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Acute combined exposure to heavy metals (Zn, Cd) blocks memory formation in a freshwater snail

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Abstract The effect of heavy metals on species survival is well documented; however, sublethal effects on behaviour and physiology are receiving growing attention. Measurements of changes in activity and respiration are more sensitive to pollutants, and therefore a better early indicator of potentially harmful ecological impacts. We assessed the effect of acute exposure (48 h) to two heavy metals at concentrations below those allowable in municipal drinking water (Zn: 1,100 µg/l; Cd: 3 µg/l) on locomotion and respiration using the freshwater snail, *Lymnaea stagnalis*. In addition we used a novel assessment method, testing the ability of the snail to form memory in the presence of heavy metals in both intact snails, and also snails that had the osphradial nerve severed which connects a chemosensory organ, the osphradium, to the central nervous system. Aerial respiration and locomotion remained unchanged by acute exposure to heavy metals. There was also no effect on memory formation of these metals when administered alone. However, when snails were exposed to these metals in combination memory formation was blocked. Severing the osphradial nerve prevented the memory blocking effect of Zn and Cd, indicating that the snails are sensing these metals in their environment via the osphradium and responding to them as a stressor. Therefore, assessing the ability of this species to form memory is a more sensitive measure of heavy metal pollution than measures of activity, and indicates that the snails' ability to demonstrate behavioural plasticity may be compromised by the presence of these pollutants.

Keywords Cadmium · Heavy metal · *Lymnaea stagnalis* · Learning and memory · Locomotion · Respiration · Zinc

Abbreviations

LTM	Long-term memory
CNS	Central nervous system
TS1	First training session
TS2	Second training session
MT	Memory test

Introduction

Toxic metals, often referred to as 'heavy metals', are generally considered to be any metal that can cause negative effects on living organisms, with some of the most common being Pb, Cd, Cr, Zn, Hg and Cu (Boyd 2010). While these elements are naturally occurring in varying concentrations and distributions, anthropogenic activity can lead to increases in the concentration of these elements in the environment. Resource extraction (e.g. mining) that causes the release of heavy metals into the environment means that toxins, including heavy metals, are continually being added to freshwater systems, for example via run off from deposit areas (Han et al. 2002; Schwarzenbach et al. 2010; Sánchez 2008; Kelly et al. 2010). Heavy metals accumulate in freshwater organisms in a dose dependant manner (Goodyear and McNeill 1999), and are known to have lethal effects (Sánchez 2008). However, behavioural responses that can reduce animal fitness may occur at much lower levels than those that cause direct mortality (Boyd 2010).

It has become increasingly recognised that sublethal effects as measures of toxicity are far more sensitive in the

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assessment of the potential impact of pollution within an ecosystem (Boyd 2010; Gerhardt 2007). For example, development is significantly altered in response to Cd pollution below lethal limits in northern leopard frog tadpoles, *Rana pipiens* (Gross et al. 2007), and Cu inhibits the production of morphological defences in Arabian toad tadpoles, *Bufo arabicus* (Barry 2011). Locomotion and respiration are commonly assessed traits to demonstrate the response to heavy metal pollution, and show changes in a wide range of aquatic species at considerably lower concentrations than those found to be lethal (Oligocheates: Gerhardt 2009; Cyprinodontiformes and Cladocera: Gerhardt et al. 2005; Decapoda: de Bisthoven et al. 2006; Amphipoda, Trichoptera and Ephemeroptera: Macedo-Sousa et al. 2008). These traits are primarily targeted due to the ease with which they can be measured; however, neurological function has been demonstrated to be affected following ingestion of low levels of heavy metals in mammals (Gilbert and Lasley 2002; Salinas and Huff 2002; Toscano and Guilarte 2005), and may therefore prove a more sensitive measure of toxicity than measures of locomotion and breathing behavior.

