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Influence of the cestode *Ligula intestinalis* and the acanthocephalan *Polymorphus minutus* on levels of heat shock proteins (HSP70) and metallothioneins in their fish and crustacean intermediate hosts

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ABSTRACT

It is a common method to analyse physiological mechanisms of organisms – commonly referred to as biomarkers – to indicate the presence of environmental pollutants. However, as biomarkers respond to a wide range of stressors we want to direct the attention on natural stressors, i.e. on parasites. After two years maintenance under controlled conditions, roach (*Rutilus rutilus*) revealed no influence on levels of metallothionein by the parasite *Ligula intestinalis*. The same was found for *Gammarus fossarum* infected with *Polymorphus minutus*. However, the heat shock protein (HSP70) response was affected in both host-parasite systems. While the infection of roach resulted in reduced levels of HSP70 compared to uninfected roach, the infection in *G. fossarum* led to higher levels of HSP70. We also analysed the effect of a 14 days Cd exposure (4 μg/L) on the uninfected and infected gammarids. The exposure resulted in induced levels for both, metallothionein and HSP70 whereas the combination of stressors, parasite and exposure, revealed a decrease for levels of HSP70 in comparison to the metal exposure only. Accordingly, parasites as natural parts of aquatic ecosystems have to be considered in ecotoxicological research.

1. Introduction

The continuous exposure of the aquatic environment with a variety of pollutants has led to the development of different evaluation tools for the assessment of the pollution's impact on aquatic organisms. Due to very low concentrations of chemicals as well as complex pollutant mixtures chemical analyses alone are often insufficient to determine environmental risks. This has led to the idea of using biological markers (biomarkers) to indicate the presence of contaminants and to unravel their effects on organisms (Livingstone et al., 1994). Unfortunately, the most commonly used biomarkers are not only sensitive to anthropogenic pollutants but might also be induced by a variety of natural stressors (Marcogliese and Pietrock, 2011; Sures, 2008a). As biomarkers are applied under field conditions, their possible modulation by naturally occurring stressors has to be evaluated. One of the most prevalent and important stressors of free living animals are parasites (Marcogliese et al., 2005; Sures, 2008b). However, the knowledge about possible interactions between parasites and pollution is limited (Marcogliese and Pietrock, 2011; Sures, 2008a).

Among biomarkers it is important to distinguish between contaminant specific markers (e.g. metallothioneins as markers for metals) and less specific markers being part of a general stress response (e.g. heat shock proteins). Metallothioneins (MTs) are considered to play a central role in regulation of tissue concentrations of essential metals (e.g. Zn and Cu) and are known to be involved in detoxification processes of non-essential toxic metals such as Cd and Hg (Kägi, 1991; Roesijadi, 1992, 1996). As the response of MT levels in organisms depends on the element and the exposure concentration, MTs have been considered as potential biomarkers for metal pollution (Amiard et al., 2004; Benson et al., 1990; Canli et al., 1997; Pedersen et al., 1997). Accordingly, several studies reported higher MT-levels in metal-exposed roach (*Rutilus rutilus*) or gammarids compared to controls (Bonwick et al., 1991; Brown et al., 1987; Geffard et al., 2007, 2010; Paris-Palacios et al., 2000; Stuhlbacher and Maltby, 1992).

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In terms of ecotoxicological research, heat shock proteins (HSPs) are used as biomarkers for a wide range of adverse stressors including anthropogenic pollution (Iwama et al., 2004; Sanders, 1993) and their induction is usually interpreted as a general sign of protein damage (Köhler et al., 2001; Lewis et al., 1999; Perceval et al., 2001; Radlowska and Pempkowiak, 2002). However, an increase in HSP70 levels is also reported as a consequence of metal exposure (i.e. Cd) in *Gammarus fossarum* (Schill et al., 2003) and in fish (Köhler et al., 2001; Misra et al., 1989).

