Influence of parasites on biomarkers in aquatic animals

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Glossary

AAS **Atomic absorption spectrometry**. This methodology is used for the quantitative determination of chemical elements analysing the absorption of optical radiation by free atoms in the gaseous state.

BCF **Bioconcentration factor**. A ratio between the metal concentrations of different tissues. For example the parasite and the host tissue $C_{[L.intestinalis]}/C_{[host\ tissue]}$.

CF **Condition factor**. This factor is used to compare fish condition by building the ratio of fish somatic mass x 100/fish total length³.

GSI **Gonadosomatic index**. This index is used for indication on the degree of gonad maturation by building the ratio of fish gonad mass/fish somatic mass x 100.

GST **Glutathione-S-transferase**. This enzyme is part of the phase II enzymes. It belongs to a multigene family which is involved in the detoxification of a wide variety of chemicals by catalyzing the conjugation of the tripeptide glutathione and electrophilic substances of exogenous origin (Eaton and Bammler, 1998).

HSI **Hepatosomatic index**. This factor is used to compare liver health status by building the ratio of fish liver mass/fish somatic mass x 100.

HSP **Heats hock protein**. This proteins are part of the general stress response, binding to cell proteins to provide for correct protein structure (for example: Sanders, 1990, 1993). Based on their molecular weight they are named HSP70 (70 kDa) for instance, whereas there exist also more HSPs with different size and function.

ICP-MS **Inductively Coupled Plasma Mass Spectometry**. This methodology includes inductively coupled plasma for ionization and mass spectrometer for detecting the ions. It is a rapid and highly sensitive technique for measuring numerous metals.

LTS **Listertalsperre**. Reservoir Listertalsperre (51° 5`38” N, 7° 50`15” E) is a mesotrophic dam built in 1912 (surface area of 0.79 km², max depth of 39 m) in the south of Meinerzhagen (Sauerland, Germany) whithout known pollution.

MS **Mueggelsee**. A polymictic and eutrophic shallow lake (5°26`N, 13° 39`E), (surface area of 7.3 km², mean depth of 5 m) in the southeast of Berlin which is flushed by the river Spree (Driescher et al., 1993).

MT **Metallothionein**. Metal-binding proteins which bind a couple of metal ions (Wang et al., 1996). They are considered to play a central role in the regulation of essential metals but also to be involved in the detoxification of non-essential, toxic metals (Kägi, 1991).

PI **Parasitisation index**. This index is used to compare the degree of parasite's influence on host's total mass as parasite mass/fish somatic mass x 100.

List of used element abriviations: arsenic (As), cadmium (Cd), chromium (Cr), cobalt (Co), copper (Cu), iron (Fe), manganese (Mn), nickel (Ni), lead (Pb), zinc (Zn).

Background

"It is well known that water is life; […] water also means livelihoods. It is the route out of poverty for individuals and communities. Managing water is essential if the world is to achieve sustainable development."

Ban Ki-moon

(World Water Assessment Programme, 2009)

It is a global phenomenon that our aquatic environment is exposed to various anthropogenic pollutants whereas information about pollution loads and changes of water quality is lacking in many countries because of inadequate monitoring systems (World Water Assessment Programme, 2009). Chemicals or other substances in concentrations exceeding natural conditions are typically referred to as pollution. Major water pollutants include microbes, nutrients, heavy metals, organic chemicals, oil and sediments as well as heat (World Water Assessment Programme, 2009).

Beside the direct detection of individual compounds in waterbodies by techniques of analytical chemistry, an interdisciplinary environmental science which is called ecotoxicology developed in the last decades. It deals with the interactions between environmental chemicals and biota, thereby focusing on adverse effects at different levels of biological organisation (Fent, 2004). To detect anthropogenic stressors in the aquatic habitat, living organisms can be used in two different ways:

Accumulation indication benefits from the fact that organic as well as inorganic contaminants are accumulated by an organism resulting either from direct uptake from the water or from ingestion (Streit, 1998). Due to consequential higher biotic than abiotic concentrations the sensitivity of an analytical procedure may therefore be increased (Beeby, 2001).

Focused on **effect indication,** the so-called biomarkers are used, reflecting the adverse biological responses towards anthropogenic environmental toxins (Bucheli and Fent, 1995). In an environmental context, biomarkers can be understood as sensitive indicators demonstrating that toxicants have entered the organism, have been distributed among tissues,

and are eliciting a toxic effect (van der Oost et al., 2003). Accordingly, biomarkers can be defined at any level of biological organisation (Figure I).

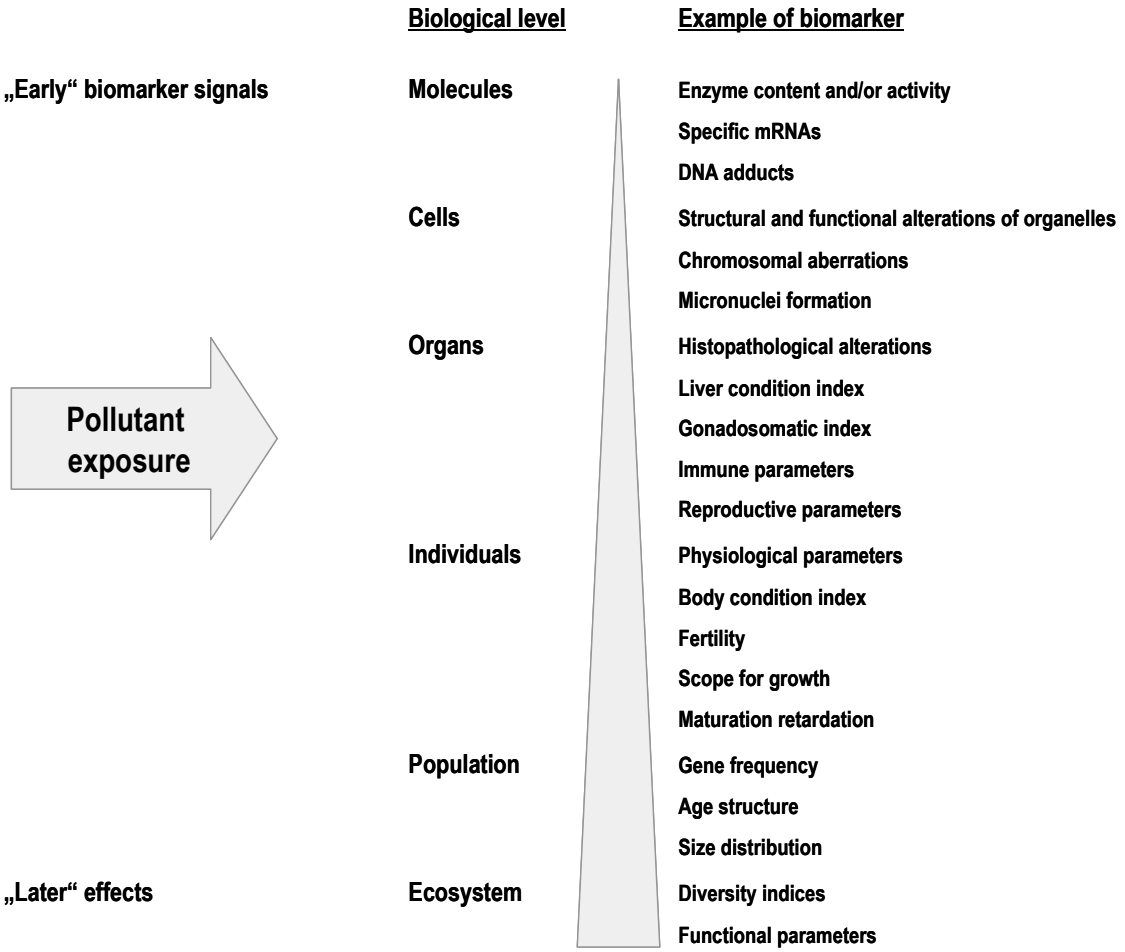

Figure I: Schematic representation of the sequential order of responses to pollutant stress within a biological system. Modified from Bucheli and Fent, 1995 and van der Oost et al., 2003.

Considering stressors of aquatic systems one should not be conscious of the fact that beside anthropogenic impacts natural stressors also exist. In that context parasites are considered as natural stressor with the same impact as anthropogenic stressors (Gunkel, 1994).

As parasitism is the most prevalent life style among organisms, it is not surprising that most organisms are exploited by parasites (Bush et al., 2002). Therefore, the incidence of parasites as part of the aquatic ecosystem is another common phenomenon. From an ecological perspective, it is well defined that host-parasite relationships include the consideration of the ecology of the host(s) in a parasite's life cycle, as well as the host as a habitat for the parasite. Thus, many of the biotic and abiotic factors affecting the ecology of the host will also affect the parasite. The parasite itself has to deal with physiological, especially immunological host's response mechanisms. As Bush et al. (2002) indicated: "It must be understood that

these [..] interactions between the parasite and the host are as 'ecological' as those involving the host's relationships with its own environment."

Thus, the health of aquatic organisms is affected by pollutants as well as by parasites. Consequently, response mechanisms against these threats may be similar, whereas also pollutant- or parasite-specific reactions occur (Figure II).

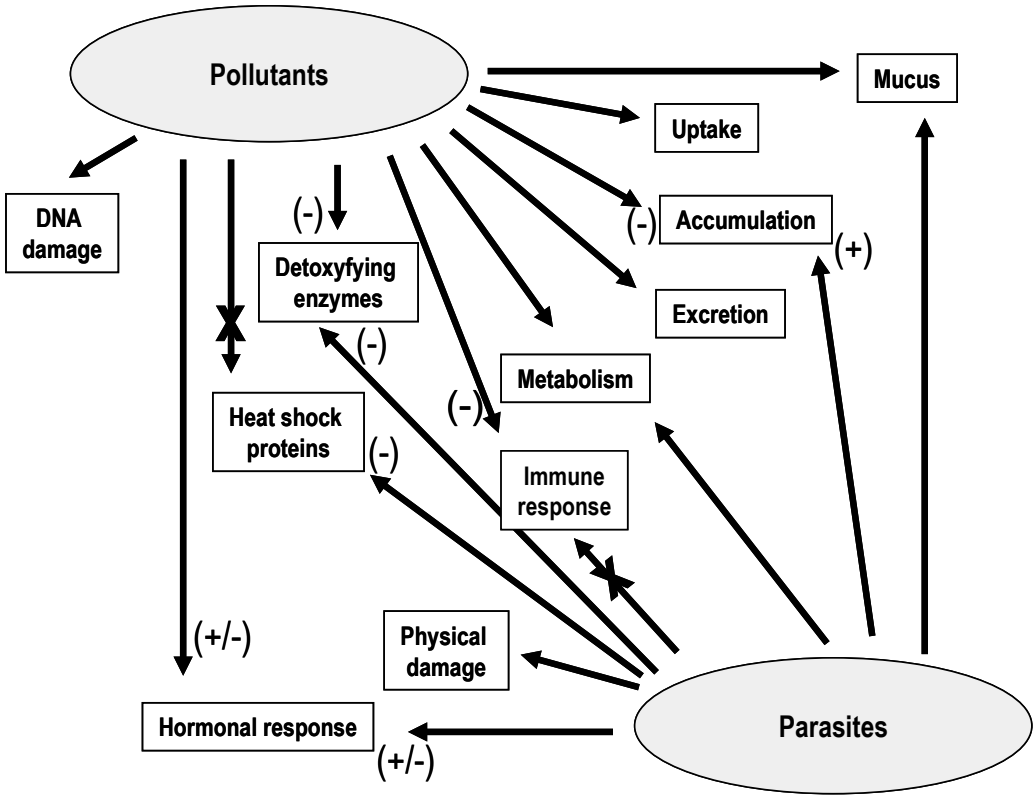

Figure II: Organism's response against isolated pollution or parasitism (Sures, 2008b).

The combination of parasitological and ecological research is mainly focused on the impact of changing environmental conditions on natural parasite communities (Bush et al., 1990; Kennedy, 1976; Sures, 2001). As it is recently accepted that pollution can favour or decrease parasitism, parasites are versatilely used as bioindicator of environmental impact (Palm and Rückert, 2009; Vidal-Martinez et al., 2010). Although there is an increasing number of studies indicating adverse effects on different biomarkers of various aquatic organisms by parasites (reviewed in Sures, 2006, 2008a, b), the knowledge about the combined effects of parasites and pollution on their host's physiology is still premature.

In fish, one of the most important organs for metabolism of xenobiotics is the liver. Here, the biotransformation of contaminants or the detoxification of endogenous metabolites takes

place (Goksoyr and Husoy, 1998). Biotransformation is subdivided into phase I and phase II metabolism aiming to convert hydrophobic lipid-soluble organic xenobiotics to water-soluble metabolites ready for excretion (Livingstone, 1998). Some of the enzymes responsible for biotransformation are used as biomarkers as their induction or reduction allows to draw conclusions on the presence and bioavailability of certain contaminants. One of the best studied biomarker is the induction of cytochrome P450-dependent monooxygenases (P450) which are part of phase I (Bucheli and Fent, 1995). Their induction is rather specific for certain xenobiotics such as polychlorinated biphenyls (PCB) (Goksoyr and Husoy, 1998). As part of the phase II enzymes, which are also inducible by exposure to organic xenobiotics, the glutathione-S-transferases are a multigene family which is involved in the detoxification of a wide variety of chemicals by catalyzing the conjugation of the tripeptide glutathione and electrophilic substances of exogenous origin (Eaton and Bammler, 1998; Livingstone, 1998). However, organisms also face a more general stress response which is able to react on a broad range of stressors (Wendelaar Bonga, 1997). Heat shock proteins are part of that general stress response, binding to cell proteins to provide for correct protein structure (for example: Sanders, 1990, 1993). Based on their molecular weight, they are named HSP70 (70 kDa) for instance, whereas there exist also more HSPs with different size and function. The ubiquitous occurring HSP70 has been proved to show higher synthesis at adverse environmental conditions why it serves as biomarker for environmental contamination (Iwama et al., 2004; Sanders, 1993). Specifically to metal contamination, metal-binding proteins exist, particularly metallothioneins (MT) which bind a couple of metal ions (Wang et al., 1996). They are considered to play a central role in the regulation of essential metals but also to be involved in the detoxification of non-essential, toxic metals (Kägi, 1991; Roesijadi, 1992, 1996). In organisms, the levels of MT vary depending on element concentrations why they have been regarded as potential specific biomarker for metal pollution (Amiard et al., 2004; Benson et al., 1990).

Taken together, it is obvious that organisms have different possibilities to react against contaminants. Some mechanisms work by supporting detoxification, accumulation or excretion of the respective chemicals. Other mechanisms work by repairing or protecting proteins to ensure their proper functions. Parasite infection always activates defence mechanisms of the host. Therefore, it cannot be ignored that this will change the physiology or even the behaviour of the host what may be repercussive also changing the protective mechanisms against contaminants (Figure).

Consequently, it raises the question if infection with parasites can act as such a stressor, that host's stress response mechanisms which are used as biomarker for environmental pollution are affected. Therefore, this thesis aims at increasing the knowledge of the influence of parasites on biomarkers used in ecotoxicology. Even if the indications for such interrelations are rising since several years (for example Sures, 2004), the recent knowledge is just premature.

The interaction of parasites and pollution is possible by two ways of action:

Due to their effects on the physiology and behaviour of the host, parasites can interfere with established bioindication procedures. Consequently, false-negative as well as false-positive indications of pollution are possible (Sures, 2007; Thilakaratne et al., 2007). As parasites also respond to anthropogenic pollution, they can correspondingly be used as effect or accumulation indicators, respectively (Sures, 2004). Furthermore, the negative effect on fish health can be greater if infection and pollution converge than either stressor alone (Marcogliese et al., 2005; Sures, 2004; Thilakaratne et al., 2007).

Accordingly, the parasite which is best-known to cause natural endocrine disruption in its intermediate fish hosts and which proved to be an invaluable model for parasitologists as well as for endocrinologists, ecologists, genetics, immunologists and pollution studies was chosen (Hoole, 2010). The diphyllobothridean cestode *Ligula intestinalis* is characterised by a three host life cycle. Fish eating birds serve as final hosts. Copepods become infected by eating the first, free swimming, larval stage which develops to the second larval stage in the copepod's hemocoel. The third larval stage, the so called plerocercoid, develops in the body cavity of fish usually from the family Cyprinidae (Dubinina, 1980). In Germany, *L. intestinalis* is often found to infect roach, *Rutilus rutilus*, why this host-parasite-system was chosen for all biomarker studies (Figure III).

Figure III: Dissected roach, *R. rutilus*, with the plerocercoid of *L. intestinalis* removed from its body cavity.

First of all, naturally infected roach were analysed for a number of metals to increase the knowledge of the accumulation capacities of *L. intestinalis* and to test if there is a competition for minerals between the parasite and its host. Although there is a number of reports certificating cestodes as useful tools in monitoring heavy metal pollution (Eira et al., 2009; Malek et al., 2007; Retief et al., 2006), there are conflicting results in case of *L. intestinalis* (Barus et al., 2001; Oyoo-Okoth et al., 2010; Tekin-Özan and Barlas, 2008; Tekin-Özan and Kir, 2008; Tenora et al., 2000). One hypothesis is that those cestodes and *L. intestinalis* in particular do accumulate metals specifically and not only without mechanisms of control. But there are no indications so far, that cestodes are capable to accumulate metals in a similar degree as for example acanthocephalans where *Pomphorhynchus laevis* even tended to reduce levels of lead in tissues of its host *Squalius cephalus* (Sures, 2003). The adequacy of *L. intestinalis* for accumulation indication is to query whereas the influence on accumulation indication capacities of its host *R. rutilus* lacks the direct comparison between uninfected and infected fish so far.

To test the parasite's influence on marker for effect indication, the activity of the phase II enzyme Glutahtione-S-transferase, the levels of metal-binding proteins, in particular metallothioneins as well as the levels of proteins indicating a general stress response, the heat shock protein 70 were checked. The qualification of GST-activity in pollution monitoring is debatable as several studies observed problems when analysing differences between fish from control and polluted sites (Krca et al., 2007; Van der Oost et al., 2003; Vigano et al., 1998). The effects of various pollutants on GST-activity in different fish species under laboratory conditions and in the wild mostly tended to raising GST-activities but they also revealed conflicting results concerning the correlation between pollution and GST-activities in fish (Van der Oost et al., 2003). For carp infected with the cestode *Ptychobothrium* sp. (Dautremepuits et al., 2002, 2003) and even for mammals infected with the trematodes *Dicrocoelium dendriticum* (Skálová et al., 2007) or *Fasciola hepatica* (Galtier et al., 1983, 1986, 1987, 1991) an enhancing influence on host's hepatic GST-activity is already demonstrated; therefore one hypothesis is that even more parasites may have effects on that biomarker response. To test whether the possible influence on GST-activity can be demonstrated under laboratory conditions, a second host-parasite system was used as the experimental infection of roach was not possible for *L. intestinalis*. Three-spined sticklebacks (*Gasterosteus aculeatus*) were bred in the laboratory and experimentally infected with *Schistocephalus solidus* as described by Scharsack et al. (2007). Because of the available techniques for culturing the parasite *in vitro* as well as the laboratory breeding of fish, this is an increasingly used laboratory model for investigating host-parasite interactions (Barber and Scharsack, 2009). As *S. solidus* is closely related to *L. intestinalis*, it is to expect that possible effects on host's GST-activity found for *S. solidus* can also be found for *L. intestinalis*. To identify possible effects on roach hepatic GST-activity induced by *L. intestinalis*, a field sampling was conducted at two different sites. At one site, additionally to roach, chub (*S. cephalus*) was acting as intermediate host for *L. intestinalis* and could therefore be analysed. Thus, it is presumed that even if not all possible influences can be validated in the wild, at least at one site or for one of the host-parasite systems, effects on host's GST-activity should become obvious as far as they exist.

The most specific and the most general biomarker were compared in the same studies. Due to their function, metallothioneins are a sensitive marker for metal pollution. They tend to increase with an increasing metal burden as they bind metals for detoxifying or regulatory processes. However, even for that biomarker it was shown, that digenean parasites or bacterial

infections can alter their host's MT response (Baudrimont et al., 2006; Baudrimont and de Montaudouin, 2007; Fazio et al., 2008; Paul-Pont et al., 2009). Contrary to the very specific way of induction for MTs, the induction of heat shock protein 70 is usually interpreted as a general sign of protein damage independent of the causative stressor (Köhler et al., 2001; Lewis et al., 1999; Perceval et al., 2001; Radlowska and Pempkowiak, 2002, Sanders, 1993). Their induction could be demonstrated by different contaminants from organic compounds (Hassanein et al., 1999; Maradonna and Carnevali, 2007; Padmini et al., 2009) to heavy metals (Köhler et al., 2001; Misra et al., 1989; Schill et al., 2003) whereas their use as indicators of stressed states in fish is criticised to be in general premature (Iwama et al., 2004). From an ecotoxicological view, the knowledge about the additional effect of parasites on host's HSP70 response is also premature just as several indications exist for heat shock proteins being a missing link in the host-parasite relationship as there exist strong associations between HSP and the immune system (Kaufmann, 1990). Interestingly, the so far observed effects of parasitisation on host's HSP70 response range from reduction (Fazio et al., 2008) to absolutely absence (Sures and Radszuweit, 2007) of an adequate HSP70 response. For excretory-secretory products from a larval stage of *Schistosoma mansoni* it was recently demonstrated that they are able to reduce HSP70 protein levels in host's defence cells (Zahoor et al., 2010). To increase the knowledge about parasites possibly influencing either their host's MT or HSP70 response, roach were sampled in the field and kept under controlled conditions for two years to assure similar conditions for uninfected as well as for infected fish. Additionally, an invertebrate host was chosen as some of the studies indicating effects of parasitisation on the interested biomarkers were conducted with mussels or gammarids. Furthermore, the second investigated host-parasite system, consisting of the amphipod *Gammarus fossarum* partly infected with the acanthocephalan larvae *Polymorphus minutus*, was not only kept for two weeks under controlled conditions, it was additionally exposed to low Cd concentrations to identify possible synergistic or antagonistic effects of parasite and pollution. As referred before, a reduction of host's HSP70 response is to assume for both host-parasite systems whereas for MTs no assumption exists.

To sum it up, the thesis is structured as follows:

- Chapter 1:

Competition for minerals between Ligula intestinalis *and its intermediate fish host roach (*Rutilus rutilus*)*

The first chapter deals with metal analyses on tissues of roach and its parasite *L. intestinalis*. Roach were captured from Reservoir Listertalsperre that is not known to be polluted. The analysed metal concentrations of Co, Cu, Fe, Mn, Ni and Zn were compared between uninfected and infected fish as well as metal concentrations in *L. intestinalis* plerocercoids were compared to those in fish tissues (muscle and intestine). To test for a possible element competition between the parasite and its host, correlation analyses were done.

- Chapter 2:

Biomarker 1: The glutathione-S-transferase-activity in three different fish species considering the infection with diphyllobothridean cestodes

This chapter includes a laboratory experiment to define parasite's influence under controlled conditions and field surveys on two sampling sites to compare laboratory results with the wild. The laboratory experiment was performed with lab-bred three-spined sticklebacks (*G. aculeatus*), experimentally infected with *S. solidus*. At two sampling sites roach (*R. rutilus*) infected with *Ligula intestinalis* were caught. Additionally, at one of these sites, chub (*S. cephalus*) also infected with *L. intestinalis* were investigated. Therefore the comparison of effects on GST-activity by *L. intestinalis* on the same host was possible between two sites as well as the comparison of effects by *L. intestinalis* on two different hosts within the same site.

- Chapter 3:

Biomarker 2: The metallothionein levels in Rutilus rutilus *and* Gammarus fossarum *considering parasite infection*

In a 14 day laboratory experiment with *G. fossarum* infected with *Polymorphus minutus*, the joint effects of Cd-exposure and parasite infection were investigated. In Chapter 3 the results are given for metallothionein levels in the analysed gammarids. Furthermore,

roach, obtained from the wild but maintained for 2 years under controlled conditions, were tested for their effects on basic metallothionein levels by infection with *L. intestinalis*.

- Chapter 4:

Biomarker 3: Levels of heat shock protein (HSP70) in Rutilus rutilus *and* Gammarus fossarum *considering parasite infection*

This chapter deals with the experimental design described for Chapter 3 but gives the results for parasite's influence on levels of heat shock protein (HSP70).

