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# Toxicity of cadmium and zinc mixtures to the decaudized cercarial life span of *Diplostomum spathaceum*

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**Abstract**

The effects of cadmium and zinc mixtures at concentrations ranging from 0.1 to 10,000 $\mu g\,l^{-1}$ on the life-span of decaudized cercarial bodies (cercariae that have shed their tails) of *Diplostomum spathaceum* (Trematoda: Diplostomatidae) was investigated. Cercariae were exposed to metal mixtures of equal and unequal concentrations, and a low-dose pre-treatment followed by a high-dose exposure mixtures. Metal mixtures demonstrated variable effects on decaudized cercariae either by increasing or reducing their life-span compared to single metal exposures dependent on concentration and the type of mixed metal treatment. Prolonged exposure to equal metal mixtures at low concentrations (0.1–100 $\mu g\,l^{-1}$) resulted in a reduction in the life-span of decaudized cercariae at 0.1 and 100 $\mu g\,l^{-1}$ in those individuals decaudized during the initial 24 h exposure period compared with those decaudized during the final 24 h period of cercarial survival, whilst in controls there was no significant life-span change between the two time periods. Decaudized cercariae which were exposed to low concentrations (0.1–100 $\mu g\,l^{-1}$) of equal metal mixtures were also evaluated for their role as an indicator of larval 'fitness' for migrating through the tissues of their target fish host for those individuals decaudized during the initial 24 h exposure period, and demonstrated only a limited change in their life-span compared to control and single metal exposures. The importance of metal mixtures in parasite establishment in the fish host is discussed.

## Introduction

The toxic effects of pollutants on parasite transmission through wild aquatic host populations has, in recent years, become an increased focus of study (e.g. Lewis & Hoole, 2003). Trematodes, in particular, have been the subject of many investigations because of their medical and veterinary importance, especially the vulnerability of free-living stages, which may be particularly susceptible to toxicants (Morley *et al.*, 2003c). Studies on the effects of toxicants on cercariae have mainly considered single-metal exposures and their effects on survival (e.g. Abd Allah *et al.*, 1996), and these results have shown only limited cercarial mortality at low toxic concentrations, especially during the initial free-living period when cercariae show maximum infectivity to the target host. Attention has thus begun to focus on other mechanisms of cercarial functional biology which may be better indicators of an increased vulnerability to toxicants.

The present paper forms part of a larger ecotoxicological study on cadmium and zinc toxicity to the cercariae of *Diplostomum spathaceum* (Morley *et al.*, 2001, 2002a,b, 2003a,b, 2005a,b), a ubiquitous parasite of fish and birds in the UK (Chubb, 1979). These studies have demonstrated a number of important physiological factors which influence parasite transmission under toxicant exposure. Morley *et al.* (2003b), in particular, have shown how studies on the life span of decaudized cercariae, an important but brief transitory life stage occurring once the cercariae have attached to the target host surface and shed their tails prior to penetration (Hoglund, 1991), under cadmium and zinc single-metal exposures can provide

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important information on mechanisms of toxicity in relation to the functional biology of cercariae. In addition, decaudized cercariae may act as a possible indicator of the relative 'fitness' of cercariae, in terms of at least glycogen utilization, to migrate into their target fish host. Morley *et al.* (2002b, 2005a) have shown how cadmium/zinc mixtures can increase survival and prolong the period of tail loss in *D. spathaceum* compared to single-metal exposures.

The aim of the present paper is to investigate how Cd/Zn mixtures administered either as equal concentrations, as high/low concentrations, or as a short pre-treatment followed by a high metal dose may influence the life span of decaudized cercariae. Measurements of the interactions between the two metals will provide a better understanding of potential toxic effects to *D. spathaceum* cercarial transmission, especially as mixtures of heavy metals are more likely to occur under natural conditions.

