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REVIEW/ ARTÍCULO DE REVISIÓN

OVERVIEW OF THE STATUS OF HEAVY METAL ACCUMULATION BY HELMINTHS WITH A NOTE ON THE USE OF *IN VITRO* CULTURE OF ADULT ACANTHOCEPHALANS TO STUDY THE MECHANISMS OF BIOACCUMULATION

UNA MIRADA GLOBAL DEL ESTADO DE LA ACUMULACIÓN POR METALES PESADOS POR HELMINTOS CON UNA NOTA EN EL USO DE CULTIVO *IN VITRO* DE ACANTOCÉFALOS ADULTOS PARA ESTUDIAR LOS MECANISMOS DE BIOACUMULACIÓN

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Abstract

Bioaccumulation of metals by helminths is a well acknowledged phenomenon that has triggered increasing research interest in the past two decades and found applications in environmental studies. The ecological literature is fairly abundant but still shows gaps with some taxa not having been studied. Variations in the ability of helminths to sequester various metals are recognized and a synthetic overview of the literature is provided herein. Adult acanthocephalans are known to be particularly efficient as bioaccumulators of heavy metals. We optimized an *in vitro* culture technique of the acanthocephalan *Moniliformis moniliformis* and initiated *in vitro* exposure to cadmium and lead. We propose to use this technique to study the mechanisms of uptake and sequestration of heavy metals, which are yet to be understood.

Key words: helminthes – acanthocephalans – bioaccumulation - heavy metals - *in vitro* culture - transmission electron microscopy.

Resumen

La bioacumulación por metales por helmintos es un fenómeno bien reconocido que ha provocado un incremento de interés en investigación en las dos décadas pasadas y ha encontrado aplicaciones en estudios ambientales. La literatura ecológica es bastante abundante pero aun muestra lagunas con algunos taxas que no han sido estudiados. Variaciones en la habilidad de los helmintos para secuestrar varios metales son reconocidos y una sintética mirada global de la literatura es proporcionada aquí. Los acantocéfalos adultos son conocidos por ser particularmente eficientes como bioacumuladores de metales pesados. Optimizamos una técnica de cultivo *in vitro* del acantocéfalo *Moniliformis moniliformis* e iniciamos una exposición *in vitro* a cadmio y plomo. Proponemos el uso de esta técnica para estudiar el mecanismo de captación y secuestro de metales, los cuales aun deben ser entendidos.

Palabras clave: helmintos – acantocefala – bioacumulación - metales pesados - cultivo *in vitro* - microscopio electrónico de transmisión.

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INTRODUCTION

The roles of parasites in ecosystems are multiple although too often neglected by scientists (Moller, 1987; Marcogliese & Cone, 1997; Lafferty *et al*., 2008). For example, the sporadic attempts at understanding the synergistic or antagonistic interactions between parasites and pollutants have (e.g., Pascoe & Cram, 1977; Brown & Pascoe, 1989) in general been ignored by the scientific community for decades. In short, pollution has typically been viewed as an added stress to hosts leading to an increased vulnerability to parasitic diseases (e.g., Zelikoff 1993; Arkoosh *et al*., 1998), or as affecting parasites' biodiversity (e.g., Dusek *et al*., 1998), but the parasites' impact proper has been ignored in evaluating the effects of environmental pollutants on organisms. Slowly, the complexity of the relationship between parasitism and pollution has begun to unravel, showing the necessity to consider parasitism in evaluating environmental stressors (e.g., Moller, 1987; Lafferty & Kuris, 1999; Sures & Siddall, 1999; Schludermann *et al*., 2003; Sures, 2006; Hudson *et al*., 2006) since parasites may in turn influence the hosts' response to pollutants by affecting their hosts physiology and tolerance of stressed conditions (MacKenzie, 1999; Marcogliese, 2002; Sures *et al*., 2002,2003a; Turceková *et al*., 2002; Sures, 2006; Sures & Radszuweit, 2007). Ignoring parasites in assessment of pollution on organisms is now recognized as a potential bias in studies, which may then lead to false conclusions (Moller, 1987; Evans *et al*., 2001). Recently, another aspect of the role of parasites in evaluating environmental pollution has emerged via the recognition of their ability to concentrate inorganic elements (heavy metals in particular) at much higher levels than free-living organisms (e.g., MacKenzie *et al.*, 1995; Sures & Siddal, 1999; Taraschewski, 2000). Heavy metals are known to have a negative impact on organisms and ecosystems (e.g, de Caralt *et al.*, 2002; Cámara *et al*., 2008; Cebrian, 2008), to bioaccumulate via the food web (e.g., Zheng *et al*., 2007; Widmeyer & Bendell-Young, 2008), and to be serious threats to human health (e.g., Di Gioacchino *et al*., 2008; Ekino *et al*., 2007). Hence, there is a continuous search for bioindicators of metal pollution (e.g., Rainbow & Philips, 1993), including helminths, as illustrated by the steep increase in manuscripts over the past two decades reporting studies of host-parasite models challenged by heavy metal exposure [see reviews by Sures *et al*. (1999) and Sures (2001, 2003, 2004].