In contrast to a wealth of reports on the modulation of MT or HSP in aquatic organisms by chemicals, only few studies are published dealing with the impact of parasites on these biomarkers. Recently, effects of digenean parasites (Baudrimont et al., 2006; Baudrimont and de Montaudouin, 2007) or bacterial infections (Paul-Pont et al., 2009) on metallothionein synthesis of *Cerastoderma edule* were described, indicating a significant alteration of the protective effect of metallothioneins towards metals. In European eel (*Anguilla anguilla*) a reduced HSP70 and MT response following infection with digenean parasites was observed, whilst infection with the swim bladder nematode *Anguillicola crassus* had a significant positive relationship with the expression level of the MT gene (Fazio et al., 2008). With respect to amphipods it was recently shown that *Polymorphus minutus* can significantly affect the physiological homeostasis of its intermediate hosts. Under uncontaminated conditions infected *Gammarus roeseli* had lower protein and lipid contents, but glycogen contents were higher compared to uninfected conspecifics (Gismondi et al., 2012a). If amphipods were similarly infected with *P. minutus* and exposed to Cd a paradoxical pattern was obvious suggesting that *P. minutus* increases cadmium toxicity in female *G. roeseli* although it tends to increase several host antitoxic defense capacities and accumulates part of the metal (Gismondi et al., 2012b). For *Gammarus roeseli* infected with *P. minutus*, no HSP70 response could be observed after exposure of infected gammarids to heat or palladium (Sures and Radszuweit, 2007). The acanthocephalan *P. minutus* is a common parasite in gammarids (Bollache et al., 2001; Hynes, 1955; Ward, 1986) and is known to change the sensitivity of individual hosts against pollutants (Brown and Pascoe, 1989). This parasite may even cause "castration" in female gammarids (Hynes, 1955; Le Roux, 1933; Ward, 1986) as well as a retardation of the development of male secondary sex characteristics (Le Roux, 1933) and thus decreases the pairing success of male gammarids (Bollache et al., 2001; Zohar and Holmes, 1998).

Another parasite known for its severe effects on host physiology is the cestode *Ligula intestinalis* which is characterized by a three-host life cycle involving copepods as first intermediate hosts, fish as second intermediate hosts and birds as final hosts (Dubinina, 1980). The larval stage in the fish intermediate host, the plerocercoid is located in the body cavity of cyprinids. *Ligula intestinalis* inhibits gonad development of its fish host and thus has been demonstrated to have significant impacts on common biomarkers for endocrine disruption in bream (*Abramis brama*), chub (*Squalius cephalus*) and roach (*R. rutilus*) (Hecker et al., 2007; Hecker and Karbe, 2005; Schabuss et al., 2005; Trubiroha et al., 2009, 2010). Additionally, Frank et al. (2011) reported a reduction of glutathione-S-transferase activity in naturally infected fish hosts, which is a clear indication of a strong interaction between parasites and pollution biomarkers in the host.

The present study aims at investigating the interactions between parasitism and generally accepted biomarkers for pollution such as metallothioneins and heat shock proteins. As model organisms the cyprinid *R. rutilus* and the amphipod *G. fossarum* were used. Part of the fish were naturally infected with the diphyllobothridean cestode *L. intestinalis* whereas approximately 50% of the amphipods were naturally infected with larvae of the palaeacanthocephalan *P. minutus*. The relative levels of heat shock protein 70 (HSP70) and MT were analysed in host tissues of both host-parasite systems, after maintaining the hosts under controlled laboratory conditions. In order to assess combined effects of parasites and pollution, the amphipod-parasite-system was exposed to Cd in the laboratory at relatively low aqueous Cd concentrations (4 μg/L). Hence, metal accumulation in gammarids and parasites was compared and levels of HSP70 as well as MT were analysed to identify possible synergistic or antagonistic effects of parasites and pollution.