1 Competition for minerals between *Ligula intestinalis* and its intermediate fish host roach (*Rutilus rutilus*)

1.1 Introduction

The aquatic environment is exposed to various anthropogenic pollutants worldwide. Recently, attempts to detect different kinds of pollution in the aquatic environment also include organisms harbouring parasites. As pollution can favour or decrease parasitism, comparisons of parasite compositions of free living organisms are often used as bioindicator for environmental impact (Palm and Rückert, 2009; Vidal-Martinez et al., 2010). In case of metal pollution, some groups of parasites are known to act as sensitive indicators because of their higher accumulation capacity compared to their host tissues (Sures et al., 1999; Sures, 2001.). For example, adult acanthocephalans emerged to have the best accumulation capacitiy (Sures, 2001) whereas for cestodes controversial observations have been made. Among freshwater fish cestodes, *Ligula intestinalis* is one of the parasites which consistently attracted attention as a potential accumulation indicator within the last decades (Barus et al., 1999, 2001; Oyoo-Okoth et al., 2010; Tekin-Özan and Barlas, 2008; Tekin-Özan and Kir, 2005, 2008; Tenora et al., 1997). While this parasite is characterised 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 located in the body cavity of cyprinids, the so called plerocercoid is known for its massive impact on host's physiology. Apart from the growth and maturation of the plerocercoids (Tenora et al., 1997), the species of fish intermediate host also affects the degree of element accumulation in *L. intestinalis* (Barus et al., 1999). In field studies with four different intermediate hosts (*Abramis brama, Blicca bjoerkna, Rastrineobola argentea, Rutilus rutilus*), *L. intestinalis* was found to be an appropriate sentinel for Pb, Cd and Cr (Barus et al., 2001; Oyoo-Okoth et al., 2010; Tenora et al., 2000) whereas in tench (*Tinca tinca*), the accumulation levels of the parasite did not exceed those of fish tissues (Tekin-Özan and Kir, 2008). As most studies focussed on element concentrations of either only the parasite or the parasite together with its infected host, studies comparing element concentrations of uninfected and infected fish or even the parasite's effect on host's metal balance are rare. In the parasite-host assemblage of *L. intestinalis* and the cyprinid *R. argentea*, an element competition for Cu and Co between fish and parasite was observed,

while Cr and Cd were found to display a partitioning with high concentrations in the parasite (Oyoh-Okoth et al., 2010).

The aim of the present study was to compare metal concentrations in uninfected and infected roach (*R. rutilus*). Furthermore, metal concentrations in *L. intestinalis* plerocercoids were compared to those in different tissues (muscle and intestine) of its fish host, the roach. Correlation analyses were used to test for element competition between the parasite and its host.

1.2 Material and methods

1.2.1 Sample collection

Roach from the water reservoir Listertalsperre were caught by electrofishing in June 2008 and transferred to the Institute in aerated tanks. The Reservoir Listertalsperre (LTS) (51° 5`38" N, 7° 50`15" E) is a mesotrophic dam built in 1912 (surface area of 0.79 km², max depth of 39 m) in the south of Meinerzhagen whithout known pollution. Roach were held in a 200 L aquarium with aerated tap water at approximately the same water temperature as in the reservoir (22°C) until they were dissected within the next three days.

After anaesthetization with ethyl 3-aminobenzoate methansulfonate (MS222, Sigma) fish were killed by decapitation. The fish were measured (to the nearest mm) and weighed (to the nearest mg), organs and parasites were removed, weighed (to the nearest mg) and immediately frozen in liquid nitrogen. All samples were stored at -80°C until further processing.

For metal analyses, tissue samples (muscle, intestine and parasite) were split into pieces using stainless steel dissecting tools, which were previously cleaned with 1% ammonium-EDTA solution and double- distilled water to avoid contamination. Parasites were split into three pieces coming from the proximal, middle and the distal part of the worms.

The sample material was rinsed with physiological solution (0.8% NaCl suprapure) and frozen at -20°C until metal analyses.

1.2.2 Heavy metal analysis

For metal analyses of fish and parasite samples a microwave assisted digestion was used following the procedure described by Zimmermann et al. (2001). Up to 300 mg (wet weight) of previously homogenized sample material was weighed and placed into 150 ml perfluoralkoxy (PFA) vessels, into which a mixture of 1.3 ml nitric acid (65% HNO_3, suprapure) and 2.5 ml hydrogen peroxide (30% H_2O_2, suprapure) was added. Using the microwave digestion system MDS-2000 (CEM GmbH, Kamp-Lintfort, Germany), the vessels were heated for 90 min at about 170°C. After digestion the clear sample solution was filled with doubly distilled water in a 5 ml volumetric glass flask to its calibrated volume and kept in polypropylene sample tubes until further analysis. The concentrations of arsenic (As), cadmium (Cd), colbalt, (Co), copper (Cu), iron (Fe), manganese (Mn), molybdenum (Mo), nickel (Ni), lead (Pb), tin (Sn), vanadium (V) and zinc (Zn) were analysed using inductively coupled plasma mass spectrometry (ICP-MS). The analyses were conducted with a quadrupole ICP-MS system (Perkin Elmer - Elan 5000) operating at 1100 W plasma power, 13.3 L/min plasma gas flow, 0.75 L/min auxiliary gas flow and 0.95 L/min nebuliser gas flow and an auto sampler system (Perkin Elmer AS-90) connected with a peristaltic pump with a sample flow of 1 ml/min. The wash time between measurements was set at 10 seconds (with 1% HNO_3, suprapure) in order to avoid contamination and memory effects. For samples a dilution of 1:10 was applied, using a solution of 1% HNO_3 (suprapure) with a concentration of 10 ng/L of yttrium (Y) and thulium (Tm) as internal standards. A standard solution (ICP Multielementstandard V solution, Merck, Darmstadt, Germany) was analysed after every ten samples in order to control the accuracy and stability during measurements. The realisation of metal calibration was conducted using a series of 11 dilutions of a standard solution (ICP Multielementstandard solution, Merck, Darmstadt, Germany). Element concentrations were calculated as mg L^{-1} using corresponding regression lines (correlation factor e 0.999). To check the accuracy of the analytical procedure, standard reference material (DORM-3, National Research Council, Canada) of dogfish (*Squalus acanthias*) was analysed and the values of ten certified elements were checked. For the investigated elements, detection limits were calculated as the three fold standard deviation of concentrations found in twelve procedural blanks.

1.2.3 Data analyses and statistical treatment

The following morphological and parasitological indices were calculated: the condition factor (CF) as fish somatic mass x 100/fish total length³, the hepatosomatic index (HSI) as fish liver mass/fish somatic mass x 100, the parasitisation index (PI) as parasite mass/fish somatic mass x 100. For HSI and CF fish somatic mass was determined without parasite mass.

Bioconcentration factors were calculated according to Sures et al. (1999) as follows: ($C_{[L.\ intestinalis]}$ / $C_{[host\ tissue]}$). The comparisons of element concentrations between host tissues and *L. intestinalis* as well as between uninfected and infected fish were performed using Willcoxon matched pair test and Mann-Whitney *U*-test, respectively, at significance level p d 0.05. To test for significant inter-element correlations in the tissues of roach as well as in or with its cestode parasite correlation analysis were performed. Only associations with a significance level of $p < 0.05$ were considered. The correlations were performed individually for the metals in the group of uninfected fish and infected fish, respectively. All statistical tests were conducted with STATISTICA 6.0.

1.3 Results

1.3.1 Fish samples

A total of 20 fish were used for metal analyses. No differences of meristic parameters could be found between uninfected and infected roach (Table 1.1).

Table 1.1: Morphological parameters as mean (± SD) for host fish and its parasite *L. intestinalis.*

	n	TL (cm)	Weight (g)	CF	HSI	PI
Roach (uninf)	10	9.3 (0.9)	7.5 (1.9)	0.92 (0.07)	1.35 (0.12)	
Roach (inf)	10	10.1 (1.1)	10.2 (4.3)	0.96 (0.12)	1.24 (0.29)	8.75 (4.92)
L. intestinalis	4.2 (3.4)		1.1 (0.7)			

CF: condition factor; HSI: hepatosomatic index; Inf: infected; PI: parasitisation index; TL: total length; Uninf: uninfected.

1.3.2 Analytical procedure

The established detection limits and mean concentrations of elements in the standard reference material (DORM-3) are listed in Table 1.2. The accuracy of certified elements in DORM-3 ranged between 87% to 106% whereas the highest accuracy was obtained for iron (100%).

Table 1.2 Trace metal concentrations in Dogfish Muscle Certified Reference Material (DORM 3), accuracy and detection limits determined by ICP-MS analyses.

Element	DORM-3 values [mg/kg] (± SD)	DORM-3 measured [mg/kg] (± SD)	Accuracy (%)	Detection limit (µg/L)
As	6.88 (0.3)	6.30 (0.4)	92%	0.008
Cd	0.29 (0.02)	0.27 (0.02)	94%	0.01
Co	n.c.	-	-	0.009
Cu	15.50 (0.63)	16.35 (0.93)	105%	0.19
Fe	347 (20)	347 (29)	100%	2.76
Mn	n.c.	-	-	0.10
Ni	1.28 (0.24)	1.21 (0.15)	94%	0.47
Pb	0.395 (0.05)	0.417 (0.04)	106%	0.26
Zn	51.3 (3.1)	44.4 (3.2)	87%	2.77

n.c.: element not certified

1.3.3 Element concentrations in roach and *Ligula intestinalis*

Element concentrations in the host-parasite-system are summarized in Table 1.3. No significant differences could be found with respect to fish sex, why this factor was not considered for further analyses. The toxic elements As, Cd and Pb were below the detection limits in all fish and parasite tissues. In order to determine the accumulation capacity of *L. intestinalis* within its fish host, the bioconcentration factors (BCF) for the six detected metals (Co, Cu, Fe, Mn, Ni and Zn) were calculated with respect to different host tissues (Table 1.4).

Table 1.3: Mean element concentrations [mg/kg] (± SD) in different roach tissues and *L. intestinalis.*

		metal concentration [mg/kg]			
		uninf		inf	
As	intestine	nd		nd	
	muscle	nd		nd	
	L. intestinalis	nd		nd	
Cd	intestine	nd		nd	
	muscle	nd		nd	
	L. intestinalis	nd		nd	
Co	intestine	0.019	(0.010)	0.015	(0.018)^{c}
	muscle	0.005	(0.002)	0.004	(0.001)^{b}
	L. intestinalis	-		0.111	(0.042)^{a}
Cu	intestine	3.51	(0.95)	4.04	(2.33)
	muscle	0.56	(0.21)	0.57	(0.12)^{b}
	L. intestinalis	-		3.05	(1.86)^{a}
Fe	intestine	42.0	(24.9)	42.2	(25.5)
	muscle	8.3	(2.5)	8.4	(1.7)^{b}
	L. intestinalis	-		24.1	(6.0)^{a}
Mn	intestine	1.07	(0.31)	0.84	(0.34)^{c}
	muscle	0.36	(0.11)	0.39	(0.15)^{b}
	L. intestinalis	-		4.35	(1.33)^{a}
Ni	intestine	0.751	(0.775)^{c}	0.006	(0.014)^{b}
	muscle	0.083	(0.042)	0.139	(0.135)
	L. intestinalis	-		0.259	(0.361)^{a}
Pb	intestine	nd		nd	
	muscle	nd		nd	
	L. intestinalis	-		nd	
Zn	intestine	159.9	(100.0)^{d}	217.8	(68.9)^{c}
	muscle	12.7	(5.9)	10.8	(2.0)^{b}
	L. intestinalis	-		43.1	(13.0)^{a}

Values not sharing a common letter for the same parameter from the same element are statistically different from each other (Mann Whitney, $p < 0.05$), with nd = not detected. Within each tissue, the uninfected were compared to the infected ones. Comparing the different tissues with *L. intestinalis*, only the infected individuals were analysed.

Table 1.4: Bioconcentration factors $C_{[L.\ intestinalis]}$ / $C_{[roach\ tissue]}$ for *L. intestinalis* calculated with respect to different host tissues.

Element	Muscle $C_{[L.\ intestinalis]}$ / $C_{[Muscle]}$	Intestine $C_{[L.\ intestinalis]}$ / $C_{[Intestine]}$
Co	27.0	7.5
Cu	5.4	0.8
Fe	2.9	0.6
Mn	11.2	5.2
Ni	1.9	42.7
Zn	4.0	0.2

Accordingly, the parasite showed a higher accumulation capacity for all analysed elements in comparison with the host muscle, whereas, in respect to the roach intestine, only Co, Mn and Ni were found in higher concentrations in the parasite samples (see also Figure 1.1 and Figure 1.2).

Differences between metal levels of uninfected and infected fish could only be detected in intestine tissue for Ni and Zn, respectively (Table 1.3).

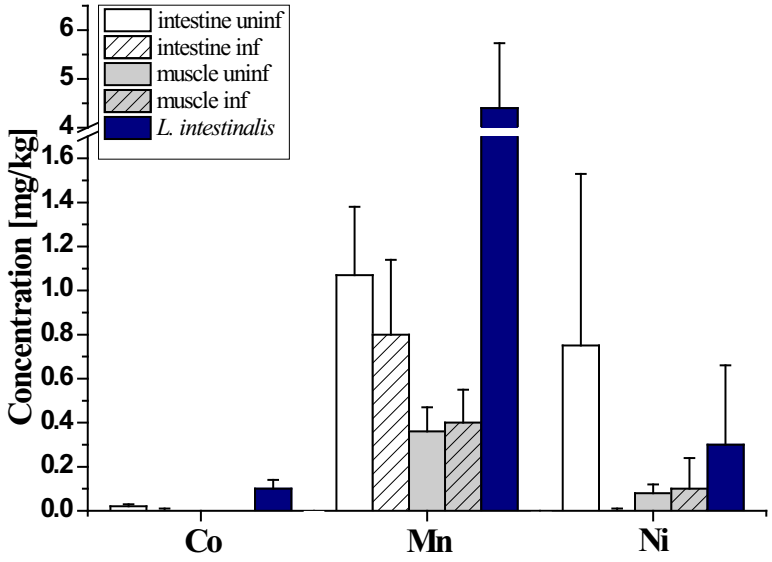

Figure 1.1: Mean element concentrations [mg/kg] (± SD) in different roach tissues and its parasite *L. intestinalis* for elements Co, Mn and Ni.

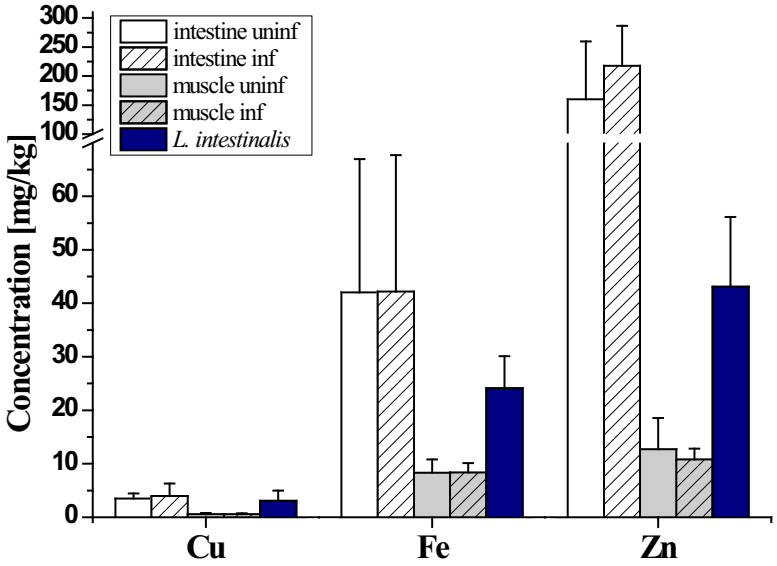

Figure 1.2: Mean element concentrations [mg/kg] (± SD) in different roach tissues and its parasite *L. intestinalis* for elements Cu, Fe and Zn.

Only positive significant inter-element correlations were found in tissues of roach as well as in or with its cestode parasite (Table 1.5).

Table 1.5: Correlation coefficients (r) between element levels in roach tissues and *L. intestinalis* for uninfected and infected group, respectively, only significant relationships are shown ($p < 0.05$).

uninfected			r	infected			r
[Co] muscle	-	[Mn] muscle	0.89	[Co] muscle	-	[Zn] muscle	0.75
[Co] muscle	-	[Fe] muscle	0.94	[Co] muscle	-	[Co] *L. intestinalis*	0.90
[Co] muscle	-	[Ni] muscle	0.75	[Co] intestine	-	[Ni] intestine	0.76
[Co] muscle	-	[Zn] muscle	0.64	[Co] intestine	-	[Cu] intestine	0.78
[Fe] muscle	-	[Ni] muscle	0.66	[Zn] *L. intestinalis*	-	[Cu] *L. intestinalis*	0.81
[Fe] muscle	-	[Mn] muscle	0.89	[Cu] intestine	-	[Ni] intestine	0.89
[Mn] muscle	-	[Ni] muscle	0.77				
[Co] intestine	-	[Ni] intestine	0.82				
[Fe] intestine	-	[Cu] intestine	0.90				

Cobalt displayed the largest number of associations with other elements. In muscle tissue of uninfected fish, it was associated with Mn, Fe and Ni, which was not found for infected fish. The regression analysis for Co with Fe, Mn and Ni (Figure 1.3) as well as for Fe with Mn and Ni (Figure 1.4) or only Mn and Ni (Figure 1.5) in muscle tissue of uninfected fish demonstrated a similar pattern of combined uptake for all these metals. The same applies to Fe and Cu in intestinal tissue of uninfected fish (Figure 1.6).

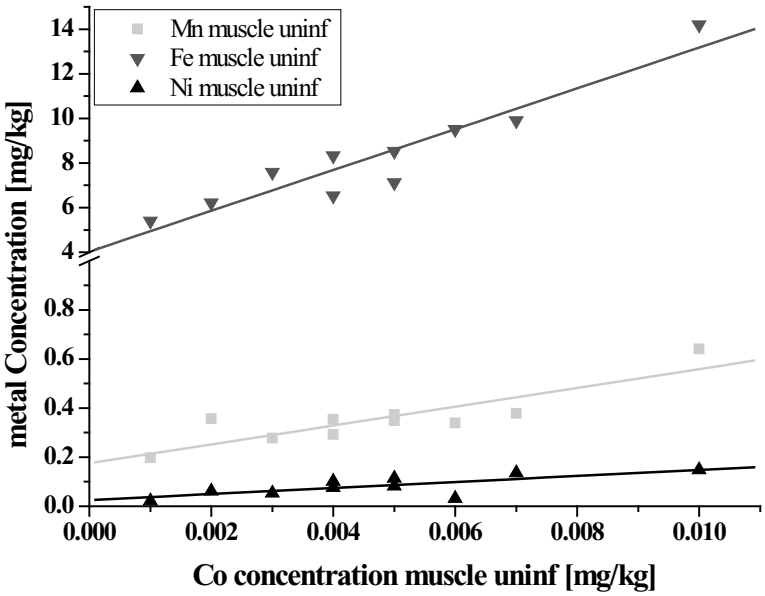

Figure 1.3: Regression analysis showing inter-element correlations in fish host muscle tissue of uninfected fish for the element Co with Mn, Fe and Ni.

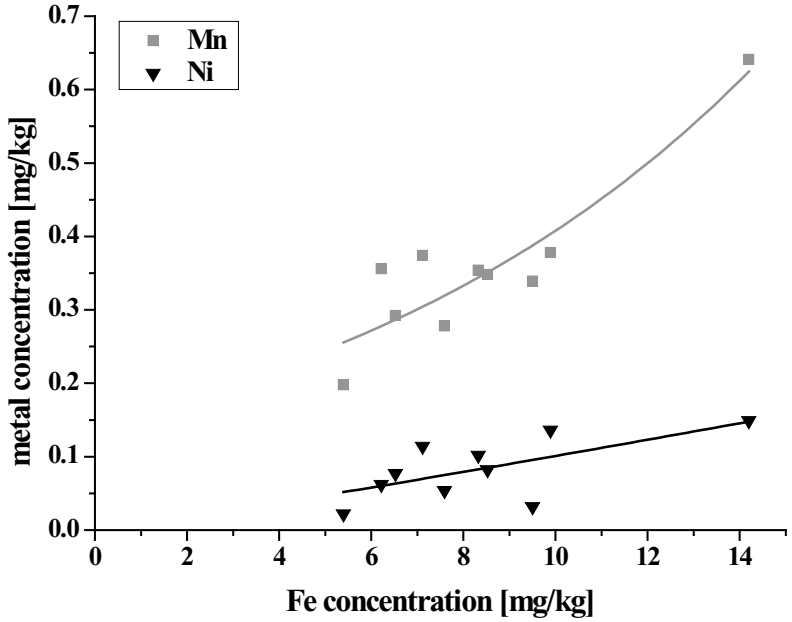

Figure 1.4: Regression analysis showing inter-element correlations in fish host muscle of uninfected fish for the elements Fe, Mn and Ni.

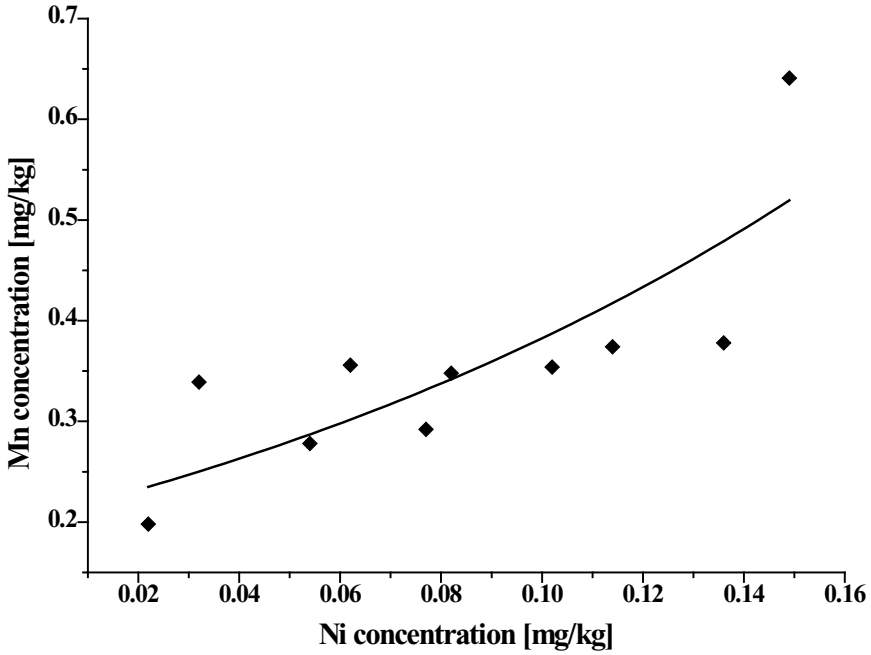

Figure 1.5: Regression analysis showing inter-element correlations in fish host muscle of uninfected fish for the elements Ni and Mn.

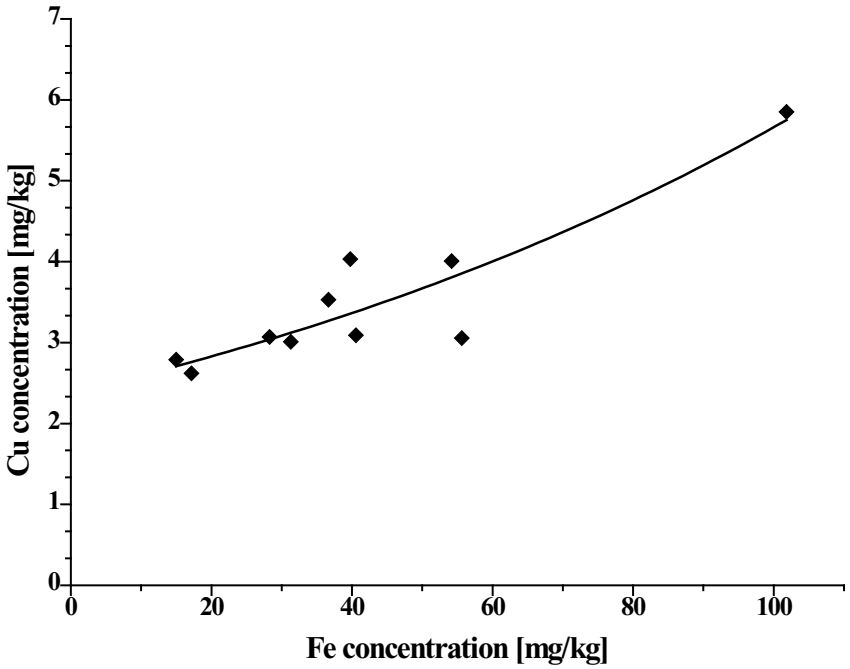

Figure 1.6: Regression analysis showing inter-element correlations in fish host intestine of uninfected fish for the elements Fe and Cu.

The association between Co and Zn in fish muscle as well as Co and Ni in fish intestine was found independent of infection. While the regression lines revealed no difference by infection for Co and Zn (Figure 1.7), the concentration of Co already increased to a higher degree at lower Ni concentrations in infected fish (Figure 1.8).

In the group of uninfected fish, most associations were found for elements from muscle tissue. Beside the Co-Ni association, the only correlation found in intestine was that between Fe and Cu. While in intestine of uninfected fish, Cu correlated with Fe, in infected fish the Cu-uptake correlated positively with Co and Ni (Figure 1.9).

For infected fish, Co from fish muscle was also associated with Co levels in the parasite, indicating rising Co-concentrations in parasite tissue simultaneous with increasing Co-concentrations in fish host muscle tissue (Figure 1.10), which was expressed by high BCF calculated for *L. intestinalis* in respect to muscle tissue (Table 1.4).