HELMINTHS AS BIOACCUMULATORS OF HEAVY METALS

Pioneering studies on the presence of heavy metals in parasites occurred as early as the late 19th century (*in* von Brand, 1952) as well as in the mid-late 20th century [Ince (1975, 1976) and Greichus & Greichus (1980) worked on an ascarid, Pascoe & Mattey (1977) studied heavy metal effects on a metacestode, Riggs *et al*. (1987) examined an adult cestode, and Brown & Pascoe (1989) cystacanths]. However, Sures *et al*. (1994a-c), Sures & Taraschewski (1995) and Zimmerman *et al.* (1999) may be considered the key-studies that provided the trigger that opened up the modern field of research into this area, which continues to attract the attention of scientists as more and more host-parasite species are being studied. The first models studied by these latter workers were aquatic and involved freshwater fish infected by adult acanthocephalans (*Paratenuis ambiguus*, *Acanthocephalus lucii*, and *Pomphorhynchus laevis*), larval *A. lucii*, and adult nematodes, *Anguillicolla (Anguillicoloides) crassus*. Adult acanthocephalans were shown to accumulate high levels of lead (Pb) and cadmium (Cd) compared to their hosts' tissues. The breadth of studies has now expanded to brackish and marine fish/parasite systems (e.g., Sures *et al*., 1997a; Bergey *et al*., 2002; Sures & Reimann, 2003; Malek *et al*., 2007), as well as to bird (Barus *et al*., 2000; Tenora *et al*., 2001, 2002), mammal (Scheef *et al*. 2000; Sures *et al*., 1998, 2002, 2003b, Barus *et al*., 2003) and crustacean systems (Bergey *et al*., 2002). The capability to bioaccumulate various metals has been tested further in acanthocephalans (Galli *et al*., 1998; Sures & Siddall, 1999; Scheef *et al*., *2000;* Sures *et al*., 2003c*;* Sures & Reimann, 2003, Zimmerman *et al*., 2005), nematodes (e.g., Szefer *et al*., 1998; Barus *et al*., 2003; Sures *et al*., 1994, 1998; Tenora *et al*., 2000; Barus *et al*., 2007; Genc *et al*. 2008), cestodes (e.g., Sures *et al*., 1997a,c; Barus *et al*. 2000, 2003; Tenora *et al*., 2000, 2001, 2002; Sures *et al*., 2003b; Tekin-Ozan & Kir, 2005; Malek *et al*., 2007; Jirza *et al*., 2008) and digeneans (Sures *et al*., 1998; Ryman *et al*., 2008). Host's tissues and organs typically tested are muscle, liver, intestine and may also include the gills and gonads of fish and the kidneys in mammals. The level of concentration of heavy metal varies depending on taxa, with cestodes and acanthocephalans being much more efficient accumulators than digeneans and nematodes. Monogeneans have not been tested. It appears also that helminths of terrestrial mammals are not as effective at heavy metal accumulation as those from fishes and birds (e.g., Barus *et al*., 2003). However, it is necessary to modulate such a general statement, since relatively few studies on even fewer species have been carried out in these hosts and because numerous factors have been detected that affect the ability of the parasites to accumulate metals. Such factors include the nature of the metal itself (e.g., Sures *et al*., 1998), the host's age and motility (*in* Tenora *et al*., 2000), the parasite's age (e.g., Barus *et al*., 2001), stage of development (e.g., Brown & Pascoe, 1977; Sures & Taraschewski, 1995; Siddall & Sures, 1998), sex (*in* Tenora *et al*., 2000), as well as, organs of the parasites examined (Barus *et al*., 2000; Sures *et al*., 2000; Taraschewski, 2000) and the location of the parasites in the host (Sures 1996; Sures & Siddall, 2001, 2003). Significant interspecific (Sures *et al*., 1997a, 1999, 2003b; Barus *et al*., 2003) and intraspecimens/intraspecific (Szefer *et al*., 1998) variations also have been found to occur.