## Materials and methods

Stock solutions of $100\,mg\,l^{-1}$ cadmium and zinc were prepared by dissolving either cadmium chloride ($CdCl_2.5/2H_2O$) or zinc chloride ($ZnCl_2$) (Sigma Chemicals) in distilled water to give the correct concentration of metal ions. Test solutions were obtained by diluting stock solutions in distilled water and adding them to the test vessels at concentrations which gave final cercarial exposures of $0.1\,\mu g\,l^{-1}$, $10\,\mu g\,l^{-1}$, $100\,\mu g\,l^{-1}$, and $10{,}000\,\mu g\,l^{-1}$. Synthetic soft water ($25\,mg\,l^{-1}$ $CaCO_3$, pH 7.85) was prepared using procedures described by HMSO (1969).

Samples of test solutions were analysed for metal loss from soft water incubated at 20°C for metal concentrations ranging from $10\,\mu g\,l^{-1} - 10{,}000\,\mu g\,l^{-1}$ ($0.1\,\mu g\,l^{-1}$ was below the limit of detection) after 0.5 h and 24 h. Solutions were analysed on a Perkin Elmer Optima 3300 Inductively Coupled Plasma-Atomic Emission Spectrometer which was calibrated with a 1% nitric acid blank and a standard consisting of $1000\,\mu g\,l^{-1}$ of cadmium and zinc in 1% nitric acid. The accuracy of data, which were assessed by analysing certified reference material (NIST SRM 1643d) along with the samples under study, was calculated to have a relative error of 0.00037% for cadmium and 0.00065% for zinc.

Specimens of *Lymnaea stagnalis* naturally infected with *D. spathaceum* were collected from Dinton Pastures, Reading, UK (National Grid Reference SU779724), maintained in aerated 8-l perspex tanks and fed on a diet of lettuce leaves. Recently emerged cercariae of *D. spathaceum* were identified according to morphological criteria described by Niewiadomska (1986). Infected snails and solutions were acclimatized to each experimental temperature in a 12 h light/dark incubator for 24 h prior to the beginning of each experiment as required.

