany) using stock solutions of 1 g Ni^{2+}/L and 1 g Zn^{2+}/L in aqua bidest. For the test solutions, these stock solutions were diluted with the same water as used for animal stock maintainance to the final nominal concentrations of 0, 0.1, 1, 10 and 100 µg Ni^{2+}/L and 0, 100, 200, 500, 1000, 2000 and 5000 µg Zn^{2+}/L, respectively. The high number of tested Zn concentrations made two independent sets of experiments necessary: in a first set, the concentrations 0, 100, 1000, and 5000 µg Zn^{2+}/L were tested, the second set focused on 0, 200, 500, and 2000 µg Zn^{2+}/L. All tests with nickel and zinc were performed in polyethylene Petri dishes, and the stock and test solutions were kept in polyethylene bottles.

Embryo toxicity test

Freshly laid egg masses were removed carefully from the walls of the aquaria and divided with a razor blade. To exclude possible effect of genetic differences, respectively 20 eggs (5 from 4 different randomly chosen egg masses each, egg masses produced during the same night) were distributed to each Petri dish for either controls or exposure groups, and incubated at 26 ± 0.5 ^{o}C for further investigations. Nine replicate Petri dishes containing 20 eggs each were investigated for each concentration and controls (n=9). The Petri dishes were covered by their transparent lids during the exposure and the control water as well as the solutions were renewed daily. The number of hatched individuals regularly differed from the number of eggs due to twin, triplet, or quad hatchlings.

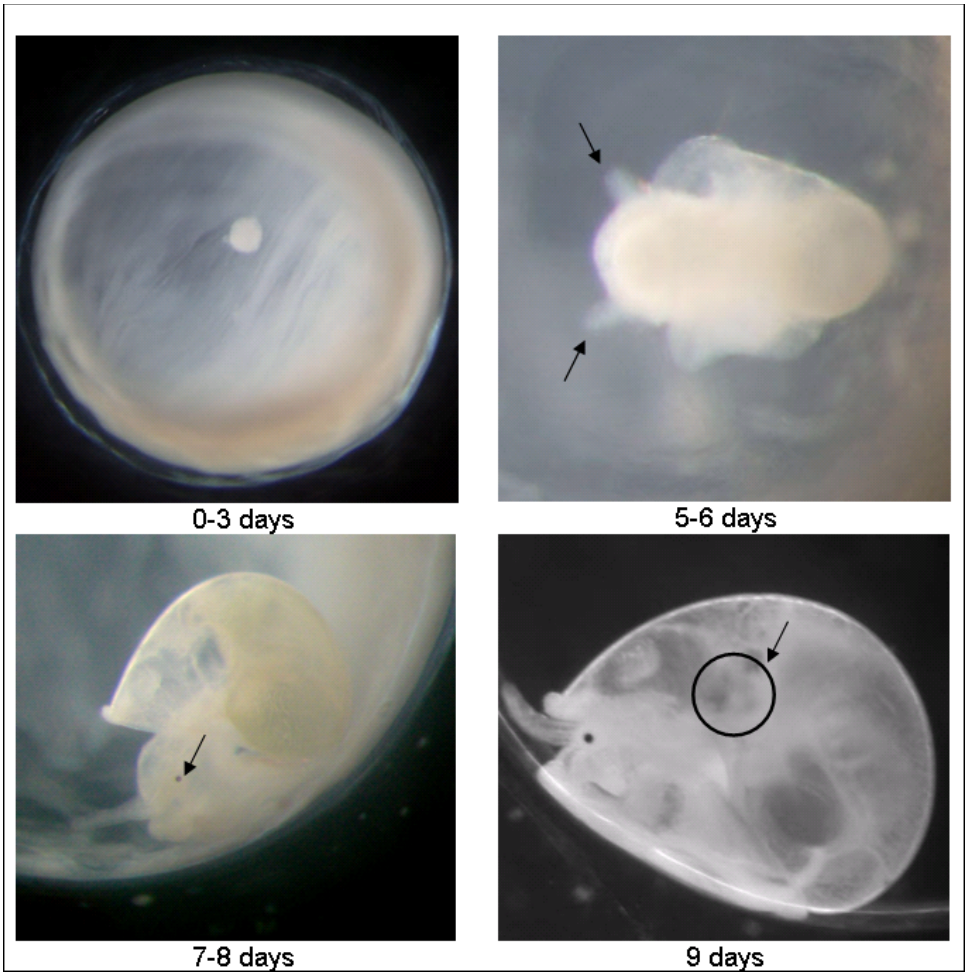

Fig. 1 *M. cornuarietis* embryonic development at different days after fertilization. Opaque egg at days 0-3, formation of tentacles (arrow) at days 5-6, formation of eyes (arrow) at days 7-8, and embryo at day 9 displaying heart (arrow) function.

The development of the snail embryos from the day of egg laying until hatching was observed using a stereomicroscope. The following endpoints were recorded: mortality (%), formation of tentacles and eyes (%), heart rate (min^{-1}), hatching (%) and weight after hatching (mg wet wt.) (Fig. 1). Mortality was recorded every day throughout the experiment, the formation of tentacles and eyes were observed from day 5 onwards, while the heart rate was recorded at day 9 only. Furthermore, possible malformations of the embryos also were recorded every day. For the tests, a mortality less than 15% in the control was accepted valid. Mortality was defined by coagulation of the embryo or cessation of the heart beat. To determine weight after hatching, five randomly chosen, newly hatched individuals from each Petri dish were transferred to the surface of soft paper tissue. After 1 minute when the adhesive water was removed completely from the hatchling, the five individuals were pooled and weighed on an analytical balance (Kern, type 770).

Statistical analysis

For statistical analysis, the software JMP® 4.0 (SAS) was used. Normally distributed data (checked by Shapiro-Wilk's test) were tested for significance with Student's *t*-test, whereas data with non-normal distribution were tested with Wilcoxon's test. The α-level for significant differences was set to $p \leq 0.05$ (*).

Results

Atrazine treatment

Both 10,000 and 30,000 µg/L atrazine significantly elevated the hatching rate at day 10, whereas embryos exposed to either 100 or 1000 µg/L atrazine showed a significant delay of hatching at day 14. (Fig. 2A). However, all four treatments did not reveal any significant changes regarding the development of tentacles and eyes, heart rate and weight after hatching in comparison to the control group.

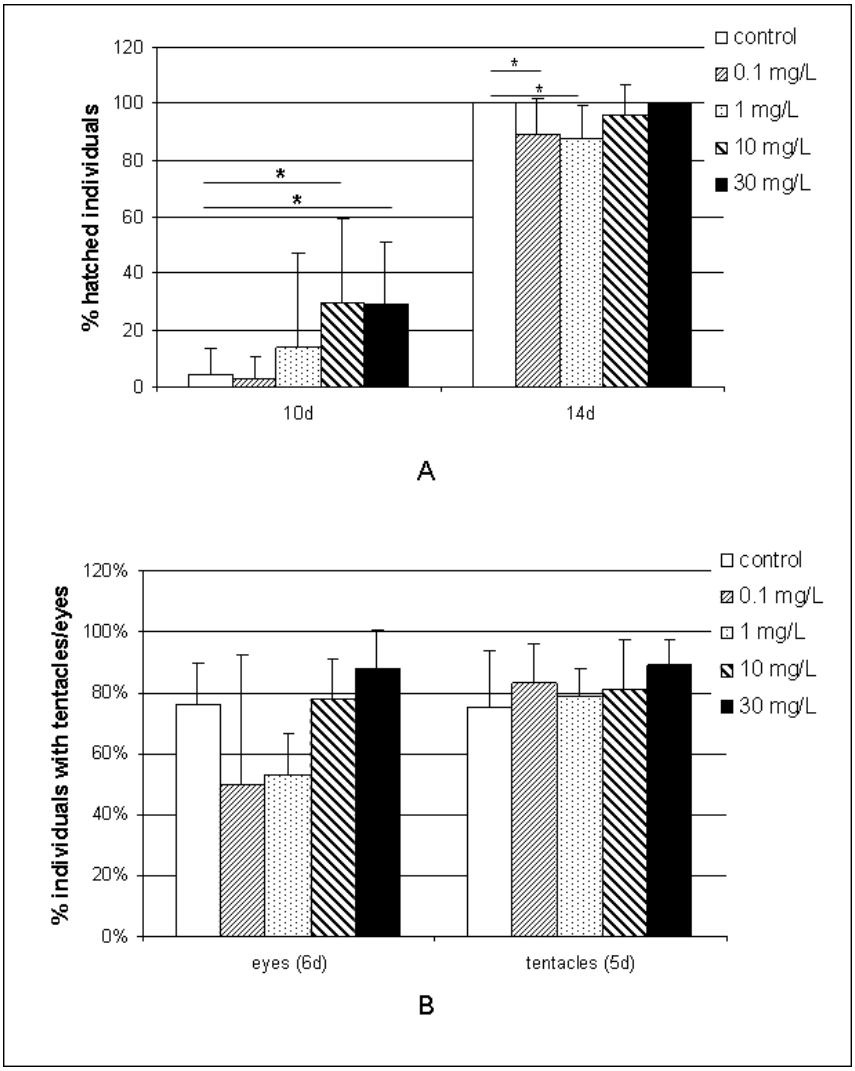

Fig. 2 Effects of atrazine on *M. cornuarietis* embryonic development, means ± SD. (A) Hatched snails after 10 and 14 days (%); (B) formation of eyes and tentacles. *Significant difference at $p \leq 0.05$ (Student's *t*-test).

For all treatments, almost 100% of the snails' tentacles and eyes were visible on day 6 and day 7, respectively. The formation of tentacles at day 5 showed a trend to being increased from 75% in the control to 89% at 30,000 µg/L atrazine. A similar trend was observed for the formation of eyes at day 6 (76% in the control *vs*. 88% at 30,000 µg/L atrazine). In both cases, however, the results were not significant (Fig. 2B).

Imidacloprid treatment

Imidaclopid (varying from 10,000 to 50,000 µg/L) was not found to induce significant changes on mortality, the formation of tentacles and eyes, hatching, and weigh after hatching. However, imidacloprid at 25,000 and 50,000 µg/L resulted in a significant decrease of the heart rate (Fig. 3).

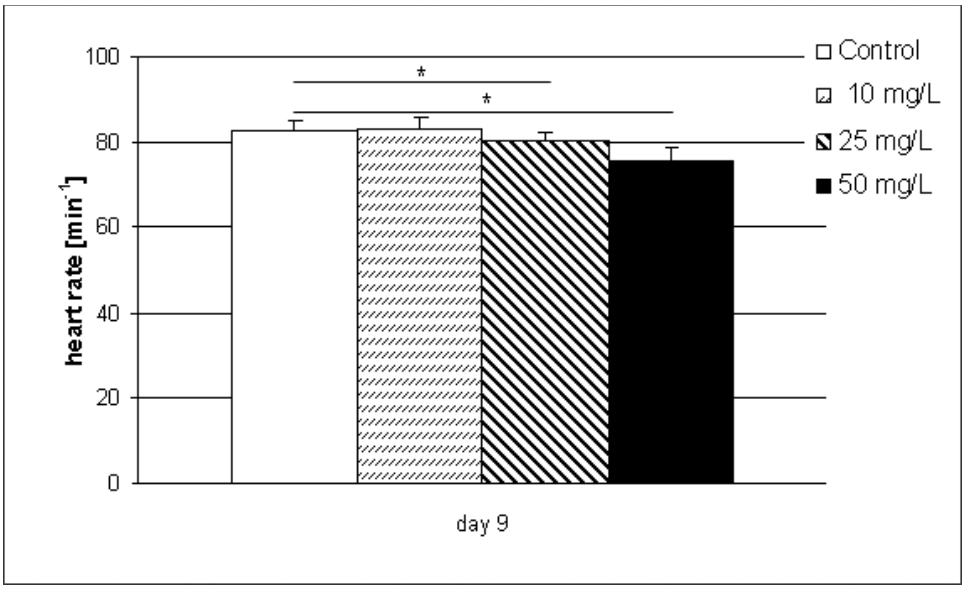

Fig. 3 Effects of imidacloprid on *M. cornuarietis* embryonic development, means ± SD. Heart rate [min^{-1}]. * Significant difference at $p \leq 0.05$ (Student's *t*-test).

Nickel treatment

At day 6, embryos treated with a concentration of 0.1 µg/L Ni^{2+} or higher showed a trend to a delayed development as indicated by a reduced mean percentage of animals with visible eyes at day 7 and tentacles at day 6. This finding, however, was significant only at the highest concentration tested (100 µg/L Ni^{2+}) (Fig. 4A). At day 10, 14% of all snails had hatched in the control and, at day 12, more than 75% had hatched. Exposure to 10 or 100 µg/L Ni^{2+} significantly reduced the hatching rate at day 10 with means of only 1% and 0%, respectively. At day 12, the snails exposed to 10 or 100 µg/L Ni^{2+} had hatched at rate of 43% and 25%, respectively (Fig. 4B). The endpoints mortality, heart rate, and weight after hatch did not differ significant between nickel treatments and the control.

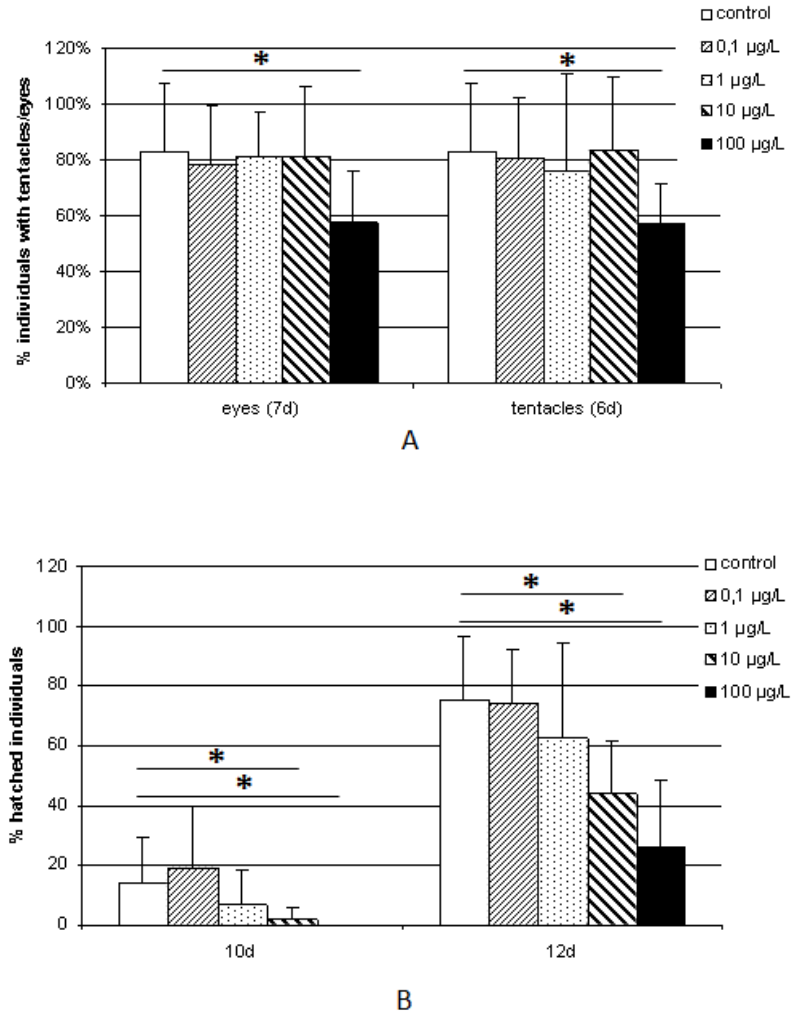

Fig. 4. Effects of Ni^{2+} on *M. cornuarietis* embryonic development, means ± SD. (A) Formation of eyes after 7 days and tentacles after 6 days (%); (B) hatched snails after 10 and 12 days (%). *Significant difference at $p \leq 0.05$ (Student's *t*-test).

Zinc treatment

Dependent on the exact time of egg clutch deposition, the observation time for the judgment on the development of eyes and tentacles differed slightly between the two experimental sets (days 5-6 for tentacles, day 6-7 for eyes). Zinc at a concentration of 1000 or 5000 µg/L significantly affected heart rate and hatching, and the formation of eyes and tentacles was delayed by 5000 µg/L (Fig. 5A, B, C). For the highest Zn concentration (5000 µg/L), significant effects were observed for all endpoints and, at day 10, mortality in this group was 100%.

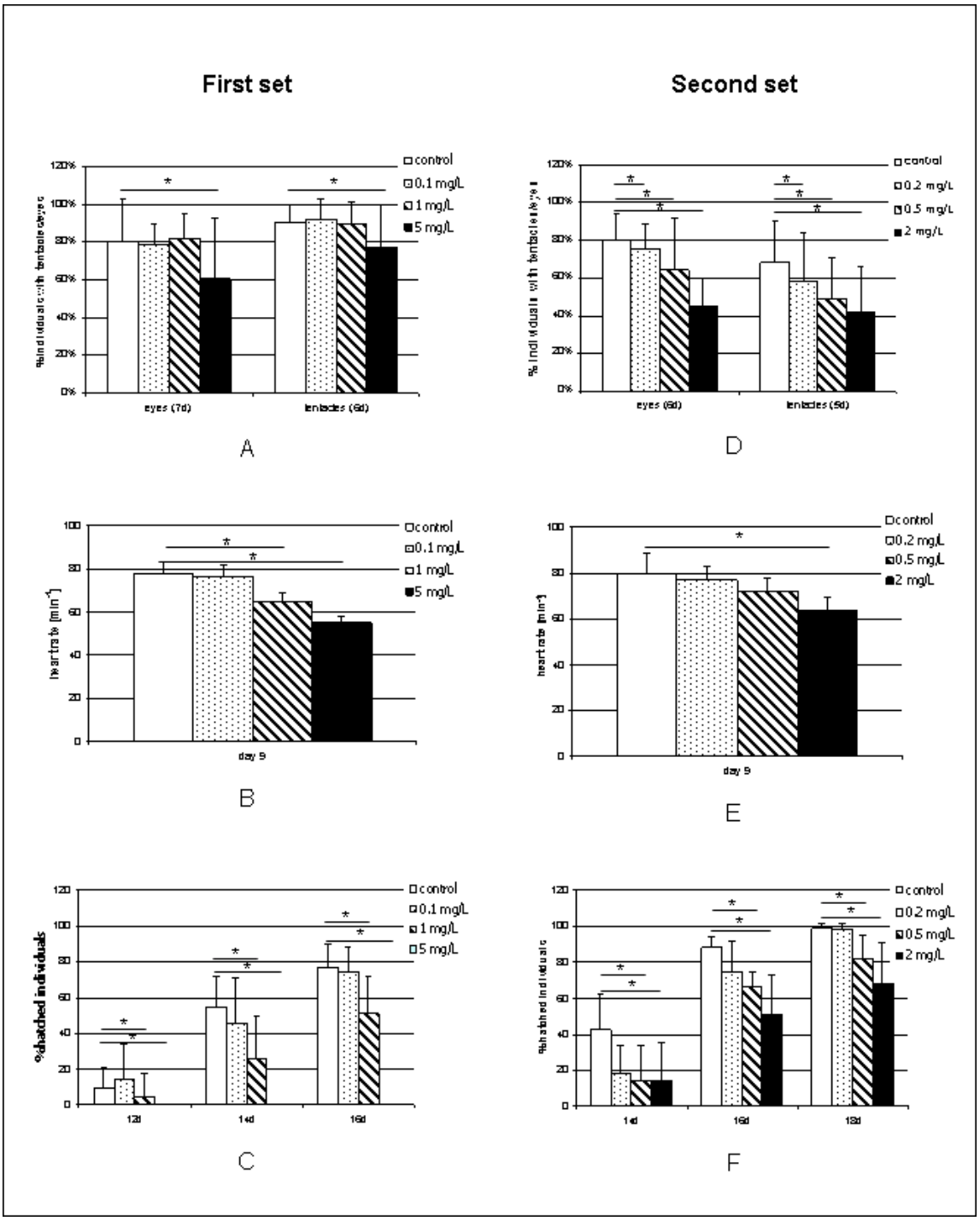

Fig. 5. Effects of Zn^{2+} on *M. cornuarietis* embryonic development, means ± SD. (A,D) Formation of eyes (%) after 6 (second set) or 7 days (first set) and tentacles (%) after 5 (second set) or 6 days (first set); (B,E) heart rate (min^{-1}) at day 9; (C,F) hatched snails (%) after 12 to 16 days (first set) or 14 to 18 days (second set). * Significant difference at $p \leq 0.05$ (Student's *t*-test for formation of eyes, heart rate, hatching; Wilcoxon test for formation of tentacles).