Freshwater gastropods are important ecosystem engineers, altering macrophyte abundance and distribution, which are susceptible to indirect effects through changes in snail behaviour (Bernot and Turner 2001) and direct effects from changes in snail abundance (Brönmark 1989). As key species within freshwater habitats, gastropods should be used as focal species in judging the potential impact of water pollution in freshwater ecosystems (Amiard-Triquet 2009). In addition to their importance in freshwater systems, aquatic gastropods are sensitive to sublethal levels of heavy metals. For example, chronic exposure to cadmium (32 µg/l) affects the feeding and growth of *Lymnaea luteola* (Das and Khangarot 2010). Similarly, chronic exposure to low levels of Cu pollutants delays hatching (Khangarot and Daas 2010) and causes reduced growth and locomotion (Das and Khangarot 2011) in *L. luteola.* Cu also inhibits the growth rate of *L. stagnalis*, with an acute exposure altering internal levels of Na and Ca (Ng et al. 2011). *Lymnaea pulustris* and *Physella columbiana* exposed to heavy metal pollutants fail to exhibit antipredator behaviour (Lefcort et al. 1999, 2000). In addition there is also some evidence that aquatic gastropods may be able to sense and avoid low levels of heavy metals in their environment as *Physella columbiana* from heavy metal sites demonstrates avoidance of these pollutants in a Y-maze (Lefcort et al. 2004).

The great pond snail, *Lymnaea stagnalis*, is commonly used as a model species to study learning and memory in neurobiology (Benjamin and Kemenes 2008; Lukowiak et al. 2003; Parvez et al. 2006). Ecologically relevant stressors can alter the ability of this species to learn and remember. Depending on the type, intensity and timing of the stress relative to the learning procedure, stress can either enhance or diminish memory formation (Lukowiak et al. 2010). Additionally, a low concentration (100 µmol/l) of an industrial pollutant, H_2S, has been previously demonstrated to block memory formation (Rosenegger et al. 2004). If heavy metals act as a stress for *L. stagnalis* it is likely that exposure to toxic metals will alter the snail's ability to demonstrate behavioural plasticity following experience. We assessed the effects of two heavy metals, Zn and Cd, on behavioural traits using *L. stagnalis*. These metals were chosen due to their synergistic effects at low levels on survival of the Columbia spotted frog tadpole, *Rana luteiventris* (Lefcort et al. 1998). We tested whether exposure to these metals alter respiration and locomotion, commonly used to assess the sublethal impacts of heavy metals (Boyd 2010). We also tested the ability of *L. stagnalis* to form long-term memory (LTM) in the presence of these metals, both singularly and in combination, as heavy metals in combination can be more toxic than when they experienced alone, even at low concentrations (Wah Chu and Chow 2002). Previous work indicated that freshwater snails may be able to sense heavy metal toxins in the water (Lefcort et al. 2004), therefore we wanted to assess whether acute responses to metal pollution are due to sensory input. The osphradium is an external chemosensory organ in *L. stagnalis* (Kamardin et al. 2001; Wedemeyer and Schild 1995), and modulates memory formation through sensing chemical cues associated with stress in the environment (Il-Han et al. 2010; Dalesman et al. 2011). As such we considered that if the snail is sensing external metal concentrations, it might use the osphradium to do so. We severed the nerve connecting the osphradium to the central nervous system to assess whether effects of heavy metals were dependant on sensory input from this organ, and therefore altering behavioural traits through sensing stressful external stimuli rather than internal poisoning.

Materials and methods

Adult *Lymnaea stagnalis* (~25 mm spire height) were used from a laboratory population that originated from snails collected in the 1950s from a Polder outside Utrecht and maintained in the laboratory for approximately 250 generations at the Vrije Universeit in Amsterdam. Snails were reared in the Biological Sciences building at the University of Calgary, maintained in artificial pond water (~0.25 g/l Instant Ocean®, Aquarium Systems Inc., Mentor, OH, USA) with the addition of $CaCO_3$ to maintain calcium concentration >50 mg/l (Hermann et al. 2009). Snails were housed in aquaria containing our standard pond water, de-ionised water combined with Instant Ocean (0.26 g/l) and calcium sulphate dihydrate (344 mg/l, Sigma-Aldrich Inc., St. Louis, MO, USA). Snails were labelled using numbers printed on waterproof paper glued onto the shell prior to experiments to allow individual behaviour within each experiment to be recorded.