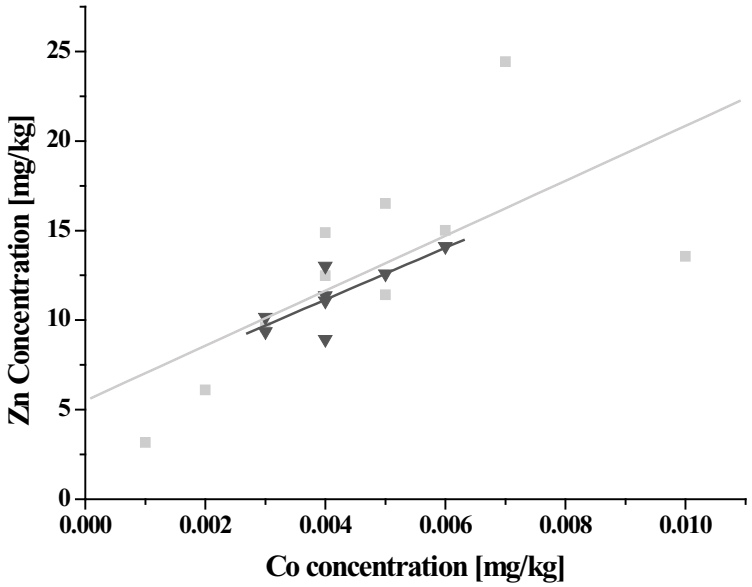

Figure 1.7: Regression analysis showing inter-element correlations in fish host muscle tissue of uninfected (light grey) and infected (dark grey) fish for the elements Co and Zn.

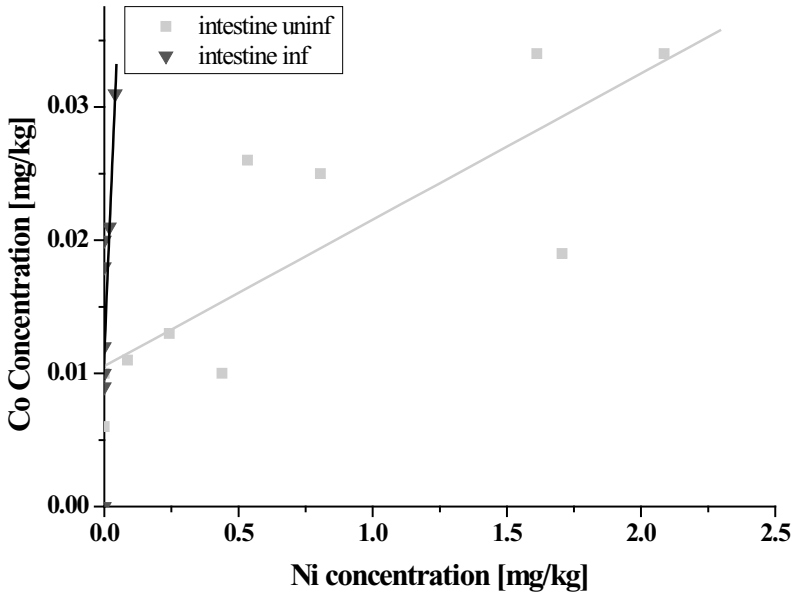

Figure 1.8: Regression analysis showing inter-element correlations in fish host intestine of uninfected and infected fish for the elements Co and Ni.

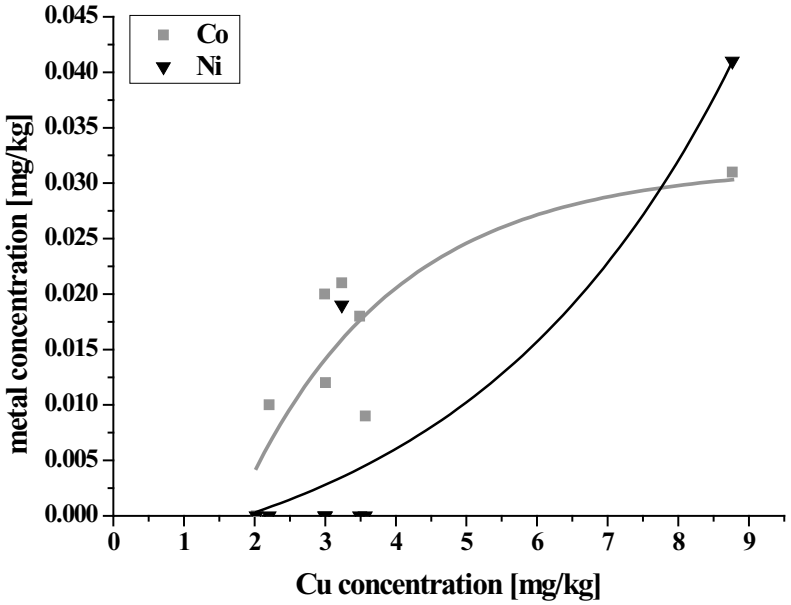

Figure 1.9: Regression analysis showing inter-element correlations in fish host intestine of infected fish for the elements Cu, Co and Ni.

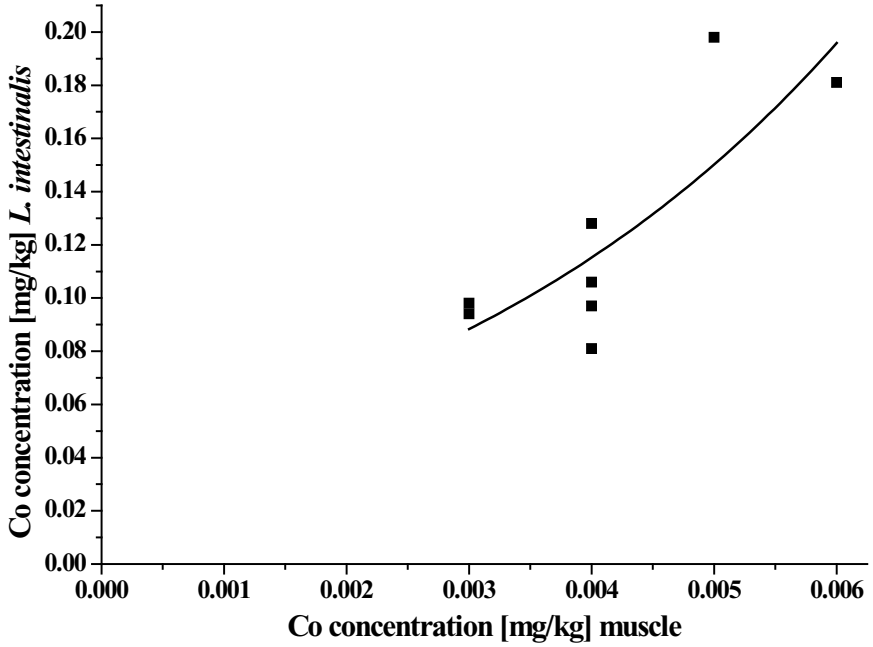

Figure 1.10: Regression analysis showing inter-element correlations in fish host muscle of infected fish and *L. intestinalis* for the element Co.

Within the parasite the only correlation found, was between the antagonists Zn and Cu, indicating either a preferred uptake of Cu or a saturation with Zn which was reached at levels

around 50 mg/kg (wet weight) Zn (Figure 1.11). To compare all the results with other studies which analysed metal levels in fish *and L. intestinalis*, Table 1.6 was added.

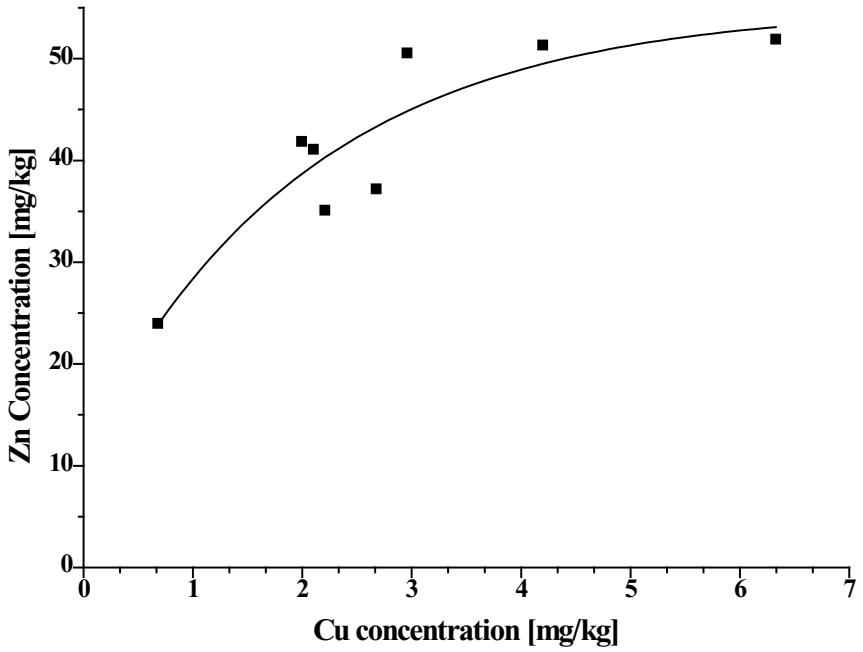

Figure 1.11: Regression analysis showing inter-element correlations in *L. intestinalis* for the elements Cu and Zn.

Figure 1.12 illustrates that with increasing Cu-concentrations in muscle tissue of uninfected roach, levels of HSI were decreasing (r = -0.74). In infected fish, the metal concentrations of Fe from intestine (r = 0.71) and Cu from muscle (r = 0.73) increased with increasing fish condition (Figure 1.13). The similar increase of metal concentrations for Mn from intestine (r = 0.74) and Ni from muscle (r = 0.74) with an increasing total number of parasite individuals is in contrast to the decreasing concentrations of Co from fish muscle (r = -0.77) (Figure 1.14), again indicating the real accumulation capacities of *L. intestinalis* for Co.

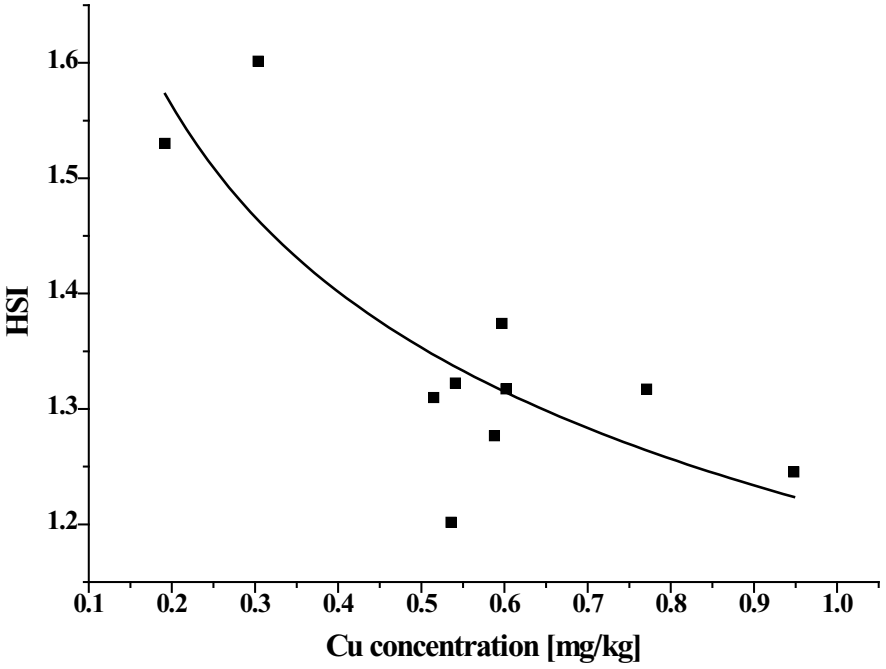

Figure 1.12: Regression analysis showing correlation of Cu concentration [mg/kg] in roach muscle and HSI of uninfected group.

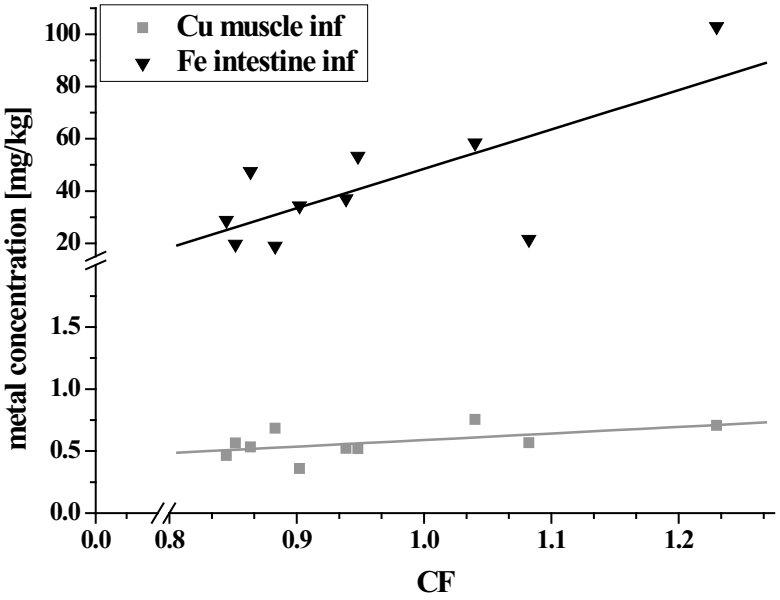

Figure 1.13: Regression analysis showing correlation between CF and concentration [mg/kg] of Cu and Fe in roach muscle/intestine of infected group.

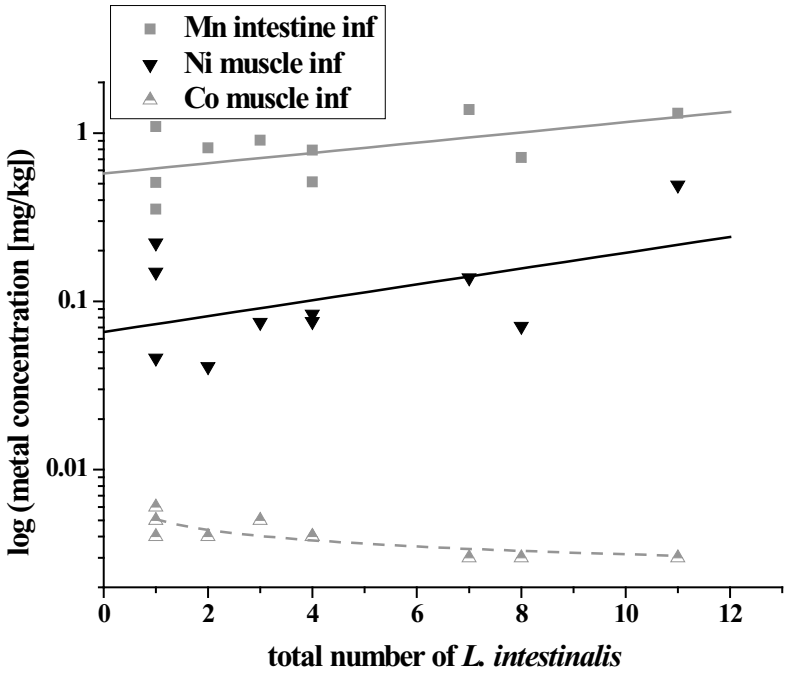

Figure 1.14: Regression analysis showing correlation between total number of *L. intestinalis* and concentration [mg/kg] of Mn, Ni and Co in roach intestine or muscle of infected group.

Table 1.6: List of recent and published data (mean and standard deviation (SD)) for elements measured in this study from *L. intestinalis* and its different fish host muscles (ww= wet weight; dw= dry weight).

	metal concentration [mg/kg]									
	Frank et al. 2010		Tekin-Özan and Kir 2005		Tekin-Özan and Kir 2008		Tekin-Özan and Barlas		Tenora et al. 1997	Barus et al. 1999
Parasite host	*R. rutilus*		*T. tinca*		*T. tinca*		*T. tinca*		*A. brama*	Pike-perch (*A. brama* ingested)
	ww	(SD)	ww	(SD)	ww	(SD)	ww	(SD)	dw	dw
Fe										
muscle	8.4	(1.7)	8.9	(4.6)			6.8	(2.2)		
L. intestinalis	24.1	(6.0)	325.1	(52.3)			4.3	(2.4)	10.4	7.1
Zn										
muscle	10.8	(2,.0)	7.0	(3.1)			8.1	(3.0)		
L. intestinalis	43.1	(13.0)	48.0	(8.0)			44.7		154.0	53.3
Cu										
muscle	0.57	(0.12)	nd				nd			
L. intestinalis	3.1	(1.9)	72.0	(11.4)			5.5	(2.6)	14.8	26.1
Co										
muscle	0.004	(0.001)								
L. intestinalis	0.111	(0.042)							0.6	0.2
Mn										
muscle	0.39	(0.15)	nd				nd			
L. intestinalis	4.4	(1.3)	8.1	(2.4)			2.1	(0.8)	3.1	1.4
Ni										
muscle	0.139	(0.135)			2.430	(2.11)				
L. intestinalis	0.259	(0.361)			0.200	(0.19)				

1.4 Discussion

In the present study the toxic metals As, Cd and Pb could not be detected, neither in tissues of roach nor in its parasite *L. intestinalis*. This is in agreement with the fact that the fish were taken from a water reservoir which serves as a source for drinking water and thus should lack high levels of toxic metals.

The idea of cestodes being used as accumulation indicators for heavy metals is controversially discussed. Malek et al. (2007) found good accumulation capacities for two intestinal cestode species in the shark *Carcharhinus dussumieri*. The system *Proteocephalus macrocephalus/Anguilla anguilla* is also proposed as a bioindicator system to detect environmental contamination with Cr, Ni, Pb and Zn (Eira et al., 2009). The cestode *Bothriocephalus acheilognathi* is considered as a potential bioindicator, because 8 of 23 elements (Li, Be, Mn, Se, Hg, Tl, Pb and U) were accumulated in a higher degree in the parasite than in the host (Retief et al., 2006). For *L. intestinalis* from tench, no considerable heavy metal accumulation was described (Tekin-Özan and Barlas, 2008; Tekin-Özan and Kir, 2008) whereas in *Rastreneobola argentea, L. intestinalis* is proposed as a promising bioindicator for evaluating environmental impact of the elements Pb, Cd and Cr (Oyoo-Okoth et al., 2010). However, in a direct comparison with the nematode *Philometra ovata*, *L. intestinalis* only showed low accumulation capacities for Pb, Cd and Cr (Tenora et al., 2000). In summary, there are two main differences between *L. intestinalis* and the other investigated cestodes: first *L. intestinalis* is located in the host's body cavity whereas the others are located in the host's intestine and secondly *L. intestinalis* lives as larval stage in its intermediate fish host, whereas the others are adult and reproduce. The stage of maturation was already demonstrated to be a reason for different amounts of Co in parasitic helminths, as e.g. immature proglottids of adult *Echinococcus granulosus* showed higher cobalt concentrations than mature and gravid proglottids (Chowdhury and Singh, 1995). In contrast, in the whale tapeworm *Diphyllobothrium macroovatum* the elements Fe, Cu, Zn, Mn and Ca showed a tendency to increase from immature to gravid proglottids (Yamane et al., 1986). The possible influence of cestode location and developmental stage on metal accumulation capacity is also indicated by Sures et al. (1997) who found 75 and 43 times more Pb and Cd in *Monobothrium wageneri* than in the muscle of its host, the tench. As no toxic metals were

found at all, this study was not able to expand the knowledge about the biondicator properties of *L. intestinalis* in its intermediate host *R. rutilus*.

For the elements Co, Cu, Fe, Mn, Ni and Zn no differences concerning roach sex could be detected whereas Burger (2007) reviewed a high number of studies where gender-related differences in metal levels occurred. This lack of gender specific differences might be due to the low number of roach being investigated from each sex. The same could also apply for the differences between uninfected and *L. intestinalis*-infected fish. Only two elements revealed differences by infection status. While Zn was significantly higer in intestine tissue of infected roach, significantly lower levels of Ni were detected in the same group. Ni also increased in muscle tissue with an increasing number of *L. intestinalis*. Unfortunately, the high variance in Ni levels of muscle tissue prevented for further findings confirming a relation between these observations.

Beside the fact that most of the correlations between metals like Co, Mn, Fe and Ni from roach muscle were not found in infected fish, differences were also found for the significant correlations between Ni and Co in roach intestine of uninfected and infected fish with higher Co- concentrations at lower Ni- concentrations for the latter. Cobalt also showed the highest BCF in the parasites in comparison to roach muscle as well as, most associations with other metals and the only negative correlation within the group of infected fish. Altogether these results emphasize the suggested need for cobalt of growing cestodes (Chowdhury and Singh, 1995). A possible anaemia of cyanocobalamin (vitamin B 12), in which cobalt constitutes the central atom, is known for mammals infected with *Diphyllobothrium latum*, a relative of *L. intestinalis*. For example, 44% of an oral vitamin B12 dose to the host could be detected in the proximal part of *D. latum* (Nyberg, 1958) and even the in vitro uptake was proved (Brante and Ernberg, 1957). As inorganic cobalt was not taken up by the parasite (Nyberg, 1958) it can be suggested that the Co detected in *L. intestinalis* is part of vitamin B12. For vitamin B12 growth promoting effects were early described (Nyberg, 1958) and the highest concentrations of cobalt as well as vitamin B12 were demonstrated in the neck or immature region of cestodes (Chowdhury and Singh, 1995; Nyberg, 1958). This could explain the association between parasite abundance and decreasing Co-concentrations in host muscle tissue in contrast to associations between parasite mass and metal concentration in studies with other parasite classes, for example acanthocephalans (Sures, 2003). The excretion of

cobalt in roach increased with the parasite number, as every individual parasite seems to need its special amount of cobalt or vitamin B12 respectively.

Another interesting phenomenon is the indicated saturation level in *L. intestinalis* for Zn. The intra-element competition between copper and zinc in parasite tissue revealed a saturation level at around 50 mg/kg (wet weight) for Zn, whereas the Cu levels seemed to reach no saturation. This is confirmed by the comparison with other studies investigating *L. intestinalis* in its host *Tinca tinca* (Tekin-Özan and Kir, 2005; Tekin-Özan and Barlas, 2008). There is no difference between the mean levels of Zn, while for example the mean levels of Fe ranged between 4 to 325 mg/kg (wet weight). For copper, one study even confirms the assumption of unlimited copper uptake by 14 times higher Cu levels at the same assumed saturated Zn level (Tekin-Özan and Kir, 2005). Zn is thought to play similarly to Co a major role for the optimal growth of cestodes (Chowdhury, 2005). Concerning cestodes, *Mesocestoides corti* for example is known to be able to concentrate a variety of cations (like Cr, Cu and Zn) in their calcerous corpuscles (Smyth and McManus, 1989). However, for *L. intestinalis* it is not clear whether the detected elements were contents of calcareous corpuscles or if they had any other clear function. Nevertheless, many inorganic elements are essential for growth, development, metabolism and immunobiology of parasites. In other cestodes like *Spirometra erinacei* (Pseudophyllidea), a Cu/Zn superoxide dismutase (SOD) may play an essential role for parasite survival not only by protecting itself from endogenous oxidative stress but also by preventing an oxidative killing of the parasite by host immune effector cells (Li et al., 2010). In comparison to the literature, Zn attracted attention in five of six studies because of its high contents in *L. intestinalis* compared with the other metals analysed (Barus et al., 1999; Oyoo-Okoth et al., 2010; Tekin-Özan and Barlas, 2008; Tekin-Özan and Kir, 2005; Tenora et al., 1997). Unfortunately its specific function has not yet been elucidated.

Whereas there was no difference concerning infection between meristic parameters or tissue's Cu-concentrations, the levels of copper in muscle tissue of uninfected roach showed a significant association to lower HSI values at higher Cu-concentrations. Unfortunately, contrary results have been observed by increasing as well as decreasing HSI at sublethal metal concentrations in fish (Gagnon et al., 2006; Sindhe and Kulkarni 2004) which is why it may only be supposed that muscle Cu-concentration may have been associated to another toxic compound not analysed in the present study but responsible for the observed effect. In the group of infected roach, there is also a positive association between muscle Cu-concentration

and fish condition factor. The same was found for Fe-concentrations in fish intestine. Additionally, the muscle Zn-concentrations as well as the condition factors from infected fish showed the tendency to decrease with an increasing total number of *L. intestinalis*, whithout being significant. As the CF decreased with parasite burden, a higher CF could stand for fish with less parasites and therefore maybe with less competition for the named elements.

In summary, zinc was found to have the highest and cobalt the lowest concentration of all analysed metals in fish intestine or muscle (both irrespective of infection status) as well as in the parasite *L. intestinalis*. The general order of concentration was: Zn>Fe>Cu>Mn>Ni>Co. Andreji et al. (2005) also analysed element concentrations in roach muscle and reported the same order of concentration as the present study; and in most cases even with the same metal mean concentrations. For *L. intestinalis* the order of metal concentration coincides for most of the metals with five of six literature comparisons (Barus et al., 1999; Oyoo-Okoth et al., 2010; Tekin-Özan and Barlas, 2008; Tekin-Özan and Kir, 2005; Tenora et al., 1997). Only one investigation resulted in a noticeable higher level of iron even exceeding Zn and Cu (Tekin-Özan and Kir, 2005). It cannot be defined if this is due to the high levels of Fe in the water the fish host lived in. But even in two different whale tapeworms, a metal order headed by Zn was found whereas the Fe concentration seemed to be highest in the scolex and neck region compared to immature as well as mature proglottids (Yamane et al., 1986).