In the second set of experiments, at days 5 and 6, embryos exposed to a concentration of 200, 500, or 2000 µg/L Zn^{2+} showed a significant delay in the formation of both tentacles and eyes, (Fig. 5D). The heart rate at day 9 was significantly reduced in the 2000 µg/L Zn^{2+} group (Fig. 5E). Furthermore, 500 or 2000 µg/L Zn^{2+} reduced the hatching rates at day 14, 16, and 18. At day 14, 42% of all snails had hatched in the control, but only 14% in the 500 or 2000 µg/L Zn^{2+} treatments. At day 16, 88% of the control snails had hatched whereas exposure to 500 or 2000 µg/L Zn^{2+} resulted in hatching rates of 65% or 50%, respectively. At day 18, the hatching rate decreased from 99% in the control to 80% and 68% in the 500 and 2000 µg/L Zn^{2+} groups (Fig. 5F). No zinc effect on weight after hatch and mortality was observed.

Discussion

Our study showed that, in comparison to the established embryo toxicity test with *D. rerio*, the sensitivity of *M. cornuarietis* to atrazine was about one order of magnitude higher: in the present study, the lowest effect concentration (LOEC) was 0.1 mg/L (hatching), whereas the LOEC on the embryonic development in *D. rerio* was 1.3 mg/L (Gorge and Nagel, 1990) (Table. 1). Also other species were reported to display a rather limited sensitivity to atrazine: according to Bringolf et al. (2004), atrazine (5 and 50 µg/L) did not cause reproductive toxicity in adult fathead minnow at environmentally relevant concentrations (10-100 µg/L). Also the size of the laryngeal dilator muscle, a sexually dimorphic muscle in larval *Xenopus laevis* remained unaffected by similar concentrations of atrazine (Carr et al., 2003). As well, atrazine was not acutely toxic and did not exhibit any behavioral effects to *Lepomis macrochirus*, *Pimephales promelus* and *Chironomus tentans* at 1 mg/L (Mehler et al., 2008), and at 10 mg/L atrazine did not exert toxicity to larval grass shrimp *Palaemonetes pugio* (Key et al., 2007). Furthermore, atrazine did not influence the expression of the CYP19 gene

Table 1 Comparison of lowest effect concentrations (LOECs) on the embryonic development in *Marisa cornuarietis* (MariETT) and *Danio rerio* (DarT)

Test substances	*Marisa cornuarietis*		*Danio rerio*	
	LOEC [mg/L]	Endpoint (this study)	LOEC [mg/L]	Reference
Ni^{2+}	0.01	hatching	10	Kienle *et al.* (2008)
Zn^{2+}	0.2	eye and tentacle formation	32.7	Senger *et al.* (2006)
Atrazine	0.1	hatching	1.3	Gorge & Nagel (1990)
Imidacloprid	25	heart rate	> 50	M. Langer (unpublished)

in *D. rerio* juveniles (Kazeto et al., 2003). In freshwater mussels, *Lampsilis siliquoidea*, atrazine did not cause significant acute toxicity in juvenile mussels even at exposure concentrations approaching water solubility limits (Bringolf et al., 2007). On the other hand, a few reports on highly sensitive endpoints do exist. In pond snail *Lymnaea stagnalis*, atrazine at concentrations from 10 to 100 µg/L induced a significant increase in the mean number of circulating hemocytes, (Russo and Lagadic, 2004). In *Daphnia magna,* a significant increase of developmental abmortalities and the molting period were observed at 0.5, and 15 mg/l atrazine, respectively (Palma et al., 2009). Adult zebrafish changed their behaviour and preferred habitats with dark substratum upon exposure to 5 µg/L (Steinberg et al., 2005). The LOEC for atrazine observed in our MariETT assay (100 µg/L) matched the high sensitivity of these tests.

In our assay as well, exposure to 25,000 µg/L imidacloprid caused a significant decrease in the heart rate of snail embryos, whereas effects on other morphological endpoints were not observed. In zebrafish (*D. rerio*), at ranges of 100 to 50,000 µg/L imidacloprid, acute toxicity was not observed (M. Langer, Tübingen, unpublished). However, the 48-h median lethal concentration (LC_{50}) in *Daphnia magna* was 10,000 to 17,000 µg/L (Song et al., 1997), indicating a similar sensitivity of this endpoint to the heart rate in the MariETT assay. The LC_{50} of imidacloprid on two earthworm species for the anecic species *Aporrectodea nocturna* and the endogeic species *Allolobophora icterica* was between 2 and 4 mg kg^{-1} dry soil, whereas the effect concentration (EC_{50}) on the burrowing behaviour of two earthworm

species was 0.5 or 1 mg kg^{-1} dry soil (Capowiez et al., 2005). The 96-h LC_{50} of imidacloprid to larval grass shrimp *Palaemonetes pugio* was 308.8 µg/L (Key et al., 2007). The toxicological data in the literature and also those from this study on *M. cornuarietis* suggested a relatively low toxicity of imidacloprid to aquatic organisms.

Marisa cornuarietis embryos seem to be particularly sensitive to heavy metals. Exposed to 250 µg/L Cd^{2+}, these animals showed significant effects on the time of hatching, and 500 µg/L Cd^{2+} caused high (up to 94%) mortality at day 12 of their embryonic development. (Schirling et al., 2005). In contrast, an embryo toxicity test with zebrafish, *D. rerio*, at concentrations up to 10,000 µg/L Cd^{2+} did not cause any visible effects on the developing zebrafish (Hallare et al., 2005).

The limited number of studies on nickel toxicity in aquatic animals was the reason for the selection of this metal to assess the sensitivity of our test. Also for nickel exposure, the sensitivity of *M. cornuarietis* was remarkably high in comparison to the embryo test with *D. rerio*. In our study, concentrations of ≥ 10,000 ng/L Ni^{2+} caused a delay in hatching after 10 days in snail embryos, whereas, in the *D. rerio* embryo toxicity test, exposure to ≥10 mg/L Ni^{2+} were necessary to induce delayed hatching (Kienle et al., 2008). Comparing the respective LOECs, the *M. cornuarietis* test thus was three orders of magnitude more sensitive than the established *D. rerio* test (Table 1). Also in respect to mortality, the MariETT assay exhibited a higher sensitivity than the established zebrafish embryo toxicity test: Dave and Xiu (1991) observed increased mortality of *D. rerio* at 360 µg/L Ni^{2+}, whereas, in the present study, 200 µg/L Ni^{2+}, were sufficient to cause 100% mortality in *M. cornuarietis*. The EC_{50} of nickel on the embryonic development of the blue mussel, *Mytilus trossolus*, was 150 µg/L Ni^{2+} (Nadella et al., 2008), whereas, in our study, its LOEC on the embryonic development on *M. cornuarietis* was found to be 10 µg/L Ni^{2+}. Several studies have shown the influence of nickel on many earthworm species and the EC_{50}, always was above 200 mg Ni/kg (Lock and Janssen, 2002; Maleri et al., 2007; Scott-Fordsmand et al., 1999). The effect of nickel exposure was also tested in another animal species. In amphibian embryos, *Bufo arenarum*, the 24 h LC_{50} and 168 h LC_{50} were 26 and 1.8 mg/L Ni^{2+}, respectively (Herkovits et al., 2000). Thus, the results from our study indicate that embryos of M. cornuarietis are very sensitive to nickel in comparison to other established test organisms.

Even though zinc is an essential structural, catalytic, and regulatory micronutrient for many enzymes and is critical for protein synthesis, growth, development, and reproduction (Vallee and Falchuk, 1993), the lethal concentration to *M. cornuarietis* was 5 mg/L Zn^{2+} at day 10 in our experiment. This value corresponds well to the 96 h LC_{50} in the marine copepod, *Tigriopus japonicus*, and in two freshwater crustaceans, *Atyaephyra desmarestii* and *Echinogammarus meridionalis*, which were 7.8, 5.43, and 4.61 mg/L Zn^{2+}, respectively (Lee et al., 2007; Pestana et al., 2007). At sublethal concentrations, of 0.2 to 2 mg/L Zn^{2+}, exposure resulted in a delayed development of the *M. cornuarietis* embryos, reflected by delayed formation of eyes and tentacles, a reduced heart rate, and a delay in hatching. The LOEC of zinc on the embryonic development of *M. cornuarietis* was 200 µg/L Zn^{2+}, whereas Senger et al. (2006) found a LOEC for *D. rerio* embryonic development of 0.5 mM (corresponding to 32.7 mg/L Zn^{2+}), more than 2 order of magnitude higher (Table. 1). Nickel LOECs given for other aquatic organisms also point out the very high sensitivity of *M. cornuarietis* embryos. In the fish ectoparasite, *Gyrodactylus turnbulli,* concentrations effects between 30 and 120 µg/ Zn^{2+} decreased the survival and reproduction (Gheorghiu et al., 2006). Franco et al. (2006; 2008) observed reduced glutathione reductase activity at 30 µM Zn (corresponding to 1.96 mg/L Zn^{2+}) in brown mussel, *Perna perna,* or at 10 µM (corresponding to .065 mg/L Zn^{2+}) in juvenile carp, *Cyprinus carpio*. Furthermore, the LOEC of zinc which caused abnormal shape larvae of *Perna perna* was 250 µg/L Zn^{2+} (Jorge and Moreira, 2005). Also other mussel species were reported to display a rather low sensitivity to zinc: according to Kraak et al. (1994), a LOEC of 382 µg/L Zn^{2+} affected the filtration rate of the zebra mussel *Dreissena polymorpha*, and mortality was observed just after exposure to high zinc concentrations (2758 µg/L). During the embryonic development of estuarine crab, *Chasmagnathus granulatus*, zinc concentrations of 10 mg/L or lower did not produce a significant mortality of females, nor a decrease in the number of hatched larvae nor a decrease in the egg incubation time. However, for concentrations of 10 mg/L Zn^{2+}, a significant number of ovigerous females with asynchronous hatching was noted (Lavolpe et al., 2004).

Although *M. cornuarietis* responded very sensitively to both test metals, our study showed that nickel was more toxic to *M. cornuarietis* than zinc.

The comparison of our *M. cornuarietis* LOEC data with corresponding literature data on zebrafish, embryotoxicity and invertebrate bioassays gives evidence for the remarkably high sensitivity of this novel MariETT assay. The difference between these two test species was at least one order of magnitude for the tested organic pesticides and up to three orders of magnitude for the tested metal cations.

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Chapter 2: The sensitivity of ramshorn snail, *Marisa cornuarietis*, embryos to the pesticides chlorpyrifos, flubendiamide, methiocarb, and Mesurol® snail pellets*

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In recent years, the contamination of the aquatic environment by pesticides is of concern throughout the world. Pesticides are among the broad range of organic pollutants that have ecological impact due to their sometimes excessive use in agriculture. Different categories of pesticides are known to exert different mode of action on living organisms and, in the case of contaminated waters, on aquatic organisms. Since the *Marisa cornuarietis* embryo toxicity test (MariETT) has been introduced some years ago by Schirling et al. (2006) it has to be regarded equally or even more sensitive to environment chemicals compared with other biotests. Particularly for some metals and insecticides, it has been shown that concentrations causing toxicity on *Marisa* embryos can be orders of magnitude lower than the effective concentrations in other biotests (Osterauer et al., 2009; Sawasdee and Köhler, 2009; Sawasdee and Köhler, 2010).

The purpose of this study was to broaden the dataset available for this rather new bioassay and hence to quantify the toxicity of selected pesticides with different modes of action: the organophosphorus insecticide chlorpyrifos, flubendiamide as a representative of a new class of phthalic diamide insecticides, and the carbamate methiocarb on the embryonic development of *Marisa cornuarietis* by monitoring the following endpoints: mortality, formation of tentacles and eyes, heart rate, hatching, and weight after hatching. This toxicological information is needed to further assess the degree of sensitivity of the *Marisa cornuarietis* embryo toxicity test (MariETT) incomparison to other established biotests.

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MATERIALS AND METHODS

The ramshorn snail, *Marisa cornuarietis* (Ampullariidae) strain used for egg production originated from a breeding stock of the Zoological Institute of Frankfurt/Main University, Germany. *M. cornuarietis* has a high egg production, a short spawning interval, and transparent eggs, which collectively make this species especially suitable as an organism for embryotoxicological studies. For our experiment, adult snails were maintained in 120L glass aquaria containing water with a conductivity of ≈ 800 µS/cm (based on tap water, conductivity adjusted with NaCl), a pH of ≈ 7.5, and a temperature of 24 ± 1 ^{o}C. The photoperiod was 12h:12h (light:dark). Aquaria were part of a circulation system with aerated and filtered water. The snails were fed commercial fish flake food (TetraMin, Tetra GmbH, Germany) or fresh vegetables once a day.

The development of the snail embryos from the day of egg laying until hatching was observed using a stereomicroscope. The following endpoints were observed: mortality (%), formation of tentacles and eyes (%), heart rate (min^{-1}), and hatching success (%). We also measured weight after hatching (mg wet wt.). *M. cornuarietis* deposits its eggs during the night in relatively large, soft, gelatinous egg masses which are usually attached to the side wall of the aquaria. Freshly laid egg masses were removed from the aquaria walls and divided with a razor blade. To exclude possible effect of genetic differences, 20 eggs (5 each from 4 randomly chosen egg masses deposited during the same night) were distributed into either control groups (with aquarium water) or exposure groups, and incubated at 27 ^{o}C for further investigations. For each treatment plus control, 9 replicate Petri dishes were used (n=9). The control water and the test solutions were renewed daily. The number of hatched individuals often differed from the number of eggs due to twin, triplet, or quad hatchlings. For the tests, mortality < 10% in the control was accepted as valid. Mortality was defined by coagulation of the embryo or cessation of the heart beat; this was recorded daily during the experiment. The formation of tentacles and eyes was observed from day 5 onwards, while the heart rate was recorded at day 9 only. Possible malformations of the embryos also were investigated. To measure snail fresh weight after hatching, five hatchlings were chosen randomly from each Petri dish, and transferred to the surface of soft paper tissue. When the adhesive water was removed completely from the hatchling, the five individuals were pooled, respectively, and weighed on an analytical balance (Kern, type 770).

Chlorpyrifos (Sigma-Aldrich, Germany) was dissolved by constant overnight stirring in de-ionized or double distilled water at a water temperature of 35°C. A stock solution (1 mg/L) was prepared in a glass bottle directly before every exchange of test media. The stock solution was diluted with the same water as used for animal stock maintainance to the final nominal concentrations of 100, 150, 200, and 350 µg/L. Flubendiamide (Sigma-Aldrich, Germany) was dissolved in the solvents dimethyl sulfoxide (DMSO) (Merck, Germany) in de-ionized or double distilled water for the flubendiamide stock solution (10 g/L). For the solvent control, DMSO was directly diluted in the same water as used for animal stock maintainance. The DMSO concentrations tested were 100 and 200 µl/L. The final nominal test concentrations of flubendiamide were 100 and 200 µg/L. Methiocarb (Sigma-Aldrich, Germany) was dissolved in de-ionized or double distilled water in a glass bottle. The stock solution (10 mg/L) was diluted with the same water as used for animal stock maintainance to the final nominal concentrations of 100, 250, and 500 µg/L. Methiocarb-containing Mesurol® snail pellets (Bayer CropScience, Germany) were crushed and dissolved with the same water as used for animal stock maintainance to the final nominal concentrations of one time application rate (0.5 g/m^2, ≅ 1270 µg/L methiocarb), three times application rate (1.5 g/m^2, ≅ 3810 µg/L methiocarb), and 10 times application rate (5 g/m^2, ≅ 12 700 µg/L methiocarb).

The software JMP® 7.0 (SAS) was used for statistical analysis. Normally distributed data (checked by Shapiro-Wilk's test) were analyzed using a Student's *t*-test to determine if there were significant ($p \leq 0.05$) differences among treatments. Data that were not normally distributed were analyzed using Wilcoxon's test. Significance here was tested at $p \leq 0.05$ as well.

RESULTS AND DISCUSSION

Toxic concentrations of chlorpyrifos to *M. cornuarietis* embryos ranged from 150 µg/L to 350 µg/L. Chlorpyrifos at 350 µg/L caused 100% mortality of snail embryos, whereas 100 µg/L did not induce significant changes on any investigated endpoint. Fig. 1(a) to (d) shows the reduction in the integrity of embryogenesis as indicated by the proxies percentage of alive embryos, as the formation of tentacles, the formation of eyes, hatching success, and weight after hatching. At days 5 and 6, embryos exposed to 150 µg/L chlorpyrifos showed a

significant delay in the formation of both tentacles and eyes. Chlorpyrifos in a concentration of 200 µg/L was found to induce significant changes on the weight of newly hatched animals. Our study showed clearly that chlorpyrifos dramatically increased mortality rates and affected embryonic development in *M. cornuarietis*. While chlorpyrifos at 150 µg/L induced adverse effects in the snail embryos already, embryogenesis of several fish has been shown to be less sensitive to chlorpyrifos: according to Kienle et al. (2009), zebrafish (*Danio rerio*) larvae exposed to chlorpyrifos showed a significant increase in the percentage of individuals with morphological deformations at 250 µg/L or higher concentrations. As well, the heart rate in the developing ambon damsel, *Pomacentrus amboinensis,* was significantly affected by chlorpyrifos concentrations at or above 500 µg/L (Humphrey et al, 2004), whereas chlorpyrifos at 350 µg/L already caused 100% mortality of snail embryos in this study. However, the lowest chlorpyrifos concentration that caused significant reduction in viable hatch and length in embryos of *P. amboinensis* was 125 µg/L (Humphrey et al, 2004), indicating a similar sensitivity to this endpoint and the MariETT assay. In respect to mortality, the MariETT assay exhibited a higher sensitivity than established fish tests (Varó et al., 2000; Humphrey et al, 2004). On the other hand, a few reports on highly sensitive endpoints do exist. In the eastern rainbow fish *Melanotaenia splendida splendida,* embryos exposed to chlorpyrifos at concentrations at or above 25 µg/L were significantly smaller in size than the control group at hatching; however, embryos exposed to chlorpyrifos at 12.5 and 6.25 µg/L showed no significant difference (Humphrey and Klumpp, 2003). In 96h juvenile toadfish *Opsanus beta,* 520 µg/L chlorpyrifos resulted in significant mortality (Clark et al. 1985). Furthermore, embryos of *M. cornuarietis* appear to be more sensitive to chlorpyrifos compared to the few other species for which data on embryos or other early life stages are available. On early larvae of the amphibian *Ambystoma mexicanum*, chlorpyrifos did not cause any lethal effect, even at the highest concentration tested (2.5 mg/L) (Robles-Mendoza et al., 2009), whereas the median lethal concentration (LC_{50}) calculated for larval *Rana boyii* was 3.0 mg/L (Sparling and Fellers, 2007). Regarding to the Frog Embryo Teratogenesis Assay-Xenopus (FETAX), the 96-h LC_{50} for premetamorphs of *Xenopus laevis* receiving a static dose of chlorpyrifos was 14.6 mg/L, and the EC_{50} associated with malformations was 1.71 mg/L (Richards and Kendall, 2002). Only the biochemical marker ChE activity, being indicative of the anticipated target action, neurotoxicity, responded to the rather low concentration of 10 µg/L chlorpyrifos in *Xenopus* embryos (Richards and

Kendall, 2002). Apart from this single biochemical parameter, the comparison between the present study and previous reports indicated a particular sensitivity of *M. cornuarietis* embryonic development to chlorpyrifos in comparison to other established test organisms.

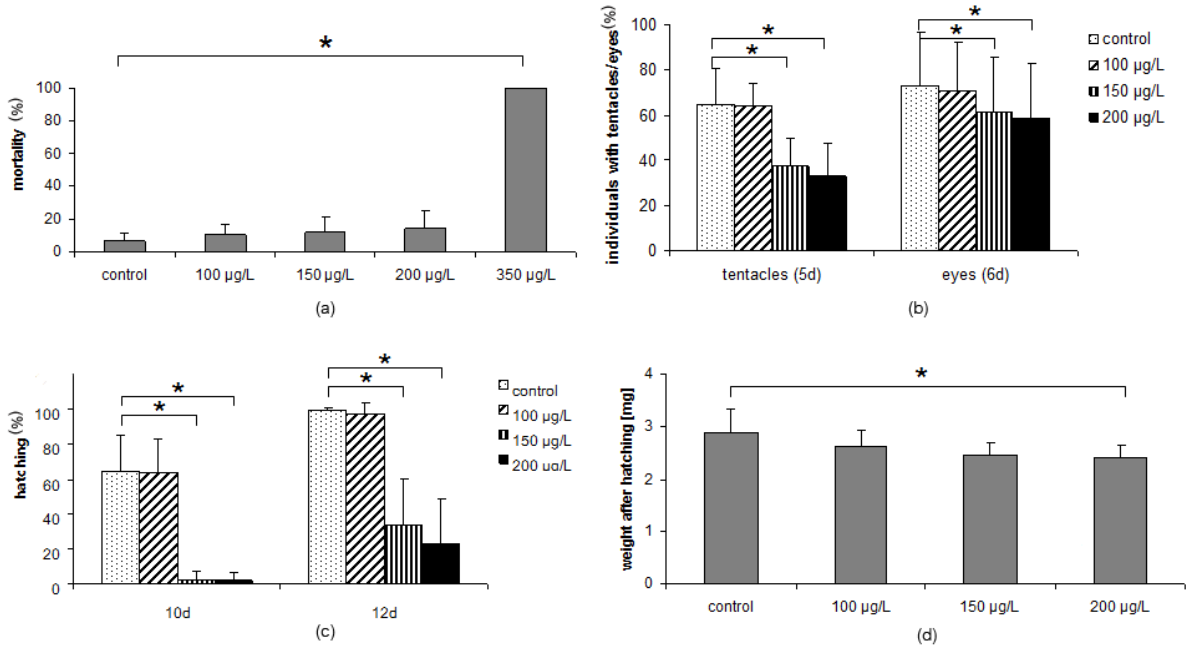

Fig. 1 Effects of chlorpyrifos on *M. cornuarietis* embryonic development, means ± SD. (a) Mortality (%); (b) formation of tentacles and eyes after 5, and 6 days exposure to chlorpyrifos (%); (c) hatched snails (%) after 10, and 12 days exposure to chlorpyrifos; (d) individual weight after hatching (mg). *Significant difference at $p \leq 0.05$ (Student's *t*-test).