Heavy metal exposure

The Canadian maximum allowable levels in municipal drinking water for zinc and cadmium are 5,000 and 5 µg/l, respectively (Toop and de la Cruz 2002).We chose to assess whether heavy metal levels below these allowable limits could alter behavioural traits in *L. stagnalis*. Zinc sulphate heptahydrate ($ZnSO_4{\cdot}7H_2O$; M.W. 287.59: Pfaltz and Bauer, Waterbury, CT. USA), and cadmium chloride ($CdCl_2$; M.W. 183.35: Fisher Scientific Company, Ottawa, Ontario, Canada) were added to our standard pond water (see above) to produce heavy metal water. A zinc concentration of 1,100 µg/l or cadmium concentration of 3 µg/l was used for the single metal exposures, with both metals added at these concentrations in the combined treatment.

Aerial breathing rate

Lymnaea stagnalis are bimodal breathers, in high oxygen conditions they breathe primarily by absorbing oxygen directly across their skin; however, when water becomes hypoxic they move to the surface to perform aerial respiration using a rudimentary lung opened to the air via the pneumostome (respiratory orifice). Initial aerial breathing behaviour prior to metal exposure (0 h) was assessed in standard pond water alone. Snails were then exposed to either control (pond water alone), single metals (Zn or Cd) or combined metals (Zn and Cd) for 48 h at the concentrations outlined above. Subsequent breathing behaviour was then assessed at 24 and 48 h. To assess aerial breathing behaviour, 500 ml of water, either control or containing metals dependant on treatment, was placed in a 1-l glass beaker and then made hypoxic to encourage aerial respiration by bubbling N_2 through the water for 20 min. Snails were acclimated to conditions in the beaker for 10 min, and then total breathing time was recorded over a period of 30 min.

Crawling rate

Crawling rates were measured for snails prior to metal exposure (0 h) and then at 24 and 48 h following exposure to either control conditions (in standard pond water) or combined metal exposure (Zn and Cd). Crawling rate was measured by placing an individual snail into a large Petri dish (14 cm diameter by 2 cm depth) in 200 ml of pond water or combined metal water depending on treatment group, giving a depth of 15 mm in the Petri dish sufficient to fully submerge the snail. Once the snail fully emerged from its shell (head completely visible) and commenced movement, the distance each snail moved was assessed by recording movement across a 2 cm × 2 cm grid on the base of the Petri dish during a 15 min period (Dalesman and Lukowiak 2010). This distance was converted into an average crawling rate of mm/s for analysis.

Learning and memory

Long-term memory formation was assessed using operant conditioning of aerial respiration in hypoxia (Lukowiak et al. 1996). Snails were exposed to either control, Zn alone, Cd alone or combined metals (Zn and Cd), and trained following 24 h exposure. They were then tested for LTM formation 24 h after training, resulting in 48 h total exposure time.

To make water hypoxic, 500 ml of water (control, Zn, Cd or Zn and Cd depending on exposure group) was placed in a one litre beaker and N_2 was bubbled through for 20 min to make water hypoxic. Snails were placed into the beaker and allowed to acclimate for 10 min. They were then trained by gently poking the snail on the pneumostome each time it attempted to breathe, such that the pneumostome closed but the snail did not fully withdraw into its shell. Each individual received two training sessions (TS1 and TS2) lasting 30 min, separated by an hour. This was followed by a memory test (MT) 24 h after TS2, using identical protocol to a single training session (Lukowiak et al. 1996).

Long-term memory was determined to be present if the number of attempted pneumostome openings in MT was significantly lower than TS1 but not significantly higher than TS2 (Sangha et al. 2002).

Osphradial nerve cuts

To assess the role that the osphradium, an external chemosensory organ, has in the snail's response to heavy metals we severed the osphradial nerve. Snails were anaesthetized, a small slit was made in the skin, and the osphradial nerve was severed proximal to the osphradium to interrupt input to the central nervous system (Dalesman et al. 2011). Sham operated animals, where the slit was made but the osphradial nerve was left intact, were used to control for the effects of surgery. Snails were allowed to recover for 1 week following surgery, then trained and tested as above ("Learning and memory" section) in either control or combined heavy metals.