1.5 Conclusions

To conclude, cobalt revealed the most indications for an existing element competition between the parasite *L. intestinalis* and its fish host (*R. rutilus*). Zn occurred in fish host tissue as well as in its parasite with the highest metal levels whereas a saturation level for Zn in *L. intestinalis* could be assumed at around 50 mg/kg wet weight. As no toxic metals were found, no conclusion could be given about the parasite's bioaccumulation capacity or the qualification as bioindicator for toxic metal pollution, respectively. *L. intestinalis* showed element concentrations higher than in muscle tissue of fish for all analysed metals and in case of Co, Mn and Ni also higher than in intestine tissue; but it cannot be excluded that these are just the normal element concentrations occurring in the parasite.

2 Biomarker 1: The glutathione-S-transferase-activity in three different fish species considering the infection with diphyllobothridean cestodes

2.1 Introduction

The variety of pollutants in the aquatic environment continues to increase, necessitating means to detect and assess the impact of pollution. Particularly low concentrations of chemicals, as well as complex pollutant mixtures, are difficult to detect applying only chemical analyses. This has generated the idea of using molecular biotic indicators (biomarkers) to unravel exposure to, and effects of, contaminants on organisms (Livingstone et al. 1994). However, the evaluation of biomarkers for environmental contaminants is usually done in laboratory experiments which inherently lack complex environmental interactions. Considering that biomarkers are also applied under field conditions, the artificial nature of laboratory analyses can be compensated for, as it should be with natural stressors which may influence a biomarker response.

Total hepatic glutathione-S-transferase (GST)-activity has been a commonly applied biomarker for assessing different groups of pollutants for several years. The GST enzyme is an important intracellular enzyme belonging to the second stage of xenobiotic metabolism by virtue of catalyzing the conjugation of the tripeptide glutathione and electrophilic substances of exogenous origin (Eaton and Bamler, 1999). In contrast to a wealth of reports on the modulation of GST-activity in organisms by chemicals, only a few studies have been published dealing with the impact of parasites on GST-activity in fish - showing an increased antioxidant response in carp parasitized by the cestode *Ptychobothrium* sp. (Dautremepuits et al. 2002, 2003). Another parasite known for its significant impact on common biomarkers used in aquatic ecotoxicology is the cestode *Ligula intestinalis.* Due to its most striking effect, the inhibition of fish host's sexual maturation, previous work focused on biomarkers for endocrine disruption such as vitellogen in bream (*Abramis brama*), chub (*Squalius cephalus*) and roach (*Rutilus rutilus*) (Geraudie et al. 2009; Hecker et al. 2007; Hecker and Karbe, 2005; Schabuss et al. 2005; Trubiroha et al. 2009, 2010a,b). In regards for using a laboratory model to investigate host-parasite interactions under controlled conditions, three-spined stickleback (*Gasterosteus aculeatus*) infected with *Schistocephalus solidus* is recommended (Barber and

Scharsack, 2010). An additive effect of Cd exposure and parasite infection was already demonstrated by decreased survival rates compared with uninfected fish (Pascoe and Cram, 1977; Pascoe and Woodworth, 1980). Both parasites are 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 parasitic stage in the fish intermediate host, the so-called plerocercoid is located in the body cavity, usually of cyprinids for *L. intestinalis* and the three-spined stickleback for *S. solidus*. Roach (Machala et al. 2000; Van der Oost et al. 1994), chub (Havelkova et al. 2008; Krca et al. 2007; Vigano et al. 1998) and three-spined sticklebacks (Björkblom et al. 2009; Sanchez et al. 2007, 2008) are recently used as sentinels for freshwater monitoring in biomarker studies. However, the knowledge of parasites as modulators of GST-activity in fish hosts is rare despite parasites being a factor commonly found in every fish population. Therefore we investigated the influence of infections with the diphyllobothridean cestodes *S. solidus* and *L. intestinalis* on GST-activity of their intermediate fish hosts *G. aculeatus*, *R. rutilus* and *S. cephalus*.

2.2 Material and methods

2.2.1 Sampling design

Three different host-parasite systems were investigated. In order to evaluate effects of parasites under controlled conditions, three-spined sticklebacks experimentally infected with *S. solidus* were chosen as a laboratory host-parasite system (LAB). To transfer the results into the wild, cyprinids, i.e. roach and chub naturally infected with *L. intestinalis* were chosen. They were collected from two sites in Germany where fish are known to be infected with the respective parasites. One sampling site was located at Lake Mueggelsee (MS) (5°26`N, 13° 39`E), a polymictic and eutrophic shallow lake (surface area of 7.3 km², mean depth of 5 m) in the southeast of Berlin which is flushed by the river Spree (Driescher et al., 1993). The second sampling site was at the Reservoir Listertalsperre (LTS) (51° 5`38” N, 7° 50`15” E), a mesotrophic dam built in 1912 (surface area of 0.79 km², max depth of 39 m) in the south of Meinerzhagen. At both sampling sites *L. intestinalis* infected roach were collected, additionally, chub infected with this parasite were also caught at LTS. Accordingly, this sampling design allowed not only to compare the same host-parasite system from different independent sites but also to compare the effects of the same parasite on two different hosts.

2.2.2 Fish collection and tissue sampling

Three-spined sticklebacks (*G. aculeatus*) originating from the lake Großer Plöner See were bred in the laboratory and experimentally infected with *S. solidus* as described by Scharsack et al. (2007), based on Smyth (1946). After two weeks of acclimatization in an 80 L aquarium, 44 fish were held under constant conditions for ten weeks in aerated tap water at a water temperature of 17°C and a photoperiod of 12h light: 12h dark. Uninfected and infected sticklebacks were maintained together in the same aquaria and fed three times a week with tubificids. The water was changed twice weekly.

Roach (*R. rutilus*) were caught by electrofishing from MS during autumn 2007 and dissected within the capture day. Roach and chub (*S. cephalus*) from the water reservoir Listertalsperre were caught by electrofishing in June 2008 and transferred to the Institute in aerated tanks. Roach were held in a 200 L aquarium with aerated tap water at approximately the same water temperature as in the reservoir (22°C) until they were dissected within the next three days. In order to reduce variations due to catching stress, chub were held for seven weeks in the laboratory at a photoperiod of 12h light: 12h dark before being killed and dissected. During maintenance water was changed once a week and fish were fed 5 times a week with tubificids.

For sampling, the dissecting procedure was the same for all fish species. After anaesthetization with ethyl 3- aminobenzoate methansulfonate (MS222, Sigma) fish were killed by decapitation. The fish were measured (to the nearest mm) and weighed (to the nearest mg), organs and parasites were removed, weighed (to the nearest mg) and immediately frozen in liquid nitrogen. All samples were stored at -80°C until further preparations.

2.2.3 GST analyses

Liver samples (60 mg) were homogenised with a sonicator (Sonoplus HD 2070, Bandelin) in adequate volumes of phosphate buffer (50 mM KH_2PO_4, 1 mM EDTA, pH = 7) and centrifuged at 10.000g for 10 min at 4°C. The glutathione-S-transferase-activity was evaluated using the GST Assay Kit from Sigma-Aldrich (Germany) based on the use of 1-chloro-2.4 dinitrobenzene (CDNB) as substrate as previously described by Habig et al. (1974). The measurements were performed with the supernatant at 25°C within 6 min with a microplate reader (Infinite M200, Tecan). The total protein concentration was determined in

each supernatant using the Pierce BCA Protein Assay Kit (Pierce Biotechnology, Rockford Illinois, USA).

2.2.4 Data analyses and statistical treatment

The following morphological and parasitological indices were calculated: the hepatosomatic index (HSI) as fish liver mass/fish somatic mass x 100, the gonadosomatic index (GSI) as fish gonad mass/fish somatic mass x 100, the condition factor (CF) as fish somatic mass x 100/fish total length^3, and the parasitisation index (PI) as parasite mass/fish somatic mass x 100. For HSI, GSI and CF fish somatic mass was determined without parasite mass. As all data were normally distributed, pair wise analysis was done by Student's t- test. Significance was accepted when p d 0.05.

After confirming normality and homogeneity of variance with Kolmogorov-Smirnow test and Levene-Test, respectively, the differences among GST-activities were assessed using a univariate analysis of variance (ANOVA) with GST-activity as dependent factor and host sex as well as infection status as independent factors. For the analysis of roach data from two different sites, the independent factor capture site was added. When necessary, the ANOVA was followed by Student's t-test to compare the uninfected fish with the infected ones within the same sex and site.

Spearman's rank correlation coefficient was used to investigate the associations between morphological parameters/indices and GST-activity, if a sufficient number of data points was given ($n > 6$). Therefore, a lack of significant correlations might simply be the result of a low number of data pairs.

All statistical analyses were performed using Statistica 6.0; except the analysis of variance including Kolmogorow-Smirnow test and Levene test were performed with SPSS, PASW 18.

2.3 Results

2.3.1 Meristic parameters

Total length, CF, HSI, GSI and PI of all fish are summarized in Table 2.1. Fish of each group consistently showed a similar size range, although significant differences occurred between groups. In the groups LAB and LTS, the uninfected females were significantly longer than the

uninfected males; for MS this was the case in infected fish as well. For LTS-chub a difference was only found between uninfected and infected males; with infected fish being significantly smaller. Roach from MS (Min/Max = 10.8/19.6 cm) were significantly larger than roach from LTS (Min/Max = 7.3/11.8 cm).

For sticklebacks, CF and HSI showed reduced values due to infection in both genders. Only infected male chub from LTS had a higher HSI than the uninfected males. In roach from MS, there was a significant reduction of GSI by parasite infection in both genders. Furthermore the GSI for MS females was higher than for males.

For sticklebacks, significant correlations were only found in infected females, for which CF showed a negative relationship with parasite burden ($r = -0.56$). For MS, negative correlations were found between CF and PI for both genders ($r = -0.67$ (female); -0.64 (male)), albeit the CF difference between the uninfected and infected fish of each sex was not significant. Roach from LTS showed a negative correlation between CF and HSI for infected females ($r = -0.93$). For uninfected male chub the CF showed significant correlations with the total length ($r = 0.72$), whereas the CF of infected females was significantly correlated with the GSI ($r = -0.67$).

Sticklebacks showed the highest PI with 37%, without differences between sexes. The PI was lowest for roach with approximately 10% for both sites and sexes. Chub infected with the same parasite as roach showed a PI around 17%.

Table 2.1: 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, na = not analysed. Within each sex group the uninfected were compared to the infected ones. Comparing the different sexes only the individuals with the same infection status were analysed.

Sites	Sex (uninf/inf)	n	TL (cm)	CF	HSI	GSI	PI
LAB	M (uninf)	5	4.2 (0.3)^{a}	0.72 (0.03)^{a}	3.3 (0.5)^{a}	na	
(stickle-	M (inf)	4	4.4 (0.5)^{ab}	0.65 (0.02)^{b}	2.6 (0.3)^{b}	na	37.5 (5.0)^{a}
back)	F (uninf)	6	4.9 (0.3)^{b}	0.74 (0.03)^{cab}	4.2 (0.5)^{cb}	na	
	F (inf)	14	4.5 (0.5)^{ab}	0.68 (0.07)^{dab}	2.6 (0.4)^{dab}	na	37.3 (6.5)^{a}
MS	M (uninf)	10	14.8 (2.5)^{ab}	0.82 (0.07)^{a}	na	3.1 (1.3)^{a}	
(roach)	M (inf)	11	13.8 (0.9)^{a}	0.76 (0.05)^{a}	na	0.4 (0.1)^{b}	10.7 (4.1)^{a}
	F (uninf)	11	14.9 (0.9)^{ab}	0.88 (0.12)^{a}	na	5.6 (2.0)^{c}	
	F (inf)	13	15.1 (1.7)^{b}	0.77 (0.08)^{a}	na	1.1 (0.4)^{d}	9.5 (1.9)^{a}
LTS	M (uninf)	5	8.6 (0.7)^{a}	0.95 (0.09)^{ab}	1.4 (0.1)^{a}	0.5 (0.2)^{a}	
(roach)	M (inf)	5	9.5 (0.8)^{ab}	0.88 (0.04)^{a}	1.2 (0.3)^{a}	0.4 (0.2)^{a}	10.0 (4.8)^{a}
	F (uninf)	5	9.9 (0.2)^{b}	0.90 (0.04)^{ab}	1.3 (0.1)^{a}	0.6 (0.2)^{a}	
	F (inf)	5	10.0 (1.1)^{ab}	1.04 (0.13)^{b}	1.3 (0.3)^{a}	0.6 (0.1)^{a}	9.5 (4.0)^{a}
LTS	M (uninf)	9	9.2 (0.7)^{a}	0.77 (0.03)^{a}	1.0 (0.4)^{a}	0.3 (0.1)^{a}	
(chub)	M (inf)	6	8.4 (0.7)^{b}	0.73 (0.07)^{ab}	1.6 (0.6)^{b}	0.4 (0.4)^{a}	15.8 (5.9)^{a}
	F (uninf)	5	8.7 (0.7)^{ab}	0.72 (0.02)^{b}	1.2 (0.3)^{ab}	0.3 (0.2)^{a}	
	F (inf)	9	8.8 (1.0)^{ab}	0.75 (0.10)^{ab}	1.2 (0.7)^{ab}	0.3 (0.1)^{a}	19.9 (9.2)^{a}

CF: condition factor; F: female; GSI: gonadosomatic index; HSI: hepatosomatic index; Inf: infected; M: male; PI: parasitisation index; TL: total length; Uninf: uninfected.

2.3.2 Total hepatic GST-activity and correlation with morphological parameters

Hepatic GST-activities in sticklebacks are presented in Figure 2.1. The analysis of variance indicated a small but significant effect of host sex ($F = 4.591$; $p = 0.042$) on the stickleback's GST-activity with females presenting 8-18% higher values than males. Whereas, the effect of infection with plerocercoids of *S. solidus* was highly significant ($F = 39.284$; $p < 0.001$). The extent of reduction on GST-activity was the same in both host sexes (39%). The only significant correlation found for sticklebacks was a positive relation between GST-activity and HSI in infected females ($r = 0.65$).

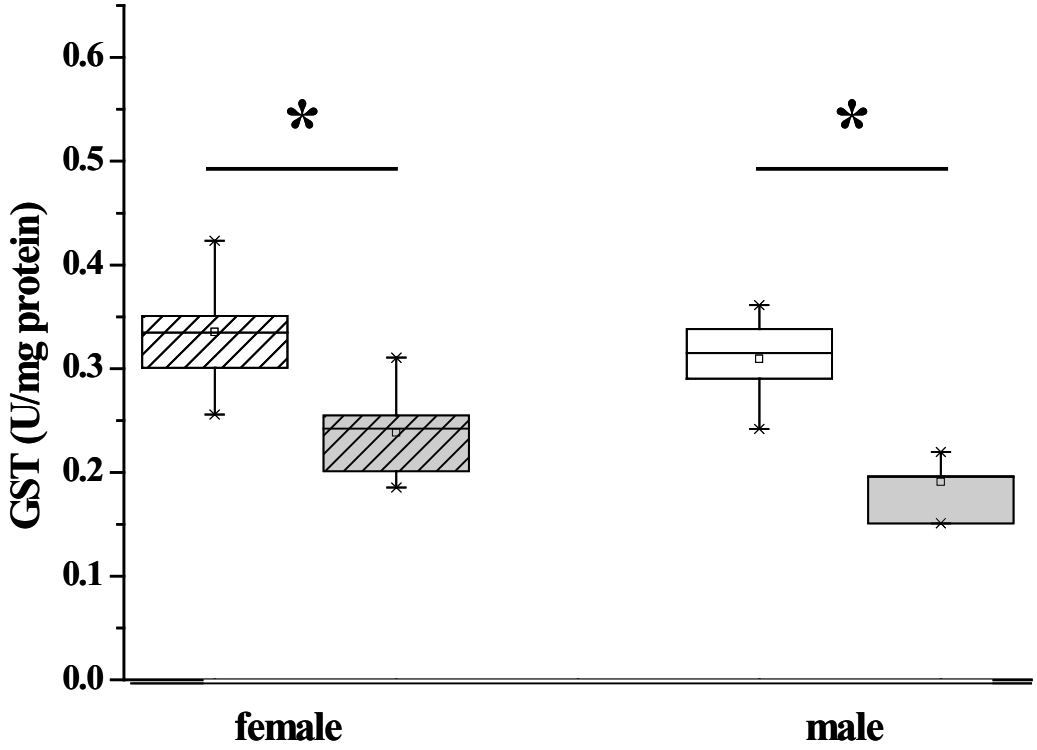

Figure 2.1: GST-activity (U/mg protein) of three- spined sticklebacks.
Differentiated by fish sex and infection status. (female = striped boxes, male = empty boxes; uninfected = white boxes and *S. solidus*-infected = grey boxes). Stars indicate significant differences between groups (* $p < 0.05$, ANOVA). Each box represents the interquartile range (box range: 25-75% percentile; whisker range: 1-99% of values) with the empty dot showing the mean and the horizontal line showing the median. The asterisks show the minimum and maximum values of the dataset. Numbers are listed in Table 2.1.

Hepatic GST-activities in roach are presented in Figure 2.2. The analysis of variance indicated no effect of factor site ($F = 3.253$; $p = 0.078$) or host sex ($F = 1.803$; $p = 0.186$). The infection

with *L. intestinalis* was the only factor indicating a significant difference in GST-activity of roach (F = 12.295; p d 0.001). The small but significant interaction between host sex and infection status (F = 4.764; p d 0.05) indicated 32% higher GST-activities for infected male roach in comparison to infected females. The differences between GST-activities within the same sex were different between the sites (F = 6.095; p d 0.05), whereas the dimension of difference between infected female or male, respectively wass the same for both sites (F = 0.477; p = 0.493). Because of the significant interaction between host sex, infection status and site (F = 16.434; p d 0.001) a Student's t-test was performed between each pair of uninfected and infected roach of each sex per site, indicating the significant reduction of GST-activity occurred at site MS only for males (25%) and at site LTS only for females (55%).

For infected females from MS, the CF was positively correlated with the GST-activity (r = 0.62). For roach from LTS the only correlation between GST-activity and fish size was found for infected females (r = -0.92). At site LTS, roach were not the only fish analyzed. Similar to LTS roach, the LTS chub showed a 35% reduction in GST-activity only for infected chub females. However, this difference was not significant (F = 1.549; p = 0.225) (Fig. 2). The GST-activity of infected chub females was significantly correlated with the GSI (r = 0.75). For male chub the GST-activity showed significant correlations with the total length ((r = 0.73 (uninfected); 0.82 (infected)).

In summary, while the results for *S. solidus* revealed a significant reduction of hepatic GST-activity in both sexes of infected sticklebacks, the impact of *L. intestinalis* was not the same for the analysed cyprinids. For female roach and chub at LTS, reduced GST-activities were found. Contrary, for MS roach a reduced GST-activity was only detected for infected males.

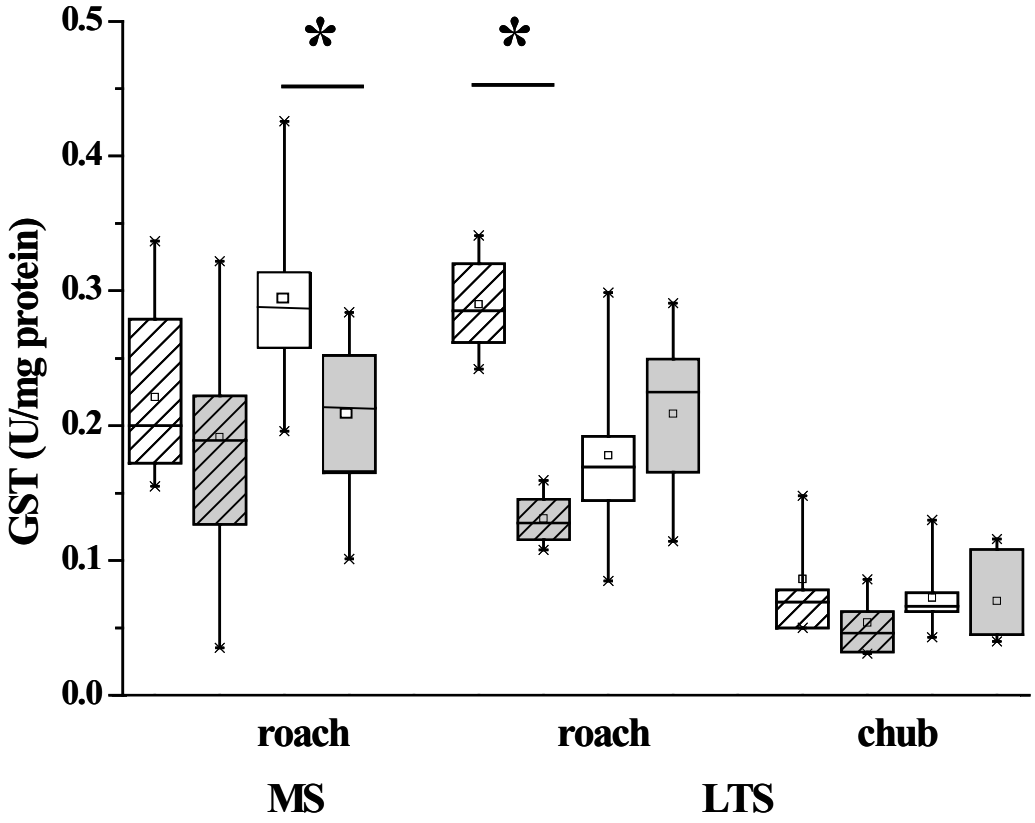

Figure 2.2: GST-activity (U/mg protein) of roach from LTS and MS.
Each time differentiated by sex (female = striped boxes, male = empty boxes) and infection (uninfected = white boxes and *L. intestinalis*-infected = grey boxes). Stars indicate significant differences between groups (* $p < 0.05$, t-test). Each box represents the interquartile range (box range: 25-75% percentile; whisker range: 1-99% of values) with the empty dot showing the mean and the horizontal line showing the median. The asterisks show the minimum and maximum values of the dataset. Numbers are listed in Table 2.1.

2.4 Discussion

The presented results confirm the assumption of parasites being a natural stressor affecting biomarker response in fish. In the present study hepatic glutathione-S-transferase (GST)-activity is reduced due to parasite infection in three different host-parasite systems. The experiment with laboratory bred and laboratory infected fish showed a significant reduction of GST-activity in sticklebacks maintained under controlled conditions. In addition, the comparison of similar host-parasite systems from two different sites and the comparison of two different host-parasite systems from the same site indicate the interference of cestode plerocercoids with host GST-activity as a general effect. The plerocercoids of *S. solidus* and

L. intestinalis are located in the body cavity of their fish hosts why, due to their large size, they are usually easy to detect. Therefore, infected fish could be disregarded in biomarker studies (Hecker and Karbe, 2005; Schabuss et al., 2005). However, even if the large plerocercoids are easily detected, there is a wealth of parasites living in nearly every organ of the host which will certainly also affect host's physiological homeostasis and thus might change biomarker levels (Sures, 2008a, b). Parasitological studies show that under natural conditions almost every fish is infected with at least one parasite species (Lamková et al., 2007; Nachev and Sures, 2009). With respect to cestodes, most adult species are dwelling in the intestine of fish. This organ is usually not checked for the presence of parasites when the expression of biomarkers e.g. in the liver is analysed. However, Dautremepuits et al. (2003) observed an increased GST-activity in carp parasitized by the intestinal cestode *Ptychobothrium* sp. when compared with uninfected fish. Because of the lacking contact between the parasite and the analysed organ it was suggested that the observed increase in different antioxidant defences (liver catalase-, activities of GST and glutathione reductase) in infected carp is due to the modulation of the metabolic activity by the parasite. A further possibility would be the also known parasitic release of molecules in order to suppress the host's oxidative response allowing a long-term survival of the parasite (Miura et al., 2001; Salzet et al., 2000). Dzik (2006) presents a broad range of enzymes and other molecules secreted by helminths subverting the host immune defence in order to maintain a long-term persistence within the host. Some examples are given for helminths secreting GST enzymes or presenting them on their body surface for a favoured parasite survival by neutralizing the toxins acting against them. For cestodes, GSTs are suggested as biochemical systems that protect the parasites against the host's immune attack (Brophy and Pritchard, 1992). Barber and Scharsack (2010) mentioned that *S. solidus* is apparently capable of manipulating (evading) immune traits that are specifically directed against parasite antigens. To what extent the possible secretion/presentation of parasite GST is linked with the host's GST response is not clear. As only the GST-activity was analysed, it is impossible to distinguish between a reduced enzyme synthesis and a reduced activity due to blocked binding sites. But reduced hepatic GST-activity is also reported in two more host-parasite systems. The infection with either *Dicrocoelium dendriticum* or *Fasciola hepatica* is known to reduce hepatic GST-activity in their respective final hosts (Galtier et al., 1983, 1986, 1987; Skálová et al., 2007). Both are parasites known to live many years in the bile ducts of their host's liver. Recently, it was shown that a sigma class glutathione transferase (rFhGST-si), a recombinant form of a

F. hepatica secreted molecule, modulates host immunity by suppressing responses associated with chronic inflammation (Dowling et al., 2010). This confirms the suggestion for the existence of more reasons for the observed effects of the present study than only incidental side effects like for example liver injury or nutritional drains. Even if for fasted rainbow trout (*Oncorhynchus mykiss*) a decreased GST-activity was described compared to the control (Gourley and Kennedy, 2009), the inhibition of roach gametogenesis by *L. intestinalis* is only attenuated but not abolished when receiving food supply *ad libitum* (Trubiroha et al., 2010a).