In a nutshell *in vivo* adult acanthocephalans (but not cystacanths), adult digeneans, (but not metacercariae), and both adult cestodes and their plerocercoids, are known to accumulate some heavy metals. Nematodes displayed the most variation in their ability to bioaccumulate heavy metals, with adult philometrids (Tenora *et al*., 2000; Barus *et al*., 2007) and adults and larvae of *Anisakis* (Pascual & Abollo, 2003) being the only ones reported to be efficient accumulators whereas other species displayed no concentration or only little concentration of certain metals and not others (e.g., Szefer *et al*., 1998; Tenora *et al*. 1999; Barus *et al*., 2003; Palikova & Barus, 2003; Genc *et al*., 2008). Adult acanthocephalans have been found to accumulate Pb, Cd, chromium (Cr), silver (Ag), nickel (Ni), and copper (Cu) (Sures *et al*., 1994a; Sures & Taraschewski, 1995; Galli *et al*., 1998; Sures & Reimann, 2003). In particular, Cd levels were reported to be as high as 400 fold over control levels and Pb levels to be as high as 2,700 fold higher than hosts' tissues (Sures *et al*., 1994c).

Up to a 27,000 fold higher than water exposure concentration has been reported (*in* Taraschewski, 2000). These extremely high concentrations of Cd and Pb make acanthocephalans better bioindicators than even the zebra mussel, *Dreissena polymorpha*, which is commonly used in monitoring water contamination (Sures *et al*., 1997b, 1999b).

ACANTHOCEPHALANS AS BIOACCUMULATORS

While acanthocephalans appear to be tolerant of these high concentrations of heavy metals, the uptake process and accumulation in the worms is still unexplained. It is possible that acanthocephalans might have devised an entirely novel mechanism to acquire heavy metals from their surroundings. However, the available observations suggest that uptake may be occurring via mechanisms similar to those described for divalent cation transport in other organisms and that this uptake may result from a lack of discrimination between Ca^{2+} ions and heavy metal ions by the parasite (Taraschewski, 2000).

Experiments involving activated cystacanths exposed *in vitro* to Pb also showed the potential role of bile salts in enhancing heavy metal uptake (Sures & Siddall, 1999). However the hypothesis that the worms absorb bile-bound heavy metals was challenged by the fact that cadmium-exposed rats infected by acanthocephalans had no decrease in the amount of Cd in their tissues (Scheef *et al*., 2000). These latter authors thus proposed that different metals may have different uptake mechanisms. The acanthocephalans' tolerance to heavy metals indicates that they may detoxify heavy metals, as has been suggested for other organisms (Rainbow & Philips, 1993; Vivjer *et al*., 2004). Where in the worms the metals are sequestered is not known, but it has been suggested, as also for organisms from other phyla, that metals are stored in the form of intracellular granules (Rainbow & Philips, 1993). Although not reported in acanthocephalans, this supports the idea of Tarachewski (2000) that metals may be stored as 'amorphous material' in the worms' tissues.

IN VITRO CULTURE OF *MONILOFORMIS MONILIFORMIS*

Because the purpose of previous studies of heavy metal intake by adult acanthocephalans was to determine the relative accumulation of heavy metals by the parasite and the host and to identify potential bioindicators, these studies were performed *in vivo*.