Toxic effects were investigated by pipetting 36 cercariae (maximum age 25 min) individually into wells of flat-bottomed 96-well microtitre plates (Life Sciences International) using a wide-bore pipette tip to prevent cercarial damage. Each well contained a final solution volume of 300 $\mu$l of either distilled water, soft water, or hard water with sufficient test solution having been added, or a control addition of distilled water, to give the appropriate concentration exposure. Cercariae were maintained in a 12 h light/dark incubator set at 20°C and all experiments were set up at the same time of day. Three replicates of each treatment were set up and observations of cercariae were undertaken at intervals of 0.5 to 3 h, depending on the concentration of the test solution used. Once the cercariae had shed their tails they were observed at the same time intervals until death occurred. Tail loss occurred at regular intervals as the cercarial population aged (Morley *et al.*, 2002a, 2005a). Death was pronounced when decaudized cercariae failed to respond to mechanical stimulation with a fine needle. Cercariae were exposed concurrently to the following mixtures: equal concentrations of $10{,}000\,\mu g\,l^{-1}$ Cd-$10{,}000\,\mu g\,l^{-1}$ Zn, $100\,\mu g\,l^{-1}$ Cd-$100\,\mu g\,l^{-1}$ Zn, $10\,\mu g\,l^{-1}$ Cd-$10\,\mu g\,l^{-1}$ Zn, $0.1\,\mu g\,l^{-1}$ Cd-$0.1\,\mu g\,l^{-1}$ Zn; unequal concentrations of $10{,}000\,\mu g\,l^{-1}$ Cd-$100\,\mu g\,l^{-1}$ Zn, $10{,}000\,\mu g\,l^{-1}$ Cd-$10\,\mu g\,l^{-1}$ Zn, $10{,}000\,\mu g\,l^{-1}$ Cd-$0.1\,\mu g\,l^{-1}$ Zn, $10{,}000\,\mu g\,l^{-1}$ Zn-$100\,\mu g\,l^{-1}$ Cd, $10{,}000\,\mu g\,l^{-1}$ Zn-$10\,\mu g\,l^{-1}$ Cd, $10{,}000\,\mu g\,l^{-1}$ Zn-$0.1\,\mu g\,l^{-1}$ Cd. In addition, cercariae were exposed for 1 h to a pre-treatment of either $10\,\mu g\,l^{-1}$ Cd or $10\,\mu g\,l^{-1}$ Zn before a high concentration of either $10{,}000\,\mu g\,l^{-1}$ Zn or $10{,}000\,\mu g\,l^{-1}$ Cd was added. For each individual mixture exposure, experiments were also undertaken concurrently with single metal exposures in the concentration range of $0.1–10{,}000\,\mu g\,l^{-1}$. Results were analysed using a Lee-Desu comparison test for differences between the entire decaudized cercarial population. This test calculates survival function directly from the continuous survival times and the resulting estimates do not depend on the grouping of data into a certain number of time intervals. In addition, we investigated whether prolonged metal exposure of aged cercariae affected the subsequent lifespan of the decaudized cercarial body at low concentrations of equal metal mixtures ($0.1–100\,\mu g\,l^{-1}$), where survival over time of the cercarial population exceeded 24 h. This was done by comparing those individuals which decaudized during the initial 24 h exposure period with those that decaudized during the final 24 h survival period in each test solution using the Lee-Desu comparison test. To investigate the relative 'fitness' of decaudized cercariae to migrate through the skin membrane of the target host, the lifespan of individuals, exposed to low concentrations of equal metal mixtures ($0.1–100\,\mu g\,l^{-1}$), which had decaudized only during the initial 24 h post emergence period was compared with controls using the Lee-Desu comparison test (glycogen utilization by cercariae during this period has been determined to primarily occur from the tail reserves rather than the body reserves as described by Ginetsinskaya, 1960, and Fried *et al.*, 1998). Statistical analysis was performed using UNISTAT 4.5 (Unistat Ltd, 1996).

## Results

Analyses of water samples showed that loss of dissolved metals occurred in most test solutions by 0.5 h, which continued up to 24 h. The highest concentrations (10,000 $\mu g\,l^{-1}$) showed the greatest initial rate of metal loss (8% by 0.5 h), although none of the test solutions exceeded a 15% loss over the 24 h period.

Under control conditions, the decaudized cercarial life-span of *D. spathaceum* showed a rapid decline in survival in the first few hours after tail loss with a minority of cercariae surviving for an extended period of time up to a maximum of 18 h (fig. 1).

The effects of mixing cadmium and zinc resulted in a variable change in the decaudized cercarial life-span compared with controls and single metal exposures, depending on the test solution. With exposure to equal metal mixture (fig. 1.) there was a significant difference between Cd/Zn mixtures and controls in all concentrations apart from 100 $\mu g\,l^{-1}$ Cd/Zn (Lee-Desu test $P \leq 0.0408$). However, mixed exposures demonstrated only limited differences from single-metal exposures dependent on the particular metal concentration, with significant differences only being recorded at 10,000 $\mu g\,l^{-1}$ between mixed and single Zn exposures (Lee-Desu test $P = 0.0227$), at 10 $\mu g\,l^{-1}$ between mixed and both single metal exposures (Lee-Desu test $P \leq 0.0082$), and at 0.1 $\mu g\,l^{-1}$ between mixed and single Cd exposures (Lee-Desu test $P = 0.0097$).