Data analysis

Data were analysed using repeated measures ANOVA in SPSS 17.0 (SPSS Inc.‘ Chicago, IL, USA). Mauchley’s test for sphericity was used to confirm homogeneity of variance prior to all analyses. Breathing rate was analysed as the total breathing time in 30 min, with zinc exposure (present/absent) and cadmium exposure (present/absent) as the between-subject factors and exposure duration (0, 24 or 48 h) as the within-subject factor. Crawling rate was analysed as the average crawling speed (mm/s), with metal exposure (control versus combined metals) as the between-subject factor and exposure duration (0, 24 or 48 h) as the within-subject factor. The response to training in intact snails was measured as the number of pneumostome opening attempts in 30 min, with zinc exposure (present/absent) and cadmium exposure (present/absent) as the between-subject factors, and the training or test period (TS1, TS2 and MT) as the within-subject factor. The effect of severing the osphradial nerve on the response to training in heavy metals was analysed using surgery (sham versus cut) and metal exposure (control versus combined metals) as between-subject factors and the response to training (TS1, TS2 and MT) as the within-subject factor. Where significant interaction effects were found, post hoc analyses were used to identify where significant differences lay among treatment groups, using Student–Newman–Keuls (SNK) tests to assess between-subject pair-wise differences and paired *t* tests for within-subject differences. *P*-value required for significance for paired *t* tests was adjusted to 0.0167 for to account for multiple comparisons. Final *N*-values are presented in the figure legends.

Results

Effect of heavy metals on respiration and locomotion

There was no significant effect of acute exposure to Zn ($F_{1,59} = 0.73$, $P = 0.40$) or Cd ($F_{1,59} = 1.29$, $P = 0.26$) or a combination of Zn and Cd ($F_{1,59} = 0.37$, $P = 0.54$), on overall aerial respiration behaviour in the absence of training (Fig. 1). Length of exposure (0, 24 or 48 h) also had no effect on breathing rate overall ($F_{2,118} = 0.18$, $P = 0.83$), interaction with individual metals (Zn: $F_{2,118} = 2.06$, $P = 0.13$; Cd: $F_{2,118} = 1.64$, $P = 0.20$) or Zn and Cd combined ($F_{2,118} = 2.23$, $P = 0.11$). Exposure to Zn and Cd did not significantly alter the mean crawling rate of snails in each exposure group (main effect of metal presence: $F_{1,28} = 2.336$, $P = 0.14$) or when assessed across different exposure periods (interaction metals × exposure period: $F_{2,56} = 0.435$, $P = 0.65$), neither was there an overall effect of duration of exposure (Fig. 2, main effect of duration: $F_{2,56} = 0.446$, $P = 0.64$). Hence, there was no effect of heavy metal exposure on either of these basic behavioural traits.

