Effects of the acanthocephalan *Polymorphus minutus* and the microsporidian *Dictyocoela duebenum* on energy reserves and stress response of cadmium exposed *Gammarus fossarum*

Hui-Yu Chen, Daniel S Grabner, Milen Nachev, Hsiu-Hui Shih, Bernd Sures

Amphipods are commonly parasitized by acanthocephalans and microsporidians and co-infections are found frequently. Both groups of parasites are known to have severe effects on their host. For example, microsporidians can modify host sex ratio and acanthocephalans can manipulate the behavior of the amphipod to promote transmission to the final host. These effects influence host metabolism in general and will also affect the ability of amphipods to cope with additional stressors such as environmental pollution, e.g. by toxic metals. Here we tested the effects of sub-lethal concentrations of cadmium on glycogen and lipid levels, as well as on the 70kDa heat shock protein (hsp70) response of field collected *Gammarus fossarum*, which were naturally infected with microsporidians and the acanthocephalan *Polymorphus minutus*. Infected and uninfected *G. fossarum* were exposed to a nominal Cd concentration of 4μg/L, which resembled measured aqueous Cd concentration of 2.9 μg/L cadmium in reconstituted water for 7 d at 15° C in parallel to an unexposed control. After exposure gammarids were snap frozen, weighed, sexed and tested for microsporidian infection by PCR. Only individuals containing the microsporidian *Dictyocoela duebenum* were used for the further biochemical and metal analyses. *P. minutus* infected amphipods were significantly smaller than their uninfected conspecifics. Mortality was insignificantly increased due to cadmium exposure, but not due to parasite infection. Microsporidian infection in combination with cadmium exposure led to increased glycogen levels in female gammarids. An increase of glycogen was also found due to interaction of acanthocephalan and microsporidian infection. Elevated lipid levels were observed in all groups infected with microsporidians, while acanthocephalans had the opposite effect. A positive correlation of lipid and glycogen levels was observed. The general stress response measured in form of hsp70 was significantly increased in microsporidian infected gammarids exposed to cadmium. *P. minutus* did not affect the stress response of its host. Lipid levels were correlated negatively with hsp70 response, and indicated a possible increased stress susceptibility of individuals with depleted energy reserves. The results of our study clearly demonstrate the importance of parasitic infections, especially of microsporidians, for ecotoxicological research.

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2 *duebenum* on energy reserves and stress response of cadmium exposed *Gammarus fossarum*

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19   **Abstract**

20   Amphipods are commonly parasitized by acanthocephalans and microsporidians and co-infections

21   are found frequently. Both groups of parasites are known to have severe effects on their host. For

22   example, microsporidians can modify host sex ratio and acanthocephalans can manipulate the

23   behavior of the amphipod to promote transmission to the final host. These effects influence host

24   metabolism in general and will also affect the ability of amphipods to cope with additional stressors

25   such as environmental pollution, e.g. by toxic metals.

26   Here we tested the effects of sub-lethal concentrations of cadmium on glycogen and lipid levels,

27   as well as on the 70kDa heat shock protein (hsp70) response of field collected *Gammarus*

28   *fossarum*, which were naturally infected with microsporidians and the acanthocephalan

29   *Polymorphus minutus*. Infected and uninfected *G. fossarum* were exposed to a nominal Cd

30   concentration of 4µg/L, which resembled measured aqueous Cd concentration of 2.9 µg/L

31   cadmium in reconstituted water for 7 d at 15° C in parallel to an unexposed control. After exposure

32   gammarids were snap frozen, weighed, sexed and tested for microsporidian infection by PCR.

33   Only individuals containing the microsporidian *Dictyocoela duebenum* were used for the further

34   biochemical analyses, while infection status of animals used for metal analyses was not tested. *P.*

35   *minutus* infected amphipods were significantly smaller than their uninfected conspecifics.

36   Mortality was insignificantly increased due to cadmium exposure, but not due to parasite infection.

37   Microsporidian infection in combination with cadmium exposure led to increased glycogen levels

38   in female gammarids. An increase of glycogen was also found due to interaction of

39   acanthocephalan and microsporidian infection. Elevated lipid levels were observed in all groups

40   infected with microsporidians, while acanthocephalans had the opposite effect. A positive

41   correlation of lipid and glycogen levels was observed. The general stress response measured in

42   form of hsp70 was significantly increased in microsporidian infected gammarids exposed to

43   cadmium. *P. minutus* did not affect the stress response of its host. Lipid levels were correlated

44   negatively with hsp70 response, and indicated a possible increased stress susceptibility of

45   individuals with depleted energy reserves. The results of our study clearly demonstrate the

46   importance of parasitic infections, especially of microsporidians, for ecotoxicological research.