Flubendiamide had no acute effect on snail embryos even at the limits of its water solubility. All investigated endpoints lacked significance. As well, the solvent DMSO did not affect any of the endpoints. According to Hall (2007), no statistical differences were detected in fish early life stage tests as well. Also invertebrates were reported to display a rather low sensitivity to flubendiamide. Based on the proposed use for flubendiamide, the US EPA did not assign any potential risk to freshwater and marine fish, marine crustaceans, marine mollusks and aquatic plants at the limit of solubility (US EPA, 2008). As described earlier, flubendiamide exerts extremely potent and broad spectrum activity within the insect order Lepidoptera (butterflies and moths), but no significant activity has been reported for insects outside this taxon (Tohnishi et al., 2005; Ebbinghaus et al., 2007; Hirooka et al., 2007; Lahm

et al., 2009). Although only very few published data are available to compare our *M. cornuarietis* test results with, the toxicological data in the literature and also those from the present study suggested a relatively low toxicity of flubendiamide to aquatic organisms.

Regarding methiocarb, no significant effects on *M. cornuarietis* embryos could be recorded for concentrations of 100 and 250 µg/L. In embryos exposed to 500 µg/L, the mortality was shown to significantly increase from 6% in the control to 14% in the 500 µg/L treatment group (Fig. 2a). Also a significant delay in the formation of tentacles was found: the percentage of individuals with developed tentacles at day 5 decreased from 95% in the control to 82% in the treatment group (Fig. 2b). The highest methiocarb concentration we tested (500 µg/L) affected the survival, and the formation of tentacles and eyes in *M. cornuarietis* embryos. Considering methiocarb concentrations of up to 100 µg/L in surface water from rice fields in The United States (Primus et al., 2001) and a safty factor of just 10, our study showed environmental relevance. In comparison to the early life stage toxicity study of Altinok et al. (2006) who reported concentrations of 4.82 to 5.43 mg/L methiocarb to kill 50% of juvenile rainbow trout (*Oncorhynchus mykiss*) within 24 to 96h we found *M. cornuarietis* to be about one order of magnitude more sensitive to methiocarb. Even in histopathological studies with *O. mykiss* exposed to 3.75 mg/L methiocarb, the only lesion observed was in the gill (Altinok and Capkin, 2007).

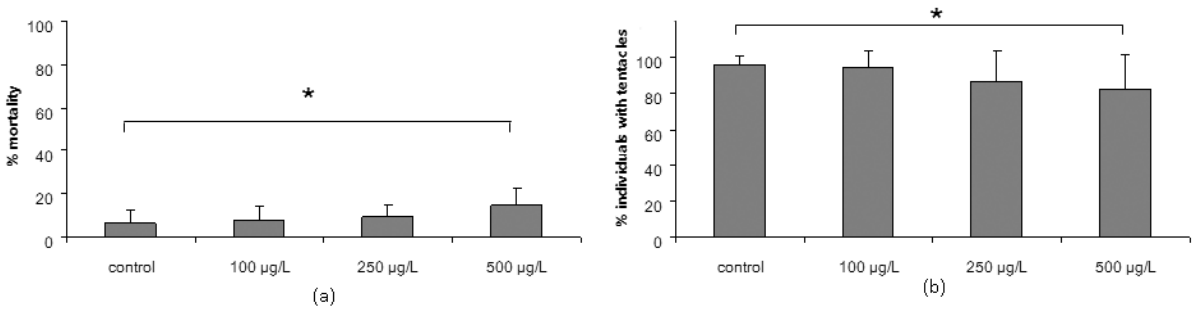

Fig. 2 Effects of methiocarb on *M. cornuarietis* embryonic development, means ± SD. (a) Mortality (%); (b) formation of tentacles and eyes after 5 days exposure to methiocarb (%). *Significant difference at $p \leq 0.05$ (Student's *t*-test).

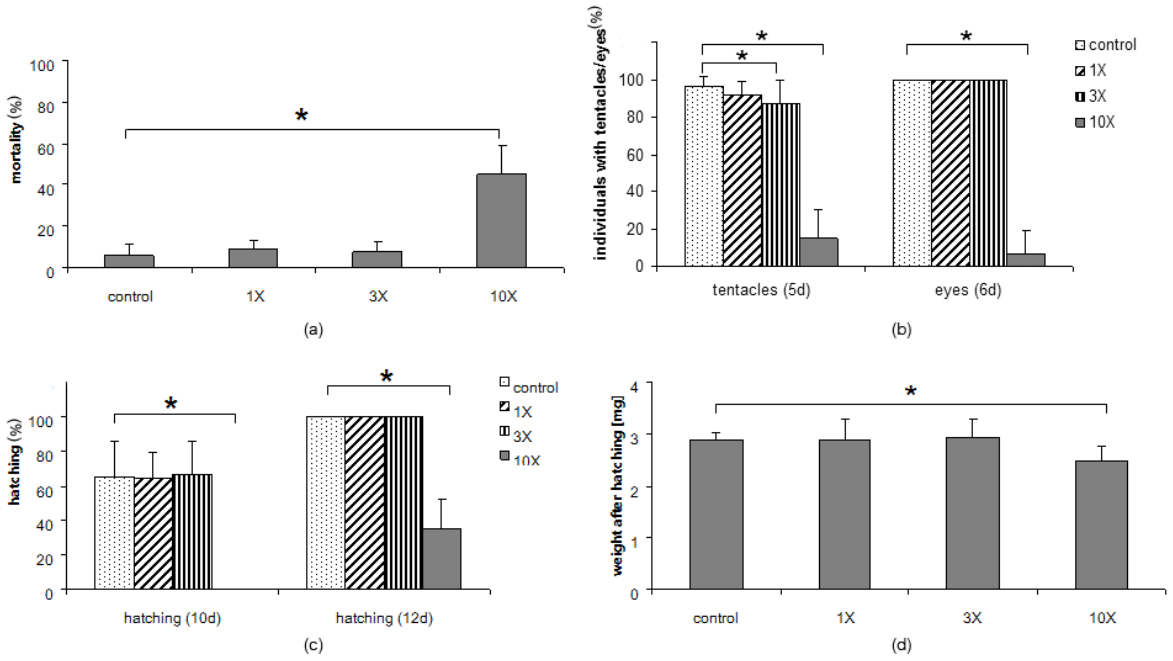

Fig. 3 Effects of Mesurol® snail pellets on *M. cornuarietis* embryonic development, means ± SD. (a) Mortality (%); (b) formation of tentacles and eyes after 5, and 6 days exposure to Mesurol® snail pellets (%); (c) hatched snails (%) after 10, and 12 days exposure to Mesurol® snail pellets; (d) individual weight after hatching (mg). *Significant difference at $p \leq 0.05$ (Student's *t*-test).

The concentration of Mesurol® at 1 time application rate (equivalent to 1270 µg/L methiocarb) did not reveal any significant changes regarding the development in comparison to the control group. Embryos exposed to Mesurol® at 3 times application rate (equivalent to 3810 µg/L methiocarb) showed a significant delay in the formation of tentacles only. Exposure to Mesurol® at 10 times application rate (equivalent to 12 700 µg/L methiocarb) resulted in a significant delay in the formation of tentacles and eyes, and increased mortality significantly. The hatchlings showed a trend to decreasing weight with increasing *Mesurol®* concentrations which was found to be significant in the 10 times application rate treatment versus the control (Fig. 3). In contrast to sole methiocarb, dissolved in water, Mesurol® snail pellets at 1 time application rate did not reveal any effects on *M. cornuarietis* embryos. It is reasonable that the methiocarb pellet formulation is less effective as a contact poison than after ingestion. Only limited information on effects of Mesurol® snail pellets on organisms has been published so far. Choo and Baker (1998) found a reduced earthworm weight when Mesurol® was applied at 10 times application rate. Similarly, in our study, *M. cornuarietis*

exposed at 10 times application rate showed significant effects on survival, the formation of tentacles and eyes, and weight after hatching.

In conclusion, this study showed the MariETT assay to be particular sensitive to selected pesticides, especially to chlorpyrifos and methiocarb, compared to the other embryo or early life stage testes with aquatic biota. As shown earlier for metal ions (Schirling et al., 2006; Sawasdee and Köhler, 2009), our data point out the suitability of the *M. cornuarietis* embryo as an invertebrate model for embryo toxicity testing.

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Chapter 3: Metal sensitivity of the embryonic development of the ramshorn snail *Marisa cornuarietis* (Prosobranchia)*

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Abstract

We investigated the effects of metal ions on the embryonic development of the ramshorn snail, *Marisa cornuarietis,* by exposing embryos to varying concentrations of copper (0, 50, 100, and 250 µg Cu^{2+}/L), lead (0, 5, 10, and 15 mg Pb^{2+}/L), lithium (0, 1, 2.5, and 3 mg Li^{+}/L), or palladium (0, 50, 100, 500 µg Pd^{2+}/L). Effects of these metals were examined by recording mortality, the rate of tentacles and eyes formation, heart rate, hatching success, and weight after hatching. Compared to the control, we found a significant delay in the formation of tentacles and eyes after treatment with 100 µg Cu^{2+}/L, 15 mg Pb^{2+}/L, 2.5 mg Li^{+}/L or 500 µg Pd^{2+}/L. The heart rate decreased significantly at 500 µg Pd^{2+}/L. At 10 mg Pb^{2+}/L, 2.5 mg Li^{+}/L, or 500 µg Pd^{2+}/L, hatching was delayed significantly; 50 µg Cu^{2+}/L induced a significantly earlier hatching, and reduced body weight. The LC_{50} values were calculated to be about 50 µg Cu^{2+}/L, 500 µg Pd^{2+}/L, 2500 µg Li^{+}/L, and 10000 µg Pb^{2+}/L. These results show that the embryonic development of *M. cornuarietis* is about as sensitive to copper and lithium, compared to the most sensitive fishes used in embryo toxicity testing. Even though the MariETT is a laboratory-based assay focusing on toxicological endpoints of a selected model species, future application is envisaged to include testing of "natural" samples such as stream water or sediment interstitial water.

Keywords: development, embryo toxicity test, metals*, Marisa cornuarietis*

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Introduction

Metals in industrial wastes are of concern because they persist in the environment and because many metals are toxic, at relatively low concentration. Metals such as copper, lead, and lithium can exert embryotoxic or teratogenic effects by affecting cellular physiology, either directly or indirectly. The biological effects of metals have been subject of many studies but, in most cases, the acute toxicity i.e. the lethal concentration, or LC_{50}, has been determined in adult or immature individuals over relatively short exposure durations (Martin and Holdich, 1984; Gopalakrishna Bhat and Vamsee 1993; Sorvari and Sillanpää, 1996; Macdonald et al., 2002; Ferrer et al., 2006; Khangarot and Das, 2009). However, to understand the sublethal effects of metal contaminants on animal health, their action should be studied during the main life cycle phases of an organism, especially during the first stages of development, which are generally considered to be particularly sensitive to toxic and teratogenic metals. Despite availability of large amounts of information on biological effects of metals, by far the highest number of studies has been conducted on model or economically important species (Hamilton 1995; Rougier et al., 1996; Clearwater et al., 2002; Grosell et al., 2006; Osman et al., 2007; Vieira et al., 2009; Yadav and Trivedi, 2009). Further, mortality of adults is the most commonly used parameter in such studies. This is justified by the advantage of obtaining an easily quantifiable answer within a short time. But there is still need for a better understanding of sublethal effects of metals on the various developmental stages of non-model taxa.

The ramshorn snail, *Marisa cornuarietis,* has been the focus of various ecotoxicological studies, and has been show to be sensitive to endocrine disrupting chemicals (Oehlmann et al., 2000; Schulte-Oehlmann et al., 2000; Schirling et al., 2006). In this context, a test based on the embryonic development of this prosobranch snail was developed by Schirling et al. (2006) and Showing a high sensitivity to various substances, this *Marisa* embryo toxicity test (MariETT) serves as a suitable alternative to the established *Danio rerio* embryo toxicity test (DarT) (Sawasdee and Köhler, 2009). Here, we use the MariETT assay to assess the toxicity of copper, lithium, lead, and palladium.

Materials and Method

Test organisms

The ramshorn snail *Marisa cornuarietis* (Prosobranchia, Ampullariidae) strain that we used for egg production originated from a breeding stock of the Zoological Institute in Frankfurt/Main, Germany (kindly donated by J. Oehlmann). *M. cornuarietis* has a short generation interval, a short spawning interval, and transparent eggs, which collectively make this species especially suitable as an organism for toxicological studies. Adult snails reach 40 to 50 mm in shell diameter; eggs are about 2 to 3 mm in diameter. During embryo development, the eggs swell up to 4 mm. They also become more transparent and the embryonic snail become visible as a spot inside the egg. Depending on the temperature, embryonic development until hatch takes from 8 to 20 days (about 12 days at 26 ^{o}C). The ontogeny was described in detail by Demian and Yousif (1973).

For our experiment, adult snails were maintained in 120-L glass aquaria containing water with a conductivity of ≈ 800 µS/cm (based on tap water, conductivity adjusted with NaCl), a pH of ≈ 7.5, and a temperature of 24 ± 1 ^{o}C. The photoperiod was 12h:12h (light:dark). Aquaria were part of a circulation system with aerated and filtered water. The snails were fed commercial fish flake food (TetraMin, Tetra GmbH, Germany) or fresh vegetables once a day.

Exposure conditions

The metals were applied as $CuCl_2$ (99%, Acros organics, Great Britain), $PbCl_2$ (≥ 98%, Fluka, Switzerland) or LiCl (≥ 99%, Fluka, USA) using stock solutions of 1 g/L Cu^{2+}/L, 1 g Pb^{2+}/L and 1 g Li^{+}/L in de-ionized or double distilled water. Palladium was applied as $PdCl_2$ standard solution (Sigma-Aldrich, Germany) using a stock solution of 1 mg Pd^{2+}/L in de-ionized or double distilled water. The test solutions were prepared by diluting the stock solutions with the same water as used for snail maintainance, at final nominal concentrations of 0, 50, 100 and 250 µg Cu^{2+}/L; 0, 5, 10, and 15 mg Pb^{2+}/L; 0, 1, 2,5 and 3 mg Li^{+}/L; and 0, 50, 100, 500 µg Pd^{2+}/L, respectively. All tests were performed in polystyrene Petri dishes (∅ 9 cm; ~50ml volume of test solution); the stock and test solutions were kept in polyethylene bottles. It

was assumed that the nominal concentrations in the present study were near the measured concentrations. Using an identical study design for metal toxicity testing (Kienle et al., 2009), reported that the detection rates for nickel were almost 100% of the nominal concentrations.

Embryo toxicity test

The development of the snail embryos from the day of egg laying until hatching was observed using a stereomicroscope. The following endpoints were observed: mortality (%), formation of tentacles and eyes (%), heart rate (min^{-1}), and hatching success (%). We also measured weight after hatching (mg wet wt.). *M. cornuarietis* deposits its eggs during the night in relatively large, soft, gelatinous egg masses which are usually attached to the side wall of the aquaria. Freshly laid egg masses were removed from the aquaria walls and divided with a razor blade. To exclude possible effect of genetic differences, 20 eggs (5 each from 4 randomly chosen egg masses deposited during the same night) were distributed into either control groups (with aquarium water) or exposure groups, and incubated at 27 ^{o}C for further investigations. For each treatment plus control, 9 replicate Petri dishes were used (n=9). The control water and the test solutions were renewed daily. The number of hatched individuals often differed from the number of eggs due to twin, triplet, or quad hatchlings. For the tests, mortality < 10% in the control was accepted as valid. Mortality was defined by coagulation of the embryo or cessation of the heart beat; it recorded daily during the experiment. The formation of tentacles and eyes was observed from day 5 onwards, while the heart rate was recorded at day 9 only. Possible malformations of the embryos also were investigated. To measure snail fresh weight after hatching, five hatchlings were chosen randomly, and transferred to the surface of soft paper tissue. When the adhesive water was removed completely from the hatchling, the five individuals were pooled and weighed on an analytical balance (Kern, type 770).

Statistical analysis

We used JMP® 4.0 (SAS) for statistical analysis. Normally distributed data (checked by Shapiro-Wilk's test) were tested for significance with Student's *t*-test, and data with non-normal distribution were tested with Wilcoxon's test. The α-level for significant differences was set at $p \leq 0.05$.

Results

All parameters which were significantly effected by a treatment were displayed in figures. These included mortality (Fig.1), tentacle formation (Fig.2), eye formation (Fig.3), heart rate (Fig.4), percentage of hatching (Fig.5), and weight after hatching (Fig.6).

Copper treatment

After 10 days of exposure to 50 or 100 µg Cu^{2+}/L, a trend to preponed hatching was evident in the copper-exposed snails, and a significant increase of the hatching rate occurred 10 d at 100 µg/L (Fig. 5a). Concomitantly, a significant decrease in hatchling weight was found after Cu exposure, even at 50 µg/L (Fig. 6a). At days 5 and 6, embryos exposed to 100 µg Cu^{2+}/L showed a significant delay in tentacle and eye formation (Fig. 2a, 3a). Copper at a concentration of 100 µg/L, resulted in 100% mortality immediately after the snails had hatched. A copper concentration of 250 µg Cu^{2+}/L caused extreme effects in snail embryo development. Tentacles and eyes were not formed during the usual time of development (Fig. 2a, 3a) and 100% of the embryos died within 10 d. (Fig. 1a). However, none of the copper treatments caused any significant changes in heart rate of snails, compared to snails the control group.

Palladium treatment

Embryos exposed to 500 µg Pd^{2+}/L had a significant delay in the formation of both tentacles and eyes: the percentage of individuals with developed tentacles at day 5 decreased from 80% in the control to 44% in the treatment group (Fig. 2b). A similar trend was observed for the formation of eyes at day 6 (71% in the control *vs*. 55% in the treatment group; Fig. 3b). Also the heart rate was shown to significantly decrease from 78 beats/min in the control to 68 beats/min in the 500 µg Pd^{2+}/L treatment (Fig. 4). At day 12, more animals (97%) hatched in the control than in the 500 µg Pd^{2+}/L group (86%; Fig. 5b). However, palladium concentrations from 50-500 µg Pd^{2+}/L did not significantly affected mortality or weight of newly hatched snails.

Lithium treatment

At 2.5 mg Li^{+}/L, mortality (23%) was higher compared to mortality in the control group (10%) (Fig. 1b). The number of snail embryos with developed tentacles and eyes at days 5 and 6, respectively, was significantly reduced at 2.5 mg Li^{+}/L (9% for tentacles and 23% for eyes) compared to the control group (100% for both tentacles and eyes) (Fig. 2c, 3c). However, no differences in heart rate was found among the five groups. Significantly more animals hatched in the control (70%) than in the 2.5 or 3 mg Li^{+}/L treatment (56% and 9%, respectively; Fig. 5c) and the weight of the hatchlings was reduced by a Li concentration of 3 mg Li^{+}/L (Fig. 6b).

Lead treatment

At day 5, embryos in the 15 mg Pb^{2+}/L group showed a significantly delayed development in comparison to the control by a reduced mean percentage of visible tentacles (98% in the control *vs*. 13% in the treatment group, Fig. 2d). The formation of eyes at day 6 also was reduced, from 99% in the control to 2% in the 15 mg Pb^{2+}/L treatment group (Fig. 3d). At 10 mg Pb^{2+}/L, a significant early hatching was found: 42% of the snails hatched in the control

and 65% in the treatment group (Fig. 5d). No effect of lead was found on the heart rate or on the fresh weight of hatchlings was observed.