Since our interest lies in understanding the mechanisms of accumulation in these parasites we first needed to optimize experimental conditions for rearing acanthocephalans. Therefore, we chose to culture adult acanthocephalans, *Moniliformis moniliformis, in vitro.* Our choice was motivated by the fact that both Cd (Scheef *et al.*, 2000) and Pb (*in* Taraschewski 2000) were reported to concentrate in these worms compared to rat host's tissues, because the life cycle of this species is fairly easy to maintain in the laboratory, and because the large size of these worms yields large amount of tissues and allowed dissection of various organs.

In vitro culture of adult acanthocephalans had been performed in the past but has been problematic (see Smyth, 1990). Techniques were either cumbersome (Nicholas & Grigg, 1965) or successful for only a short period of time that would not be long enough for heavy metal exposure (in Crompton & Lassière, 1984; Smyth, 1990; Polzer & Taraschewski, 1994). Optimization of the technique of rat-collected adult *Moniliformis moniliformis* and culture of individual worms was successful for up to 8 days in Krebs Ringer medium enriched with glucose (Sigma) at 37°C, at pH 7.2 and under 5%CO_2/95%N_2 gas conditions. Culture solution was changed once after 4 days of culture. Trial experiments involved taking six week old worms harvested from rat duodenum that were exposed for 4 or 8 days to lead ($Pb(NO_2)_2$) and cadmium ($CdCl_2$) (100 µg/L, equivalent to 0.3 µM and 0.4 µM, respectively). The concentrations of each heavy metal (mg/g dry weight) after 4 days exposure in culture were quantified using atomic absorption spectrometry (Fig. 1). Preliminary results showed accumulation of both heavy metals, with accumulated Cd levels approximately 100 fold those of Pb. Both metals accumulated to a sufficient degree to detect their presence in whole worms using x-ray absorption spectroscopy (Fig. 2). Given the insensitivity of this technique in general (a solution spectrum requires a minimum concentration of ~300µM metal to observe a signal), the ability to measure this spectrum indicates a substantial accumulation of the heavy metal within the tissue of the worm. Structural studies are underway to determine the *in vivo* coordination and speciation of the heavy metal sequestered in the worms. Comparison of the absorptive surface of control worms taken from rats' intestines, control cultured worms, and heavy metal exposed cultured worms was done using transmission electron microscopy (TEM) and showed that no structural damage occurred in the worms exposed to heavy metals (Fig. 3). We thus, propose the use of this combination of *in vitro* –TEM techniques to not only quantify heavy metals in individuals but to allow visualization of the form they are being sequestered in as well as their exact location in the various organs of the worms.

CONCLUSIONS

Identification of the process by which these parasites accumulate heavy metals could find application in new techniques for detection of heavy metals, in bioremediation, and in improved alternative techniques to current methods of heavy metal therapy and detoxification. The ability of helminths to concentrate heavy metals has raised the controversial question of whether it might be beneficial to the vertebrate host to be infected by these worms that appear to act as a heavy metal sanitizer for the host (Sures & Siddall, 1999; Taraschewski, 2000; Malek *et al*., 2007). However fascinating and challenging this hypothesis is, it must be balanced, however, by the idea that bioaccumulation by helminths may be the reflection of a higher ability of the host to clear heavy metals (Szefer *et al.*, 1998). Thus, more studies must be carried out focusing not only on uptake pathways but also upon sequestration mechanisms.

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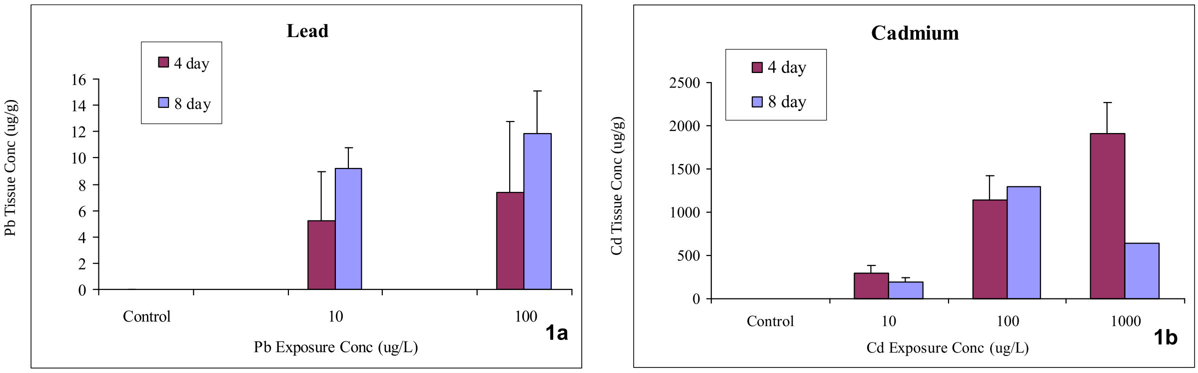