Introduction

Parasites are natural and ubiquitous parts of ecosystems and their role for ecosystem function and stability has been recognized in recent years (Marcogliese, 2005; Hudson, Dobson & Lafferty, 2006; Kuris et al., 2008). Nevertheless, parasites are often neglected in ecotoxicological research, although results of recent studies showed that they may influence the host response to pollution (Sures, 2004, 2008a,b; Marcogliese & Giamberini, 2013) and can impact the stress response of their host, especially on the biochemical level (Sures, Lutz & Kloas, 2006; Sures & Radszuweit, 2007; Frank et al., 2011, 2013; Gismondi, Cossu-Leguille & Beisel, 2012a; Gismondi et al., 2012a,b; Filipović Marijić, Vardić Smrzlić & Raspor, 2013; Grabner, Schertzinger & Sures, 2014). Such impacts are particularly relevant when using stress responses on the biochemical level as biomarkers in environmental monitoring programs to indicate the presence of contaminants and to unravel their effects on organisms. As biomarkers are applied under field conditions, their possible modulation by naturally occurring stressors such as parasites has to be evaluated preferably under laboratory conditions as the knowledge about possible interactions between parasites and pollution is still limited (Sures, 2008a; Marcogliese & Pietrock, 2011).

Amphipods are important elements of aquatic ecosystems due to their widespread distribution, high biomass, and their ecological function as shredders of organic material (Schirling et al., 2005; Platvoet et al., 2006; Navel et al., 2010; Piscart et al., 2011). Because of their sensitivity to contaminants, they are often used as test organism in ecotoxicological studies and particularly in metal toxicity tests (Schill, Görlitz & Köhler, 2003; Sures & Radszuweit, 2007; Lebrun et al., 2015). Amphipods are also affected by several parasite groups including acanthocephalans and microsporidians (Taraschewski, 2000; Stentiford et al., 2013).

72   Acanthocephalans are a group of unique helminth parasites that have an invertebrate intermediate

73   and a vertebrate final host (Taraschewski, 2000). They often grow to a large size in the body cavity

74   of their amphipod intermediate hosts and can impact them in various ways. For example, they can

75   influence the activity of the host and its predator avoidance behavior (Bauer et al., 2000; MacNeil

76   et al., 2003a,b; Benesh et al., 2008) or affect its reproduction rates (Ward, 1986; Zohar & Holmes,

77   1998; Bollache, Gambade & Cézilly, 2001). Furthermore, acanthocephalans can influence the

78   energy metabolism of the host and its vulnerability to metals (Gismondi, Cossu-Leguille & Beisel,

79   2012a,b; Gismondi, Beisel & Cossu-Leguille, 2012b).

80   Microsporidians are a diverse group of endoparasitic fungi of invertebrates and vertebrates (Smith,

81   2009; Stentiford et al., 2013). Their transmission can be horizontal from one individual to another

82   and/or vertical, directly from mother to offspring (Dunn & Smith, 2001). High prevalences and

83   co-infection cases with up to three microsporidian species underline the importance of these

84   parasites for amphipods (Haine et al., 2004; Grabner, Schertzinger & Sures, 2014; Grabner et al.

85   2015). Due to intense proliferation of sporogonic stages microsporidians can modulate the host

86   metabolism or its hormone homeostasis leading to feminization of male individuals (Bandi et al.,

87   2001; MacNeil et al., 2003a; Smith, 2009). Both effects are likely to influence the host response

88   to toxic stressors compared to uninfected individuals. Even under asymptomatic conditions,

89   microsporidians were found to influence the stress response and energy reserves of their amphipod

90   host, when an additional chemical stressor was present (Gismondi et al., 2012a).

91   Cadmium is released to the environment by various human activities and is frequently found in

92   freshwater ecosystems in concentrations up to several μg/L. It can affect aquatic organisms and

93   whole communities (EPA, 2001). The combination of exposure to metals like cadmium and

94   simultaneous parasite infections can influence the immune response of the host (Cornet et al.,

95   2009) and can increase energy consumption for maintenance of the basic metabolic functions

96   (Gismondi et al., 2012b). Both glycogen and lipids are important energy reserves in animals and

97   their levels can be used as biomarkers for metabolic stress (Plaistow, Troussard & Cézilly, 2001).