2. Material and methods

2.1. Fish collection and tissue sampling

Roach were collected by electrofishing from Lake Mueggelsee (Berlin, Germany), in November/December 2006 and were transferred to the laboratory. The immediate investigation of a fish subsample revealed a fish age of 1+ and 2+ ($n = 12$, females and males pooled). Fish were maintained in an aerated 1000 L tank under natural photoperiod and a constant flow of tab water of 15 °C temperature. Roach were fed daily *ad libitum* with commercial trout pellets (DAN-Ex 1750, Dana Feed) and *Chaoborus* sp. larvae. After two years of maintenance, roach were sacrificed. Tissue samples were stored at −80 °C until further processing. All experimental procedures were conducted in compliance with the institutional guidelines for the care and use of animals.

The following biometric and parasitological parameters were measured: fish total length (to the nearest mm); fish total mass and somatic mass (to the nearest 0.1 g); fish gonad mass and parasite mass (to the nearest mg); number of parasites per fish. Morphological and parasitological indices were calculated as follows (see Table 1): the gonadosomatic index (GSI) as (fish gonad mass/fish somatic mass) × 100, the condition factor (CF) as fish somatic mass × 100/(fish total length)^3, the parasitisation index (PI) as (parasite mass/fish somatic mass) × 100. For GSI and CF fish somatic mass was determined without parasite mass.

2.2. Field sampling of gammarids, laboratory exposure experiments, sample processing and metal analyses

Uninfected and naturally infected *Gammarus fossarum* harbouring cystacanths of *Polymorphus minutus* were collected with a pond net from the brook Ruthertalbach close to Essen, Germany in August 2008. They were transported to the laboratory in aerated river water. Thereafter, gammarids were divided into four groups, with 145–172 individuals each. They were placed in 20-L plastic tanks with 15 L of aerated, dechlorinated tap water. As cystacanths of *P. minutus* are usually visible by eye as oval orange structures in the haemocoel of *G. fossarum*, obviously infected gammarids were pooled in two groups (unexposed control and Cd exposure group). Final information on the infection status was obtained after the animals were killed and dissected at the end of the experiment. To avoid heat stress, handling and exposure of the crustaceans were performed at the same water temperature as measured in the brook (15 °C). Amphipods were fed with alder and horse chestnut leaves. The water was replaced twice a week.

Gammarids were divided into four groups: one infected and one uninfected group were used as unexposed controls (CoUninf and CoInf). The other two groups were exposed to nominal Cd concentrations of 4 μg/L (CdUninf and CdInf). Cd exposure was performed by adding 60 μL of Cadmium standard solution (Cd 1000 μg/mL, Kraft) to the tank water at the beginning of the experiment and after each water replacement. Gammarids were maintained under these conditions for 14 days. Water characteristics and data on amphipods are summarized in Table 2.

For analyses of Cd in tank water, water samples were taken from each tank before and after water replacement according to the following procedure: 10 mL

Table 1
Morphological parameters of host fish (TL = total length, CF = condition factor, GSI = gonadosomatic index, PI = parasitisation index) and mean infection intensity with *L. intestinalis*.

R. rutilus	*L. intestinalis*	*n*	TL (cm)	CF	GSI	PI
Female	–	17	16.5 (1.0)^a	0.79 (0.04)^a	7.8 (2.9)^a	10.1 (3.3)^a
Female	1.6 (1.1)	9	18.5 (1.6)^{bc}	0.81 (0.07)^a	1.7 (1.1)^{bc}	
Male	–	10	15.5 (1.3)^b	0.80 (0.07)^a	2.4 (0.7)^b	11.6 (1.9)^a
Male	2.0 (0.9)	7	17.8 (1.6)^c	0.79 (0.06)^a	1.2 (0.7)^c	

Data are given as mean (±SD). Values not sharing a common letter for the same parameter from the same site are statistically different from each other (Student *t*-test, $p < 0.05$), with n = number. Within each gender the uninfected fish were compared to the infected ones. Comparison between genders was done separately for uninfected and infected fish.