Figure 1. Concentrations of heavy metals by 8 days old adult acanthocephalans *Moniliformis moniliformis* cultured *in vitro* four days and quantified using atomic absorption spectrometry. a: lead exposure. b: cadmium exposure.

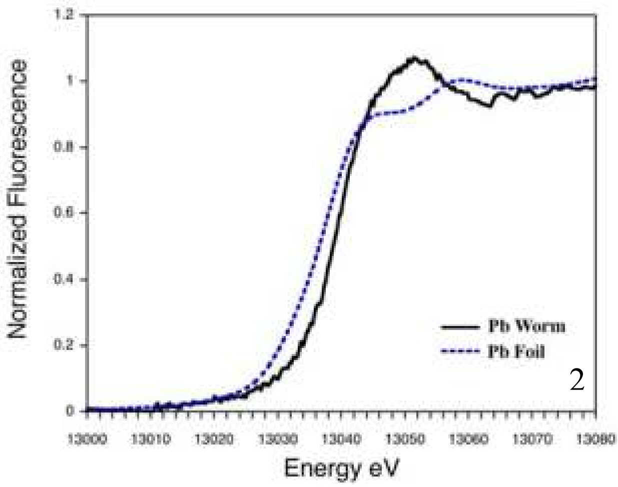

Figure 2. XANES (X-ray Absorption Near-Edge Spectrum) spectrum at the Pb L_{III} edge of a whole worm recovered from host and cultured in media containing 1000µg·L^{-1} of Pb salt. The spectrum was collected on a whole worm that had been rinsed extensively to remove any excess culture media. Data was collected at both the Stanford Synchrotron Radiation Laboratory (SSRL) and the National Synchrotron Light Source (NSLS) at cryogenic temperatures (less than 30K). A solid state Ge-detector was used to monitor the x-ray fluorescence and scans were energy calibrated using a lead foil.

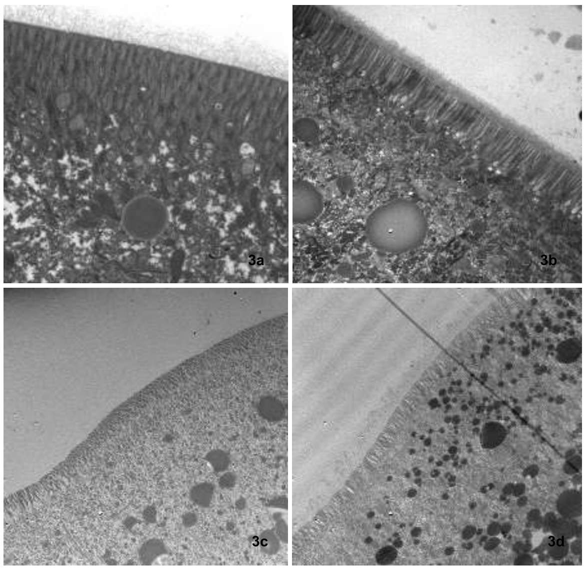

Figure 3. Transmission electron micrographs of the absorptive surface of 8 days old adult acanthocephalan *Moniliformis moniliformis*. a: Control worm removed from the intestine of the rat (x 15000). b: *In vitro* cultured worm for four days (x 5000). c: *In vitro* cultured worm for four days exposed to100 µg/L of lead (x 3000). d: *In vitro* cultured worm for four days exposed to100 µg/L of cadmium (x 3000).

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