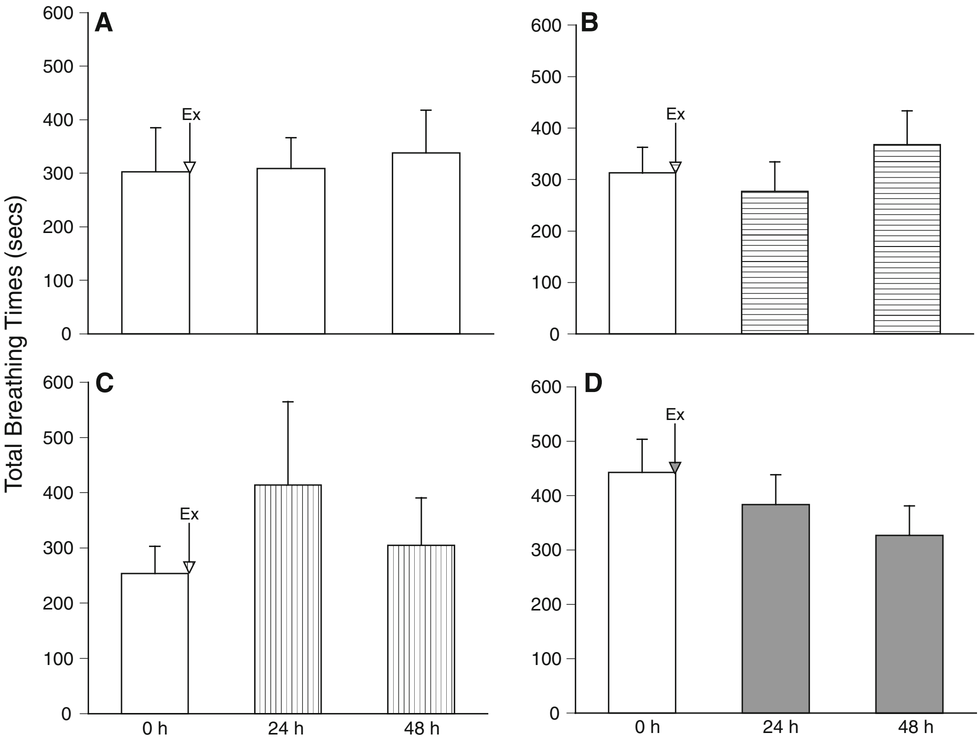


Fig. 1 Mean total breathing time (s ± SEM in 30 min) in hypoxia. Exposure to control conditions only (*white column*) at 0 h, followed by 48 h exposure (*Ex*) to **a** control pond water, *white columns* ($N = 16$); **b** zinc, *horizontal stripe* ($N = 18$); **c** cadmium, *vertical stripe* ($N = 16$) and **d** combined zinc and cadmium, *dark grey* ($N = 13$)

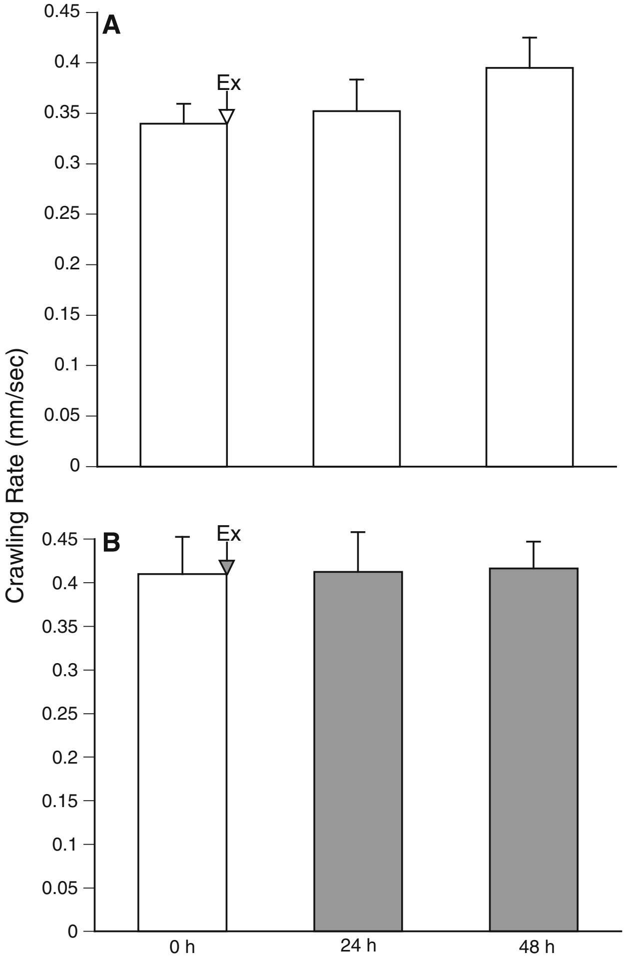


Fig. 2 Mean crawling rate (mm/s ± SEM) over 15 min. Exposure to control conditions only (*white column*) at 0 h, followed by 48 h exposure (*Ex*) to **a** control pond water, *white columns* ($N = 14$) or **b** combined zinc and cadmium, *dark grey* ($N = 16$)

Effect of heavy metals on learning and memory

Neither Zn or Cd exposure alone altered the ability of *L. stagnalis* to learn and form memory relative to the controls; however, the snails exposed to combined metals did not demonstrate intermediate or LTM following training, i.e. memory formation was blocked (Fig. 3; three-way interaction between metal presence, Zn and Cd, and effect of training: $F_{2,94} = 5.38$, $P = 0.006$). Control and single metal exposure groups learnt and formed intermediate-term memory, demonstrated by a significant decline in aerial respiration attempts during the second training (TS1 vs. TS2: control: $t = 7.71$, $P < 0.001$; Zn alone: $t = 5.61$, $P < 0.001$; Cd alone: $t = 4.98$, $P = 0.001$), and also formed LTM at 24 h (TS1 vs. MT: control: $t = 6.43$, $P < 0.001$; Zn alone: $t = 7.73$, $P < 0.001$; Cd alone: $t = 6.03$, $P < 0.001$). However, the response to operant conditioning to reduce aerial respiration was significantly altered by the presence of the two metals combined, such that snails did not demonstrate learning and intermediate-term memory (TS1 vs. TS2: $t = 1.24$, $P = 0.24$) or LTM at 24 h (TS1 vs. MT: $t = 0.18$, $P = 0.86$). The effect of the two metals on memory formation was not due to a change in initial number of pneumostome opening attempts, as this did not differ among treatment groups (SNK: $P > 0.05$ for all pair-wise tests), though the number of opening attempts was significantly greater during both TS2 and MT following training in both Zn and Cd compared to both the control group and groups exposed to each metal individually (Fig. 3; SNK: TS2: $P < 0.05$; MT: $P < 0.05$ for all pair-wise tests between combined metals and other groups).