At exposures to high cadmium/low zinc concentrations (fig. 2) there was a significant difference in the decaudized cercarial life span between 10,000 $\mu g\,l^{-1}$ Cd single exposure and all mixed exposures (Lee-Desu test $P \leq 0.0458$) and between all mixed exposure concentrations apart from between 10,000 $\mu g\,l^{-1}$ Cd/100 $\mu g\,l^{-1}$ Zn and 10,000 $\mu g\,l^{-1}$ Cd/0.1 $\mu g\,l^{-1}$ Zn (Lee-Desu test $P < 0.0001$). At exposures to high zinc/low cadmium concentrations (fig. 3) there was a significant difference in the life span between 10,000 $\mu g\,l^{-1}$ Zn single metal exposure and all mixed exposures apart from 10,000 $\mu g\,l^{-1}$ Zn/100 $\mu g\,l^{-1}$ Cd (Lee-Desu test $P \leq 0.0444$) and between all the mixed exposure concentrations (Lee-Desu test $P \leq 0.0042$). With pre-treatment exposures (fig. 4) the was a significant difference in the decaudized cercarial life span between pre-treatment and concurrently administered metal mixture exposures (Lee-Desu test $P \leq 0.0012$) but no significant difference occurred between pre-treatment and single metal exposures.

To determine whether the life span of decaudized cercariae was affected by the age of the cercariae prior to decaudization, an examination was undertaken at low concentration equal metal exposures (0.1–100 $\mu g\,l^{-1}$) of the decaudized cercarial life-span between those decaudized in the initial 24 h exposure period compared to those in the final 24 h period of survival of the experimental population. This demonstrated that in both 100 $\mu g\,l^{-1}$ and 0.1 $\mu g\,l^{-1}$ equal metal mixture exposures there was a significantly reduced life-span in the latter 24 h period compared with the initial 24 h period (Lee-Desu test $P \leq 0.0119$). In contrast, there was no significant difference for neither control nor cadmium single metal exposures between the two time periods, although all zinc single metal exposures demonstrated a significantly reduced life span in the latter 24 h period (Lee-Desu test $P \leq 0.0064$).