98   Additionally, heat shock proteins (hsp) can be used as general stress markers, suitable to detect

99   effects of parasites and metal exposure in amphipods (Schill, Görlitz & Köhler, 2003; Frank et al.,

100 2013). The effect of cadmium exposure in combination with infection with the acanthocephalan

101 *Polymorphus minutus* and microsporidians was already investigated in detail for the amphipod

102 *Gammarus roeseli* (Gismondi et al., 2012b). Both *G. roeseli* and *G. fossarum* are suitable hosts

103 for *P. minutus*, however it was found that they respond differently (e.g. on biochemical and

104 behavioral level) to infection (Bauer et al., 2000; Helluy, 2013). We assumed that *G. fossarum*

105 might also react differently to the combination of stressors such as metal pollution and parasite

106 infections. Therefore, the aim of the present study was to investigate the combined effects of

107 parasitism and cadmium pollution on ecotoxicological biomarkers such as energy metabolism

108 (total lipid and glycogen content) and general stress response (hsp70) of *G. fossarum*. For this

109 purpose, we conducted laboratory exposure experiments with amphipods, which were naturally

110 infected with microsporidians and acanthocephalans.

111

112 **Material and Methods**

113 **Sampling of gammarids and exposure design**

114 Gammarids were collected by kick-sampling from a small stream (Rumbach) near Mülheim (N51°

115 25' 28.628"; E6° 54' 25.553"), NRW, Germany in March 2014. This site is characterized by a

116 mixed population of *Gammarus pulex* and *Gammarus fossarum,* and high prevalence of the

117 acanthocephalan *Polymorphus minutus* in both amphipod species (unpublished results). After

118 sorting the collected gammarids under a binocular a total of 382 *G. fossarum* was used for the

119 experiment.

120 The gammarids were grouped by acanthocephalan infection (only amphipods infected with single

121 cystacanths were used) and kept in the laboratory for 2 days at 15°C in reconstituted water (7.36

122 μM NaCl, 231.39 μM KCl, 117.13 μM NaHCO$_3$, 707.05 μM, MgSO$_4$·7H$_2$O, 303.70 μM). Alder

123 leaves from the sampling site were used as food source. The exposure experiment was conducted

124 in six 2 L-tanks for control and cadmium-exposed groups, respectively. Each tank contained both

125 acanthocephalan-uninfected *G. fossarum* (n = 16-17) and gammarids infected with *P. minutus* (n

126 = 14-16). All animals were kept in reconstituted water and cadmium was added to the exposed

127 groups to achieve a nominal concentration of 4 μg/L (diluted from a 1 g/L Cd standard, Cd(NO$_3$)$_2$

128 in 0.5 M HNO$_3$, Bernd Kraft GmbH). The duration of the exposure was 7 d at 15° C in a climate

129 chamber with 12/12 light/dark-cycle. This exposure time was considered to be long enough, as

130 Gismondi, Cossu-Leguille & Beisel (2012b) found effects of cadmium on energy reserves of

131 gammarids already after 96h exposure. Experimental tanks were aerated to saturation. No food

132 was provided during the experimental exposure. Water had a temperature of 14.5° C (± 0.5), a pH

133 of 8.1 (± 0.1) and a conductivity of 1010.1 μS/cm (± 7.8) in the control tanks and 14.3° C (± 0.5),

134 pH 8.0 (± 0.2) and 1015.1 μS/cm (± 17.2) in the cadmium tanks. Water samples for metal analysis

135 were taken from each tank at different time points: once before cadmium was added and after 5,

136 10, 20, 30, 60, 120, 240 min, followed by daily sampling until the end of the experiment. A sample

137 of 5 mL was filtered (cellulose nitrate filter, pore size 0.45 μm, Sartorius AG), acidified with 5 μL

138 HNO$_3$ (65% suprapure, Merck) and stored at -20° C until metal analysis. If present, dead

139 gammarids were removed from the tanks twice daily and were discarded. At the end of the

140 exposure period, gammarids were removed from the aquaria with a sieve, blotted dry and weighed.

141 Cystacanths were dissected from infected amphipods and stored in separate tubes. All samples

142 were snap frozen individually in 1.5 mL reaction tubes in liquid nitrogen and stored at -80° C.

143

Metal analyses

145 Cadmium concentrations in water samples, gammarids and cystacanths were determined by

146 electrothermal atomic absorption spectrometry (ET-AAS) as described in Sures, Taraschewski &

147 Haug (1995). Water samples from 2 tanks were pooled resulting in three samples for each group.

148 The acidified water samples were determined without any further pretreatment in duplicate. For

149 tissue analysis, whole gammarids (not tested for microsporidian infection status) or cystacanths

150 were pooled to achieve sample wet weight ranging from 55 to 80 mg for gammarids (2 males and

151 2 females with and without *P. minutus* from control and cadmium-treatment) or 11mg for

152 cystacanths (one pool of 69 for control and 66 for cadmium-treatment). Samples were digested in

153 a mixture of 1.3 mL nitric acid (65% HNO_3, suprapure, Merck) and 2.5 mL hydrogen peroxide

154 (30% H_2O_2, AppliChem) using a microwave digestion procedure described by Zimmermann et al.