Role of the osphradium in the response to heavy metals

In sham groups, snails exposed to control conditions significantly reduced the number of pneumostome opening attempts in response to training (TS1 vs. TS2: $t = 9.54$, $P < 0.001$; TS1 vs. MT: $t = 6.07$, $P < 0.001$), where as those in heavy metals did not (TS1 vs. TS2: $t = 0.54$, $P = 0.62$; TS1 vs. MT: $t = 0.24$, $P = 0.82$). However, in snails with the osphradial nerve severed both those exposed to control (TS1 vs. TS2: $t = 5.09$, $P < 0.001$; TS1 vs. MT: $t = 4.46$, $P = 0.001$) and Zn and Cd together (TS1 vs. TS2: $t = 6.14$, $P < 0.001$; TS1 vs. MT: $t = 6.03$, $P < 0.001$) demonstrated a significant decrease in pneumostome opening attempts following training (Fig. 4; three-way interaction: surgery × metal exposure × response to training: $F_{2,92} = 3.12$, $P = 0.049$). The initial number of pneumostome opening attempts (TS1) did not differ among treatment groups (SNK: $P > 0.05$ for all pair-wise comparisons), and therefore does not explain the difference in the response to training.

Discussion

Here we present the first results demonstrating that toxic heavy metal pollution has a negative effect on cognition in an invertebrate, and that this measure of the potential ecological impact is more sensitive to low pollutant levels than using standard measures of locomotion and respiration (Gerhardt 2009; Gerhardt et al. 2005; de Bisthoven et al. 2006; Macedo-Sousa et al. 2008). Importantly, this memory deficit is only seen at these low concentrations when Zn and Cd are presented together, and not when presented alone. Additive and synergistic effects of combinations of toxic metals are a common complication in studies involving the potential impact of heavy metals (Boyd 2010). Natural environments usually contain more than one heavy metal pollutant released through anthropogenic