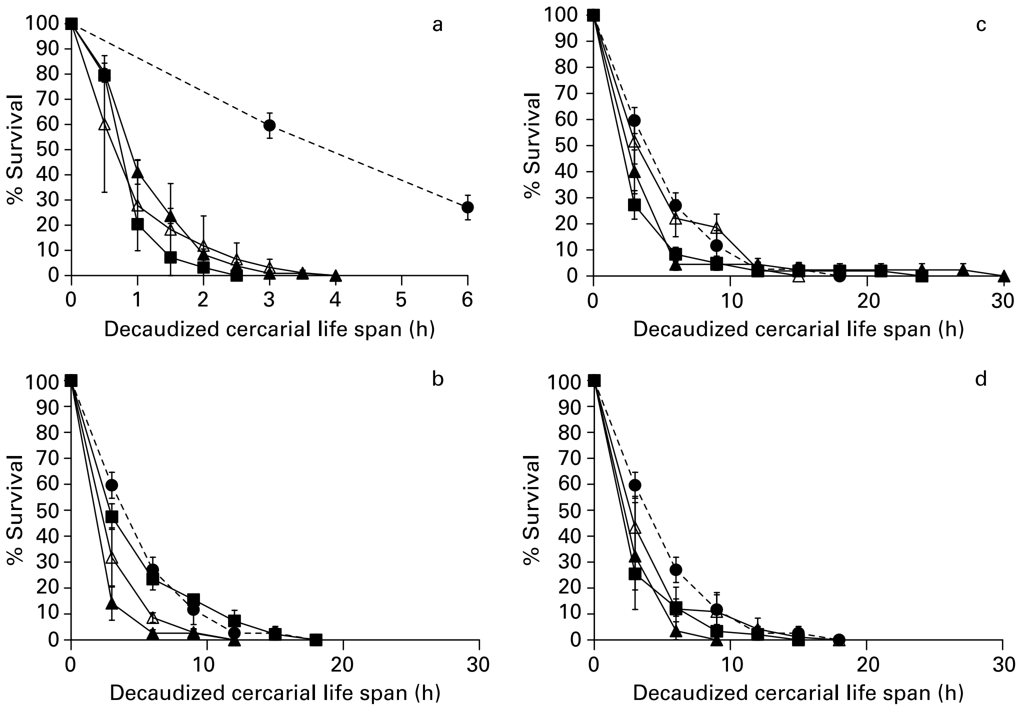


Fig. 1. The decaudized cercarial life-span of *Diplostomum spathaceum* exposed to cadmium and zinc either as single metals or metal mixtures of equal concentration (a, 10,000 $\mu g\,l^{-1}$; b, 100 $\mu g\,l^{-1}$; c, 10 $\mu g\,l^{-1}$; d, 0.1 $\mu g\,l^{-1}$). ●, Control; ■, Cd/Zn mixture; △, Cd single-metal exposure; ▲, Zn single-metal exposure.

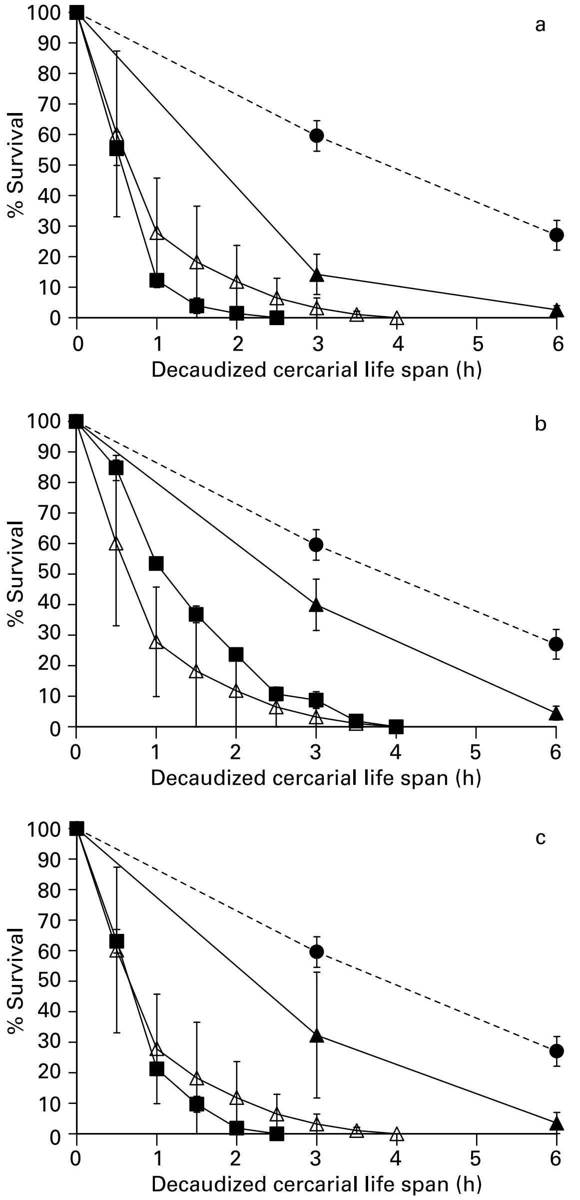


Fig. 2. The decaudized cercarial life-span of *Diplostomum spathaceum* exposed to cadmium and zinc either as single metals or metal mixtures of unequal concentration (high cadmium concentration with low zinc concentration: a, 10,000 $\mu g\,l^{-1}$ Cd + 100 $\mu g\,l^{-1}$ Zn mixture with 10,000 $\mu g\,l^{-1}$ Cd and 100 $\mu g\,l^{-1}$ Zn single-metal exposures; b, 10,000 $\mu g\,l^{-1}$ Cd + 10 $\mu g\,l^{-1}$ Zn mixture with 10,000 $\mu g\,l^{-1}$ Cd and 10 $\mu g\,l^{-1}$ Zn single-metal exposures; c, 10,000 $\mu g\,l^{-1}$ Cd + 0.1 $\mu g\,l^{-1}$ Zn mixture with 10,000 $\mu g\,l^{-1}$ Cd and 0.1 $\mu g\,l^{-1}$ Zn single-metal exposures). ●, Control; ■, Cd/Zn mixture; △, Cd single-metal exposure; ▲, Zn single-metal exposure.