The driving force for biomarker research is their use to study pollution effects in wild animals. But all populations of aquatic organisms are usually confronted with parasites (Kuris et al., 2008). Van der Oost et al. (2003) reviewed more than 40 papers dealing with effects of various pollutants on GST-activity in different fish species under laboratory conditions and in the wild. They mostly reported conflicting results concerning the correlation between pollution and GST-activities in fish. According to the results on GST-activity in sticklebacks, it became obvious that significant differences between sticklebacks were not to observe before the consideration of the infection with *S. solidus*. This underlines the extent of parasite's impact for the use of biomarkers. Thus, for environmental studies it is not only difficult to find pristine reference conditions in order to describe the expression level of a biomarker at the organism's physiological homeostasis, but it is also important to evaluate the effects of parasites on the level of biomarkers in fish and to describe biomarker levels of infected unexposed fish. In summary, it is conceivable that even more parasites are interacting with different physiological parameters commonly used as biomarker for pollution.

Therefore it is hard to understand why exposure studies are usually done without considering parasites, although their effects on the physiological homeostasis of their hosts are repeatedly documented (Sures 2008a, b; Sures et al. 2006). The results of the present study accentuate the need for more integrative approaches in environmental pollution research, similarly raising the question how the two parasites manipulate their host's physiology.

2.5 Conclusions

Several studies reported problems when hepatic glutathione-S-transferase-activity is used as a biomarker for anthropogenic contamination in freshwater fish due to inconsistency of the results. In contrast to other biomarkers, the GST-activity often lacks a clear correlation with pollution gradients in field studies. The presented results show a significant impact of two

parasites on host's GST-activity. Sticklebacks experimentally infected with *S. solidus* show significantly reduced GST-activities in both genders. But also in field investigations on roach and chub, a reduction of GST-activity could be observed as a result from infection with the parasite *L. intestinalis*. Thinking in a more integrative way in ecotoxicological studies and considering possible effects parasites might have on the physiological homeostasis of their hosts is therefore to suggest. Neglecting parasites could lead to severe misinterpretations of a biomarker's response.

3 Biomarker 2: The metallothionein levels in *Rutilus rutilus* and *Gammarus fossarum* considering parasite infection

3.1 Introduction

The aquatic environment has been exposed to a variety of pollutants for several years now which has led to the development of evaluation tools for the assessment of pollutant impact on aquatic organisms. Particularly low concentrations of chemicals as well as complex pollutant mixtures are hardly to detect applying solely chemical analyses. 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 (Sures, 2008a, b). As biomarkers are applied under field conditions, their possible modulation by natural 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, 2008a, b). However, the knowledge about possible interactions between parasites and pollution is restricted (Sures, 2008a, b).

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., 2006; Benson et al., 1990; Canli et al., 1997; Pedersen et al., 1997). Accordingly, several studies reported increased MT-levels in metal-exposed roach (*Rutilus rutilus*) or gammarids compared to controls (Brown et al., 1987; Geffard et al., 2007, 2010; Bonwick et al., 1991; Paris-Palacios et al., 2000; Stuhlbacher and Maltby, 1992).

In aquatic organisms, the modulation of MT by chemicals is well reported whereas only few studies are published dealing with the impact of parasites on this biomarker. Recently, impacts 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 the European eel (*Anguilla anguilla*), infection with digenean parasites reduced MT response, whilst infection with *Anguillicola crassus* was associated with an induced MT response (Fazio et al., 2008).

Another parasite known for its severe effects on host's physiology is the cestode *Ligula intestinalis* which is characterised 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 so called plerocercoid is located in the body cavity of cyprinids. *L. intestinalis* inhibits gonad development of its fish host and thus, this parasite 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, 2010b).

The acanthocephalan *Polymorphus minutus* is a common parasite in gammarids (Bollache et al., 2001; Hynes, 1955; Ward, 1986) and is known to affect the ecology of its intermediate host population or 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).

The present study aims at investigating the interactions between parasitism and biomarker response of the respective host. As model organisms the cyprinid *R. rutilus* partly infected with the diphyllobothridean cestode *L. intestinalis* and the amphipod *Gammarus fossarum* partly infected with larvae of the acanthocephalan *P. minutus* were used. For both host-parasite systems the relative levels MT were analysed in host tissues after keeping the hosts under controlled laboratory conditions for a longer period of time. In order to assess combined effects of parasites and pollution, the amphipod-parasite-system was exposed under laboratory conditions to Cd at relatively low concentrations (4 µg/L). Hence, metal accumulation in gammarids and parasites was compared and levels of MT were analysed to identify possible synergistic or antagonistic effects of parasites and pollution.

3.2 Material and methods

3.2.1 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. 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 *Chaoberus* spec. larvae. After two years of maintenance, roach were sacrificed in November 2008. 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: the gonadosomatic index (GSI) as (fish gonad mass/fish somatic mass) x 100, the condition factor (CF) as fish somatic mass x 100/(fish total length)^3, the parasitisation index (PI) as (parasite mass/fish somatic mass) x 100. For GSI and CF fish somatic mass was determined without parasite mass.

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

Unparasitized and naturally infected *G. fossarum* harbouring cystacanths of *P. minutus* were collected by 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 hemocoel of *G. fossarum,* the infected gammarids were separated provisionally. Final information on the infection status was obtained after dissecting animals at the end of the exposure period. To avoid heat stress, handling and exposure of the crustaceans was 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 was 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 1000µg/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.

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 tank water was filtered (cellulose nitrate filter, pore size 0.45 µm, Sartorius AG, Göttingen, Germany), acidified with 10 µL HNO_3 (65% suprapure quality; Merck, Darmstadt, Germany) and stored at -21°C until metal analysis. The pH-value (pH 340, WTW) and conductivity (LF 33, WTW) was also determined before and after water replacement in each tank. After an exposure period of 14 days, crustaceans were collected from each group, dried on a paper towel, divided into aliquots with 40 individuals each, transferred in plastic tubes and killed by deep freezing with subsequent storage at -80°C until further preparation. After killing, the cystacanths were separated from the host tissue. In order to avoid metal contamination, all instruments used for sampling were cleaned with 0.1% EDTA-solution and bidestilled water.

Concentrations of Cd in water, gammarids and cystacanths were determined by electrothermal atomic absorption spectrometry (ET-AAS) as described in Sures et al. (1995). The water samples were measured without any pre-treatment in duplicate. For metal analyses, three aliquots of the pooled gammarid tissues (approximately 50 mg wet weight) or 3 mg (wet weight) of cystacanth tissue were digested with 1.3 mL nitric acid (65%, suprapure, Merck) and 2.5 mL hydrogenperoxid (30%, Aplichem) using a microwave digestion procedure described by Sures et al. (1995). In order to determine the analytical detection limits, blanks were prepared without insertion of sample material. After the digestion, the clear solutions were filled in volumetric flasks up to 5 mL with bidestilled water. Metal concentrations of the digested tissues as well as of the water samples were determined with ET-AAS using a Perkin-Elmer model 4100ZL spectrometer (Ueberlingen, Germany) equipped with a Zeeman effect background correction system. Calibration was performed by standard addition. Cd concentrations in each sample were calculated by fitting linear regression lines to the points by the spiked concentration values and the corresponding integrated peak areas. Correlation coefficients were always better than 0.99.

3.2.3 Metallothionein analysis

For determination of MT, fish liver samples (60 mg) were homogenised with a sonicator (Sonoplus HD 2070, Bandelin) in adequate volumes of phosphate buffer (50 mM KH_2PO_4, 1mM EDTA, pH = 7) and centrifuged at 10.000g for 10 min at 4°C. An amount of 200 µl of the supernatant was mixed on ice with 360 µl of a 0.25 M sucrose solution. An aliquot of 300 mg gammarid tissue (cystacanths were previously removed) was placed in an Eppendorf tube and mixed on ice with 900 µl 0.25 M sucrose solution using a homogenizer (VDI 12, VWR) followed by UltraTurrax (VW 2070, Bandelin Electronic). After adding the sucrose solution, both homogenates were centrifuged at 20,000 g for 20 min at 4°C (Eppendorf Centrifuge 5810 R, Eppendorf, Germany). From the supernatant (fish liver/gammarid) three aliquots of 125/180 µl were processed for MT analysis by means of the silver saturation method (Scheuhammer and Cherian, 1985) with slight modifications. Samples were incubated with 125/150 µl of a 20 mg/l silver solution for 20 min at room temperature in order to saturate the metal binding sites of MT. Excess silver ions were removed by adding 30 µl bovine red blood cell hemolysate followed by heat treatment in a water bath (100°C for at least 10 min). The heat treatment caused precipitation of silver-bound hemoglobin and other proteins, except for MT, which are heat-stable. The denaturized proteins were removed by centrifugation at 1,200 g for 10 min (Eppendorf Centrifuge 5810 R, Eppendorf, Germany). Addition of the hemolysate, heat treatment and centrifugation was performed three times for each sample. Finally, the supernatant was centrifuged at 16,000 g for 15 min. The silver concentration in the final supernatant, which is proportional to the amount of MT, was determined by electrothermal atomic absorption spectrometry (AAS) using a Perkin-Elmer model 4100ZL spectrometer equipped with a Zeeman Effect background correction system.

3.2.4 Data analyses and statistical treatment

Cd concentrations in crustacean and cystacanth tissue were determined as µg/g (wet weight). For the evaluation of the MT increase in the animal tissue, the extinction value (E) of the Ag-analysis was calculated as E/g (wet weight) and is given as relative MT level.

For statistical analyses of the differences between groups, the student-t-test was applied as normality was given for all data. Significance was accepted when P d 0.05.

3.3 Results

3.3.1 *Rutilus rutilus* infected with *Ligula intestinalis*

Total length, CF, GSI and PI are summarized in Table 3.1. Length of infected female and male roach was significantly higher compared to uninfected conspecifics. Furthermore, length of uninfected females was significantly higher compared to uninfected males. No difference in CF was detected between sexes or infection status. GSI was significantly lower in infected roach of both genders. The PI was similar for both sexes.

Table 3.1: Morphological parameters of host fish.
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 sex group the uninfected were compared to the infected ones. Comparing the different sexes, only the individuals with the same infection status were analysed.

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

CF: condition factor; GSI: gonadosomatic index; PI: parasitisation index; TL: total length.

3.3.2 Effect of *L. intestinalis* on hepatic levels of metallothionein in roach

The MT content of roach liver was not significantly different between uninfected and *L. intestinalis*-infected conspecifics. However, sex-specific differences in liver MT content were observed, with male fish showing 17% higher levels of metallothioneins than females (Figure 3.1).

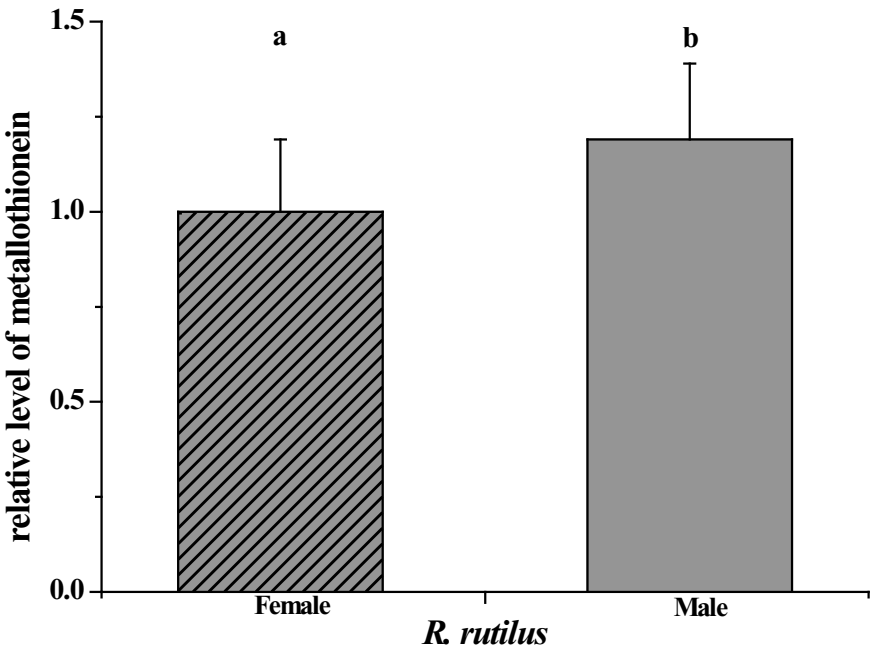

Figure 3.1: Relative level of metallothionein (mean ± SD).
In female (striped bar) and male (blank bar) *Rutilus rutilus* without determination of infection status with *L. intestinalis*. Different letters identify significant differences between groups ($p < 0.05$, t-test; n = 10 per group).

3.3.3 Gammarus fossarum infected with *Polymorphus minutus*

Mean infection intensity of *P. minutus* in *G. fossarum* was found to be one cystacanth per gammarid. Infection or Cd exposure had no influence on mortality (mortality was about 20%) (Table 3.2). Only infection combined with simultaneous Cd exposure resulted in a higher mortality (39%).

Table 3.2: Experimental design and physico-chemical characteristics of the tank water. Values for pH, water temperature and conductivity are means (± SD), for four observations made through time during the 14 days' exposure.

			G. fossarum		Water characteristics			
	Numbers (n)	Cd Exposure	*P. minutus*	Mortality rate (%)	pH	Temperature (°C)	Conductivity (µS/cm)	Cd Concentration (µg/L)
Group								
CoUninf	172	-[a]	-	22	8.2 (0.1)	14.7 (0.1)	1075 (89)	0.1 (0.1)
CoInf	149	-[a]	+	17	8.2 (0.1)	14.8 (0.1)	1078 (85)	0.2 (0.2)
CdUninf	149	4 µg/L	-	17	8.2 (0.1)	14.6 (0.2)	1076 (88)	3.3 (0.3)
CdInf	145	4 µg/L	+	39	8.2 (0.1)	14.6 (0.1)	1075 (89)	3.6 (0.5)

[a]No cadmium (Cd) added to tank water.

3.3.4 Cd concentration in water and animal tissues

The detection limit for cadmium (3xSD) in animal tissue was determined as 279 ng/g (wet weight), respectively.

Metal levels in water of the unexposed control groups were low (0.1 µg/l). In the metal exposure groups, Cd levels of approximately 3.5 µg/l were measured during the entire exposure period, which was still below the threshold value for tapwater in Germany (DVGW, 2011). Metal accumulation in gammarids and their cystacanths is summarized in Figure 3.2. Concentrations of Cd in gammarids and parasites from the unexposed groups were always below 1 µg/g. Following Cd exposure, gammarids as well as cystacanths showed significantly higher Cd concentrations with approximately 5 µg/g and 2 µg/g, respectively. The presence of cystacanths did not affect Cd concentrations in gammarids.

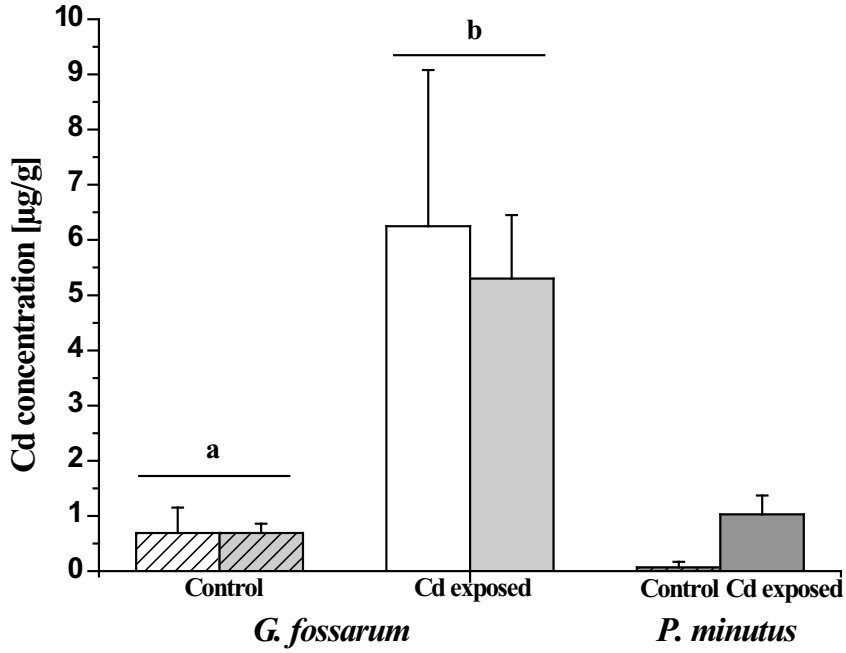

Figure 3.2: Mean concentration (mean ± SD) of cadmium [µg/g (wet weight)].
In uninfected (white bars) and with *P. minutus* infected (grey bars) *G. fossarum* and the parasite itself (dark grey bars); for control (striped bars) and after exposure to Cd (blank bars). Different letters identify significant differences between gammarid groups ($p < 0.05$, t-test; number of pooled samples: Control uninfected = 6; Control infected = 4; Cd uninfected = 10; Cd infected = 8; Control *P. minutus* = 2; Cd *P. minutus* = 2).

3.3.5 Metallothionein response in *G. fossarum*

The parasite infection did not affect metallothionein levels neither in exposed gammarids nor in controls. Relative metallothionein levels in crustacean tissue increased significantly (11%) for the exposed animals when compared with the controls (Figure 3.3).

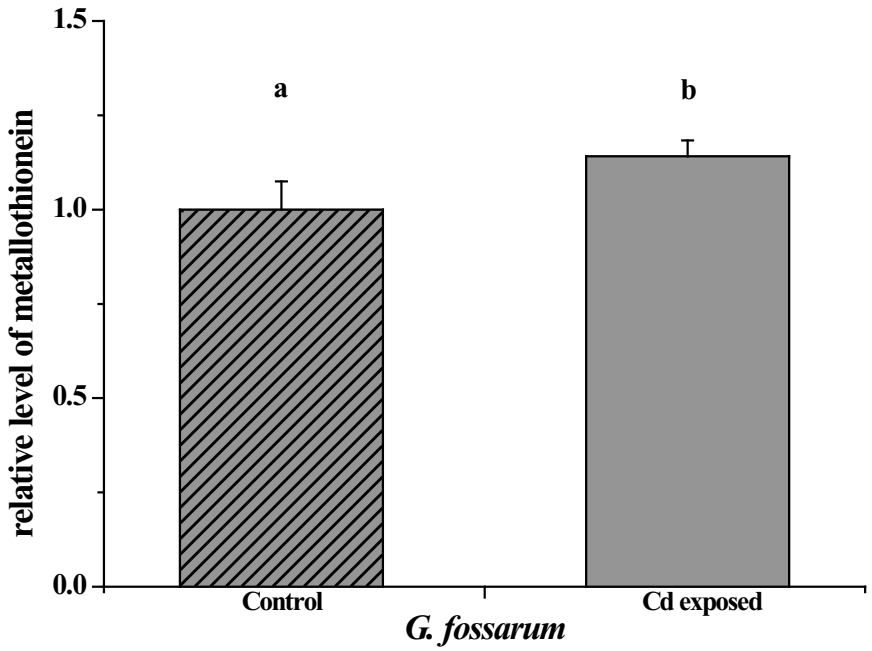

Figure 3.3: Relative level of metallothionein (mean ± SD).
In control (striped bar) and Cd- exposed (blank bar) *G. fossarum* without determination of infection status with *P. minutus*. Different letters identify significant differences between groups ($p < 0.05$, t-test; number of pooled samples = 6 per group).

3.4 Discussion

Results of the present study show no effect by parasitisation on MT response.

An impact of *L. intestinalis* on hepatic MT levels in roach was not to observe, but interestingly, sex-specific differences were found with higher MT levels in male roach. On the contrary, in chub (*S. cephalus*) or brown trout, no differences between male and female fish could be discovered for MTs (Dragun et al., 2009; Linde et al., 1999). One reason for the sex specific differences in MT levels could be an interaction between MTs and sex specific hormones such as estrogens or androgens. However, this seems unlikely since *L. intestinalis* causes endocrine disruption and infected roach showed significantly reduced GSI and lower sex steroid levels compared to uninfected conspecifics of the same sex (Trubiroha et al., 2010a, b). While endogenous MT inducers like cytokines and glucocorticoids are known in mammals (Coyle et al., 2002), there is no clear pattern of hormonal MT control in teleosts. As fish were kept together in the same tank and under the same conditions, a modulation of MT levels by external factors can also be excluded. To recollect the fact that MTs are metal

binding proteins, sex specific differences in metal homeostasis could be a further explanation for the observed effect. In roach muscle and intestine tissue, no gender-related differences were observed for the metals Co, Cu, Fe, Mn, Ni and Zn (Chapter 1). Unfortunately, gender-related differences in metal levels of fish have been rarely investigated and the data available so far provides no clear pattern (Burger, 2007). Thus, the reason for the demonstrated difference between liver MTs in roach of different sex remains elusive.

Similar to the results from roach, infection with acanthocephalan larvae had no effect on MT levels in *G. fossarum*. Metal exposure resulted in a clear accumulation of Cd in gammarids and their parasites. The accumulation of Cd in *G. fossarum* infected with *P. minutus* did not differ from uninfected gammarids as was also described earlier (Brown and Pascoe, 1989). In addition to its accumulation, Cd also elicit a clear stress response as measured by a significant increase in metallothionein levels which was as well as independent of infection as for the unexposed gammarids. Nevertheless, MT should not be generally concluded as a biomarker which is unaffected by parasites. In contrast to the present findings, there are nevertheless examples for parasite-induced changes in MT levels/induction in a vertebrate as well as in an invertebrate host (Baudrimont et al., 2006; Baudrimont and de Montaudouin, 2007; Fazio et al., 2008; Paul-Pont et al., 2009). Just as parasite infection could have a direct impact on metallothionein biosysnthesis, it should be mentioned that because of their high accumulation capacities, parasites can act as a metal sink (Sures and Siddal, 1999) and thus may alter MT levels via such indirect ways. Therefore, in terms of MTs as biomarker for ecotoxicological monitoring in the field it is to emphasize the need to consequently integrate parasitism.

3.5 Conclusions

In conclusion, the present chapter confirms that parasites have to be considered as natural stressors which are able of influencing biomarker responses of their hosts. Biomarkers such as MTs, which respond rather specific to metals, seem to be less affected by parasites.

4 Biomarker 3: Levels of heat shock protein (hsp70) in *R. rutilus* and *G. fossarum* considering parasite infection

4.1 Introduction

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). 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 gammarids (Schill et al., 2003; Sures and Radszuweit, 2007) and in fish (Köhler et al., 2001; Misra et al., 1989). In contrast to a wealth of reports on the modulation of HSP in aquatic organisms by chemicals, only few studies are published dealing with the impact of parasites on this biomarker. Recently, the evidence emerges that the most commonly used biomarkers are not only sensitive to anthropogenic pollutants but might also be induced by a variety of natural stressors (Sures, 2008a, b). Among parasitological research, parasites are considered to be one of the most prevalent and important stressors of free living animals (Marcogliese et al., 2005; Sures, 2008a, b).

In the European eel (*Anguilla anguilla*), a reduced HSP70 response following infection with digenean parasites was observed (Fazio et al., 2008). For *Gammarus roeseli* infected with *Polymorphus minutus,* no HSP70 response could be found after exposure of infected gammarids to heat or pollution (Sures and Radszuweit, 2007).

The present study aims at investigating the interactions between the acanthocephalan *P. minutus* or the cestode *Ligula intestinalis* and the HSP70 response of their respective host. These two parasites are already described in chapter 3.1. In order to assess only the effect of infection, both host-parasite systems were analysed after keeping them under controlled laboratory conditions for a longer time period. Additionally, an exposure experiment with low Cd-concentrations (4 µg/L) was conducted for the amphipod-parasite system. Therefore, in gammarids the identification of possible synergistic or antagonistic effects of parasite and pollution on their host's HSP70 response was possible.

4.2 Material and methods

4.2.1 Fish collection and tissue sampling

The same roach sampling was used as described in 3.2.1. Just as well as the biometric and parasitological parameters are the same.