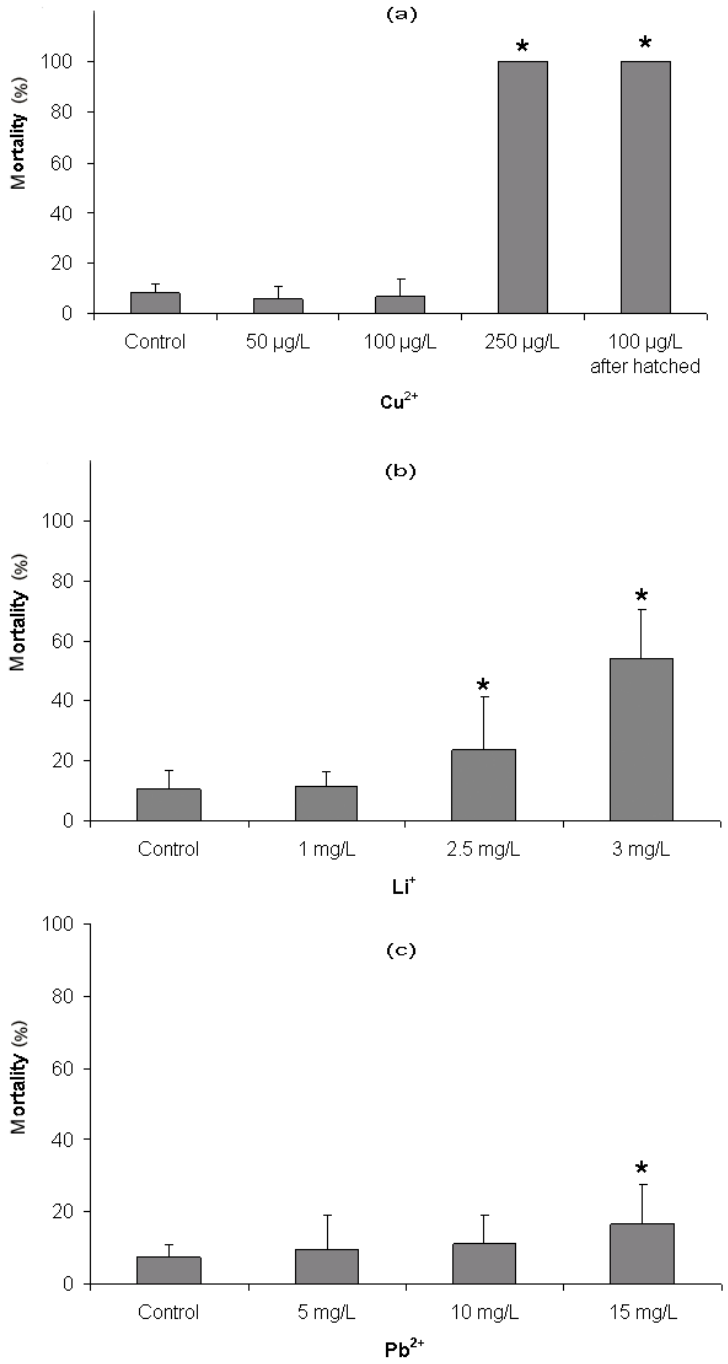

Fig.1 Mortality of *M. cornuarietis* embryos exposed to varying concentrations of Cu, Li, and Pb. Data are means (± SD) for (a) Cu^{2+}; (b) Li^{+}; and (c) Pb^{2+}. Asterisks designate differences where $p \leq 0.05$ (Student's *t*-test).

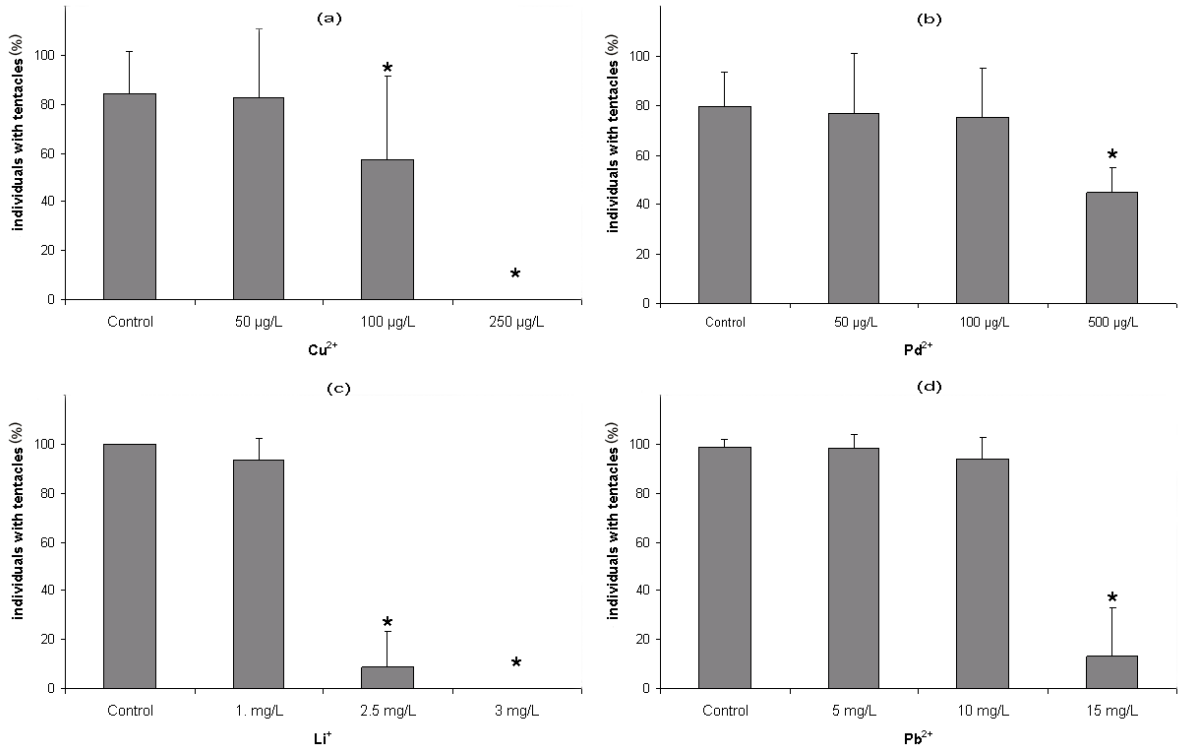

Fig. 2 Effects of metals on the formation of tentacles in *M. cornuarietis* (at day 5) during embryonic development. Data are means (± SD) for (a) Cu^{2+}; (b) Pd^{2+}; (c) Li^{+}; (d) Pb^{2+}. Asterisks designate differences where $p \leq 0.05$ (Student's *t*-test).

Discussion

Previous studies have shown that *Marisa cornuarietis* provides opportunity to follow embryo development, from three to four days after fertilization until hatching. Thus, the *M. cornuarietis* test permits investigators to easily track sublethal effects caused by exposures to various chemicals (Schirling et al., 2006).

A copper concentration of 50 µg/L caused earlier hatching and reduced the weights of newly hatched snails. The effect of Cu on snail weight may be due to the inhibition of Cu on metabolic rate. MacInnes and Thurberg (1973) reported that Cu reduced oxygen consumption rate of mud snails. *Marisa cornuarietis* with a LOEC of 50 µg Cu^{2+}/L, is slightly more sensitive than many other aquatic species (Table 1). In the mussel, *Anodontites trapesialis*, Cu concentrations from 60 to 2000 µg Cu^{2+}/L did not significantly affected

mortality (Loayza-Muro and Elías-Letts, 2007), whereas, in the present study, copper at a concentration 100 µg Cu^{2+}/L already resulted in 100% mortality after hatching in *M. cornuarietis* embryos. In the only other available study on snails (test species: garden snail *Helix aspersa*) no relationship between the mortality of juveniles or adults and maximum Cu concentrations of 1250 µg/g (on a dry mass basis) was detected (Laskowski and Hopkin, 1996b). An embryo toxicity test with the zebrafish, *Danio rerio*, resulted in a LOEC of 50 µg Cu^{2+}/L (Rougier et al., 1995, endpoint: listerial infection).

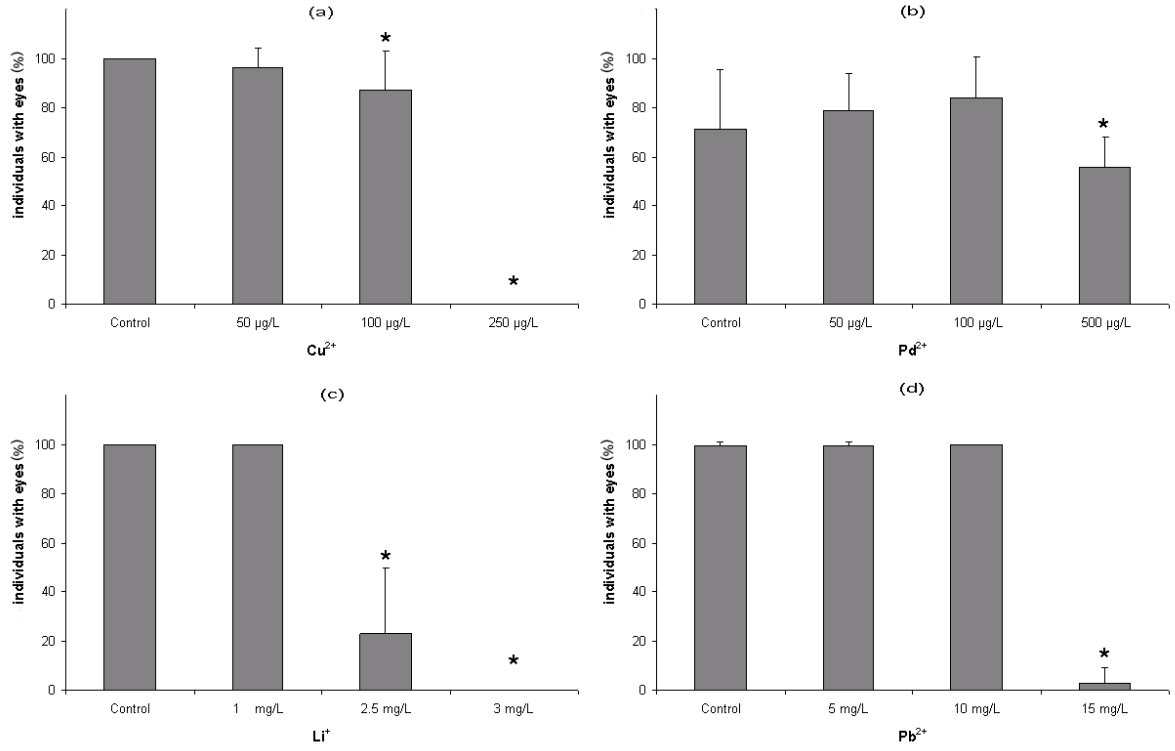

Fig. 3 Effects of metals on the formation of eyes in *M. cornuarietis* (at day 6) during embryonic development. Data are means (± SD) for (a) Cu^{2+}; (b) Pd^{2+}; (c) Li^{+}; (d) Pb^{2+}. Asterisks designate differences where $p \leq 0.05$ (Student's *t*-test).

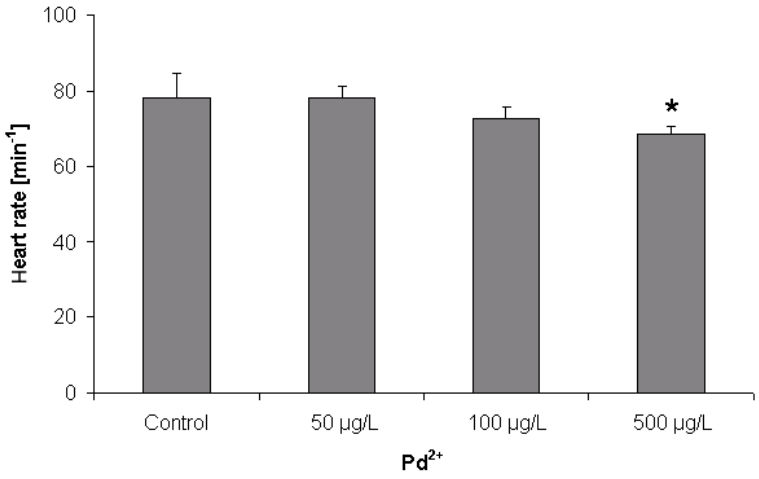

Fig. 4 Effects of Pd on the heart rate [min^{-1}] of *M. cornuarietis* embryos development. Data are means (± SD). Asterisks designate differences where $p \leq 0.05$ (Student's *t*-test).

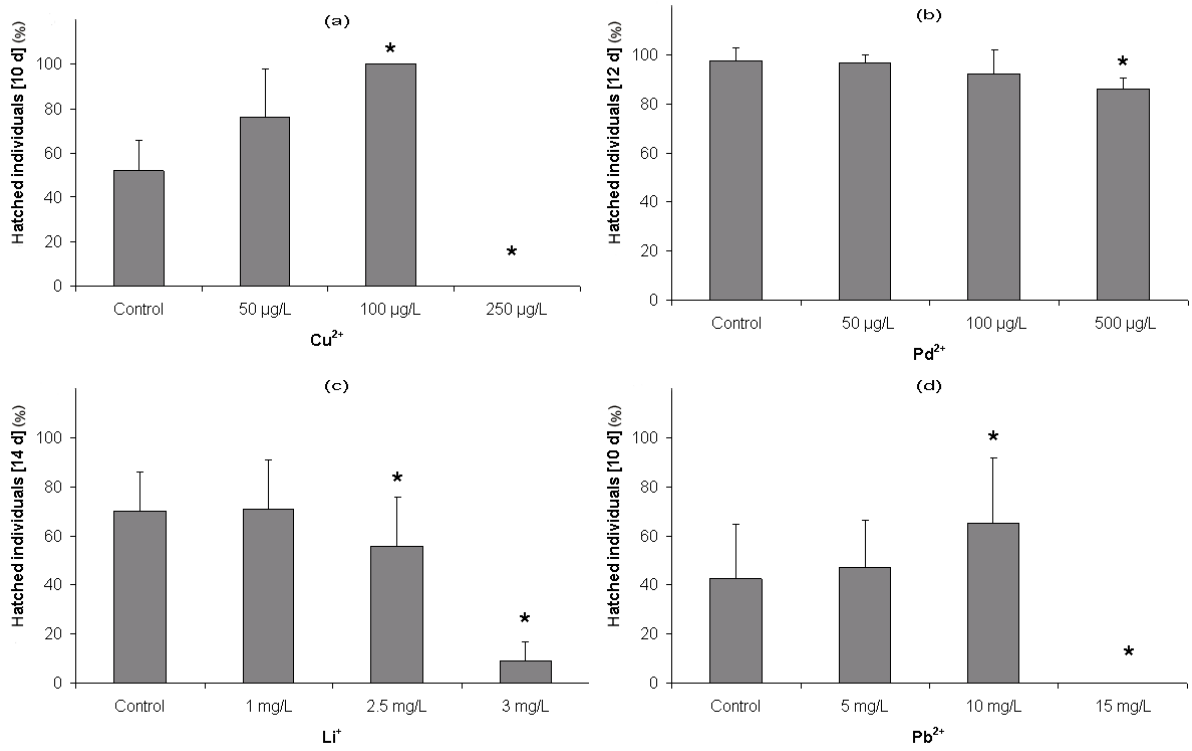

Fig. 5 Effects of Cu, Pd, Li, and Pb on *M. cornuarietis* embryonic development. Data are means (± SD) for (a) hatched snails (%) after 10 days exposed to Cu^{2+}; (b) hatched snails(%) after 12 days exposure to Pd^{2+}; (c) hatched snails (%) after 14 days exposure to Li^{+}; (a) hatched snails (%) after 10 days exposure to Pb^{2+}. Asterisks designate differences where $p \leq 0.05$ (Student's *t*-test).

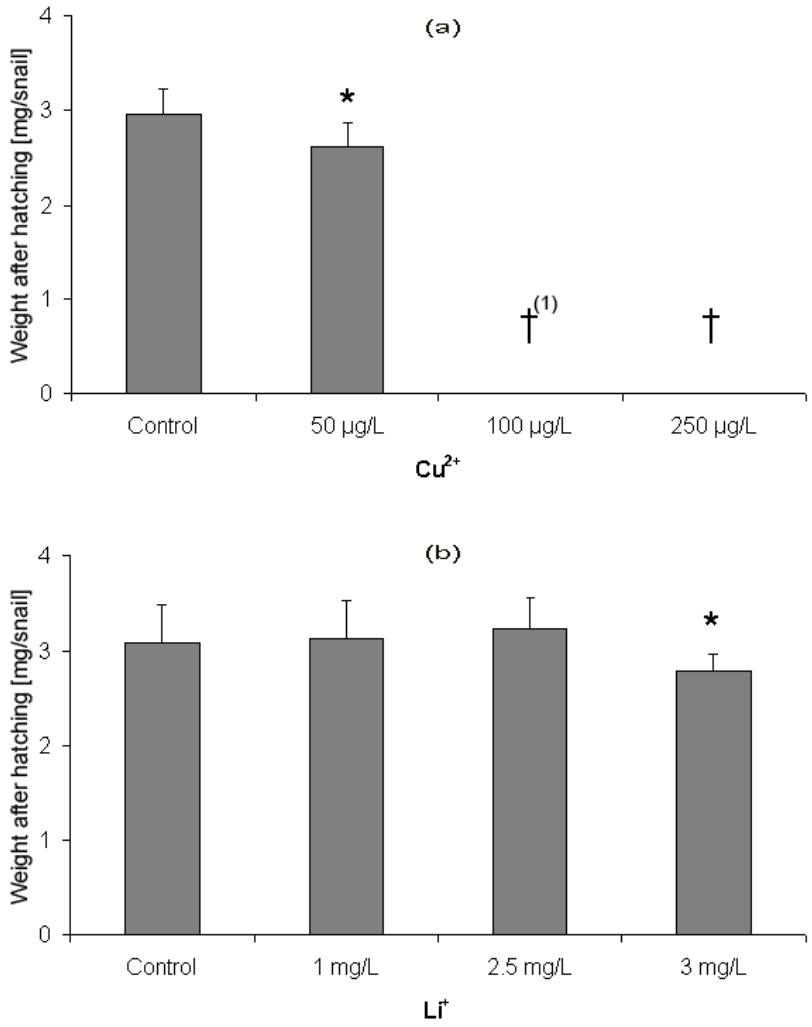

Fig. 6 Individual weight of snails (*M. cornuarietis*) after hatching (mg). Data are means (± SD) for (a) Cu^{2+}; (b) Li^{+}. Asterisks designate differences where $p \leq 0.05$ (Student's *t*-test). (1) At 100 µg Cu^{2+}/L, all embryos hatched but died (and disintegrated) shortly after hatching.

Palladium, along with platinum, rhodium, ruthenium, iridium and osmium form a group of elements referred to as the platinum group metals (PGMs). Platinum group elements are noble metals, which belong to the rarest elements in the upper earth crust. The largest part of PGE emissions can be attributed to automobile traffic due to the use of PGE in catalytic converters (Hoppstock and Sures, 2004). Among the PGM, Pd seems to be the most important metal in terms of its mobility, biological availability and its toxicological effects (Sures et al., 2001, 2006; Zimmermann and Sures, 2006).

Palladium occurs at very low concentrations (<1 µg/kg) in the Earth's crust, and concentrations of palladium in surface water generally range from about 0.4 to 22 ng/L in fresh water (WHO, 2002). The effects of palladium on aquatic organisms were established so far (Table 2). Because of the clear effects of Pd on the embryonic development of *M. cornuarietis* in this study, the MariETT may be suitable assay for PGE contaminant in organisms. Although the LOEC of Pd on snail embryos in this study (500 µg Pd^{2+}/L) is still above environmental Pd concentration, the chronic effects due to chronic Pd exposure at contaminated sites such as sites which receive road runoff and even chronically damaging effects in organisms with high accumulation levels cannot be excluded.

Table 1 Effect of Cu^{2+} on the embryonic development of *M. cornuarietis* in the MariETT assay, plus corresponding data for established organisms (obtained from the literature).

Organisms	Concentrations (µg/L)	Endpoints	References
Perna viridis	25	oxygen consumption	Krishnakumar et al., 1999
Marisa cornuarietis	50	hatching time and weight after hatching	this study
	100	mortality (100%)	this study
Anodontites trapesialis	64	filtration rate	Loayza-Muro and Elias-Letts, 2007
Utterbackia imbecillis	520	LC_{50}	Milam et al., 2005
Gammarus aequicauda	820	LC_{50}	Prato et al., 2006
Corophium insidiosum	470	LC_{50}	Prato et al., 2006
Sphaeroma serratum	4600	LC_{50}	Prato et al., 2006
Idotea baltica	1450	LC_{50}	Prato et al., 2006

Concentrations of lithium in surface water are typically low (1-100 µg/L; Martinez and Carrillo-Rivera, 2006; Rubio-Arias et al., 2007). Lithium is a classic tool for the study of phosphoinositide-dependent cellular process. It also is teratogenic (Berridge et al., 1989) and known to respecify the dorsoventral axis in zebrafish (Stachel et al., 1993). A review of the lithium resources, distribution, and toxicity to aquatic biota is provided in Kszos and Stewart (2003).

Table 2 Effect of Pd^{2+} on the embryonic development of *M. cornuarietis* in the MariETT assay, plus corresponding data for established organisms (obtained from the literature).