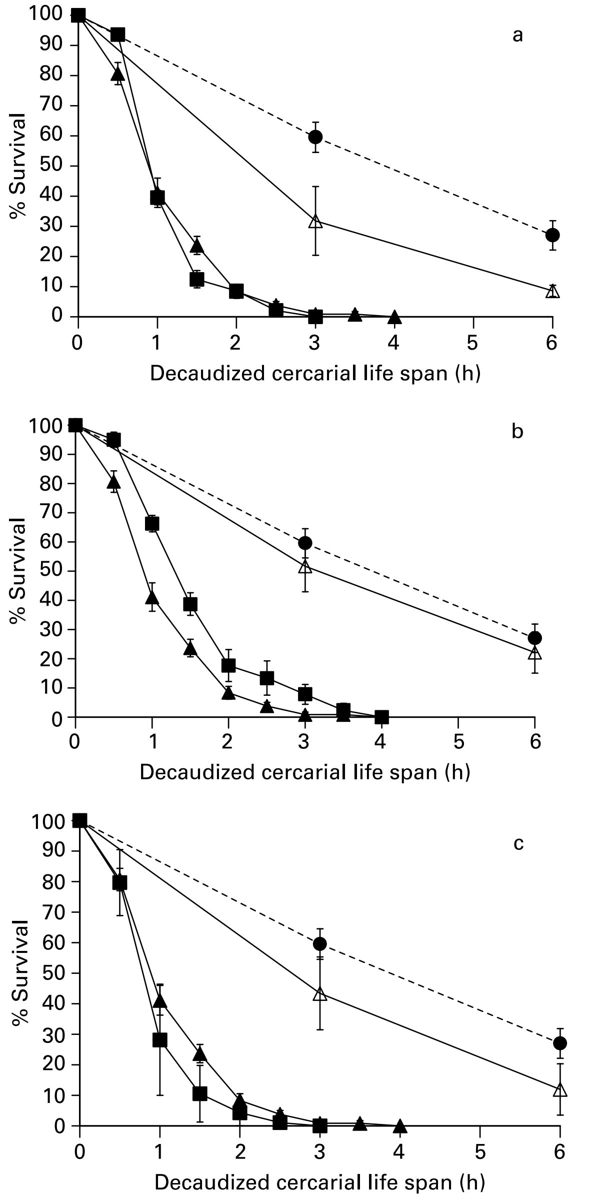


Fig. 3. The decaudized cercarial life-span of *Diplostomum spathaceum* exposed to cadmium and zinc either as single metals or metal mixtures of unequal concentration (high zinc concentration with low cadmium concentration: a, 10,000 $\mu g\,l^{-1}$ Zn + 100 $\mu g\,l^{-1}$ Cd mixture with 10,000 $\mu g\,l^{-1}$ Zn and 100 $\mu g\,l^{-1}$ Cd single-metal exposures; b, 10,000 $\mu g\,l^{-1}$ Zn + 10 $\mu g\,l^{-1}$ Cd mixture with 10,000 $\mu g\,l^{-1}$ Zn and 10 $\mu g\,l^{-1}$ Cd single-metal exposures; c, 10,000 $\mu g\,l^{-1}$ Zn + 0.1 $\mu g\,l^{-1}$ Cd mixture with 10,000 $\mu g\,l^{-1}$ Zn and 0.1 $\mu g\,l^{-1}$ Cd single-metal exposures). ●, Control; ■, Cd/Zn mixture; △, Cd single-metal exposure; ▲, Zn single-metal exposure.