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

The sampling of gammarids, the laboratory exposure experiment, and the sample processing and metal analyses are the same as described in 3.2.2.

4.2.3 Heat shock protein analysis

The detection of HSP70 was performed in accordance with Schill et al. (2003) and Sures and Radszuweit (2007). For protein quantification, fish liver (around 50 mg) was placed in Eppendorf tubes, balanced to the nearest mg (Libror 220, AEG) and mixed on ice with 200 µL of a buffered extraction solution (80 mM potassium acetate, 5 mM magnesium acetate, 20mM HEPES; ROTH, Germany, at pH 7.5; 2% protease inhibitor cocktail, Sigma). The same procedure was applied to individuals of frozen gammarids (cystacanths were removed; besides this, uninfected and infected gammarids were treated similarly) using 50 µL of the buffered extraction solution. The mixture (fish liver as well as gammarid) was homogenized using a micropestle (Eppendorf) and centrifuged at 20,000 g for 10 min at 4°C. The total protein concentration was determined in each supernatant using the Pierce BCA Protein Assay Kit (Pierce Biotechnology, Rockford Illinois, USA). Constant protein weights (20 µg for fish liver, 40 µg for gammarids) of the supernatant were separated by SDS-PAGE (12% acrylamide-bisacrylamide) in two steps (15 min at 80 V and 120 min at 120 V) in a Mini protean cell 3 (Bio-Rad) and transferred to a nitrocellulose filter for 120 min with 90 mA per gel. Each sample was processed two times and a protein marker was used to visualize protein weight (Bio-Rad Perfect Stain, Bio-Rad, Munich, Germany). After the transfer of the proteins, the filter was blocked for 90 min with 50% horse serum (Sigma,) in Tris-buffered saline (Sigma) and subsequently incubated with a primary antibody against HSC/HSP70 (for fish liver: mouse anti-HSP70/Hsc70 monoclonal antibody, SPA-820, Stressgen, Germany; for gammarids: mouse anti-HSP70 monoclonal antibody, H5147,

Sigma-Aldrich, Siegen, Germany) at room temperature overnight. The filter was washed 5 min with TBS and then incubated for 90 min with a second antibody (peroxidase-conjugated goat anti-mouse IgG; P0447, Dako A/S, Denmark) at room temperature. After washing in TBS for 5 min, the antibody cross reaction was visualized by 4-chloro(1)naphtol. The grey value intensity of the HSP70 bands was quantified by a densitometric image analysis (imageJ).

4.2.4 Data analyses and statistical treatment

For evaluating the HSP70 induction, it was necessary to normalize the data of each gel to a constant reference. Therefore, an HSP70-positive sample has been run as an internal standard in all gels. In order to calculate the relative HSP70 in the tissue samples, the following equation was used: $rel.c_{sample} = (d_{sample}/d_{std,\ gel})x100(\%)$ where $rel.c_{sample}$ is the relative HSP70 concentration in the tissue sample related to the HSP70 positive control sample; $d_{std,\ gel}$ is the mean density of the double analysis of the HSP70 internal standard on the gel; and d_{sample} is the mean density of the double analysis of the sample.

For statistical analyses of the differences between groups, the student-t-test was applied as normality was given for all data. Significance was accepted when P d 0.05.

4.3 Results

4.3.1 *Rutilus rutilus* infected with *Ligula intestinalis*

Total length, CF, GSI and PI of investigated roach are summarized and described in 3.3.1.

4.3.2 Effect of *L. intestinalis* on hepatic levels of HSP70 in roach

In uninfected fish, HSP70 levels were significantly 30% lower in males than in females (Figure 4.1). Furthermore, in both sexes the infection with *L. intestinalis* led to a significant reduction of HSP70 levels (48% in females, 43% in males compared to uninfected conspecifics).

4.3.3 Gammarus fossarum infected with *Polymorphus minutus*

Data of the investigated gammarids and the exposure experiment is summarized and described in 3.3.3 and 3.3.4.

4.3.4 HSP70- response in *G. fossarum*

Heat shock proteins were detected in all groups (Figure 4.2). Infection as well as Cd exposure resulted in significantly increased HSP70 levels in *G. fossarum* compared to the uninfected, unexposed controls (33 fold and 100 fold, respectively). Combination of both stressors led to a significant decrease of HSP70 levels compared to the gammarids which were exposed to Cd only, and the level were comparable to those detected in infected *G. fossarum*. However, the HSP70 levels were still higher than those of the control group (17 times higher).

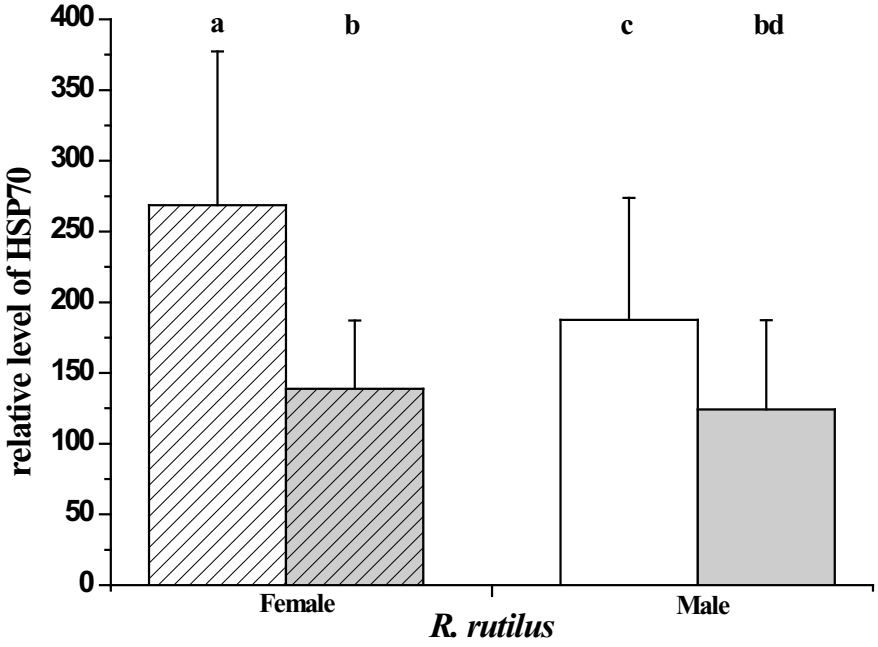

Figure 4.1: Relative level of HSP70 (mean ± SD).
In uninfected (white box) and with *L. intestinalis* infected (grey box) *R. rutilus* after determination in female (striped) and male (blank) hosts. Different letters identify significant differences between groups whereas within each sex group the uninfected were compared to the infected ones. Comparing the different sexes, only the individuals with the same infection status were analysed ($p < 0.05$, t-test; numbers are listed in Table 3.1).

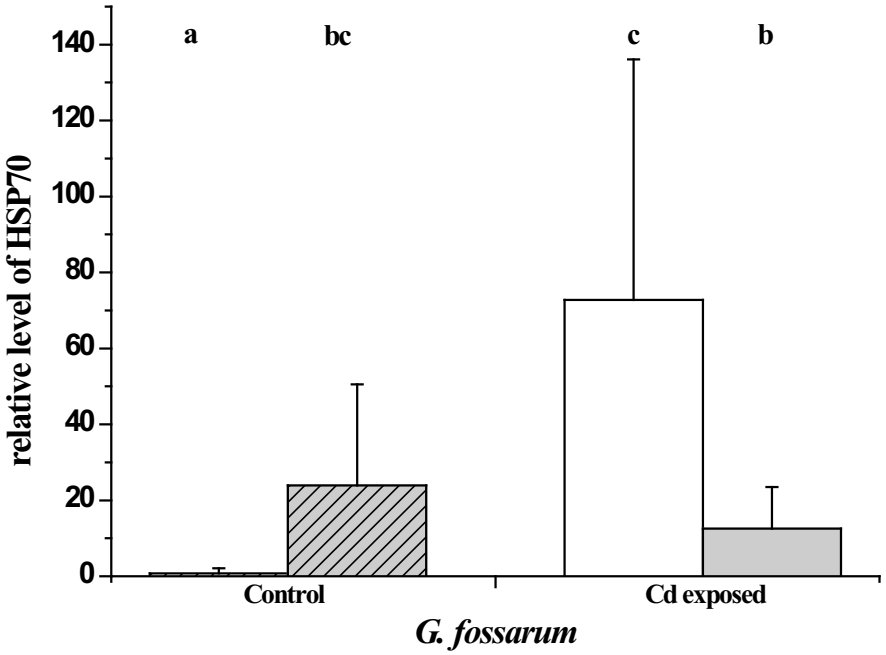

Figure 4.2: Relative level of HSP70 (mean ± SD).
In uninfected (white box) and with *P. minutus* infected (grey box) *G. fossarum* after determination in control (striped) and Cd- exposed (blank) hosts. Different letters identify significant differences between groups ($p < 0.05$, t-test; number of individuals: Control uninfected = 7; Control infected = 11; Cd uninfected = 5; Cd infected = 14).

4.4 Discussion

Parasites play an important role in aquatic ecosystems (Kuris et al. 2008). Therefore, they have to be considered as natural stressors when effects of anthropogenic pollution are monitored under field conditions. Parasites interfere with the physiology of exposed animals and may therefore modify their protection mechanisms (Sures, 2008a, b). Results of the present study show a significant influence of different parasites on heat shock levels of their respective hosts. With respect to the combination of parasite and pollution, a reduced HSP70 response was obvious compared to gammarids which were solely exposed to the metal.

Metal exposure resulted in a clear accumulation of Cd in gammarids and their parasites. The accumulation of Cd in *G. fossarum* infected with *P. minutus* did not differ from uninfected gammarids as was also described earlier (Brown and Pascoe, 1989). In addition to its accumulation, Cd also elicit a clear stress response as measured by a significant increase in

metallothionein levels which was as well as independent of infection as for the unexposed gammarids.

HSP70 levels in roach and *G. fossarum* were significantly affected by the presence of *L. intestinalis* and *P. minutus,* respectively. The observed sex-specific difference between HSP70 levels in uninfected roach is in accordance with findings of Afonso et al. (2003), who demonstrated higher HSP70 levels in female than in male chinook salmon (*Oncorhynchus tshawytscha*). Sex-specific differences might appear as a consequence of different hormonal regulations in males and females. However, effects of sex steroid hormones are rarely described (Afonso et al., 2003) although the influence of steroid hormones such as cortisol on the HSP70 response in fishes was repeatedly demonstrated (Basu et al., 2001, 2003). Infection with *L. intestinalis* led to reduced HSP70 levels in both sexes of roach. Fazio et al. (2008) also reported a reduced hepatic HSP70 gene expression in eels infected with intestinal digenean parasites. As parasites are normally considered as stressors to their host, an induced stress response measured by induced levels of HSP70 was to be expected (Basu et al., 2002). To discuss reduced levels of HSP70 there are two major considerable possibilities. On the one hand, parasites are known to interfere with the host's immune system and a reduced stress response could be related to altered immune function. In rainbow trout (*Onchorynchus mykiss*), an interaction between HSP70 and the immune system was reported, where HSP70 has been observed in the glucocorticoid receptor complex (Basu et al., 2003). Recently, the differential expression profiles of heat shock proteins and glucocorticoid receptors appear of physiological importance to mediate a balanced immune system after pathogen exposure in carp (*Cyprinus carpio*) (Stolte et al., 2009). Furthermore, it is also known that a broad range of enzymes and other molecules secreted by helminths can subvert the host immune defense in order to realize a long-term persistence within the host (Dzik, 2006). Parasites are even known to produce and alter hormone concentrations in their host to exploit immunological differences which exist between sexes (Klein, 2004). As the influence of *L. intestinalis* on hosts' endocrine system is most striking with regard to an inhibition of gonad development, previous research was concentrated on the parasite's effect on the reproductive system. However, the mode of action underlying reproductive dysfunction in roach remains elusive and combined effects between involving both, the endocrine and the immune system, are also possible. On the other hand, the parasite's metabolism taxes the host's nutritional status and therefore its energy reserves (Bush et al., 2002). Concerning *Oniscus asellus* (Isopoda), Eckwert and Köhler (1997) already suggested an energy deficit to

lower the HSP70 level as a result of a general metabolic decline. However, the roach investigated in the present study were apparently in a good condition as indicated by CF and the deposition of perivisceral fat. Therefore, it is to suspect that there are other factors responsible for the detectable effects of *L. intestinalis* on hepatic HSP70 in roach than just incidental side-effects of parasitisation such as energy drains. Nevertheless, further investigations are necessary in order to prove possible specific interactions.

Similar to the already discussed fish-parasite system, the amphipod-parasite system also demonstrates the strong impact parasites can have on host's biomarker response. Particularly joint effects of parasite and pollution become obvious.

Even when an impact on gammarid's HSP70 response was found by parasitisation, in contrast to the results on roach, the solely infection with *P. minutus* led to increased HSP70 levels in *G. fossarum* compared to uninfected conspecifics. However, exposure of infected gammarids to Cd, resulted in significantly lower HSP70 levels than in uninfected Cd exposed animals. Despite the infected gammarids did not seem to accumulate more Cd than the uninfected ones, a difference in the mortality rate of these two groups was obvious. Similar to *G. pulex* infected with *Pomphorhynchus laevis* (Brown and Pascoe, 1989), *G. fossarum* infected with *P. minutus* showed a 20% higher mortality when additionally exposed to cadmium. The decreased HSP70 response might propably result from stressors that become overly severe. This is known if organisms face a combination of stressors as reported for *G. fossarum* exposed to different Cd concentrations (Schill et al., 2003) or for fish (*Salmo trutta* f. *fario*, *Barbatula barbatula*) exposed to mixtures of environmental pollutants (Köhler et al., 2001). Interpreting the present data with HSC/HSP70 induction kinetics (Köhler et al., 2001) it is to assume that the solely infection with *P. minutus* belongs to mild stress but adding another stress factor led to a sharp decrease of HSP70 levels reflecting a stress response system which is overhelmed. Therefore, the contrary findings of Sures and Radszuweit (2007), who described a total suppression of HSP70 in *G. roeseli* infected with *P. minutus*, irrespectively of metal exposure are maybe indicating cystacanths of *P. minutus* as stronger stressor for *G. roeseli* than for *G. fossarum*. Alternatively, the higher mortality of infected and metal exposed *G. fossarum* might be a reflection of the impaired stress response, i.e. HSP70 induction, which in turn could have resulted in higher Cd toxicity compared to uninfected conspecifics.

4.5 Conclusions

In conclusion, the biomarker HSP70 which responds to a broad range of environmental, physical and biological stressors, confirmed that parasites have to be considered as natural stressors which are able of influencing biomarker responses of their hosts. As long as parasites are disregarded in field studies, a high variation is the most harmless effect on biomarker results. Both, false positive (HSP70 induction in infected *G. fossarum*) as well as false negative results (HSP70 induction in infected *G. fossarum* + Cd; HSP70 in infected roach) are possible as shown here for two different host-parasite systems.

Summary, conclusions and future prospects

In the field of aquatic ecotoxicology, it is a common method to use biomarkers for detecting anthropogenic stressors in the waterbodies. The current thesis aimed at pointing out that parasites as natural stressors are also capable of changing their host's biomarker responses. The main focus was concentrated on the host-parasite system *Rutilus rutilus* – *Ligula intestinalis*. This fish-cestode system serves as a useful scientific model since the last 60 years. First of all, the parasite was analysed for its own accumulation capacities and it was tested, if a competition for minerals exists between the parasite and its host (Chapter 1). In total, three biomarkers for effect indication were investigated. The analyses were conducted with different samplings and for each biomarker additional host-parasite systems were analysed either to get the chance of experimental conditions or to increase the knowledge to an invertebrate host. Thus, on the one hand the enzymatic activity of hepatic glutathione-S-transferase was tested with laboratory bred three-spined sticklebacks (*Gasterosteus aculeatus*) which were experimentally infected with the cestode *Schistocephalus solidus*. On the other hand the roach-*L. intestinalis* system was investigated during field samplings from two different sites whereas at one site the results could be compared with a second intermediate host, the chub (*Squalius cephalus*) (Chapter 2). For the further biomarkers, the roach – *L. intestinalis* system was manipulated in this respect, as they were kept for two years under controlled conditions after sampling from the field. This handling provided same conditions for all fish. Furthermore, as second host-parasite system the amphipod *Gammarus fossarum* infected with the acanthocephalan larvae *Polymorphus minutus* was chosen. They were studied after two weeks of maintenance whereas additionally a Cd exposure was conducted to detect possible joint effects of parasite and pollution. Compared to the metal specific biomarker, metallothioneins (Chapter 3), the more general biomarker, heat shock protein 70 (Chapter4) was investigated in both host-parasite systems.

Summary of the most important results:

Chapter 1: Competition for minerals between *Ligula intestinalis* and its intermediate fish host roach (*Rutilus rutilus*)

- The toxic metals As, Cd and Pb were neither detected in the parasite nor in its host.
- *L. intestinalis* showed higher levels for the elements Co, Cu, Fe, Mn, Ni and Zn compared to fish host's muscle tissue (BCF ranging from 2 to 27). Compared to fish host's intestine

tissue, the parasite only showed higher levels for Co, Mn and Ni (BCF ranging from 5 to 43).

- No differences concerning fish sex or infection status were detected in host's metal concentrations. Only Ni from intestine tissue was lowered in infected fish.
- The general order of metal concentration in fish tissues as well as in the parasite was: Zn>Fe>Cu>Mn>Ni>Co.
- Co was the element with the highest BCF compared to fish muscle and revealed to be an essential element for the parasite competing with its host.
- In parasite tissue, an intra-element competition between copper and zinc was indicated. Comparisons with other studies confirmed the assumption of an existing saturation level for Zn at around 50 mg/kg (wet weight).

Chapter 2: The glutathione-S-transferase-activity in three different fish species considering the infection with diphyllobothridean cestodes

- The cestode parasites *S. solidus* and *L. intestinalis* have reducing impact on their fish host's GST-activity.
- Under laboratory conditions, *S. solidus* reduced GST-activity in both sex of *G. aculeatus.*
- The comparison of roach from two different sites only revealed reduced GST-activities for either males or females when infected with *L. intestinalis*.
- The additional intermediate host, the chub, tends also towards reduced GST-activity when infected with *L. intestinalis*. This occurred in the same sex as for roach from the same site.

Chapter 3: The metallothionein levels in *Rutilus rutilus* and *Gammarus fossarum* considering parasite infection

- The infection with *L. intestinalis* had no effect on hepatic metallothionein levels of roach.
- Male roach had higher levels of MT than females.
- The infection with *P. minutus* had no effect on MT levels of *G. fossarum*.

- The Cd exposure resulted in increased MT levels in *G. fossarum* independent of infection.

Chapter 4: The levels of HSP70 in *Rutilus rutilus* and *Gammarus fossarum* considering parasite infection

- The infection with *L. intestinalis* reduced levels of hepatic HSP70 in both genders of roach.
- Uninfected female roach had higher levels of HSP70 than uninfected males.
- The infection with *P. minutus* increased levels of HSP70 in *G. fossarum.*
- The Cd exposure increased levels of HSP70 in *G. fossarum.*
- When *G. fossarum* was stressed by Cd exposure and parasite together, the levels of HSP70 were reduced compared to the solely Cd exposure.

Conclusions and future prospects

The results of the different studies confirmed the assumption that there exist more parasite species that exert influence on biomarkers for aquatic pollution. The knowledge about the biasing of biomarker responses by the parasite *L. intestinalis* was expanded by its reducing influence on GST-activity and HSP70. In case of metal accumulation and metal specific biomarker response (metallothionein), *L. intestinalis* did not provoke mentionable changes.

Even if the two studied parasites (*L. intestinalis* and *P. minutus*) did not change metal accumulation or levels of metallothionein of their respective host, it is not to conclude, that parasites are not to consider when studying these aspects. It is rather to suggest that more research is needed focusing on fish infected with adult acanthocephalans which are known to accumulate toxic metals to a high degree and which are even known to reduce their host's metal burden (Sures, 2003; Nachev et al., 2010). When parasites act as a metal sink in their host it is to assume that consequently host's metal response mechanisms like metallothioneins may also be changed.

All of the analysed metal concentrations in *L. intestinalis* exceeded those of fish host's muscle but the only indication of a competition between the two organisms was given for cobalt. As cestodes are known for their preference for cyanocobalamin (vitamin B12) (Chowdhury and Singh, 1995; Nyberg, 1958) it would be interesting to see if the detected mineral competition is linked to a competition for vitamin B12. The association between the decreasing Co-

concentration in fish muscle and the parasite number demands further studies as it indicates a regulated uptake for Co by this cestode. A regulated uptake was definitely demonstrated for copper and zinc. They are known antagonists but it remains to study why the parasite tissue never exceeded a Zn concentration of about 50 mg/kg (wet weight) contrary to apparent unlimited raising Cu concentrations.

Due to its affiliation to phase II metabolism of xenobiotics, the GST-activity is often used as biomarker in studies for organic pollution (Van der Oost et al., 2003). Platyhelminthic parasites seem to have enormous influence on host's hepatic GST-activity independently of the parasite's habitat. The current thesis added two more parasite species to the already known intestinal cestode and the two liver flatworms. Most work was focused on *Fasciola hepatica* so far, why for that parasite the secretion of a molecule modulating host immunity by suppressing responses associated with chronic inflammation was proofed (Dowling et al., 2010). This should encourage future research to resume the search for molecules released by *L. intestinalis*. The reason for the reduction of GST-activity by *L. intestinalis* and *S. solidus* remains unclear whereas it is to suggest that there are more reasons than incidental side effects just like energy drains or liver injuries. The same is given for the observed modifications of HSP70 response. HSP70 is known to increase when organisms are stressed by various reasons just as it was observed for *G. fossarum* stressed by either infection or metal exposure. Both stressors together reduced the HSP70 response what is demonstrative for the case when the stress response is overwhelmed. If this is also the reason for the reduced HSP70 response in case of roach infected with *L. intestinalis* is not clear. But these results coincide with other studies which all observed a reduced or even deleted HSP70 response in case of parasite infection. For the platyhelminthic parasite *Schistosoma mansoni*, it was demonstrated, that parasitic excretory/secretory products reduce HSP70 protein levels in their host's defence cells (Zahoor et al., 2010). Therefore it is again to suggest that *L. intestinalis* also owns mechanisms to ensure its longevity in its fish intermediate host.

All together, for future research the presented results matter in two different modes:

1) The use of biomarkers in pollution monitoring:

The affiliation of parasites to natural ecosystems is not to deny. To obtain an integrated view about the health status of an aquatic environment, it is not enough to analyse an organism's stress response without taking into account a ubiquitous stressor, parasitism. Parasites can alter trophic activities as the respective parasite burden can either cause higher trophic costs

or even change the selection of food. These changes will be particularly important for contaminants which become accumulated by oral uptake and digestion, for example organic compounds like PCB. Thus, the consideration of parasites could at least lead to smaller variances between groups from different sites.

Therefore, a more integrative research is needed, regarding parasites as:

- natural stressors with influence on the same physiological endpoints as anthropogenic pollution.
- organisms which also react on anthropogenic pollution, why they can also be used for indication of contaminants particularly by accumulation indication.
- organisms which alter their host's behavior and may influence the organism's contaminant exposure.

2) Investigation of host-parasite interactions:

Interactions between parasites and their hosts belong to the oldest strategy of life "Live and let live". They significantly pushed on the development of their host's immune system. The continual existence of parasites implies developing strategies of survival within the host by the parasite. The observed effects on host's stress response mechanisms may all be linked to parasite's interaction with its host's immune system. Therefore, future research concerning the parasite *L. intestinalis* should add the question into focus how the parasite ensures its longevity in its intermediate fish host.

Zusammenfassung

Der Einfluss von Parasiten auf Biomarker aquatischer Organismen

Hintergrund

Alles Leben benötigt Wasser. Daher ist es eines der kostbarsten Güter dieser Erde. Im Laufe der letzten Jahrzehnte musste man jedoch erkennen, dass durch die Aktivitäten des Menschen verschiedenste Stoffe in das Ökosystem eingeführt werden und letztendlich auch im Wasser zu finden sind. Aquatische Organismen sind daher Stoffen ausgesetzt, die sie natürlicherweise gar nicht kennen oder an deren Quantität sie nicht angepasst sind.

Auf Grund der Vielzahl möglicher Schadstoffe und ihrem häufig zeitlich begrenzten Auftreten im Gewässer kann die analytische Chemie nur einen Teil zur Aussage über den Belastungzustand eines Gewässers und seiner Biota beitragen. Die interdisziplinäre Wissenschaft der Ökotoxikologie befasst sich daher mit den Interaktionen zwischen Schadstoffen und Organismen.

Die **Akkumulationsindikation** beruht auf der Tatsache, dass organische sowie anorganische Schadstoffe von Organismen angereichert werden können (Streit, 1998). Die Anreicherung mancher Schadstoffe führt sogar zu Vorteilen in deren analytischen Nachweisverfahren (Beeby, 2001).