Organisms	Concentrations (µg/L)	Endpoints	References
Dreissena polymorpha	5	Metallothionein response	Frank et al., 2008
Tubifex tubifex	90	96-h LC_{50}	Khangarot, 1991
Oncorhynchus mykiss	190	96-h LC_{50}	WHO, 2002
Oncorhynchus mykiss	200	pigmentation	WHO, 2002
Dreissena polymorpha	500	induction of hsp70	Singer et al., 2005
Marisa cornuarietis	500	heart rate and formation of eyes and tentacles	this study
Oryzias latipes	4200	24-h lethal concentration	Doudoroff and Katz, 1953

The results of our study show that lithium is toxic to embryos of *M. cornuarietis* at concentrations in the range of 2.5-3 mg/L. However, not much attention has been paid to the effects of lithium on embryonic development, and very few published data are available with which to compare our *M. cornuarietis* test results. According to Dwyer et al. (1992), tested striped bass, *Morone saxatilis* found the 96-hr LC50 was >105 mg/L. On the other hand, comparing our results with those available in the literature, we can see that the sensitivity of *M. cornuarietis* to lithium seems to be lower than embryo of the fathead minnow, *Pimephales promelas* and the freshwater snail *Elimia clavaeformi* (Table 3).

Table 3 Effect of Li^{+} on the embryonic development of *M. cornuarietis* in the MariETT assay, plus corresponding data for established organisms (obtained from the literature).

Organisms	Concentrations (mg/L)	Endpoints	References
Elimia clavaeformi	0.15	consumption rate	Kszos et al., 2003
Pimephales promelas	0.89	movement	Long et al.,1998
Pimephales promelas	1.43	LC_{50}	Long et al.,1998
Marisa cornuariets	2.5	formation of eyes and tentacles	this study
Morone saxatilis	105	96-h LC_{50}	Dwyer et al., 1992

Lead does not bioconcentrate significantly in fish but does in some shellfish, such as mussels (US EPA, 2009). Some information is available on the effects of lead in fish embryos and larvae (Buhl, 1997; Jezierska et al., 2000; Osman et al., 2007). However, relatively few studies investigated effects on the embryonic development of freshwater snails. In the present study, lead exposure reduced the mean percentage of visible tentacles and eyes after exposure to 15 mg Pb^{2+}/L, and resulted in an increased hatching rate of *M. cornuarietis*, from 46% in the control to 65% in the group exposed to 10 mg Pb^{2+}/L. Concomitantly, Gomot (1998) and Ansaldo et al. (2009) pointed out that the reduction in hatching success was correlated with the harmful effect of metals on embryonic development in freshwater gastropod snails, *Lymnaea stagnalis* and *Biomphalaria glabrata*. The effects of lead on different aquatic organisms were investigated by several authors, including Beeby and Richmond (2001); Beeby et al. (2002); and Pyatt et al. (2002) (Table 4).

Table 4 Effect of Pb^{2+} on the embryonic development of *M. cornuarietis* in the MariETT assay, plus corresponding data for established organisms (obtained from the literature).

Organisms	Concentrations	Endpoints	References
Clarias gariepinus	500 µg/L	hatching	Osman et al., 2007
Helix aspersa	500 µg/g	juvenile shell mass	Beeby et al., 2002
Lymnaea stagnalis	5 mg/L	behavioural activities	Pyatt et al., 2002
Marisa cornuarietis	10 mg/L	hatching	this study
Ptychocheilus lucius	>170 mg/L	no effects on early life stages	Buhl, 1997
Gila elegans	>170 mg/L	no effects on early life stages	Buhl, 1997
Xyrauchen texanus	>170 mg/L	no effects on early life stages	Buhl, 1997

Due to limitations in data availability, it is hard to compare the sensitivity of embryogenesis in *M. cornuarietis* and the established model organism, the zebrafish *Danio rerio*. While *M. cornuarietis* had a LOEC of 10 mg Pb^{2+}/L for the endpoint hatching rate, the 96-h LC_{50} in *D. rerio* was 474.1 mg Pb^{2+}/L (Labrot et al., 1999). However, at the highest lead concentrations applied in this study (15 mg Pb^{2+}/L) *M. cornuarietis* did not show significant effects on survival. For the reason of environmental relevance (< 0.5 mg/L) (Stansley et al., 1992;

Srinivasa Gowd and Govil, 2008), we refrained from further increasing lead concentrations in our experiment and, hence, we cannot compare lethality between both species.

Even though all experiments were conducted at constant temperature, the hatching success in the controls set up for the different experiments showed some minor variation, perhaps due to seasonal variability (June-July for Pd experiments, September-October for Pb and Cu, and December for Li). To account for this variation, LOEC data always are given relative to the respective controls.

Table 5 Lowest observed effect concentrations (LOECs) of the tested metals on the embryonic development of *M. cornuarietis* in the MariETT assay, plus corresponding LOEC data for fishes (obtained from the literature).

Metals	*Marisa cornuarietis*		*Fishes*		
	LOEC (µg /L)	Endpoint	LOEC (µg /L)	*Species*	References
Cu^{2+}	50	hatching time and weight after hatching	50	*Danio rerio* 2- 4 mm	Rougier et al. 1995
Pd^{2+}	500	heart rate and formation of eyes and tentacles	200	*Oncorhynchus mykiss*	WHO, 2002
Li^{+}	2500	formation of eyes and tentacles	5400	*Pimephales promelas* embryo	Long et al. 1998
Pb^{2+}	10000	hatching time	300	*Clarias gariepinus* embryo	Osman et al. 2007

Our results indicate that the MariETT assay is sensitive to copper and lithium compared to data reported for fish species (Table.5). Table 6 summarizes MariETT metal toxicity data recorded so far. According to this ranking of MariETT LOEC data, the least toxicity of all metals tested so far can be attributed to lead, which is in accordance with other studies which have compared metal toxicity on invertebrate performance (Knigge & Köhler, 2000). However, further research is needed for a fully comprehensive assessment of metal impact on the early development of *M. cornuarietis.*

Even though the MariETT is a laboratory-based assay focusing on toxicological endpoints of a selected model species, future application is envisaged to include testing of "natural" samples such as stream water or sediment interstitial water. Similar to the zebrafish, *Danio rerio* embryo toxicity test (DarT) which also has started as a pure toxicological test with

single substances but now is even used for natural sediment assessment (Hollert et al., 2003; Hallare et al., 2005a; Hallare et al., 2005b; Wu et al., 2010), we see a great potential of the MariETT for ecotoxicological application in the future.

Table 6 Lowest observed effect concentrations (LOECs) of the metals on embryonic development of *M. cornuarietis*.

Metals	LOECs (µg /L)	Endpoints*	References
Pt^{2+}	0.1	c	Osterauer et al., 2009
Ni^{2+}	10	d	Sawasdee and Köhler, 2009
Cu^{2+}	50	d	this study
Zn^{2+}	200	a,b	Sawasdee and Köhler, 2009
Cd^{2+}	250	d	Schirling et al., 2006
Pd^{2+}	500	a,b,c,d	this study
Li^{+}	2500	a,b,d,e,f	this study
Pb^{2+}	10000	d	this study

* (a) formation of tentacles; (b) formation of eyes; (c) heart rate; (d) hatching success; (e) mortality; and (f) weight after hatching.

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Chapter 4: Turning snails into slugs: induced body plan changes and formation of an internal shell*

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Abstact

The archetypal body plan of conchiferan molluscs is characterized by an external calcareous shell, though internalization of shells has evolved independently in a number of molluscan clades, including gastropod families. In gastropods, the developmental process of torsion is regarded as a hallmark that is associated with a new anatomical configuration. This configuration is present in extant prosobranch gastropod species, which predominantly bear external shells. Here, we show that short-term exposure to platinum during development uncouples at least two of the processes associated with torsion of the freshwater snail *Marisa cornuarietis*. That is, the anus of the treated snails is located anteriorly, but the gill and the designated mantle tissue remains in a posterior location, thus preventing the formation of an external shell. In contrast to the prosobranchian archetype, platinum treatment results in the formation of a posterior gill and a cone-shaped internal shell, which persists across the lifetime. This first finding of artificially induced snail-slug conversion was also seen in the pulmonate snail *Planorbarius corneus* and demonstrates that selective alteration of embryonic key processes can result in fundamental changes of an existing body plan and – if altered regulation is inherited – may give rise to a new one.

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Introduction

It is commonly assumed that the first mollusc-like animals in the early Cambrian were shell-less and only protected by a cuticle with aragonitic spicules or scales (Scheltema and Schander, 2006). Later a calcified shell evolved in the conchiferan clade, which includes the extant Tryblidiida, Gastropoda, Bivalvia, Scaphopoda, and Cephalopoda. Secondary reduction and internalization of the shell has evolved repeatedly and independently in several gastropod taxa (Vitrinidae, Arionidae, Limacidae, Nudibranchia, Velutinidae, Titiscaniidae, Fissurellidae), notably in the cephalopods (except for *Nautilus*), and most likely for greater motility (Furbish and Furbish, 1984). The ontogeny of internal shells has been described in detail for a selection of gastropod species (Furbish and Furbish, 1984; Page, 2000). Even though mechanisms of shell internalization vary between different evolutionary lines, always the interactions between mantle and shell growth have been shown to be modified in the early individual ontogeny.

Extant species of the paraphyletic and globally distributed group of prosobranch gastropods (nonheterobranch gastropods) mostly develop an outer shell (except for, e.g., a few species of Velutinidae, Titiscaniidae, Fissurellidae). Their ontogeny also includes the process of torsion, which is crucial for the anatomical configuration of these snails. Torsion is defined as a process in gastropod ontogenesis that rotates the visceral body 180° relative to the larval headfoot region. As a consequence the digestive tract is U-shaped and the anus is located anteriorly, the mantle cavity is located anteriorly over the back of the head, and the gills are located anteriorly in front of the heart. This process is the key character defining the gastropod class.

It is generally thought that torsion involves a counterclockwise simultaneous movement of the outgrowing mantle, the shell, and the visceral sac, and – following Ernst Haeckel's theory of ontogeny recapitulating phylogeny – that this developmental process recapitulates evolutionary events at the rise of prosobranch gastropods.

In growing snails, the mineralization of the teleoconch takes place at the peripheral edge of the mantle fold and over the entire inner surface of the shell in order to increase shell thickness. Usually, invagination of the dorsal epithelium first occurs to form the shell gland, followed by shell field evagination and the migration of shell material-secreting cells toward

the mantle edge (Kniprath, 1981; Waller, 1981). The shell gland originates at the aboral end of the embryo, later shifts to the left and forms the mantle (Demian and Yousif, 1973a). The helical growth of the shell occurs in parallel to the onset of the counterclockwise movement of the outgrowing mantle due to the process of torsion. The mantle overgrows the visceral sac in the right dorso-lateral direction and opens to the anterior, forming the mantle cavity sheltering the organs such as the gill(s).

The mollusc shell consists of an organic matrix and calcium carbonate ($CaCO_3$), which is formed from calcium and bicarbonate ions present in the extrapallidal fluid (Rousseau et al., 2003). Catalyzing the reaction $CO_2 + H_2O \leftrightarrow HCO_3^- + H^+$ and being responsible for reversible hydration of CO_2, the enzyme carbonic anhydrase (CA) is associated with tissues involved in calcification and mineralization processes such as the formation of the embryonic shell (Costlow, 1959; Maren, 1967; Wilbur and Saleuddin, 1983; Takaichi et al., 2003).

The course of anatomical modifications during embryonic development of the prosobranch freshwater snail, *Marisa cornuarietis* (Ampullariidae), is well documented (Demian and Yousif, 1973a–d, 1975). Earlier studies revealed its embryos to be particularly sensitive to metals (Schirling et al., 2006; Sawasdee and Köhler, 2009). Because of increasing importance of platinum group elements in ecotoxicology resulting from their use in automobile catalytic converters, we tested the effect of platinum (Pt) on the embryogenesis of this gonochoric species. Because we found that exposure to Pt separates the process of torsion from mantle cavity formation and therefore prevents the formation of an external shell in *M. cornuarietis* we used different methological approaches to study this effect. To investigate the variability of this effect we also tested other metals like the physicochemical similar element palladium (Pd) and the alkaline metal lithium (Li) on embryos of *M. cornuarietis*. Furthermore, we also tested the effect of Pt on the pulmonate snail *Planorbarius corneus* to compare effects on different snail species.

Materials and methods

Test animals

Test animals used in the present study were the freshwater snails *M. cornuarietis* (Ampullariidae, prosobranch gastropod) and *P. corneus* (Planorbidae, pulmonate). Origin and maintenance of the lab stock culture of the gonochoric species *M. cornuarietis* were described in Osterauer et al. (2009). The breeding stock of the hermaphroditic snail *P. corneus* was gathered in a pond near Tübingen. *P. corneus* were kept in 30 L aquaria containing oxygenized tap water in the following conditions: temperature: 20 ± 1 °C, pH: 8, conductivity: 800 µS/cm, and 12 h/12 h light/dark regime. To ensure optimal water quality, the water of the aquaria was exchanged every week. The snails were fed once a day with commercially available artificial diet (Nutrafin Max flakes, Hagen, Germany) and casually with fresh carrots or lettuce.

Exposure experiments

Test substances used in the present study were $PtCl_2$ (Ultra Scientific, Wesel, Germany), $PdCl_2$ (Sigma-Aldrich, München, Germany), LiCl (≥ 99%, Fluka, Buchs, Switzerland), and, in combination with $PtCl_2$, $CaCl_2$ (Merck, Darmstadt, Germany). For both, exposure and pulse experiments, single eggs were separated from the egg masses laid during the night and distributed to Petri dishes containing the respective substance or the control medium which was tap water taken from the snail aquaria. The described effects occurred independently of using either tap/aquaria water or reconstituted water after the OECD Test Guideline 203 (1992), modified for *M. cornuarietis*, as a solvent. Concentrations used for chronic exposure (from the day of fertilization until hatch) were 100 and 200 µg/L $PtCl_2$, 50, 100, and 500 µg/L $PdCl_2$, 2.5 and 3 mg/L LiCl for *M. cornuarietis*, and 300, 400, and 500 µg/L $PtCl_2$ for *P. corneus*. For the pulse exposure experiments, *M. cornuarietis* eggs were exposed to 200 µg/L $PtCl_2$ for either 1 (at days 3, 4, 5, or 6 postfertilization) or 2 days (at days 3+4, 4+5, 5+6, 6+7, or 7+8 postfertilization) and subsequently returned to tap/aquaria water again. Furthermore, binary combinations of $PtCl_2$ (200 µg/L) and $CaCl_2 \times 2H_2O$ (0.54 g/L) in which Pt^{2+} and Ca^{2+} concentrations were equimolar, were tested on *M. cornuarietis* (exposure

from the day of fertilization until hatch). Negative controls were exposed to tap/aquaria water, positive controls were exposed to 200 µg/L $PtCl_2$ during the whole study.

For all experiments, exposure media were exchanged daily. Throughout the exposure period, embryos were kept at 26 °C in a climate chamber and were only removed for monitoring their development using a stereomicroscope. The numbers of snails without external shell were counted at day 9 postfertilization (dpf) in chronic exposure experiments and at day 11 postfertilization in pulse experiments. To study the postembryonic development of *M. cornuarietis*, hatched snails either were continuously exposed to 100 or 200 µg/L $PtCl_2$ or 200 µg/L $PtCl_2$ plus 0.54 g/L $CaCl_2 \times 2H_2O$ or returned to tap/aquaria water but, in any case, hatched snails were fed with equal portions of commercially available artificial diet (Nutrafin Max, Hagen, Germany) ad libitum once a day. The chronic exposure experiments with *M. cornuarietis* embryos and Pt, Pd, and Li were conducted with nine replicate groups (à 20 individuals), the pulse experiments and the exposure experiments with Pt plus Ca with four replicate groups (except for control and Pt-control in the first run of the 2-day pulses with eight replicate groups) (à 20 individuals) and the exposure experiments with *P. corneus* with two replicate groups at 300 µg/L $PtCl_2$ (à 13 individuals) and at 400 µg/L $PtCl_2$ (à 6 and 11 individuals) and, due to limitations in egg production, 1 group of four individuals at 500 µg/L $PtCl_2$. The Pt-induced internalization of the shell in the investigated gastropod species was found to be highly reproducible and allowed us to induce the described body plan modifications ad libitum ever since.

Reconstituted water composition for M. cornuarietis

Reconstituted water consisted of double-distilled water supplemented with KCl (17.94 mg/L), $MgSO_4 \times 7H_20$ (0.19 g/L), $NaHCO_3$ (98.42 mg/L), $CaCl_2 \times 2H_2O$ (0.45 g/L), and NaCl (0.43 mg/L).

Histology

Whole embryos of *M. cornuarietis* at the age of 26 dpf that had been exposed to 200 µg/L $PtCl_2$ throughout were fixed in Bouin´s solution for 1 week. Detailed information on the histological techniques is given in Osterauer et al. (2010). Histological methodology was based on Triebskorn et al. (2005).

Analysis of platinum in M. cornuarietis and in the exposure media

After 26 dpf of exposure to 100 or 200 µg/L $PtCl_2$ or 200 µg/L $PtCl_2$ + 0.54 g/L $CaCl_2 \times 2H_2O$ (equimolar Pt^{2+} and Ca^{2+} concentrations), *M. cornuarietis* were frozen in liquid nitrogen and stored at -20 °C. Depending on the estimated Pt concentrations, quantity of bioaccumulated Pt in the organisms was measured with adsorptive cathodic stripping voltammetry (ACSV) after digestion via high-pressure ashing according to Zimmermann et al. (2001, 2003) or with electrothermal atomic spectrometry (ET-AAS) after microwave-assisted digestion according to Sures et al. (1995). $PtCl_2$ concentrations above 100 µg/L were analyzed by ET-AAS, while lower concentrations were analyzed by ACSV. For controls and *M. cornuarietis* exposed to 100 µg/L $PtCl_2$ the replicate number n was 8, for *M. cornuarietis* exposed to 200 µg/L $PtCl_2$ n=9, for *M. cornuarietis* exposed to 200 µg/L $PtCl_2$ plus equimolar Ca n=5. Depending on the estimated Pt concentrations in the exposure medium for *M. cornuarietis*, concentrations of Pt were measured with inductive coupled plasma mass spectrometry (ICP-MS, Perkin Elmer model Elan 5000, PerkinElmer Inc., Wellesley, MA, USA) or with ET-AAS (Perkin Elmer model 4100ZL). $PtCl_2$ concentrations above 100 µg/L were analyzed by ET-AAS, while lower concentrations were analyzed with ICP-MS. Each sample was analyzed in triplicate. Detailed descriptions of analytical procedures for ACSV and ICP-MS have been published by Osterauer et al. (2009).

Electrothermal atomic absorption spectrometry (ET-AAS)

Tissue samples of about 20 mg fresh weight each were digested by adding 1.8 mL HNO_3 (65 vol. %, subboiled) into 100 mL perfluoralkoxy vessels. Using a microwave digestion oven (CEM Model MDS-2000, 650 ± 50 W; Spectralab Scientific Inc., Toronto, ON, Canada) samples were digested according to the description of Sures et al. (1995). The resulting solution was filled up to 2 mL with bidistilled water. Analytical measurements were conducted with an atomic absorption spectrophotometer (Perkin Elmer Model 4100ZL). Therefore, 20 µL of each sample, priorily diluted with bidistilled water, were injected into a pyrolytic graphite furnace tube with L`vov platform by an autosampler AS 70. Operation parameters and further procedural descriptions have been published by Zimmermann et al. (2003). The detection limit for Pt in the tissue samples was defined to be threefold the standard deviation of the measurements of procedural blanks. For the average sample weight of 25 mg for *M. cornuarietis* it was found to be 62 ng/g.

For Pt analysis in the exposure medium 0.5 mL of the medium were topped up to 1 mL with bidistilled water and analysed as described above. The detection limit for Pt was defined threefold the standard deviation of the measurements of blanks and was found to be 1.6 µg/L.

Carbonic anhydrase activity

M. cornuarietis at the age of 10 dpf (control animals and animals treated with 200 µg/L $PtCl_2$) were homogenized in 60 µL phosphate buffer (25 mM, pH 7.4). Homogenates were centrifuged for 5 min at 2000 × *g* and 4 °C according to the protocol of Giraud (1981). The supernatant served as enzyme source. The Δ pH method of measuring CA activity was conducted according to the description of Henry (1991) and Vitale et al. (1999). For activity measurement 7.5 mL reaction medium (mannitol, 225 mM; saccharose, 75 mM and tris-phosphate, 10 mM at pH 7.4), 50 µL supernatant and 1 mL of CO_2 containing sparkling mineral water (Selters, Löhnberg, Germany) were mixed and the pH-drop was measured for 25 s with a high-precision pH meter (WTW ph 391, WTW, Weilheim, Germany). A linear regression of pH data against time was calculated and the estimated slope was adopted as

the catalyzed reaction ($b_{catalyzed}$). For control measurements, phosphate buffer was used instead of enzyme containing supernatant and the same procedure was performed. Total protein content was determined according to Bradford (1976). To determine the specific CA activity the formula according to Burnett et al. (1981) was used: SCA = ($b_{catalyzed}$ / $b_{noncatalyzed}$ –1)/mg of total protein. Five snails per sample and 3 replicates were used. All preparations were conducted at low temperature (vessels were kept on ice).