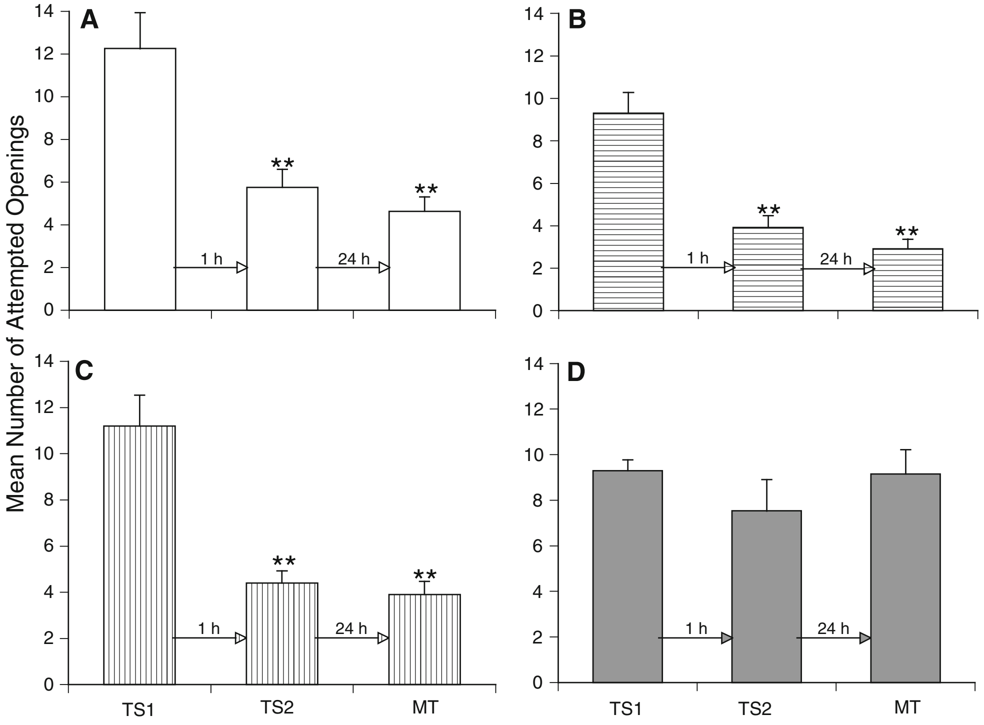
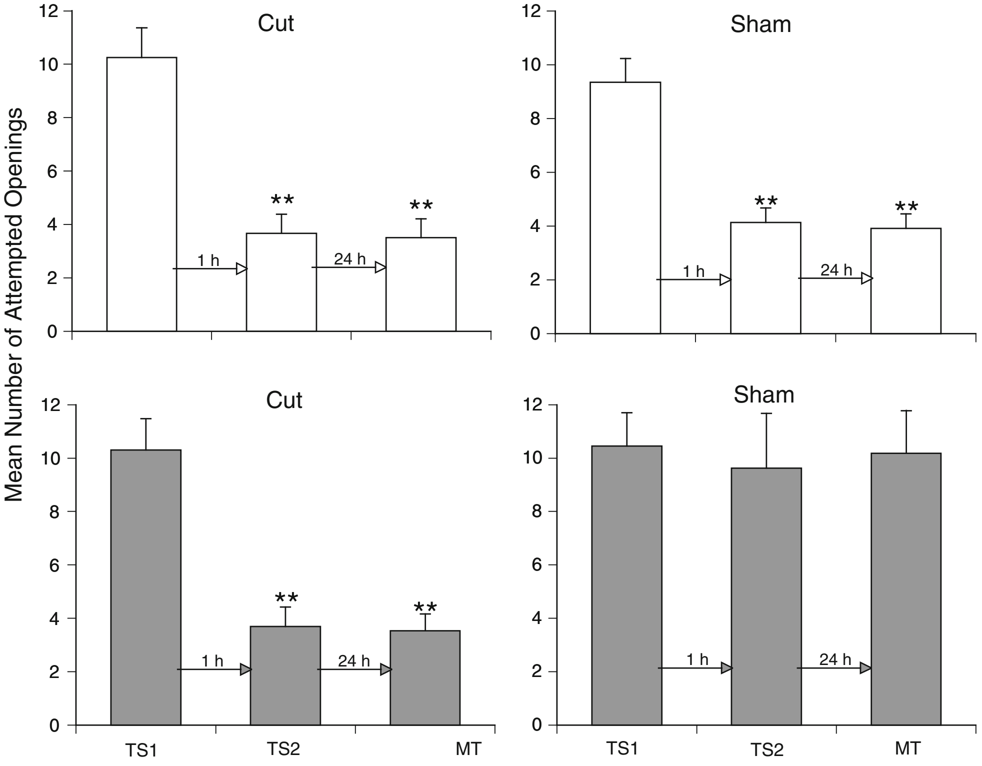


Fig. 3 Mean number of attempted pneumostome openings (±SEM) in 30 min in the first (TS1) and second (TS2) training sessions and the memory test (MT) 24 h later following exposure to **a** control pond water, *white columns* ($N = 16$); **b** zinc, *horizontal stripe* ($N = 12$); **c** cadmium, *vertical stripe* ($N = 10$) and **d** combined zinc and cadmium, *dark grey* ($N = 13$) 24 h prior to training and between training and testing. **Significantly different from TS1 (paired *t* test, $P < 0.01$)

Fig. 4 Mean number of attempted pneumostome openings (±SEM) in 30 min in the first (TS1) and second (TS2) training sessions and the memory test (MT) 24 h later following exposure to either control pond water (*white columns*) or combined zinc and cadmium (*dark grey columns*) 24 h prior to training and between training and testing. Snails either underwent sham surgery (sham) or had the osphradial nerve severed (cut) 1 week prior to TS1. *N*-values: sham/control ($N = 14$), sham/metal exposed ($N = 11$), cut/control ($N = 12$) and cut/metal exposed ($N = 13$). **Significantly different from TS1 (paired *t* test, $P < 0.01$)



activity (Kelly et al. 2010); hence it is important to develop an understanding of how different heavy metals interact to affect an organism.