Im Zuge der **Reaktionsindikation** kommen die sogenannten Biomarker zum Einsatz, welche verschiedenste biologische Antworten auf anthropogene Umweltschadstoffe reflektieren sollen (Bucheli and Fent, 1995). Biomarker können auf jeder Stufe der biologischen Organisation definiert werden. Sie werden als sensitive Indikatoren verstanden, die darauf hinweisen, dass Schadstoffe vom Organismus aufgenommen wurden und einen toxischen Effekt hervorrufen (van der Oost et al., 2003). Einen bedeutenden Anteil machen dabei veränderte biochemische und physiologische Faktoren bei aquatischen Organismen aus, die als Effekt- oder Expositionsmarker für Schadstoffe Verwendung finden.

Wenn nun anthropogene Schadstoffe als Stressoren von aquatischen Systemen und deren Organismen angesehen werden, dann sollte für die Auswertung von Biomarkern berücksichtigt werden, dass natürliche Stressoren ebenso Reaktionen im untersuchten

Organismus hervorrufen können. Hierzu zählen Parasiten, die ein natürlicher Bestandteil des aquatischen Ökosystems sind (Bush et al., 2002).

Das Potenzial von Fischparasiten, als Indikatoren für die Beurteilung der Wasserqualität zu dienen wurde schon vor einigen Jahren entdeckt und intensiv beforscht (Bush et al., 1990; Kennedy, 1976, 1997; Lafferty, 1997; Lafferty and Kuris, 1999; MacKenzie et al., 1995; Overstreet, 1997; Sures, 2001; Valtonen et al., 1997). Derzeitig wird das Wissen darüber, dass Verschmutzung parasitische Lebensgemeinschaften dezimieren oder auch begünstigen kann genutzt, indem bestimmte Parasitenarten oder Parasitengemeinschaften als Bioindikator für Umweltverschmutzung herangezogen werden (z.B.: Nachev und Sures, 2009; Palm and Rückert, 2009; Vidal-Martinez et al., 2010). Ebenso ist die Eignung von einigen Fischparasiten als Akkumulationsindikatoren bekannt, wobei sie sogar die Akkumulationseigenschaften von frei lebenden Organismen übertreffen können (Nachev et al., 2010; Sures et al., 1999; Sures, 2001). Welche Auswirkungen eine Parasitierung in Kombination mit Schadstoffbelastung auf die Wirtsphysiologie und damit auf entsprechende Biomarker hat, ist jedoch noch nicht ausreichend erforscht. Immer mehr Untersuchungen deuten jedoch auf erhebliche Effekte von Parasiten auf Biomarker hin (Sures, 2006, 2008a, b).

Ein Biomarker im Schadstoffmonitoring ist die Glutathion-S-Transferase (GST). GST ist ein Enzym der Phase II des Fremdstoffwechsels und unterstützt die Konjugation von elektrophilen Substanzen an das Tripeptid Glutathion. Die Analyse der GST-Aktivität im Schadstoffmonitoring ist jedoch umstritten, da sich in vielen Freilandstudien keine eindeutigen Unterschiede zwischen Gewässern verschiedener Belastungsgrade finden ließen (Krca et al., 2007; Van der Oost et al., 2003; Vigano et al., 1998). Von einigen Parasiten ist jeoch bekannt, dass sie die GST-Aktivität ihrer Wirte beeinflussen. So weiß man von den Trematoden *Dicrocoelium dendriticum* und *Fasciola hepatica*, dass sie die GST-Aktivität in der Leber ihrer Wirte reduzieren (Galtier et al., 1983, 1986, 1987, 1991; Skálová et al., 2007). Für Karpfen welche mit dem Bandwurm *Ptychobothrium* sp. infiziert waren, konnte wiederum eine erhöhte GST-Aktivität im Vergleich zu uninfizierten Fischen festgestellt werden (Dautremepuits et al., 2002, 2003).

Das Protein Metallothionein bindet Metalle und wird demnach als spezifischer Biomarker für Metallbelastungen benutzt. Doch auch für diesen Biomarker gibt es Studien, die belegen, dass die Metallothioneingehalte durch Parasiteninfektionen beeinflusst werden können

(Baudrimont et al., 2006; Baudrimont and de Montaudouin, 2007; Fazio et al., 2008; Paul-Pont et al., 2009).

Das Hitzeschockprotein 70 (HSP70) ist hingegen ein eher unspezifischer Indikator für auftretende Proteinschäden, unabhängig von den möglichen Ursachen. Die bisher bekannten Auswirkungen von Parasiten auf die HSP70 Gehalte ihrer Wirte reicht von einer Reduktion des HSP70 (Fazio et al., 2008) bis zur völligen Abwesenheit (Sures und Radszuweit, 2007). Insgesamt geben die Ergebnisse für die soeben beschriebenen drei Biomarker Anlass zu weiteren Untersuchungen, um zu klären, inwieweit in anderen Parasit-Wirt Systemen ebenfalls Einfluss auf diese Biomarker genommen wird.

Freilebende Organismen haben verschiedene Möglichkeiten sich vor Parasiten zu schützen. Das Immunsystem kann zum einen die Infektion stoppen bevor sie Schaden anrichtet oder zum anderen das Wachstum des Parasiten soweit wie möglich reduzieren. Solche komplexen Aufgaben des Immunsystems können dann wiederum durch erhöhte Energiekosten den Lebenszyklus des Wirtes beeinflussen. Dazu gehört beispielsweise ein verändertes Ernährungs- oder auch Fortpflanzungsverhalten (Thomas et al., 2009). Ein Parasit, der die Lebensweise seines Wirtes deutlich beeinflusst, ist der Bandwurm *Ligula intestinalis*. Dieser Parasit benötigt für seinen Lebenszyklus drei Wirte (Dubinina, 1980). Im Darm fischfressender Vögel, den Endwirten von *L. intestinalis*, kommt es zur Geschlechtsreife des Parasiten, so dass unsporulierte Eier mit dem Kot des Wirtes in die Umwelt gelangen können. Im Wasser entwickelt sich noch in den Eiern das erste Larvenstadium, das Coracidium, welches nach dem Schlüpfen vom ersten Zwischenwirt, wenige Copepoden-Arten, oral aufgenommen werden muss. In der Leibeshöhle des Copepoden entwickelt sich der Parasit zur Procercoidlarve weiter. Diese ist für eine Vielzahl von Fischarten infektiös. Nach oraler Aufnahme eines infezierten Copepoden durch einen Fisch wandert die Larve in die Leibeshöhle dieses zweiten Zwischenwirtes und entwickelt sich dort zur Plerocercoidlarve. Dieses Stadium zeichnet sich durch ein enormes Wachstum aus und führt zu erheblichen Veränderungen in der Reproduktionsphysiologie sowie den Verhaltensmechanismen des Wirtes (Arme, 1968; Hecker and Karbe, 2005; Hecker et al., 2007; Loot et al., 2001; Trubiroha et al., 2009, 2010a, b; Schabuss et al., 2005).

Im Fokus der Ökotoxikologie sind unter anderem Substanzen, die eine hormonähnliche Wirkung auf Organismen haben und damit deren Hormonsystem aus der Balance bringen. Man bezeichnet solche Stoffe insgesamt als Endokrine Disruptoren (ED). Für verschiedene

Fischarten konnte in Feldversuchen gezeigt werden, dass der Parasit *L. intestinalis* als ein natürlicher Endokriner Disruptor bezeichnet werden kann, da er auf die selben Biomarker Einfluss nimmt wie anthropogene EDs (Hecker and Karbe, 2005; Hecker et al., 2007, Schabuss et al., 2005; Trubiroha et al., 2009, 2010a,b,). Weil sich *L. intestinalis* als sehr gutes Model für viele Fragestellungen der Biologie und auch in Schadstoffanalysen bewährt hat (s. Hoole, 2010), ist das Parasit-Wirt System von *L. intestinalis* und *Rutilus rutilus* (Rotauge) das Untersuchungsmodel für alle Studien der vorliegenden Arbeit.

Die Untersuchung von mit *L. intestinalis* infizierten Rotaugen beschränkte sich auf die Analyse von Tieren aus dem Freiland. Auch wenn deren Infektion und Lebensbedingungen somit nicht zu kontrollieren waren, so spiegeln sie doch die Bedingungen wider, die man auch bei ökotoxikologischen Freilanduntersuchungen vorfindet. Der vorliegenden Arbeit wurden experimentelle Studien mit anderen Parasit-Wirt Systemen hinzugefügt, um nicht nur Aussagen für ein bestimmtes System treffen zu können. Vielmehr sollte auch getestet werden, ob sich die Ergebnisse auch in einem anderen Parasit-Wirt System finden oder auf Grund von kontrollierteren Bedingungen besser darstellen lassen.

Im Rahmen dieser Arbeit wurden somit folgende Fragestellungen berücksichtigt:

(1) Wie sind die Akkumulationseigenschaften von *Ligula intestinalis* und konkurriert der Parasit mit seinem Wirt, *Rutilus rutilus,* um Metalle?

Rotaugen (*R. rutilus*) wurden im Jahr 2008 in dem Trinkwasserreservoir Listertalsperre (Sauerland, Deutschland) gefangen und zusammen mit ihrem Parasiten *L. intestinalis* auf ihre jeweiligen Metallgehalte analysiert. Hierzu wurden die Gewebeproben mittels Mikrowellenaufschluss in Lösung gebracht (Zimmermann et al., 2001). Anschließend wurden die Metallgehalte von As, Cd, Co, Cu, Fe, Mn, Ni, Pb und Zn mit Hilfe der Massenspektrometrie (ICP-MS) bestimmt. An Hand des Biokonzentrationsfaktors wurde die Anreicherungsfähigkeit des Parasiten gegenüber den Wirtsgeweben Muskel und Darm verglichen. Für die Ermittlung einer möglichen Konkurrenz um bestimmte Metalle, wurden Korrelationsanalysen durchgeführt.

(2) Biomarker 1: Wird die Enzymaktivität der Glutathion-S-Transferase (GST) in der Leber von verschiedenen Fischen durch Bandwürmer der Familie Diphyllobothridea beeinflusst?

Die Bandwürmer *L. intestinalis* und *Schistocephalus solidus* gehören zur Familie der Diphyllobothridea. Das Parasit-Wirt System von *S. solidus* und dem dreistacheligen Stichling *Gasterosteus aculeatus* ist vollständig im Labor des Max-Planck-Instituts (MPI) in Plön etabliert. Die Fische aus dem Großen Plöner See (Deutschland) werden im Labor des MPI nachgezüchtet und experimentell mit dem Bandwurm infiziert. Daher wurde dieses System genutzt, um den Einfluss des Parasiten auf die GST-Aktivität der Wirtsleber unter kontrollierten Bedingungen zu erfassen. Nach dem Transfer infizierter Fische vom MPI an die Universität Duisburg-Essen wurden die Tiere für zehn Wochen unter kontrollierten Bedingungen im Aquarium gehältert und anschließend untersucht.

Der Einfluss von *L. intestinalis* auf die GST-Aktivität seines Wirtes wurde unter Freilandbedingungen erfasst. Hierfür wurden Rotaugen von zwei verschiedenen Standorten untersucht. Rotaugen aus dem Müggelsee (Berlin, Deutschland) wurden im Herbst 2007 gefangen und beprobt. Die Befischung der Listertalsperre im Sommer 2008 ergab den Fang von infizierten Rotaugen und Döbeln (*Squalius cephalus*) weshalb zum einen zwei Standorte mit demselben Parasit-Wirt System und zum anderen zwei Wirte mit demselben Parasiten verglichen werden konnten.

Die GST-Aktivität der Fischleberproben wurde mit Hilfe des GST Assay Kit von Sigma-Aldrich (Deutschland) photometrissch am Mikrotiterplattenreader bestimmt. Die Methodik dieses Kits basiert auf der Verwendung von 1-chloro-2.4 dinitrobenzene (CDNB) als Substrat nach einer Beschreibung von Habig et al. (1974). Für die Analyse der Gesamtproteinkonzentrationen wurde das Pierce BCA Protein Assay Kit (USA) verwendet.

(3) Biomarker 2: Werden die Metallothioneingehalte (MT) von *R. rutilus* oder *Gammarus fossarum* durch eine Parasiteninfektion beeinflusst?

Die Analyse der Biomarker 2 und 3 erfolgte am selben Probenmaterial weshalb die Beprobung nur einmal beschrieben wird.

Das Rotaugen-*L. intestinalis* System wurde für die folgenden Untersuchungen manipuliert, da die Fische nach dem Fang aus dem Freiland für zwei Jahre unter

kontrollierten Bedingungen gehältert wurden. Hierfür wurden Rotaugen im Winter 2006 aus dem Müggelsee entnommen und in eine Wanne (1000 L) überführt. Im Winter 2008 wurden die Fische dann beprobt und auf die verschiedenen Biomarker untersucht.

Als zusätzliches Parasit-Wirt System wurde ein Vetreter der Wirbellosen, infiziert mit einem Parasiten des Stammes Acanthocephala herangezogen. Zu diesem Zweck wurden Bachflohkrebse der Art *Gammarus fossarum* im Herbst 2008 aus dem Ruthertalbach (Essen, Deutschland) gefangen. Die Tiere wurden in vier Gruppen aufgeteilt und im Labor für zwei Wochen unter kontrollierten Bedingungen gehältert. Die Aufteilung der Gruppen erfolgte nach optischer Begutachtung des Infektionszustandes, da die Cystacanthlarve von *Polymorphus minutus* als orangener Punkt im Hämocoel der Gammariden zu erkennen ist. Je eine Gruppe mit uninfizierten und eine mit infizierten Tieren wurden als Kontrollgruppen definiert wohingegen dieselbe Kombination für einen Expositionsversuch mit 4 µg/L Cd verwendet wurde. Nach Versuchsende wurden die Gammariden getötet und im gefrorenen Zustand die Parasitenlarven entnommen.

Es wurden die Cd-Konzentrationen im Hälterungswasser und dem Gewebe der Gammariden sowie der Parasiten durch ET-AAS bestimmt (Sures et al., 1995). Hierfür wurden die Gewebeproben zuvor mittels Mikrowellenaufschluss in Lösung gebracht.

Für die Bestimmung der MT-Gehalte der Gewebeproben kam die Silbersättigungsmethode von Scheuhammer und Cherian (1985) zum Einsatz. Als Ausgangsmaterial dienten hierfür homogenisierte Fischleber oder Gammaridengewebe nach vorheriger Entfernung der Parasiten. Die Bestimmung der Ag-Konzentrationen, welche proportional zu den MT-Gehalten sind, erfolgte ebenfalls mittels ET-AAS.

(4) Biomarker 3: Werden die Gehalte des Hitzeschockproteins 70 (HSP70) in *R. rutilus* oder *G. fossarum* durch eine Parasiteninfektion beeinflusst?

Die Beprobung und Versuchsdurchführung des hiefür verwendeten Ausgangsmaterials wurde unter (3) beschrieben.

Die Bestimmung der HSP70- Gehalte der Gewebeproben erfolgte mittels SDS-PAGE und anschließendem Western Blot (Schill et al., 2001; Sures und Radszuweit, 2007). Wegen der besseren Bindungseigenschaften wurden zwei verschieden Antikörper für die jeweiligen Organismen benutzt. So kam für die Fischleber der Maus-anti-Hsp70/Hsc70 monoclonale Antikörper (SPA-820) von Stressgen (Deutschland) als erster Antikörper

zum Einsatz. Für die Gammaridenproben fand hingegen der Maus-anti-HSP70 monoclonale Antikörper (H5147) von Sigma-Aldrich (Deutschland) Verwendung. Der zweite Antikörper (Peroxidase konjugietre Ziege anti-Maus IgG (P0447) von Dako A/S (Dänemark)) konnte wiederum für beide Probenarten verwendet werden. Die Visualisierung der Proteinbanden wurde über die Zugabe des Substrates 4-chloro(1)naphtol ermöglicht. Für die Auswertung der verschiedenen Intensitäten der HSP70 Banden wurde eine desitometrische Bildanalyse mit dem Programm ImageJ durchgeführt.

Wichtige Ergebnisse und Erkenntnisse

(1) Wie sind die Akkumulationseigenschaften von Ligula intestinalis *und konkurriert der Parasit mit seinem Wirt,* Rutilus rutilus*, um Metalle?*

Weder im Darm- oder Muskelgewebe von *R. rutilus* noch im Gewebe von *L. intestinalis* konnten toxische Elemente wie As, Cd und Pb nachgewiesen werden. Dies deutet darauf hin, dass die Probenahmestelle nicht oder nur sehr schwach mit diesen Elementen kontaminiert ist. Es gibt somit keine weiteren Hinweise auf die Eignung des untersuchten Parasiten als Akkumulationsindikator von toxischen Metallen. Im Bezug auf essentielle Elemente wie Co, Cu, Fe, Mn, Ni und Zn zeigt *L. intestinalis* bis zu 27-fach höhere Gehalte als im Fischmuskel. Im Vergleich zum Fischdarm sind die Metalle Co, Mn und Ni ebenfalls im Parasiten in bis zu 43-fach höheren Konzentrationen zu finden. Das Geschlecht oder der Infektionsstatus der Fische hatten keine Auswirkung auf den Metallgehalt im Fischgewebe. Für Fisch- wie Parasitengewebe gilt die gleiche Rangfolge von Elementkonzentrationen: Zn>Fe>Cu>Mn>Ni>Co. Dies deutet auf eine ähnliche Bedeutung und ähnliche Transportmechanismen für die jeweiligen Elemente in beiden Organismen hin. Einzig das Element Co lässt auf Grund von Korrelationsanalysen und einem hohen Biokonzentrationsfaktor (BCF) im Vergleich zum Muskelgewebe auf eine besondere Bedeutung für den Parasiten schließen. Des Weiteren scheinen Wirt und Parasit um dieses Element zu konkurrieren. Das Parasitengewebe selbst verdeutlicht die konkurrierende Aufnahme der Antagonisten Cu und Zn. Hierbei scheint die Aufnahme von Cu bevorzugt, während Zn auf eine Maximalkonzentration von ca. 50 mg/kg (Frischgewicht) begrenzt ist.

(2) Biomarker 1: Wird die Enzymaktivität der Glutathion-S-Transferase (GST) in der Leber von verschiedenen Fischen durch Bandwürmer der Familie Diphyllobothridea beeinflusst?

Die durchgeführten Untersuchungen an drei verschiedenen Fischarten mit zwei verschieden Parasiten zeigen eine Reduktion der GST-Aktivität in der jeweiligen Wirtsleber. Dieser Effekt wurde unter Laborbedingungen am Deutlichsten. In beiden Geschlechtern des dreistacheligen Stichlings konnte eine um ca. 40% niedrigere GST-Aktivität durch eine Infektion mit *S. solidus* festgestellt werden. Im zweiten Parasit-Wirtsystem war die Reduktion der GST-Aktivität je nach Standort nur in jeweils einem Geschlecht erkennbar. Zudem war dies für die beiden Standorte unterschiedlich. Es ist davon auszugehen, dass *L. intestinalis*, einen vergleichbaren Effekt auf die GST-Aktivität seiner Wirtsfische hat wie *S. solidus*, unabhängig vom Wirtsgeschlecht. Da die Rotaugen aber aus dem Freiland stammen, konnte hier die GST-Aktivität durch weitere Faktoren beeinflusst werden, welche damit die Varianz der Ergebnisse erhöhten Ohne Berücksichtigung des Infektionsstatus wäre die Varianz für die GST-Aktivät bei *G. aculeatus* sehr hoch. Die Werte trennen sich jedoch deutlich, wenn man die Parasitierung bei der Gruppierung der Daten berücksichtigt. Daher verdeutlichen die Ergebnisse ebenso den Informationsgewinn, den die Beachtung einer Infektion auf die Auswertung von Biomarkeranalysen haben kann. In ökotoxikologischen Freilanduntersuchungen wird bei der Verwendung der GST-Aktivität als Biomarker häufig eine mangelnde Reflexion von Schadstoffgradienten kritisiert. Die Berücksichtigung von Parasitierungen könnte demnach zu einer besseren Verteilung der Daten führen, die dann wiederum Schadstoffbelastungen besser reflektiert.

(3) Biomarker 2: Werden die Metallothioneingehalte (MT) von R. rutilus *oder* Gammarus fossarum *durch eine Parasiteninfektion beeinflusst?*

In Leberproben von *R. rutilus* konnte kein Einfluss durch die Parasitierung mit *L. intestinalis* auf die jeweiligen Wirtsmetallothioneingehalte festgestellt werden. Rotaugenmännchen hatten jedoch 17% höhere MT-Gehalte als die Weibchen. Da die Fische beiderlei Geschlechts für zwei Jahre unter kontrollierten Bedingungen in derselben Wanne gehältert wurden, lassen sich keine äußeren Faktoren für eine Erklärung dieses Unterschieds heranziehen. Es lässt sich demnach vermuten, dass diese Beobachtung auf geschlechtsspezifischen physiologischen Unterschieden beruht.

Die Cd-Exposition spiegelte sich unabhängig von der Infektion in ca. 6-fach höheren Cd-Gehalten im Gammaridengewebe wider. Auch die Parasiten aus Cd-exponierten Wirtstieren

zeigten eine deutlich höhere Cd- Konzentration als jene aus der Kontrollgruppe. Bei exponierten Gammariden stieg der MT-Gehalt um ca. 11% im Vergleich zu den unexponierten Tieren. Auch dies war unabhängig von einer Parasitierung mit *P. minutus*.

(4) Biomarker 3: Werden die Gehalte des Hitzeschockproteins 70 (HSP70) in R. rutilus *oder* G. fossarum *durch eine Parasiteninfektion beeinflusst?*

Die Infektion mit *L. intestinalis* reduzierte die HSP70-Gehalte in der Leber von Rotaugen beiderlei Geschlechts um mehr als 40%. Bei unfizierten Fischen zeigten die Weibchen ca. 30% höhere HSP70-Gehalte als die Männchen.

In *G. fossarum* führten die Infektion mit *P. minutus* sowie die Exposition mit Cd zu erhöhten HSP70-Gehalten im Vergleich zu den Kontrolltieren (33-fach und 100-fach). Bei Gammariden welche beiden Stressoren gleichzeitig ausgesetzt waren (Parasit + Metall) reduzierte sich der HSP70-Gehalt wieder deutlich im Vergleich zur alleinigen Cd-Exposition, blieb aber immer noch höher als bei den Kontrolltieren (17-fach).

Schlussfolgerungen

Die im Rahmen dieser Dissertation durchgeführten Untersuchungen lieferten weitere Belege für die Beeinflussbarkeit von Biomarkern aquatischer Organismen durch Parasiten. Für *L. intestinalis* wurde eine reduzierende Wirkung auf die Biomarker GST-Aktivität und HSP70 in der Rotaugenleber nachgewiesen. Weder auf die Akkumulation der untersuchten Metalle (Co, Cu, Fe, Mn, Ni, Zn) in Darm- und Muskelgewebe noch auf die Metallothioneingehalte in der Leber hatte die Parasitierung mit *L. intestinalis* einen Einfluss.

L. intestinalis wies im Vergleich zu den Wirtsgeweben Muskel und Darm höhere Metallkonzentrationen auf. Hierbei scheint aber lediglich das Element Co kompetetiv vom Parasiten aufgenommen zu werden. Da Co das Zentralatom in Cobalamin (Vitamin B12) darstellt und bekannt ist, dass nahe Verwandte des untersuchten Parasiten bei infizierten Säugern zu einem Vitamin B12 Mangel führen, bleibt zu klären, ob es sich in diesem System ebenfalls um einen Vitamin B12–Entzug handelt.

Die GST-Aktivität in Fischleberproben ist schon seit langem ein umstrittener Biomarker in Freilandbeprobungen, da die Ergebnisse oftmals zu sehr streuen und auch nicht den Verschmutzungsgradienten der Gewässer reflektieren (Van der Oost et al., 2003). Die Annahme, dass die in dieser Arbeit untersuchten Parasiten (*S. solidus* und *L. intestinalis*),

ähnlich wie *Dicrocoelium dendriticum* und *Fasciola hepatica*, die GST-Aktivität erniedrigen, konnte bestätigt werden. Der Grund für diese Beeinflussung bleibt jedoch noch unklar. Wegen seiner hemmenden Wirkung auf die Wirtsreproduktion war *L. intestinalis* bereits Objekt intensiver Forschung bezüglich der Entdeckung möglicher parasitenproduzierter Substanzen, welche auf das Hormonsystem wirken könnten (Arme, 2002). Bislang konnte jedoch noch keine Substanz entdeckt werden. Für *F. hepatica* konnte aktuell ein parasitenproduziertes GST-Molekül nachgewiesen werden, welches die Wirtsimmunität unterdrückt und daher das Überleben des Parasiten auf lange Zeit begünstigt (Dowling et al., 2010). Daher sollte auch für *L. intestinalis* nicht ausgeschlossen werdern, dass ähnliche Mechanismen vorhanden sein könnten, weshalb die Suche nach ausgeschiedenen Molekülen wieder intensiviert werden sollte. Dass sich die Berücksichtigung der Infektion in der Auswertung der Daten positiv bemerkbar macht, ließ sich am Stichlingsmodell sehr gut erkennen. Dies sollte eine weitere Motivation sein, Parasiten als natürlichen Stressor für freilebende Organismen in ökotoxikologischen Untersuchungen zu berücksichtigen.