Diamino-benzidine (DAB) staining of mantle edge tissue

DAB staining usually is used as negative control for antibody labelling procedures. In other experiments we found DAB to specifically stain the mantle edge of developing *M. cornuarietis* embryos. Different stages of control embryos and embryos exposed to 200 µg/L $PtCl_2$ (day 3, 3.5, 4, 5 and 6 post fertilization) were mechanically removed from the chorion and fixed in 3.7% formaldehyde in phosphate-buffered saline (PBS) for 30 min. After washing for 4 × 5 min with PTw (PBS with 0.01% Tween 20, Roth, Karlsruhe, Germany) embryos were incubated with PTw+N (PTw with 5% Goat Serum, Jackson ImmunoResearch, West Grove, PA, USA) overnight at 4 °C.

For horseradish peroxidase (HRP) reaction embryos were washed with PTw for 4 × 5 min and 4 × 15 min and afterwards incubated in 0.3 mg/mL DAB solution for 20 min. The reaction was started by adding H_2O_2 to a final concentration of 0.03%. When the brown signal became visible, as could be visually detected under a stereomicroscope, reaction was stopped by washing 2 × 5 min with PTw. Finally, embryos were mounted in a 50% glycerol/DAPI (4´,6-diamidino-2-phenylindole) mix. For visual analysis and photography were used a light microscope and a stereomicroscope (both Zeiss, Jena, Germany).

Synchrotron X-ray phase contrast tomography and holotomography

Whole intact embryos of *M. cornuarietis* exposed to 100 µg/L $PtCl_2$ for 26 dpf were fixed in 100 % ethanol overnight, critical point dried (CPD 020, Balzers, Wiesbaden, Germany) and mounted on specimen holder stubs. X-ray tomography was conducted at beamline ID19

(ESRF, Grenoble, France) at an energy of 20.0 keV. Measurements were performed at three different sample-detector distances, i.e. 16 mm, 100 mm, and 841 mm. The effective pixel resolution amounted to 5.05 µm. Otherwise, instrument settings and further data treatments were done according to the description of Heethoff and Cloetens (2008). Due to limitations in beam time, measurements were conducted with a single animal each of the Pt treated group and of the control group.

Scanning electron microscopy

Embryos of *M. cornuarietis* of different age and exposure (100 or 200 µg/L $PtCl_2$, and controls) were fixed overnight in 2% glutaraldehyde in 0.01 M cacodylate buffer at pH 7.4. Subsequently, the organisms were washed three times with 0.01 M cacodylate buffer and stored in 1% osmium tetroxide overnight. The next day, the specimens were dehydrated in an ascending series of ethanol dilutions. After critical point drying they were fixed on specimen holder stubs, sputter-coated with gold, and viewed with a scanning electron microscop (SEM) (Cambridge Stereoscan 250 Mk2, Cambridge Scientific, Cambridge, UK).

Statistical analyses

Normally distributed data (Shapiro-Wilk test, JMP 4.0, SAS Systems, USA) were tested with the parametric one-way t-test (JMP 4.0, SAS Systems, USA) to detect significant differences between the treatment group and the control. Data not corresponding to normal distribution were tested using the nonparametric distribution-independent Wilcoxon´s test (JMP 4.0, SAS Systems, USA) to detect significant differences between the respective treatment groups and the control group. The alpha level was set at 0.05. Differences were considered to be significant for $p \leq 0.05$ (*) and highly significant for $p \leq 0.01$ (**), and $p \leq 0.001$ (***).

Results

Platinum accumulation and induced body plan changes in M. cornuarietis

To quantify Pt accumulation snails were exposed to 100 or 200 µg/L $PtCl_2$ for the first 26 dpf. Chemical analytical data already have been partly published by Osterauer et al. (2009), and showed exposure of *M. cornuarietis* embryos (within the egg) and juveniles to result in exceptionally high Pt concentrations in the animals: 74.2 ± 5.3 µg/L Pt in the medium (nominal concentration of 100 µg/L $PtCl_2$ corresponding to 73.4 µg/L Pt) led to 53.7 ± 19.2 µg/g Pt wet weight in the animals (bioaccumulation factor 724) and 163.4 ± 2.7 µg/L Pt (nominal concentration of 200 µg/L $PtCl_2$ corresponding to 146.8 µg/L Pt) resulted in a tissue concentration of 90.3 ± 9.9 µg/g Pt wet weight (bioaccumulation factor 553), both indicating a high potential of Pt to interact with developmental processes in this species.

In response to Pt treatment 29.9 ± 32.78% of surviving *M. cornuarietis* juveniles continuously exposed to 74.2 µg/L Pt (85.1 ± 9.41% survival) did not form an external shell, whereas at exposure to 163.4 µg/L Pt (84.4 ± 6.23% survival), all surviving animals were 'shell-less' (100 ± 0%).

To detect the most sensitive stage in embryogenesis in which Pt interferes with the formation of the shell, we conducted two separate runs of a pulse-exposure experiment. These experiments included pulses of 200 µg/L $PtCl_2$ with a duration of either 1 day or 2 days. Data varied between the two experimental runs but indicated the embryonic stages at days 4 and 5 postfertilization to be most susceptible to Pt action on shell formation (Fig. 1).

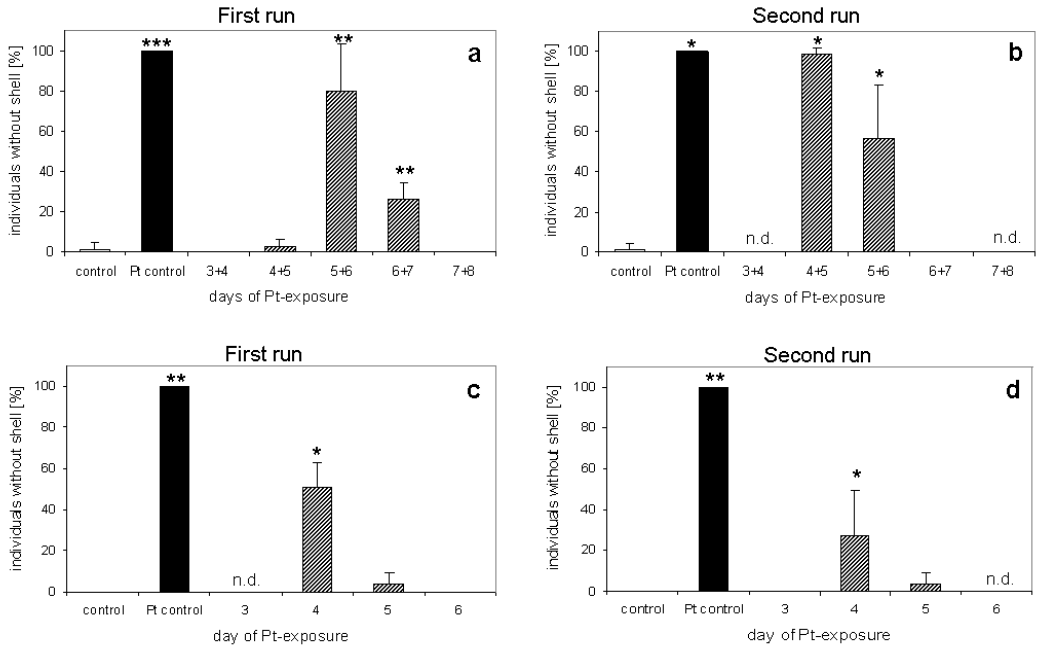

Fig. 1. Pulsed Pt-exposure of *Marisa cornuarietis* to 200 µg/L $PtCl_2$ at different days of embryonic development. Percentages of surviving individuals without external shell at day 11 post fertilization (means ± SD). (a) and (b) two-days-pulses. (c) and (d) one-day-pulses. For each experiment and run, n = 4 replicates with 20 individuals per replicate (except for control and Pt-control in the first run of two-days-pulses n = 8). Control: water exposure; Pt control: continuous exposure to $PtCl_2$ during the entire embryonic phase; n.d.: not determined. Significance vs. control : *** $p \leq 0.001$, ** $p \leq 0.01$, * $p \leq 0.05$.

We repeatedly raised large numbers of *M. cornuarietis* embryos without external shell by pulse exposure to nominal concentrations of 200 µg/L $PtCl_2$ at days 4 and 5 postfertilization. Subsequently, the embryos in their egg capsules were transferred to uncontaminated water and cultivated at 26 °C. We never obtained reversal of the Pt pulse-induced body plan changes under these conditions and, thus, none of these individuals ever developed an external shell. Without exception, all juveniles shared the following alterations relative to the nontreated snails (Fig. 2a): no mantle cavity was formed at all and hence the gill, which remained posterior to the heart at the hind part of the visceral sac, protruded from the visceral sac into the surrounding water (Fig. 2b). Nevertheless, as indicated by the deflected intestine, the position of the anus at the lower right side (Fig. 2b), and by a slight movement of the gill from hind left to hind right, part of developmental processes associated with torsion was accomplished in all individuals. The hepatopancreas, as usual, surrounds the

mid part of the intestine. Dorsal of the hepatopancreas, the hindgut bends to the front. In addition, the visceral sac surface of these snails bloats and blisters to varying degree, forming hemocoel cavities of different sizes (Fig. 2, b, d, and f). An operculum was present (Fig. 2c) and the morphology of the head and ventral part of the body, including the operculum, did not differ from the archetypical body plan (Fig. 2, a, b, and e).

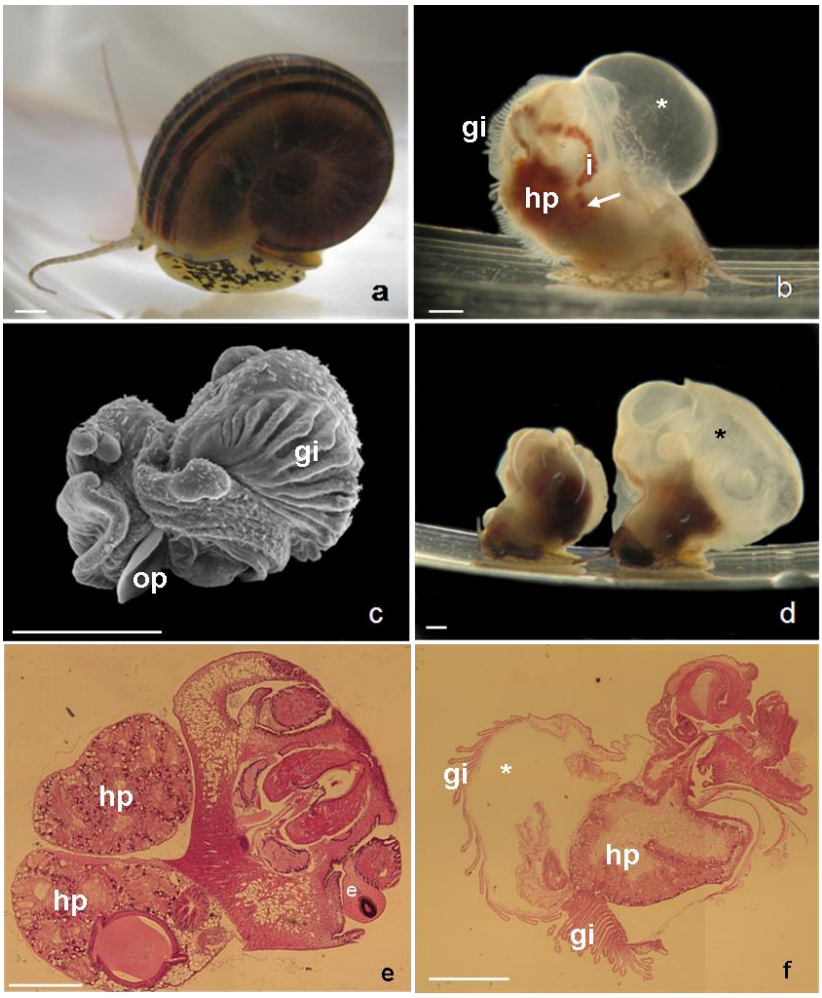

Fig. 2. Images of *Marisa cornuarietis* exposed to 200 µg/L $PtCl_2$ without external shell and control. (a) Control, 3 months old. (b) and (d): Pt-exposed snails without external shell, 2 months old. Arrow indicates position of the anus. (c) Scanning electron micrograph of an embryo without external shell, 14 days post fertilization (e) Horizontal section of a control animal, 26 days post fertilization, ventral part of the body. (f) Sagittal section of a Pt-exposed animal, 26 days post fertilization, median part of the body. gi: gill, i: intestine, hp: hepatopancreas, op: operculum, asterisks: bloats and blisters of the hemocoel. Scale bars are 500 µm.

Up to now, *M. cornuarietis* individuals with induced body plan changes due to Pt exposure reached a maximum age of 7 months and a maximum length of about 1.5 cm. During their lifetime, the animals steadily grew and gained mass but did not change their outer appearance. So far, the only known way to partly protect embryos from the action of Pt is to supplement the Pt solution with equimolar concentrations of bivalent calcium ions. In experiments with *M. cornuarietis* embryos exposed to 200 µg/L $PtCl_2$ plus 0.54 g/L $CaCl_2 \times 2H_2O$ (equimolar Pt^{2+} and Ca^{2+} concentrations) for 9 days postfertilization, 84 ± 12% of the individuals formed an external shell and 16 ± 12% did not. However, a considerable number of the shelled animals in this experiment only developed a small, cap-like external shell, which was not sufficiently large to cover completely the gill (Fig. 3a).

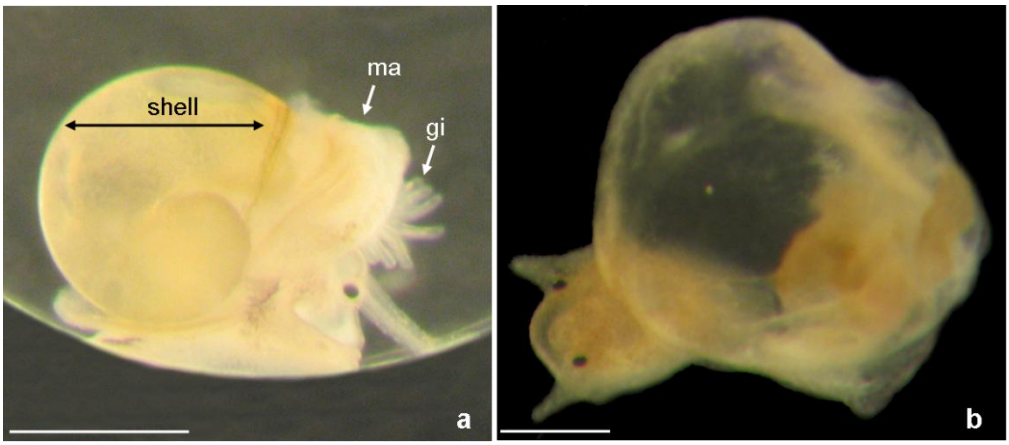

Fig. 3. Pt effect in Ca-supplemented *Marisa cornuarietis* and in *Planorbarius corneus*. (a) *M. cornuarietis* with a small, external shell, 11 days post fertilization, exposed to 200 µg/L $PtCl_2$ and equimolar concentrations of Ca^{2+}, ma: mantle, gi: gills. (b) *P. corneus* without external shell, 23 days post fertilization, exposed to 300 µg/L $PtCl_2$. Scale bars are 500 µm.

Ca supplements did not diminish Pt uptake: snails without an external shell exposed to 200 µg/L $PtCl_2$ accumulated 90.3 ± 9.9 mg/g Pt, snails without an external shell exposed to Pt and equimolar concentrations of Ca accumulated 115 ± 28.1 µg/g Pt, and snails exposed to Pt and equimolar concentrations of Ca, which developed an external shell accumulated 82.5 ± 26.4 µg/g Pt.

In contrast to Pt^{2+}, the physicochemical similar ion Pd^{2+} did not affect shell formation during embryogenesis. Only occasionally were we able to induce shell-less *M. cornuarietis* embryos with high concentrations of Li^{+}: 2.5 mg/L $LiCl_2$ caused 10.0 ± 6.0% shell-less snails and 3 mg/L $LiCl_2$ caused 20.0 ± 9.5% shell-less snails.

The fate of the shell secreting edge of the mantle fold and the formation of an internal shell

Since we found DAB to specifically stain the shell-secreting peripheral edge of the mantle fold, we were able to follow the fate of the tissue, which usually forms the mantle fold edge during embryogenesis and to visualize the presumably shell-secreting region of Pt-exposed *M. cornuarietis*. In contrast to the controls, the tissue archetypically designated to form the mantle edge did neither evaginate nor overgrow the visceral sac but remained at the posterior end of the embryo and invaginated into the body (Fig. 4, c–f), thus closing the aperture of the shell gland.

Using SEM, the morphology of the shell gland and the initial protoconch of continuously Pt-exposed embryos and water controls did not differ substantially (Fig. 4, a and b). However, while the shell grows rapidly during the subsequent days of embryogenesis in controls, the opening of the shell gland of Pt-exposed individuals was reduced in size. To test whether this effect and the subsequent lack of an external shell was possibly based on a lack or a drastic diminution of biomineralization activity during the days subsequent to the Pt-sensitive stages, CA activity was measured in embryos continuously exposed to 200 µg/L $PtCl_2$ at day 10 postfertilization. Even though Pt exposure decreased CA activity from 13.8 ± 2.6/mg in controls to 8.5 ± 3.6/mg in shell-less *M. cornuarietis* embryos, this difference was not significant ($p = 0.107$).

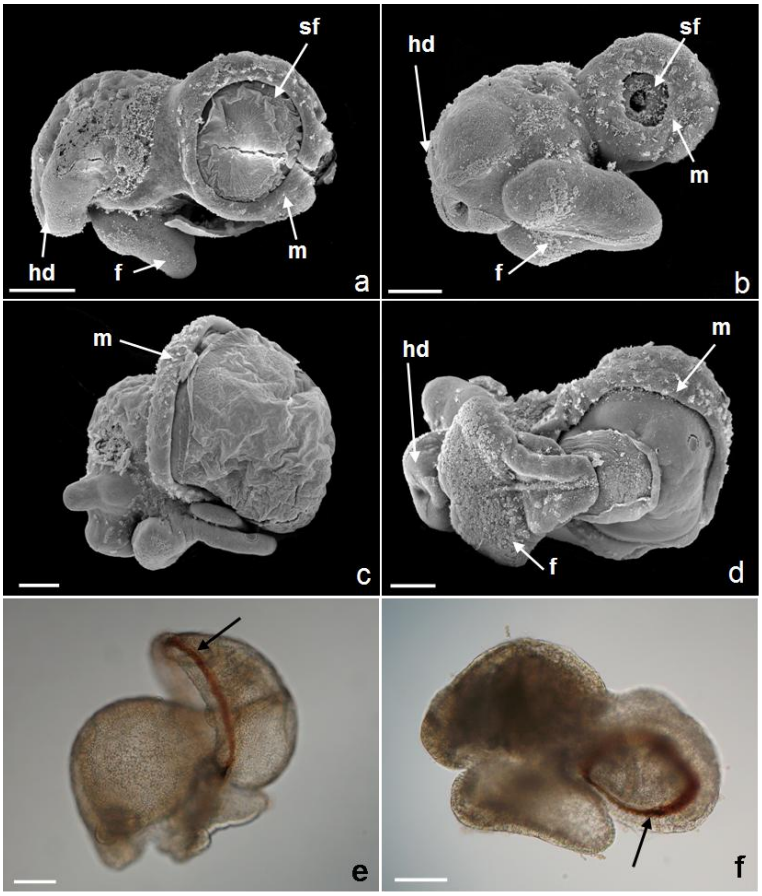

Fig. 4. Location of the shell-secreting peripheral edge of the mantle fold in *Marisa cornuarietis* exposed to 200 µg/L $PtCl_2$ and in controls. Scanning electron micrographs of Pt-exposed (200 µg/L $PtCl_2$) *M. cornuarietis* and controls. (a) Control, 3 days post fertilization. (b) Pt-exposed *M. cornuarietis*, 4 days post fertilization. (c) Control, 5 days post fertilization. (d) Pt-exposed *M. cornuarietis*, 6 days post fertilization. DAB staining (arrows) of the shell-secreting mantle edge of a control individual (e) and a Pt-exposed individual (f), both 5 days post fertilization. Scale bars are 100 µm.

Synchrotron X-ray phase-contrast microtomography applied on a 26 dpf old Pt-exposed juvenile revealed the formation of an internal solid circular structure in the ventral part of the visceral sac, right at the position of the invaginated ectodermal tissue that has been formed the shell gland (Fig. 5a). Starting from this solid structure, with increasing age the snails developed an internal calcareous shell in the shape of a hollow, nonsegmented cone, which surrounds the hepatopancreas in its ventralmost part (Fig. 5).