Changes in the decaudized cercarial life-span at low concentrations of equal metal mixtures, which may indicate their relative 'fitness' to infect and migrate through their target host were assessed in those that were decaudized during the initial 24 h exposure period. Only at a concentration of 100 $\mu g\,l^{-1}$ Cd/Zn equal metal mixture was there a significant increased life-span compared to single metal exposures (Lee-Desu test $P \leq 0.0289$), but there was no difference compared with controls (Lee-Desu test, $P = 0.4564$). All other mixed

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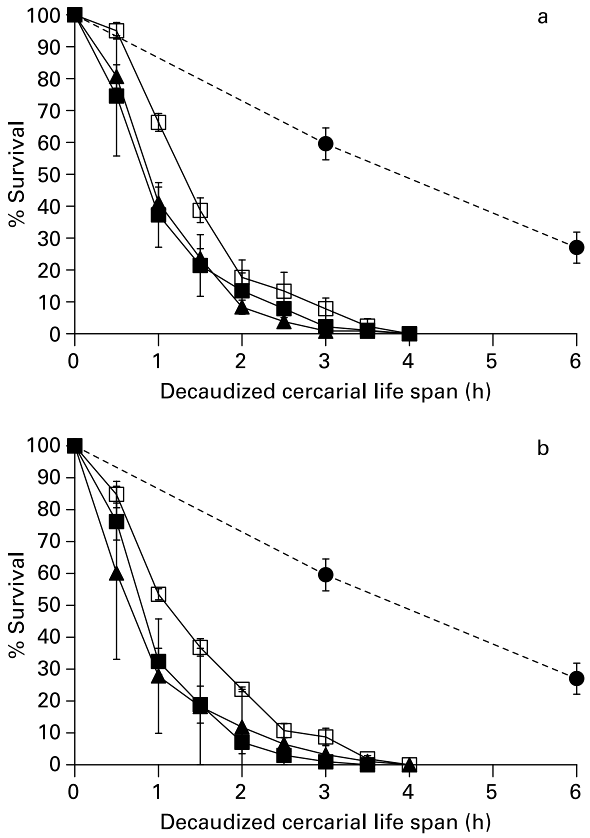


Fig. 4. Influence of low metal concentration (Cd or Zn) pre-treatment on the decaudized cercarial life-span of *Diplostomum spathaceum* exposed to a subsequent high metal concentration (Zn or Cd) compared with single-metal and concurrently administered metal mixture exposures (a, 10 $\mu g\,l^{-1}$ Cd pre-treatment + 10,000 $\mu g\,l^{-1}$ Zn with 10,000 $\mu g\,l^{-1}$ Zn single-metal exposure and 10,000 $\mu g\,l^{-1}$ Zn + 10 $\mu g\,l^{-1}$ Cd mixture; b, 10 $\mu g\,l^{-1}$ Zn pre-treatment + 10,000 $\mu g\,l^{-1}$ Cd with 10,000 $\mu g\,l^{-1}$ Cd single-metal exposure and 10,000 $\mu g\,l^{-1}$ Cd + 10 $\mu g\,l^{-1}$ Zn mixture). ●, Control; ■, 10 $\mu g\,l^{-1}$ pre-treatment + 10,000 $\mu g\,l^{-1}$; ▲, 10,000 $\mu g\,l^{-1}$; □, 10,000 $\mu g\,l^{-1}$ + 10 $\mu g\,l^{-1}$.

exposures demonstrated no significant differences between single metal exposures and controls.