Insgesamt zeigten die beiden untersuchten Parasit-Wirt Systeme keine Beeinflussung der MT-Gehalte durch eine Infektion. Dennoch belegen andere Beispiele, dass auch dieser metallspezifische Biomarker von Parasiteninfektionen beeinflusst werden kann (Baudrimont et al., 2006; Baudrimont and de Montaudouin, 2007; Fazio et al., 2008; Paul-Pont et al., 2009). Der Grund für die verschiedenen Auswirkungen in verschiedenen Parasit-Wirt-Systemen ist noch nicht geklärt, da für keines der bekannten Systeme, der Wirkmechanismus erfasst wurde.

HSP70 wird als Marker für einen gestressten Organismus benutzt, da der Proteingehalt bei verschiedensten Arten von Stressoren ansteigt. In dieser Arbeit bestätigte sich dies für *G. fossarum*, bei Exposition mit geringen Cd- Konzentrationen oder der Infektion mit Larven von *P. minutus*. Bei Rotaugen hatte eine Infektion mit *L. intestinalis* allerdings eine Reduktion der HSP70-Gehalte zur Folge. Dies könnte darauf hindeuten, dass *L. intestinalis* seinen Wirt so sehr stresst, dass dessen Schutzmechanismen, wie z.B das Hitzeschockprotein 70, schon überlastet sind. Ähnliches kann man aus der Doppelbelastung von *G. fossarum* mit Metallexposition und gleichzeitiger Parasiteninfektion schließen. Auch hier scheint der Stress für den Wirt so hoch zu sein, dass die HSP70-Produktion wieder nachlässt. Allerdings stehen auch das Immunsystem und HSP70 in einem sehr engen Zusammenhang. Daher ist hier nicht auszuschließen, dass *L. intestinalis* seinen Fischwirt über andere Mechanismen beeinflusst,

welche die Ursache für die Reduktion der HSP70-Gehalte sein könnten. Für *Schistosoma mansoni* konnte aktuell gezeigt werden, dass parasitische Exkretions/Sekretionsprodukte die HSP70-Gehalte in Abwehrzellen ihres Wirts reduzieren (Zahoor et al., 2010).

Zusammenfassend konnte mit dieser Arbeit gezeigt werden, dass der Einfluss von Parasiten auf die Wirtsphysiologie auch solche Parameter verändern kann, die zur Beurteilung der Gewässerqualität herangezogen werden. Daher kann es für zukünftige ökotoxikologische Untersuchungen nur von Vorteil sein, Parasitierungen von Organismen aus dem Freiland zu beachten. Berücksichtigt man den Umstand, dass Parasiten ein Teil des natürlichen Ökosystems sind, stellt sich die Frage nach der Physiologie unter „normalen“ Bedingungen. Gerade in Untersuchungen, bei denen verschiedene Gewässer miteinander verglichen werden sollen, kann es auf die Physiologie in erheblichem Maße einwirken, mit wie vielen und welchen Parasiten ein Organismus belastet ist. Somit kann auch derselbe Belastungsgrad mit Schadstoffen in unterschiedlichem Maße auf die Physiologie wirken, je nach Vorbelastung durch Parasitenbefall. Da Parasiten ebenso auf Schadstoffbelastung reagieren, sind dies Wechselweirkungen, die zum einen nicht mit Laborversuchen zu vergleichen sind und zum anderen nicht unberücksichtigt bleiben sollten.

Für den Parasiten *L. intestinalis* konnte gezeigt werden, dass er auch mit physiologischen Schutzfunktionen seines Wirtes in Verbindung steht. Der Vergleich mit neuesten Erkenntnissen zu Exkretions/Sekretionsprodukten anderer Plathelminthen sollte eine Motivation für die zukünftige Forschung sein, auch *L. intestinalis* diesbezüglich wieder genauer unter die Lupe zu nehmen. Das Ziel jahrzehntelanger Forschung zu diesem Parasiten war meist die Klärung der Frage, wie die Hemmung der Wirtsreproduktion erfolgt. Es ist an der Zeit, dies mit der Klärung der Frage, wie er seine Langlebigkeit im Wirtskörper sichert, zu kombinieren.

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Appendix

Appendix I. Morphological data of roach sampling from Listertalsperre in 2008 and element concentrations in fish and parasite tissues used in Chapter 1.

Used abbreviations in this index:

nr	individual sample number of fish
F/M	female/male
uninf	uninfected
inf	infected with *L. intestinalis*

Table I-A. Element concentrations [mg/kg] in roach intestine tissue.

nr	status	Mn	Fe	Co	Ni	Cu	Zn
12	F uninf	1.507	28.262	0.025	0.805	3.069	164.779
15	F uninf	0.992	17.160	0.026	0.534	2.621	130.275
18	F uninf	0.925	36.638	0.010	0.439	3.531	283.355
20	F uninf	0.784	39.763	0.010	0.000	4.033	372.781
22	F uninf	0.665	54.165	0.034	1.612	4.009	127.605
14	F inf	0.513	18.837	0.012	0.000	3.005	245.560
27	F inf	0.509	53.300	0.009	0.000	3.569	217.769
30	F inf	1.092	102.956	0.021	0.019	3.237	283.060
40	F inf	0.790	58.442	0.031	0.041	8.767	202.621
48	F inf	0.353	21.447	0.000	0.000	2.023	307.577
16	M uninf	1.071	55.615	0.006	0.000	3.057	182.636
28	M uninf	1.263	101.821	0.034	2.086	5.851	71.013
36	M uninf	1.270	31.277	0.019	1.706	3.010	59.722
37	M uninf	1.527	14.985	0.011	0.087	2.788	66.886
47	M uninf	0.709	40.554	0.013	0.242	3.090	139.532
17	M inf	1.379	47.489	0.015	0.000	3.207	109.735
19	M inf	1.310	28.722	0.018	0.000	3.489	245.839
21	M inf	0.815	37.044	0.010	0.000	2.206	182.036
29	M inf	0.714	34.329	0.020	0.000	2.992	276.696
42	M inf	0.906	19.604	0.011	0.000	7.940	107.574

Table I-B. Element concentrations [mg/kg] in roach muscle tissue. (nr. = individual sample number of fish; F/M = female/male; uninf = uninfected; inf = infected with *L. intestinalis*)

nr	status	Mn	Fe	Co	Ni	Cu	Zn
12	F uninf	0.198	5.400	0.001	0.022	0.192	3.158
15	F uninf	0.278	7.590	0.003	0.054	0.948	9.606
18	F uninf	0.641	14.196	0.010	0.149	0.602	13.551
20	F uninf	0.292	6.527	0.004	0.077	0.536	12.473
22	F uninf	0.378	9.892	0.007	0.136	0.771	24.430
14	F inf	0.266	8.539	0.004	0.076	0.684	8.931
27	F inf	0.423	9.316	0.006	0.222	0.518	14.111
30	F inf	0.399	10.260	0.005	0.149	0.707	12.585
40	F inf	0.425	10.579	0.005	0.075	0.565	10.446
48	F inf	0.277	8.474	0.004	0.046	0.568	11.368
16	M uninf	0.356	6.216	0.002	0.062	0.304	6.106
28	M uninf	0.339	9.503	0.006	0.032	0.588	15.001
36	M uninf	0.354	8.324	0.004	0.102	0.515	14.886
37	M uninf	0.374	7.115	0.005	0.114	0.597	11.405
47	M uninf	0.348	8.525	0.005	0.082	0.541	16.510
17	M inf	0.323	6.492	0.003	0.138	0.533	7.365
19	M inf	0.347	9.330	0.003	0.490	0.464	9.359
21	M inf	0.349	5.946	0.004	0.041	0.522	11.073
29	M inf	0.292	5.902	0.003	0.071	0.358	10.167
42	M inf	0.790	9.122	0.004	0.084	0.754	13.015

Table I-C. Element concentrations [mg/kg] in tissue of *L. intestinalis*. (nr. = individual sample number of host fish)

nr.	Mn	Fe	Co	Ni	Cu	Zn
14	3.811	19.803	0.097	0.094	4.199	51.324
19	3.109	22.401	0.098	0.334	2.957	50.564
21	4.946	20.321	0.081	0.331	2.102	41.093
27	6.433	22.869	0.181	0.000	2.208	35.098
29	3.839	37.532	0.094	0.000	0.678	23.993
40	5.045	26.025	0.106	0.499	2.677	37.210
48	3.505	24.551	0.128	0.189	1.993	41.859
30	4.316	31.281	0.198	0.254	6.328	51.905

Appendix II. Morphological data of stickleback experiment and roach/chub sampling from Listertalsperre in 2008 as well as roach sampling from Mueggelsee 2007 with levels of GST used in Chapter 2.

Used abbreviations in this index:

nr	individual sample number of fish
F/M	female/male
uninf	uninfected
inf	infected with *S. solidus* (for sticklebacks)/*L. intestinalis* (for roach or chub)
TL	total length
TW	total weight
LW	liver weight
GW	gonad weight
n	number of parasites
PW	parasites weight
GST	activity of Glutathione-S-transferase

Table II-A. Morphological data of stickleback sampling.

nr	status	TL (cm)	TW (g)	LW (mg)	*S. solidus* (n)	PW (g)	GST (U/mg protein)
6	F uninf	4.9	0.92	41.0	0	0.00	0.347
11	F uninf	4.4	0.59	21.0	0	0.00	0.335
26	F uninf	4.9	0.86	42.8	0	0.00	0.256
29	F uninf	5.0	0.91	42.3	0	0.00	0.301
38	F uninf	5.5	1.14	43.5	0	0.00	0.424
39	F uninf	4.8	0.78	26.5	0	0.00	0.351
1	F inf	4.9	0.98	16.3	1	0.25	0.242
2	F inf	3.7	0.45	6.0	1	0.13	0.195
3	F inf	4.0	0.58	9.2	1	0.16	0.201
8	F inf	4.7	0.93	18.4	1	0.24	0.250
10	F inf	4.2	0.69	11.0	1	0.22	0.185
12	F inf	4.8	1.05	21.5	1	0.31	0.255
13	F inf	4.5	1.03	23.9	1	0.23	0.198
14	F inf	5.1	1.20	31.5	1	0.24	0.264
17	F inf	5.2	1.22	23.5	1	0.34	0.251
21	F inf	4.3	0.80	17.6	1	0.27	0.280
25	F inf	3.9	0.50	9.9	1	0.13	0.250
27	F inf	4.6	0.92	19.6	1	0.26	0.311
30	F inf	4.6	0.90	15.2	1	0.23	0.238
31	F inf	4.8	1.07	18.8	1	0.26	0.219
9	M uninf	4.4	0.56	20.8	0	0.00	0.361
19	M uninf	4.2	0.52	14.7	0	0.00	0.242
22	M uninf	3.9	0.42	13.2	0	0.00	0.290
23	M uninf	3.9	0.43	13.5	0	0.00	0.315
36	M uninf	3.9	0.41	10.2	0	0.00	0.338
5	M inf	4.0	0.60	10.0	1	0.18	0.196
18	M inf	4.0	0.59	9.6	1	0.17	0.196
20	M inf	4.9	1.01	23.1	1	0.25	0.151
24	M inf	4.9	0.99	20.4	1	0.25	0.220

Table II-B. Morphological data of roach sampling from Listertalsperre in 2008.

nr.	status	TL (cm)	TW (g)	LW (g)	GW (g)	*L. intestinalis* (n)	PW (g)	GST (U/mg protein)
12	F uninf	10.2	9.51	0.15	0.05	0	0.00	0.341
15	F uninf	10.1	9.92	0.12	0.08	0	0.00	0.285
18	F uninf	9.8	8.26	0.11	0.02	0	0.00	0.320
20	F uninf	9.7	8.15	0.10	0.05	0	0.00	0.262
22	F uninf	9.9	8.43	0.11	0.07	0	0.00	0.242
14	F inf	11.8	16.26	0.25	0.07	4	1.75	0.108
27	F inf	9.8	9.19	0.12	0.05	1	0.27	0.145
30	F inf	11.7	21.75	0.18	0.15	1	2.05	0.115
40	F inf	10.2	12.62	0.12	0.04	4	1.73	0.128
48	F inf	9.6	10.72	0.11	0.06	1	1.15	0.159
16	M uninf	9.4	7.51	0.12	0.04	0	0.00	0.299
28	M uninf	8.8	6.59	0.08	0.02	0	0.0	0.192
36	M uninf	7.3	3.86	0.05	0.01	0	0.0	0.085
37	M uninf	8.6	5.21	0.07	0.04	0	0.0	0.169
47	M uninf	8.8	7.12	0.09	0.04	0	0.0	0.144
17	M inf	10.0	8.97	0.08	0.01	7	0.34	0.249
19	M inf	9.6	8.80	0.09	0.01	11	1.32	0.114
21	M inf	10.5	11.95	0.13	0.04	2	1.08	0.291
29	M inf	8.6	6.52	0.10	0.03	8	0.79	0.166
42	M inf	8.7	5.61	0.06	0.03	3	0.00	0.225

Table II-C. Morphological data of chub sampling from Listertalsperre in 2008.

nr.	status	TL (cm)	TW (g)	LW (g)	GW (g)	*L. intestinalis* (n)	PW (g)	GST (U/mg protein)
8	F uninf	8.4	4.27	0.07	0.01	0	0.00	0.050
20	F uninf	9.0	5.36	0.05	0.02	0	0.00	0.148
23	F uninf	9.0	5.41	0.06	0.02	0	0.00	0.078
25	F uninf	7.6	3.03	0.04	0.00	0	0.00	n.a.
29	F uninf	9.3	5.58	0.06	0.03	0	0.00	0.069
3	F inf	10.1	9.55	0.09	0.02	1	2.08	0.057
5	F inf	9.2	7.37	0.08	0.02	2	1.65	0.042
12	F inf	9.2	9.01	0.04	0.01	4	2.91	0.031
14	F inf	7.7	4.92	0.13	0.01	3	0.43	0.032
15	F inf	9.7	7.76	0.09	0.02	3	1.96	0.046
16	F inf	9.1	6.14	0.05	0.02	3	1.34	0.101
17	F inf	7.9	4.62	0.04	0.01	1	1.1	0.086
24	F inf	7.1	3.65	0.01	0.01	1	0.8	0.076
27	F inf	9.4	5.88	0.05	0.02	2	0.1	0.062
2	M uninf	8.4	4.55	0.06	0.01	0	0.0	0.062
4	M uninf	9.2	6.00	0.07	0.01	0	0.00	0.051
9	M uninf	9.4	6.32	0.09	0.02	0	0.00	0.064
10	M uninf	8.3	4.14	0.05	0.01	0	0.00	0.066
11	M uninf	8.5	4.71	0.06	0.02	0	0.00	0.043
13	M uninf	10.3	8.88	0.08	0.01	0	0.00	0.130
18	M uninf	9.1	5.65	0.03	0.01	0	0.00	0.073
21	M uninf	9.4	6.16	0.02	1.55	0	0.00	0.087
26	M uninf	10.0	8.05	0.09	0.03	0	0.00	0.076
1	M inf	7.6	3.75	0.06	0.03	2	0.65	0.040
6	M inf	7.8	4.33	0.06	0.01	2	0.5	0.045
7	M inf	8.8	6.52	0.08	0.01	3	1.7	0.116
19	M inf	9.0	6.78	0.16	0.05	2	0.8	0.108
22	M inf	9.0	6.14	0.05	0.01	3	0.7	0.066
28	M inf	7.9	3.66	0.03	0.00	1	0.60	0.045

Table II-D. Morphological data of roach sampling from Mueggelsee in 2007.

nr.	status	TL (cm)	TW (g)	*L. intestinalis* (n)	PW (g)	GST U/mg Protein
3	F uninf	14.9	32.9	0	0	0.279
11	F uninf	14.2	27.3	0	0	0.337
14	F uninf	15.6	33	0	0	0.175
18	F uninf	15.8	47.8	0	0	0.197
22	F uninf	16.7	42.9	0	0	0.155
24	F uninf	15.2	43.5	0	0	0.168
26	F uninf	14.3	31.9	0	0	0.172
28	F uninf	13.7	27.2	0	0	0.200
31	F uninf	14	32.6	0	0	0.230
45	F uninf	14.7	30.3	0	0	0.282
54	F uninf	14.3	25.5	0	0	0.240
13	F inf	17.6	58.7	1	2.61	0.298
25	F inf	13.5	23.8	3	2.47	0.226
38	F inf	14.1	27.7	2	3.2	0.156
49	F inf	13.6	26.4	4	5.3	0.222
50	F inf	14.3	29.7	1	3.2	0.322
51	F inf	15.4	33.2	1	3.7	0.035
52	F inf	13.3	23.3	1	2.3	0.218
60	F inf	14.7	32.2	6	2.8	0.095
66	F inf	18.6	62.6	3	8.1	0.188
67	F inf	14.9	29	2	3.6	0.189
68	F inf	16.8	40.4	4	5.4	0.127
69	F inf	13.4	24.2	1	1.6	0.220
2	M uninf	17	45.2	0	0	0.288
8	M uninf	14.9	30.5	0	0	0.426
10	M uninf	16.4	42.3	0	0	0.272
12	M uninf	13.6	22.3	0	0	0.313
17	M uninf	15.8	40.4	0	0	0.209
32	M uninf	13.3	22.8	0	0	0.380
39	M uninf	13	20.5	0	0	0.258
46	M uninf	13.5	21.4	0	0	0.305
53	M uninf	10.8	10.9	0	0	0.109
21	M inf	13.7	24.2	3	4.3	0.213
29	M inf	12.9	20.5	1	1.4	0.200
30	M inf	13.2	23.2	3	4.7	0.165
33	M inf	12.4	18.2	1	1.6	0.284
41	M inf	13.3	23	2	3.1	0.240
42	M inf	14.5	31.6	3	2.9	0.225
61	M inf	13.1	22	2	4	0.101
62	M inf	14.4	25.9	3	3.4	0.563
63	M inf	14.9	29.9	2	4	0.279
64	M inf	13.5	22	1	2.2	0.147
65	M inf	15.4	36	2	5.3	0.252

Appendix III. Morphological data of roach sampling from Mueggelsee in 2006 and levels of HSP70 and MT in roach and gammarids used in Chapter 3 and 4.

Used abbreviations in this index:

nr	individual sample number of fish
F/M	female/male
uninf	uninfected
inf	infected with *L. intestinalis* (for roach) or *P. minutus* (for gammarids)
TL	total length
TW	total weight
n	number of parasites
PW	parasites weight
HSP70	heat shock protein 70
MT	metallothionein

Table III-A. Morphological data of roach sampling from Mueggelsee in 2006.

nr.	status	TL (cm)	TW (g)	*L. intestinalis* (n)	PW (g)	HSP70	MT
7	F uninf	17.2	46.7	0	0.0	231	0.299
8	F uninf	18.3	54.3	0	0.0	106	0.292
31	F uninf	16.1	34.9	0	0.0	123	0.404
57	F uninf	16.2	39.1	0	0.0	158	0.402
28	F uninf	17.5	46.7	0	0.0	82	0.404
53	F uninf	16.3	38.0	0	0.0	332	
56	F uninf	17.7	49.3	0	0.0	299	
48	F uninf	15.0	31.3	0	0.0	272	
45	F uninf	14.4	26.3	0	0.0	266	
43	F uninf	16.3	40.0	0	0.0	420	
42	F uninf	15.7	35.5	0	0.0	230	
41	F uninf	16.9	42.7	0	0.0	327	
40	F uninf	16.1	39.3	0	0.0	313	
37	F uninf	17.1	46.0	0	0.0	466	
36	F uninf	15.7	33.0	0	0.0	325	
21	F uninf	16.3	42.0	0	0.0	275	
18	F uninf	16.9	39.4	0	0.0	342	
22	F inf	17.5	59.4	1	8.4	114	0.496
24	F inf	19.7	80.3	3	10.7	128	0.384
39	F inf	18.8	71.4	1	4.6	142	0.259
30	F inf	17.6	51.4	1	5.8	141	0.327
27	F inf	17.5	54.7	1	7.5	77	0.380
29	F inf	19.5	76.9	4	14.2	106	0.302
54	F inf	16.7	41.5	1	2.9	112	
46	F inf	17.1	50.0	1	5.9	237	
17	F inf	21.8	117.6	1	7.7	178	
52	M uninf	14.2	25.8	0	0.0	102	0.497
59	M uninf	14.3	22.2	0	0.0	146	0.405
19	M uninf	13.7	21.5	0	0.0	98	0.347
20	M uninf	15.0	32.6	0	0.0	83	0.518
51	M uninf	17.4	45.3	0	0.0	179	
50	M uninf	16.7	44.3	0	0.0	201	
44	M uninf	17.1	44.2	0	0.0	258	
35	M uninf	16.5	40.0	0	0.0	289	
33	M uninf	14.6	30.1	0	0.0	332	
32	M uninf	15.1	27.5	0	0.0	188	
6	M inf	17.9	54.0	3	8.5	108	0.397
23	M inf	19.4	77.3	1	9.5	82	0.491
38	M inf	17.1	49.3	2	7.6	86	0.492
25	M inf	15.5	36.2	1	4.5	220	
26	M inf	17.5	61.3	3	14.4		0.329
11	M inf	19.3	74.2	2	7.3		0.377

Table III-B. Element concentrations [µg/g] in tissue of (a) gammarid or (b) cstacanths of *P. minutus* from Rutherbach in 2009.

(a)

Cd exposure	status	Cd µg/g
no	uninf	0.530
no	uninf	0.625
no	uninf	0.876
no	uninf	0.574
no	uninf	1.471
no	uninf	0.072
no	inf	0.915
no	inf	0.667
no	inf	0.680
no	inf	0.491
yes	uninf	4.936
yes	uninf	6.013
yes	uninf	9.110
yes	uninf	12.576
yes	uninf	5.875
yes	uninf	3.174
yes	uninf	3.809
yes	uninf	6.274
yes	uninf	6.945
yes	uninf	3.786
yes	inf	5.522
yes	inf	7.257
yes	inf	5.686
yes	inf	4.242
yes	inf	5.085
yes	inf	3.687
yes	inf	5.639

(b)

Cd exposure	Cd µg/g
no	0.146
no	0.000
yes	1.269
yes	0.787

Table III-C. Levels of (a) metallothionein (MT) or (b) heats hock protein 70 (HSP70) in samples of gammarids from Rutherbach in 2009.

(a)

Cd exposure	status	MT
no	uninf	0.166
no	uninf	0.171
no	uninf	0.173
no	uninf	0.180
no	uninf	0.195
no	uninf	0.192
no	inf	0.165
no	inf	0.168
no	inf	0.159
no	inf	0.189
no	inf	0.194
no	inf	0.194
yes	uninf	0.200
yes	uninf	0.198
yes	uninf	0.208
yes	uninf	0.200
yes	uninf	0.214
yes	uninf	0.218
yes	inf	0.193
yes	inf	0.203
yes	inf	0.204
yes	inf	0.201
yes	inf	0.198
yes	inf	0.212

(b)

Cd exposure	status	HSP70
no	uninf	1.1
no	uninf	0.4
no	uninf	3.7
no	inf	13.6
no	inf	50.9
no	inf	27.0
yes	uninf	77.7
yes	uninf	16.1
yes	uninf	34.0
yes	uninf	100.8
yes	uninf	21.2
yes	uninf	36.3
yes	inf	11.3
yes	inf	22.1
yes	inf	3.9
yes	inf	5.3
yes	inf	12.6
yes	inf	22.2

Lebenslauf

Aus Datenschutzgründen wurde in der Online-Version auf die Darstellung des Lebenslaufs verzichtet.

Erklärung:

Hiermit erkläre ich, gem. § 6 Abs. 2, Nr. 7 der Promotionsordnung der Math.-Nat.-Fachbreiche zur Erlangung der Dr. rer. nat., dass ich das Arbeitgebiet, dem das Thema *„Influence of parasites on biomarkers in aquatic animals“* zuzuordnen ist, in Forschung und Lehre vertrete und den Antrag von *(Sabrina N. Frank)* befürworte.

Essen, den _____ _____

Unterschrift eines Mitglieds der Universität Duisburg-Essen

Erklärung:

Hiermit erkläre ich, gem. § 6 Abs. 2, Nr. 6 der Promotionsordnung der Math.-Nat.-Fachbereiche zur Erlangung des Dr. rer. nat., dass ich die vorliegende Dissertation selbständig verfasst und mich keiner anderen als der angegebenen Hilfsmittel bedient habe.

Essen, den _____ _____

Unterschrift des/r Doktoranden/in

Erklärung:

Hiermit erkläre ich, gem. § 6 Abs. 2, Nr. 8 der Promotionsordnung der Math.-Nat.-Fachbereiche zur Erlangung des Dr. rer. nat., dass ich keine anderen Promotionen bzw. Promotionsversuche in der Vergangenheit durchgeführt habe und dass diese Arbeit von keiner anderen Fakultät/Fachbereich abgelehnt worden ist.

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