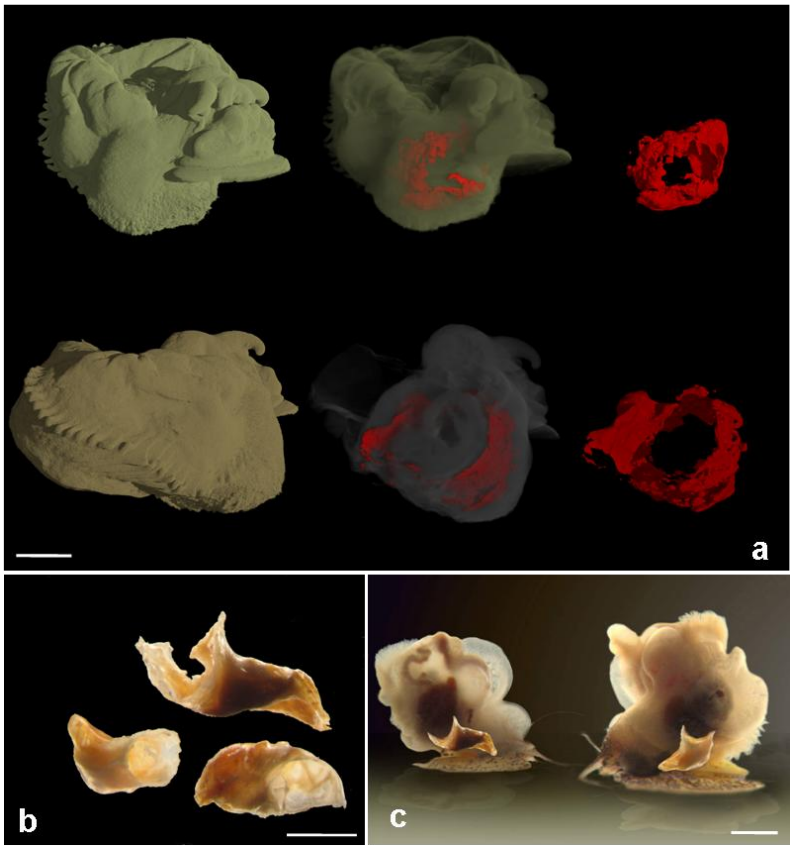

Fig. 5. Internal shells of Pt-exposed *Marisa cornuarietis*. (a) Synchrotron X-ray phase-contrast microtomographs of snails exposed to 100 µg/L $PtCl_2$, 26 days post fertilization. Upper row: view from the right, ahead; lower row: view from the left, rear. Respective left pictures: whole body; pictures in the middle: semi-transparent view of the body; respective right pictures: internal shell fragment. Scale bar is 500 µm. (b) Three examples of internal shells of snails exposed to 200 µg/L $PtCl_2$, about 2 months old. Scale bar is 500 µm. (c) Approximate position of the internal shell in *M. cornuarietis* exposed to 200 µg/L $PtCl_2$, about 2 months old, photo editing. Scale bar is 1 mm.

The phenomenon of Pt-induced body plan changes is not restricted to *M. cornuarietis* but could also be observed in embryos of the pulmonate snail *P. corneus* exposed to nominal concentrations of ≥ 300 µg/L $PtCl_2$ from fertilization until hatch. Also shell-less *P. corneus* did not form a mantle cavity (Fig. 3b). In contrast to *M. cornuarietis*, the longevity of unshelled *P. corneus* was restricted to a maximum of about 2 weeks postfertilization.

Discussion

The present study describes and investigates artificially induced shell internalization in *M. cornuarietis* due to Pt exposure, which corresponds with fundamental body plan changes. In contrast to Pt^{2+}, the physicochemical similar ion Pd^{2+} and other bivalent metals (Zn^{2+}, Ni^{2+}, Cd^{2+}; Schirling et al., 2006; Sawasdee and Köhler, 2009) did not interfere with shell formation during embryogenesis. Only occasionally high concentrations of Li^{+} (2.5 and 3 mg/L $LiCl_2$), a metal which has been shown to interact with the positional system of predominantly ectodermal tissue in *Xenopus* and *Loligo* (Kao et al., 1986; Crawford, 2003), prevented the formation of an external shell in *M. cornuarietis*. So far, solely the heavy metal Pt seems to specifically interact with key processes during early embryonic development, which inhibits the mantle to evaginate and to overgrow the visceral sac, hence, leading to the growth of an internal shell as it could be traced by the staining of the mantle edge.

Pulse experiments revealed days 4 and 5 postfertilization to be most susceptible to Pt action on shell formation. Data probably varied due to slight variation in culture temperature (26 ± 1 °C) and depending on the exact time of fertilization during the night. During these days of embryonic development the initial stages of the embryonic shell are formed (Demian and Yousif, 1973a). One hypothetical approach to explain the Pt-induced internalization of the shell relies on a possible interaction of Pt with the Ca metabolism and its uptake via Ca trans-membrane transport. Because Ca supplements did not diminish Pt uptake, we conclude that Pt likely interacts with Ca signalling pathways involved in the positional system, which may be stabilized by increasing intracellular levels of Ca. Some heavy metals have been shown to act as inhibitors of the ezyme CA (Christensen and Tucker, 1976; Morgan et al., 1997; Vitale et al., 1999) or to reduce shell mass in snails (Beeby et al., 2002). We therefore tested the CA activity in Pt exposed *M. cornuarietis*. The results indicated a diminuation but no significant difference of the activity in Pt exposed snails. Hence, the mechanisms of the internal shell production seem to be almost as effective as those involved in the growth of the external shell, and the reduction in shell size could probably only be attributed to a compressed shape due to the limited space inside the gastropod's body.

The effect of body plan changes due to Pt exposure could also be observed in embryos of the pulmonate snail *P. corneus*. Also shell-less *P. corneus* did not form a mantle cavity which might indicate that Pt action on the re-direction of presumptive mantle tissue is likely universal in gastropods. In contrast to prosobranch gastropods, however, pulmonates lack a gill but use their mantle cavity to form a lung. A re-direction of mantle tissue here consequently leads to a lack of any respiratory organ and, therefore, to a short life span in *P. corneus* embryos.

Descriptions of various authors about the time span of torsion (from about 2 min until about 200 h) and its main cause(s) (muscular activity, differential growth, hydraulic activity) in different groups of gastropods vary to a high degree (for detailed informations see Wanninger et al., 2000). Therefore, it can be assumed that ontogenetic torsion of gastropods has been highly modified within different groups subsequent to its rise in early phylogeny.

Because no external shell is formed after exposure to Pt, torsion occurs independently from retractor muscle action on the larval shell in *M. cornuarietis* as it was shown for other gastropod species in earlier studies of Hickman and Hadfield (2001) and Page (2002) who provided evidence for this view. As shown for other organisms (for an overview see Wanninger et al., 2000), our results provide additional independent evidence that several processes are involved in the ontogenetic process of torsion, in contrast to Garstang (1929) and Crofts (1937, 1955) who proclaimed contraction of asymetric larval retractor mussels to be the cause of developmental rotation. We could show that at least two of the processes associated with torsion can be uncoupled during the development of *M. cornuarietis*. That is, the anus of the treated snails is located anteriorly, but the mantle tissue and gill remains in a posterior location. Hence, the process of torsion is neither inevitably connected to mantle cavity formation nor to the translocation of its aperture together with the gill into a frontal position but rather developmentally separated from the distal outgrowth of the mantle epithelium, which is also the prerequisite for an external shell. Both freshwater model species, *M. cornuarietis* and *P. corneus*, go through a ‘direct development’ lacking a

trochophora or veliger larva. Therefore, differential growth may play a crucial role in torsion because muscles are differentiated after the torsion process only.

The fact that only the position of the mantle tissue and the gills but not the anus in Pt-treated *M. cornuarietis* can be uncoupled from torsion processes compared to nontreated animals, might be due to the observations made by Demian and Yousif (1973b) who described that the intestine of this species is entirely endodermal and opens into the mantle cavity at a relatively late stage.

This is the first report on snail–slug conversion and experimentally induced shell internalization in gastropods. Even though the morphological similarity of these artificial internal shells with internal shell derivatives in extant or fossil molluscan taxa is striking, we do not claim to be able physiologically to trigger exactly what has evolved in cephalopods, nudibranchs, and pulmonate slugs. The mechanisms of shell–mantle interactions in the formation of internal shell derivatives in extant molluscs are manifold and do not follow exactly the same developmental pattern, even though, in all cases and also in our experiments, a shell precursor is overgrown by ectodermal (mantle) tissue (Kniprath, 1981; Page, 2000; Gibson, 2003). Particularly in extant taxa of opisthobranchs, there is evidence for a stepwise reduction of the shell (Wägele and Klussmann-Kolb, 2005) which is contradicting a hypothesis of 'macromutation'- based radical developmental shifts underlying body plan modifications such as detorsion in these heterobranch gastropods. Nevertheless, it is evident from our study that minimal changes in the blueprint for molluscan ontogeny (or the corresponding signal transduction machinery of the positional system) may lead to sudden body plan shifts. This observation is consistent with the notion of modularity, the idea that a subset of variables in a system may be changed independent of the remaining variables in the system (Lipson et al., 2002) – in this case Pt leads to a drastic change during early development to one part of the body without lethal consequences to the whole organism.

We cannot exclude that similar, mutation-based body plan alterations have contributed to the evolution of shell internalizations in several molluscan taxa as we know them today.

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Chapter 5: Histopathological effects of copper and lithium in the ramshorn snail, *Marisa cornuarietis* (Gastropoda, Prosobranchia)*

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Abstract

The aim of this study was to determine and quantify effects of copper and lithium in tissues of early juveniles of the ramshorn snail, *Marisa cornuarietis.* For this purpose, hatchlings of *M. cornuarietis* were exposed for 7 days to a range of five different sublethal concentrations of copper (5, 10, 25, 50, and 75 µg Cu^{2+}/L) and lithium (50, 100, 200, 1000, and 5000 µg Li^{+}/L). Both metals changed the tissue structure of epidermis, hepatopancreas, and gills, varying between slight and strong reactions, depending on the copper and lithium concentration. The histopathological changes included alterations in epithelial and mucous cells of the epidermis, swelling of hepatopancreatic digestive cells, alterations in the number of basophilic cells, abnormal apices of digestive cells, irregularly shaped cilia and changes in the amount of mucus in the gills. The most sensible organ in *M. cornuarietis* indicating Cu or Li pollution is the hepatopancreas (LOECs were 10 µg Cu^{2+}/L, or 200 µg Li^{+}/L). In epidermis, mantle and gills relevant effects occurred with higher LOECs (50 µg Cu^{2+}/L, or 1000 µg Li^{+}/L). Base on LOECs, our results indicated that histopathological endpoints are high sensitivity to copper and lithium compared to endpoints for embryonic developmental toxicity.

Keywords: Histopathology; metal toxicity; prosobranch snail; copper; lithium; *Marisa cornuarietis*

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*Chemosphere, in press

Introduction

The present study, focused on effects of two metals: copper and lithium on the ramshorn snail *Marisa cornuarietis*. Copper is an essential metal which plays various physiological roles. In arthropods and molluscs, e.g. it is involved in haemocyanin metabolism (Gullick et al., 1979; Gullick et al., 1981; White and Rainbow, 1985), however, when biological requirements are exceeded, it is known to become harmful for aquatic organisms (US EPA, 2007). The copper concentration in aquatic ecosystem varies within a range, from 0.2 µg/L to 100 µg/L (Teasdale et al., 1996; Johnson, 2007), which concentrations caused effects on embryonic development of aquatic snails, *M. cornuarietis* and reduced oxygen consumption rate of the mussel, *Perna viridis* (Krishnakumar et al., 1990; Sawasdee and Köhler, 2010). Lithium presents essential properties (Nielsen, 1988), although it is not an essential metal. In rats, it is known to cause structural alterations in different tissues (Sharma and Iqbal, 2005). Lithium is a classical tool for the study of phosphoinositide dependent cellular process, it is known to be teratogenic (Berridge et al., 1989), and to respecify the dorsoventral axis in zebrafish (Stachel et al., 1993). Concentrations of lithium in surface water are typically low (1-100 µg/L; Martinez and Carrillo-Rivera, 2006; Rubio-Arias et al., 2007). Lithium was able to induce shell-less *M. cornuarietis* embryos with concentration of 2.5 mg Li^+/L (Osterauer et al., 2010). In addition, at 2.5 mg Li^+/L, resulted in 23% mortality and showed a significant delay in tentacle and eye formation (Sawasdee and Köhler, 2010).

Generally, many metals have been described as persistent and toxic for several aquatic species. In this context, mostly acute toxicity, measured by means of the LC_{50} values, has been determined (Mathur et al., 1981; Khangarot and Ray. 1988; Macdonald et al., 2002; Ferrer et al., 2006; Khangarot and Das, 2009). In order to understand interactions of metals with molecular or cellular targets, however effects should be studied at the sub-organ levels administering sublethal concentrations. In this context histopathological studies are useful to reveal effects in those tissues of exposed organisms, which are relevant for their essential function in response to metal exposure. In molluscs, the organ which plays the most important role in the metabolism of endogenous and xenobiotic compounds is the digestive gland (Wilbrink et al., 1990). Histopathological changes in the hepatopancreas of snails are important indicators of the effects of environmental threats, and they are frequently used in monitoring research (Triebskorn 1989; Elangovan et al., 2000; Otludil et al., 2004; Radwan et

al., 2008; Zaldibar et al., 2008; Dittbrenner et al., 2009). Although several researchers have studied toxic effects of metals in snails and bivalves (Ravera, 1991; Laskowski and Hopkin, 1996; Abd Allah et al., 1997; Gomot, 1998; Soto et al., 2000; Beeby et al., 2002; Pyatt et al., 2002; Kszos et al., 2003; Loayza-Muro and Elías-Letts, 2007; Grosell and Brix, 2009; Hoang and Rand., 2009), up to now, there are not many data on metal-induced histopathological changes in gastropods which might serve as basic indicators to assess hazards in molluscs.

The prosobranch snail, *Marisa cornuarietis*, has been in the focus of various ecotoxicological studies, and has been shown to respond in a sensitive way to endocrine disruptive chemicals (Oehlmann et al., 2000; Schulte-Oehlmann et al., 2000; Schirling et al., 2006). Since previous investigations revealed embryonic development of this species to be highly sensitive to metals (Schirling et al., 2006; Osterauer et al., 2009; Sawasdee and Köhler, 2009; Sawasdee and Köhler, 2010; Osterauer et al., 2010), the purpose of this study was (1) to describe and quantify sublethal effects of copper and lithium in newly hatched snails by means of histopathology and (2) to compare the sensitivities for the different endpoints for *M. cornuarietis.*

Materials and methods

Test animals

The ramshorn snail *Marisa cornuarietis* (Prosobranchia, Ampullariidae) strain used for egg production originated from a breeding stock of the Zoological Institute in Frankfurt/Main, Germany (gratefully donated by J. Oehlmann). For our experiments, adult snails were maintained in 120 L glass aquaria containing tap water (conductivity ≈ 800 µS/cm; pH ≈ 7,5; temperature 24 ± 1 ^{o}C; photoperiod 12 h:12 h light:dark). Aquaria were part of a circulation system with aerated and filtered water. Stock animals were fed a commercial fish flake food (TetraMin, Tetra GmbH, Germany) or fresh vegetables once a day. Ages of snail used in this study were approximately 14-16 days from the day of egg laying until hatching.

Exposure conditions

The metals copper and lithium were applied as $CuCl_2$ (99%, Acros Organics, Great Britain), and LiCl (≥ 99%, Fluka, USA) using stock solutions of 1 g Cu^{2+}/L, and 1 g Li^{+}/L in de-ionized or double distilled water. For the test solutions, these stocks were diluted with the same water as used for animal stock maintenance to the final nominal concentrations of 0, 5, 10, 25, 50 and 75 µg Cu^{2+}/L or 0, 50, 100, 200, 1000 and 5000 µg Li^{+}/L, respectively. The stock and test solutions were kept in polyethylene bottles. Each concentration of $CuCl_2$ and LiCl was tested on newly hatched snails (n=10) for 7 days. During the exposure periods, the animals were maintained in polystyrene Petri dishes (100 mm x 15 mm) containing 50 mL of the different concentrations of metal and fed a commercial fish flake food once a day. The control water as well as the test solutions were replaced daily.

Histology

After 7 days of exposure, the snails (shell diameter of about 2 mm) were fixed in Bouin's solution for 3 days. Samples were washed for 3 × 10 min with 70% ethanol containing one drop of 32% NH_3, dehydrated in a graded series of ethanol, and embedded in Techno-vit (Heraeus Kulzer, Germany). Samples were cut into 5 µm section using an automatic microtome (2050 Supercut, Reichert-Jung, Germany) and spread on microscope slides. Finally, the sections were stained with hematoxylin-eosin (HE) or periodic acid schiff reagent (PAS) and alcian blue, and covered with Eukitt (Roth, Germany).

Histopathological changes in hepatopancreas, epidermis, and gills of the snails were studied under a light microscope (Axioskop 2, Zeiss, Germany). For semi-quantitative assessment of the reactions, histopathological symptoms were classified into five categories according to the state of cellular pathology (1: control status; 3: status of reaction; 5: status of destruction; 2 and 4 were chosen as intermediate stages between 1 and 3 or 3 and 5, respectively) (Table 1). Three different sections per organ per snail were qualitatively described, and semi-quantitatively assessed by calculation of mean assessment values (MAV) which were calculated for n = 10 animals per treatment according to Triebskorn and Köhler (2003), Dittbrenner et al. (2009) and Osterauer et al. (2010).

Table 1 Classification of histopathological symptoms in tissues of *Marisa cornuarietis* as a basis for the semi-quantitative assessment. According to Osterauer et al. (2010).

	Category 1: control status	Category 2: status of reaction	Category 3: status of destruction
Epidermis	Prismatic cells with basally located nuclei	Irregular apical surfaces	Desquamation
	Filled mucocytes	Dilatation of intercellular spaces	Very large intercellular spaces
		Atrophy or hypertrophy of cells and/or nuclei	Destroyed epidermis
		Empty and/or destroyed mucocytes	
		Higher or lower amount of mucocytes	
Hepatopancreas	Small hemolymph spaces between the tubules	Large Hemolymph spaces between the tubules	Destroyed tubules
	Small tublue lumen	Enlarged tubule lumen	Necrosis of digestive and basophilic cells
	Digestive cells with smooth apex and regulary shaped microvilli	Dilatation of intercellular spaces	
	Regular compartmentation of digestive cells (small apical and large basal vacuoles)	Flattened epithelium	
	Basophilic cells with dense cytoplasm and large roundish nucleus with high amount of heterochromatin	Irregular shape of cells	
		Apical surface blebs of digestive cells	
		Increased amount and/or enlarged vacuoles in digestive cells	
		Irregular compartmentation of digestive cells	
		Varying amount and shape of basophilic cells	
		Altered density of the cytoplasm	
Gills	Columnar cells with long cilia	Enlargement of nuclei	Necrosis of cells
	Round to oval nuclei with dense heterochromatin	Increase of mucus cells	Very large interlamellar spaces
	Single mucus-secreting goblet cells	Varying amount of mucus-secreting cells	Pycnotic nuclei
		Irregular shape of cells	
		Enlarged interlamellar space	

Data analysis

For statistical analysis JMP® 4.0 (SAS) was used. Normally distributed data (checked by Shapiro-Wilk's test) were tested for significance with Student's *t*-test, and data showing non-normal distribution were tested with Wilcoxon's test. The α-level for significant differences was set at $p \leq 0.05$.

We used TableCurve 5.01 for calculation of EC_{50} value, the best fitting regression saturation curve ($R^2 > 0.98$) was used. Also data were not of continuous type, we calculated EC_{50} value for all data set. The 50% effect was defined as the arithmetic mean between the minimum assessment value (1.0) and the maximum value (5.0), equalling 3.0.

Results

Histopathological alterations observed in snails exposed to Cu^{2+} or Li^{+} include effects in epidermis, hepatopancreas, and gills. In the following, these symptoms are qualitatively described for the three tissues under investigation.

Epidermis and mantle tissues

In the epidermis, cells of control snails were characterized by regular apical surfaces (Fig. 1a). After Cu^{2+} and Li^{+} exposure, epithelial cells mainly showed irregular apical surfaces (Fig. 1b), and apical surfaces were often covered by a mucous layer (Fig. 1c). The mantle tissue of control snails contains muscle fibers, connective tissue elements, hemolymph spaces and cell bodies of mucous cells. The mantle of snails exposed to 75 µg Cu^{2+}/L and 5000 µg Li^{+}/L showed a strong dilation of hemolymph spaces and large areas with empty mucous cells.

According to the semi-quantitative assessment, epidermis and mantle of controls in snails exposed to 1000 or 5000 µg Li^{+}/L, the condition of epidermis and mantle were significantly impaired (Fig. 2a). Significantly strong effects occurred in snails exposed to Cu^{2+} concentrations of 50 µg Cu^{2+}/L or higher.

Hepatopancreas

The hepatopancreas is composed of numerous tubules, which consist of digestive cells and basophilic cells. Between the tubules, hemolymp spaces are present (Fig. 3a).