## Discussion

The present study has demonstrated that cadmium and zinc mixtures can induce variable changes in the life span of *Diplostomum spathaceum* decaudized cercariae. The two metals in combination may either have an antagonistic, synergistic or no significant effect compared to single metal exposures dependent on the type of mixed exposure and metal concentration used. These complex interactions, which are taking place in mixed exposures, appear to demonstrate little evidence of any pattern in the changes induced in the decaudized cercarial life-span, suggesting that it would be difficult to make any broad generalizations of the mechanisms of toxicity, although it is likely that these changes are associated with competition for cellular binding sites between cadmium and zinc. Metal ion stability whilst binding to cellular targets is governed by ionic and covalent indices. Cadmium and zinc have a similar low covalent index which may produce variable effects in combination during toxicity tests (Wah Chu & Chow, 2002). The complexity of the results is not unexpected in view of variability in the life span of decaudized cercariae under control conditions and their status as a brief transition life stage, usually only occurring after cercariae have attached to the surface of the target fish host and before penetration begins (Hoglund, 1991). Indeed, Howells *et al.* (1975) reported that an increase in permeability of cercariae that had shed their tails may lead to an increased absorption of toxicants. In mixed metal exposures, where two metals are competing for cellular binding sites, this increasing permeability may determine how toxicity influences the decaudized life-span of cercariae, although little is known about their permeability in *D. spathaceum*. Nevertheless, it is likely that changes in the decaudized cercarial life-span are associated with direct inhibition of enzymes involved in glycogen utilization and to a change in cercarial activity, in turn leading to an alteration in the rate of consumption of the finite glycogen reserves. Heavy metals are known to be major enzyme inhibitors in parasitic helminths (e.g. Goil, 1978; Goil & Harpur, 1979) and have been demonstrated to significantly affect cercarial activity (Cross *et al.*, 2001; Morley *et al.*, 2003a).

The pre-treatment of cercariae led to a reduced life-span compared to concurrently administered metal mixtures. This result is in contrast to many pre-treatment exposures (e.g. Howell, 1985; Willuhn *et al.*, 1996) where a protective effect was induced, associated with the probable induction of metallothionein, or metallothionein-like, protein synthesis. These metal-binding proteins assist in reducing metal toxicity, with exposure to one metal increasing resistance to another leading to co-tolerance (Webb, 1972; Brown, 1978). Interestingly, Morley *et al.* (2002b) showed that overall cercarial survival of *D. spathaceum* under Cd/Zn metal mixture exposures demonstrated an increased survival after pre-treatment. It is possible that during pre-treatment if metallothionein is being synthesized in cercariae it may be doing so only in the tail, which would not benefit the subsequent decaudized cercaria's life-span. This contrast in possible metallothionein synthesis between cercarial bodies and tails would be in accordance with previously recorded differences in their physiology and metabolism (e.g. Coles, 1973; Horemans *et al.*, 1991; Nanduri *et al.*, 1991) but needs to be experimentally determined.

Morley *et al.* (2003b) discussed the validity of examining the decaudized cercarial life-span as an *in vitro* assay for the penetration and initial migration 'fitness' of larvae and contrasted it with some of the difficulties associated with undertaking experimental *in vivo* studies. It was concluded that, at present, the data may provide important information on 'fitness' in terms of glycogen utilization, but direct comparisons with experimental infections of fish would be required to determine if such data could be used as a general indicator of penetration 'fitness'. The investigations of this parameter undertaken in the present study have revealed only slight changes in life-span compared to single metal and control exposures. The potential effects of metal mixtures on cercarial establishment in the target host therefore remain unclear. Nevertheless, studies by Morley *et al.* (2002c) on the transmission of *Echinoparyphium recurvatum* cercariae into snail hosts exposed to Cd/Zn mixtures found reduced parasite establishment compared to single metal exposures with both water hardness and target host species significantly influencing transmission success. As *D. spathaceum* cercariae may infect a range of fish hosts demonstrating differing susceptibilities to the parasite (Wootten, 1974), influenced also by host size (Hoglund, 1995), the effects of metal exposure, either singly or in combination, on transmission are likely to be variable, dependent on a range of biotic and abiotic conditions.

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