Previous work on vertebrates has demonstrated that ingestion of a heavy metal, specifically Pb, can alter cognitive function at sublethal levels (Gilbert and Lasley 2002; Salinas and Huff 2002; Toscano and Guilarte 2005). These effects appear to be due to direct changes in neuron functioning, for example exposing rats to lead causes changes in the glutamate, dopamine and NMDA receptor systems (Lasley and Gilbert 2000; Lasley et al. 2001). The effect of Zn and Cd on cognitive function in *L. stagnalis* is due to the snail sensing these metals in the pond water, and these pollutants acting as a stressor blocking memory formation. We reached this conclusion since snails exposed to Zn and Cd, but whose osphradial nerve had been severed, formed LTM. The osphradium has been shown previously to communicate with the central nervous system in order to modulate behaviour in response to other environmental stressors (Dalesman et al. 2011; Il-Han et al. 2010). Therefore, the effect of combined metals (Zn and Cd) on cognitive function in *L. stagnalis* is due to the snail sensing these metals and metals acting as a stressor blocking memory formation, i.e. the metals are not having a direct effect in the snail central nervous system. Another pulmonate snail, *Physella columbiana*, demonstrated orientation away from heavy metal pollutants (Cd, Zn and Pb), therefore the ability to detect heavy metals may be present throughout this group (Lefcort et al. 2004). Additionally, effects of Fe, Cr and Zn heavy metals on fecundity in a closely related gastropod, *Lymnaea pulustris*, whilst relating to concentration of these pollutants in the water, did not relate to internal concentrations, indicating that this species may also have been responding to metal pollutants as a stressor rather than via direct effects on physiology (Coeurdassier et al. 2005).

Heavy metals tend to accumulate in aquatic invertebrates over time in a dose dependant manner (Goodyear and McNeill 1999), therefore at low levels of a pollutant effects may not be seen in acute studies, but only become apparent following chronic exposure. For example, at levels below those that cause mortality, but still higher than those used in this experiment, cadmium can have a negative impact on the growth and sexual maturity of two pond snails, *L. stagnalis* and *L. pulustris*, following chronic exposure (Coeurdassier et al. 2003). The ability of *L. stagnalis* to sense toxic heavy metals, and therefore respond following acute exposure as a memory blocking stressor, demonstrates the sensitivity and speed with which this species can be used in assessing low levels of heavy metal contamination.

Although the heavy metals affected the normal memory formation in the snails, it did not change the locomotion or breathing behaviour. Previously, *L. stagnalis* was shown to decrease activity after chronic exposure to lead (Pyatt et al. 2002). Most studies showing changes in metabolic rate have investigated chronic exposure to metals, which might be why there was no change in breathing rate seen in the animals in this study. For example, freshwater shrimp exposed to heavy metals for 8 months demonstrating normal survival show increased metabolic rate as measured by oxygen consumption (Rowe 1998). It appears that assessing memory formation in *L. stagnalis* is more sensitive for low, acute doses of heavy metals than locomotion or breathing rate. Chronic effects may be due to metabolic costs of expressing metallothionein proteins (Houlihan et al. 1995). These proteins are used to protect the organism against accumulation of heavy metals that would cause mortality (Janssens et al. 2009), but may increase metabolic rate and decrease energy available for locomotion if costly to produce. We predict that chronic exposure of *L. stagnalis* will result in changes in locomotory and respiratory behaviour, common in other aquatic invertebrates, potentially due to the production of metallothionein proteins.

Indirect effects, including those from pollution, can significantly alter community structure (reviewed in: Fleeger et al. 2003), though these studies assessed changes in overall abundance in key species, rather than changes in behaviour and habitat use. Aquatic gastropods can have an integral role shaping habitat, for example by altering macrophyte abundance, either directly through changes in gastropod abundance (Brönmark 1994) or indirectly via changes in gastropod behaviour and habitat use (Bernot and Turner 2001). Aquatic snails rely on learning and memory to assess predation risk (Dalesman et al. 2006, 2009) and learn to recognise heterospecific alarm cues (Dalesman and Rundle 2010). The presence of heavy metals reducing the snail's ability to learn and form memory may therefore impact both directly on snail fitness (i.e. probability of survival when encountering a predator) and also indirectly on their distribution within the habitat.

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