Both Cu^{2+} and Li^{+} were shown to cause significant histopathological changes in the hepatopancreas of the exposed snails. The degree of effects was dose-dependent. In snails exposed to 10 µg Cu^{2+}/L or 200 µg Li^{+}/L, an increase in the number of large vacuoles in the digestive cells was detected, accompanied by an altered compartmentation of the digestive cells (Fig. 3b). In snails exposed to 50 µg Cu^{2+}/L or 1000 µg Li^{+}/L, a dilatation in the hemolymph spaces between tubules, a degeneration of digestive cells, enlarged tubular lumina, enlarged basophilic cells, and irregular shapes of the apices of the digestive cells

were observed (Fig. 3c). Snails exposed to 75 µg Cu^{2+}/L or 5000 µg Li^{+}/L, displayed drastic symptoms of pathology such as the dilation of hemolymph spaces between the tubules, a flattened digestive cells, and large and dark basophilic cells (Fig. 3d).

The semi-quantitative assessment of the metal effects in the digestive cells revealed significant differences between control snails and individuals exposed to 10, 25, 50, and 75 µg Cu^{2+}/L and 200, 1000, and 5000 µg Li^{+}/L, respectively. Hepatopancreas of snails exposed to 50 µg Cu^{2+}/L, 75 µg Cu^{2+}/L, 1000 µg Li^{+}/L, or 5000 µg Li^{+}/L showed particularly strong reactions and were assessed between categories 3 and 4 (Figs. 2a and b).

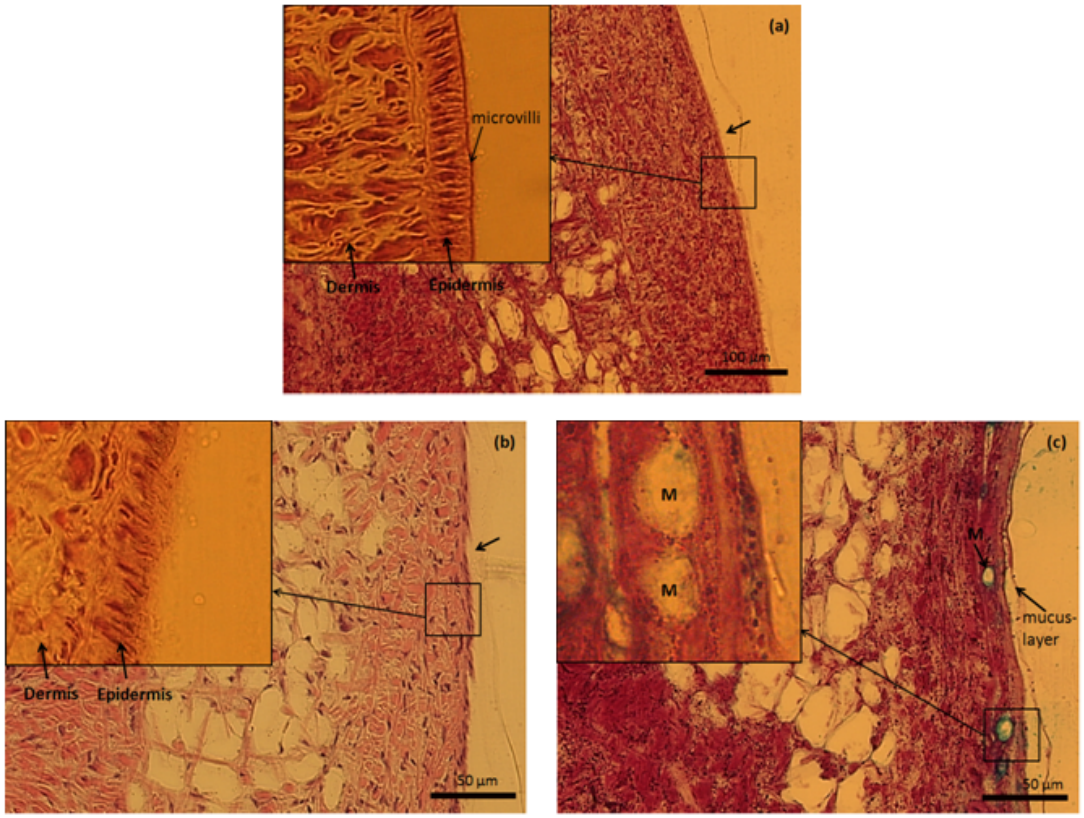

Fig. 1 (a) Epidermis of a control snail. The arrow indicates the regular surface of the epithelium, H&E; (b) epidermis of a snail exposed to 5000 µg Li^{+}/L showing an irregular apical surface of the prismatic cells (arrow), H&E; (c) epidermis of a snail exposed to 75 µg Cu^{2+}/L showing the surface of the epithelium covered with mucus released by mucous cells (M), PAS.

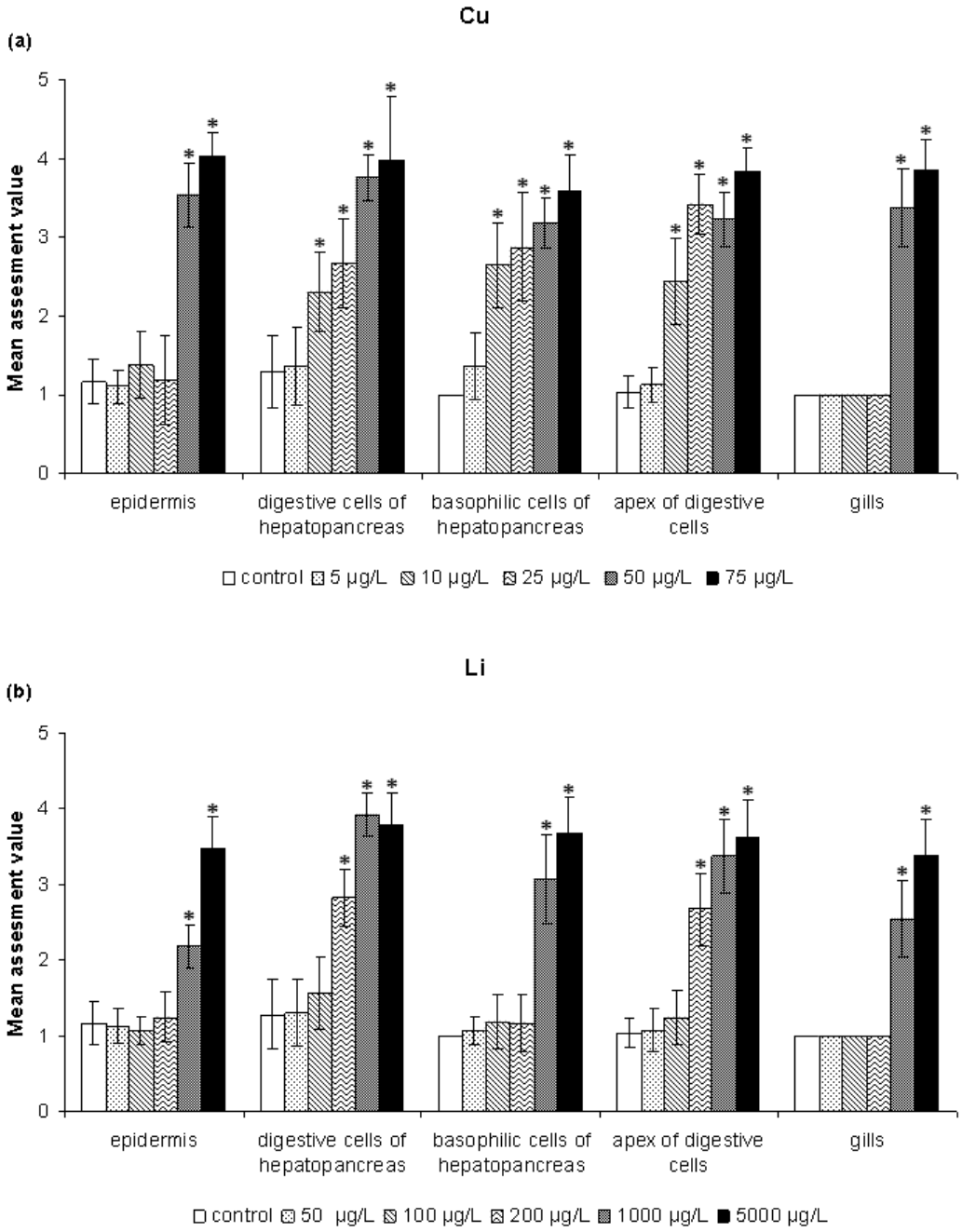

Fig. 2 (a) Mean assessment values for the conditions of epidermis, hepatopancreas, and gills of *Marisa cornuarietis* exposed to different concentrations of Cu^{2+} (mean ± SD), n = 10. Significance level $p \leq 0.05$ (*). (b) Mean assessment values of epidermis, hepatopancreas, and gills of *Marisa cornuarietis* exposed to different concentrations of Li^{+} (mean ± SD), n = 10. Significance level $p \leq 0.05$ (*).

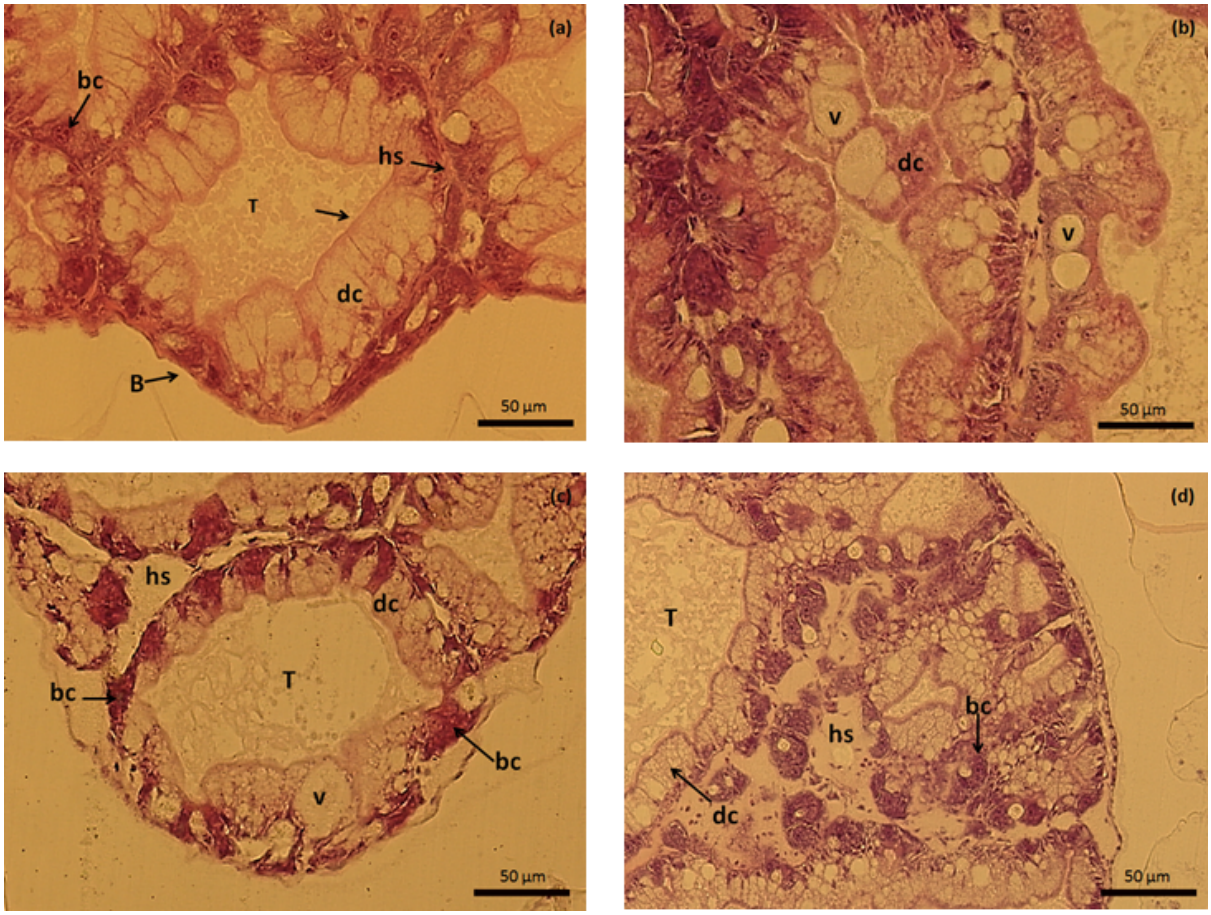

Fig. 3 (a) Hepatopancreas of a control snail with basophilic cells (bc), digestive cells (dc), tubulus lumen (T), hemolymphe spaces (hs), and regular appearance of the cell bases (B); (b) hepatopancreas of an individual exposed to 1000 µg Li^{+}/L displaying enlarged vacuoles (v) and altered compartmentation of digestive cells (arrow); (c) hepatopancreas of a snail exposed to 75 µg Cu^{2+}/L showing an irregular shape of digestive cells (dc), enlarged vacuoles (v), enlarged hemolymphe spaces (hs), large lumen (T), and large basophilic cells (bc); (d) hepatopancreas of a snail exposed to 5000 µg Li^{+}/L displaying enlarged hemolymphe spaces (hs), a large lumen (T), dark basophilic cells (bc), and a flattened epithelial consisting of digestive cells (dc), H&E.

Table 2 Half maximal effective concentration (EC_{50}) of copper and lithium in different tissues of *M. cornuarietis* in histopathological study ($R^2 > 0.98$)

Treatments	Epidermis	Hepatopancreas			Gill
		Digestive cells	Basophilic cells	Apex	
Copper (µg/L)	41.50	30.60	26.73	15.80	43.60
Lithium (µg/L)	2523.90	220.80	967.60	231.80	1402.50

Gills

In control snails, the cells of the gill filaments were of columnar shape and bearded long cilia. Some mucus-secreting goblet cells could also be found in gill filaments (Fig. 4a). After both Cu^{2+} and Li^{+} exposure, the gill epithelium showed a reduction in the length of cilia, the mucous vacuoles appeared to be more numerous, epithelial cells were irregularly shaped, and hypertrophy of nuclei was observed. In addition, the structure of the epithelial layer was heavily destroyed with condensed nuclei and irregularly shaped cells visible (Fig. 4b). These alterations occurred most frequently in snails exposed to the highest concentrations of Cu^{2+} and Li^{+}.

The semi-quantitative assessment of the reactions in the gills revealed significant differences between the controls snails and snails exposed to 50 Cu^{2+}/L, 75 µg Cu^{2+}/L, 1000 Li^{+}/L, or 5000 µg Li^{+}/L (Figs. 2a and b). Histology in snails exposed to 25 µg Cu^{2+}/L or 200 µg Li^{+}/L did not differ from control individuals.

The half maximal effective concentration (EC_{50}) value of the respective symptoms is displayed in Table 2.

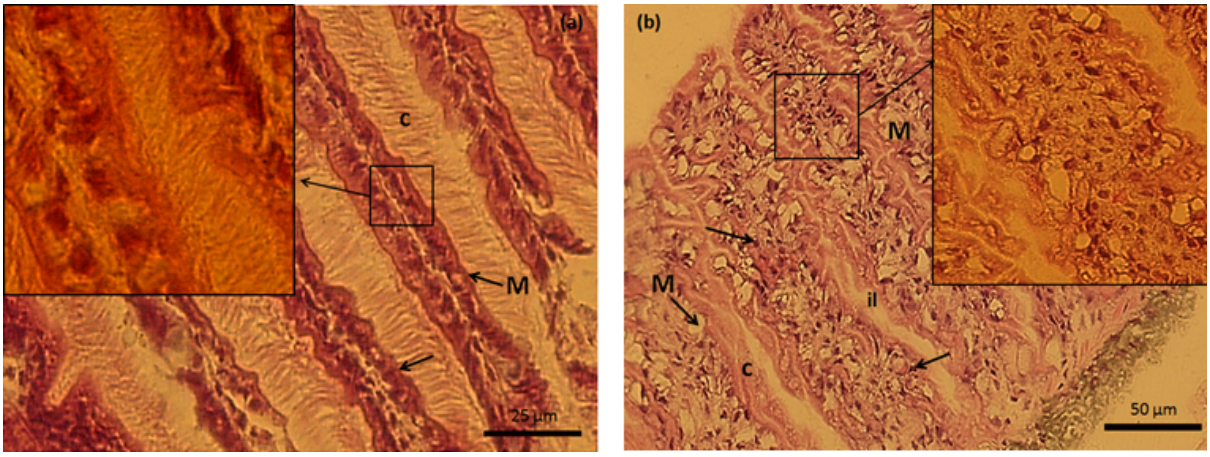

Fig. 4 (a) Gills of a control snail with cilia (c), nuclei with dense heterochromatin (arrow), and mucocytes (M); (b) gills of an individual exposed to 75 µg Cu^{2+}/L showing dilated inter lamellar spaces (il), an increased amount of mucocytes (M), and scattered nuclei (arrows), H&E.

Discussion

Several studies emphasize gastropods as useful monitor organisms for the assessment of pollution in aquatic ecosystems (Jonnalagadda and Rao, 1996; Abd Allah et al., 1997; Gomot, 1998; Oehlmann et al., 2000; Otludil et al., 2004; Mazurová et al., 2008; Hoang et al., 2008; Grosell and Brix, 2009; Hoang and Rand, 2009). Previous studies on *Marisa cornuarietis* also demonstrated this species' capability to reveal embryo toxicity of metals and organic chemicals (Sawasdee and Köhler, 2009; Sawasdee and Köhler, 2010; Osterauer et al., 2010). The results of the present study make evident that in *M. cornuarietis* histopathological alterations occur in all tissues investigated after exposure to copper concentrations and to lithium concentrations in the range between 10 and 75 µg Cu^{2+}/L, and 200 and 5000 µg Li^{+}/L, respectively.

The most severe reactions became evident in the hepatopancreas, which is the main metabolic organ in snails and also involved in detoxification and accumulation of heavy metals (Dallinger and Wieser, 1984; Tanhan et al., 2005). Histopathological effects similar to those reported in the present study, such as dilatation in hemolymph spaces and an increase in the number of vacuoles and basophilic cells have also been shown to occur in the

hepatopancreas of *M. cornuarietis* exposed to $PtCl_2$ (Osterauer et al., 2010) and in *Amphimelania holandri* exposed to phenol (Lajtner et al., 1996).

The gill plays an important role in the transport of respiratory gases and regulates osmotic and ionic balances (Willmer et al., 2000). Damage in gills, therefore, likely results in a reduction of oxygen consumption and in a disruption of the osmoregulation (Ghate and Mulherka, 1979). In the present study, exposure of *M. cornuarietis* to Cu and Li resulted in histopathological alterations of gill including reduction in the length of cilia, an increased number of mucous cells, and dilation of nuclei. Similar symptoms have also been observed in the gill of the snail *Babylonia aerolata* after exposure to cadmium (Tanhan et al., 2005) and in *M. cornuarietis* exposed to $PtCl_2$ (Osterauer et al., 2010).

In the mantle tissue of *M. cornuarietis*, Cu and Li exposure resulted in moderate to strong reactions e.g. mucocytes as well. Increased mucus production followed by increased mucus secretion is among the first unspecific reactions of gastropods to many kinds of stressors (Triebskorn et al., 1989). Similar to our results, alterations of the epidermis such as an irregular surface, and desquamation of parts of the epidermis have also been reported in *M. cornuarietis* exposed to $PtCl_2$ (Osterauer et al., 2010). In addition, Jonnalagadda and Rao (1996) reported disorganization of the mantle tissues of the snail, *Bellamya dissimilis* exposed to the pesticides endosulfan, methylparathion, quinalphos, and nuvan (DDVP).

In this study, Cu was found to be more toxic than Li. This observation is in agreement with results obtained in previous experiments of Sawasdee and Köhler (2010) who reported lithium to be consistently the least toxic test metal when compared to Pt, Ni, Cu, Zn, Cd and Pd, but it was more toxic than Pb, however. The high toxicity of Cu shown in the present study is also in agreement with the results of Dallinger and Wieser (1984) who have demonstrated copper to be more toxic than lead in *Helix aspersa.*

According to Sawasdee and Köhler (2010), conducted *M. cornuarietis* embryo toxicity test (MariETT) found the LOEC of copper was 50 µg Cu^{2+}/L, whereas, the LOEC for copper observed in the histopathological study was low (10 µg Cu^{2+}/L). On the other hand, the LOEC of lithium, indicating a similar sensitivity of both endpoints to *M. cornuarietis.* However, the combination of histopathological study and embryo toxicity test are needed for ecotoxicological application in the future.

Gastropods are known to be able to accumulate metals exceedingly (Dallinger and Wieser, 1984; Osterauer et al., 2009). According to Osterauer et al. (2009), *M. cornuarietis* accumulated about 50 times more platinum than *D. rerio.* Although the LOEC of both metals on snail in this study is still above environmental concentration, the effects due to chronic exposure in snail with high accumulation levels cannot be excluded.

Conclusions

It can be concluded from the present study that (1) the toxicity of copper is about 10 times higher than the toxicity of lithium, (2) the most sensible organ in *M. cornuarietis* indicating Cu or Li pollution is the hepatopancreas (LOECs were 10 µg Cu^{2+}/L, or 200 µg Li^{+}/L), (3) also in epidermis, mantle and gills relevant effects occurred with higher LOECs (50 µg Cu^{2+}/L, or 1000 µg Li^{+}/L), and (4) although effect data were higher than environment concentrations, chemical influence of Cu and Li should not be underestimated.

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