155 (2001). To determine the detection limits, blanks were prepared without addition of sample.

156 Cadmium concentrations of samples were calculated by fitting linear regression lines to the points

157 defined by spiked samples and the corresponding integrated peak areas.

158

Molecular identification of microsporidians

160 After thawing, the sex of gammarids was determined according to male genital papillae or female

161 oostegites. Acanthocephalan infected individuals were dissected, cystacanths removed and stored

162 at -20° C for metal analysis. Each *G. fossarum* specimen was homogenized with micropestles in

163 0.1 M sodium phosphate buffer with 0.1 M KCl. The buffer volume was adjusted to the threefold

164   wet weight of every individual (e.g. 60 µL of homogenization buffer for 20 mg of wet weight

165   tissue). Samples were centrifuged at 14.000 x g for 15 min at 4°C. The supernatant was used for

166   quantification of total protein concentration and analysis of glycogen, lipid and 70 kDa heat shock

167   protein (hsp70) (see below). The remaining pellet was used for molecular diagnosis of

168   microsporidian infections.

169   DNA was extracted from pellets with a JETQUICK Tissue DNA spin kit (Genomed) according to

170   manufacturer's instructions. For molecular detection of microsporidian infections, the primers V1

171   5'-CAC CAG GTT GAT TCT GCC TGA C-3' (Zhu et al., 1993) and Micro_rev 5'-GAG TCA

172   AAT TAA GCC GCA CAA TCC AC-3' (Krebes et al., 2010), amplifying a part of the small

173   subunit ribosomal RNA gene (ss rDNA) of microsporidians, were used. For initial identification

174   of microsporidian species infecting the gammarids, PCR bands of 10 randomly selected samples

175   were gel purified with a JETQUICK PCR Product Purification Spin Kit (Genomed) according to

176   manufacturer's instructions and were sent for sequencing (GATC). All obtained sequences were

177   tested for matches in the GenBank by blast-search (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

178   According to the obtained sequences, specific primers for each of the three microsporidian species

179   detected were designed (see table 1) to test the remaining positive samples. All PCR reactions

180   contained 4 µL of 5x OneTaq Hot Start Buffer (New England Biolabs), 0.2 mM dNTP mix (New

181   England Biolabs), 0.5 µM of each primer (MWG Eurofins), 0.5 U OneTaq Hot Start (New England

182   Biolabs) and 1 µL template DNA. The mix was topped up to 20 µL with PCR grade water. The

183   DNA was amplified by a Labcycler (SensoQuest). PCR conditions for the V1/Micro_rev primers

184   were 94° C for 3 min, 40 cycles of 94° C for 30s, annealing at 60° C for 35s, elongation at 68° C

185   for 1min and a final elongation at 68° C for 3 min. The other primers were used under the following

186   conditions: initial denaturation at 94° C for 5 min, 35 cycles of 94° C for 30s, annealing at 60° C

187 for 35s and elongation at 68° C for 40s followed by a final elongation of 68° C for 3 min. PCR

188 bands were analyzed by conventional agarose gel electrophoresis. Specificity of diagnostic primers

189 was assessed by sequencing of three randomly selected PCR products of each primer pair and

190 comparison to the sequences obtained with the primers V1/Micro_rev.

191

192 **Biochemical analyses**

193 *Total protein analysis*

194 The amount of total protein in the samples supernatants was measured with a BCA Protein Assay

195 Kit (Pierce) according to manufacturer's instructions. A 10 µL aliquot of the supernatant was

196 diluted 1:10 with homogenization buffer and 25 µL of the diluted samples were subsequently

197 measured in triplicates in 96-well plates together with a dilution series of a bovine serum albumin

198 standard in a plate reader (Tecan infinite M200).

199

200 *Glycogen analysis*

201 The pellet obtained after the methanol/chloroform-extraction (see below) was dissolved in 200 µL

202 of deionized water. Of this solution, 100 µL were transferred into clean 15mL culture tubes with

203 4.9 mL of anthrone reagent (0.2 g of anthrone in 100 mL of 95% H_2SO_4). The mixture was heated

204 in a water bath at 95°C for 17 min, and was subsequently cooled on ice. The optical density was

205 measured at 625 nm in 96-well plates in triplicates. As a reference, a standard of 25 - 800 µg/mL

206 of glucose was used. Measurements were expressed as µg glycogen/µg total protein.

207

208 *Lipid analysis*

209 To measure total lipid and glycogen content we used the method described by Gismondi et al.

210 (2012a), which was adapted from Plaistow, Troussard & Cézilly (2001). After homogenization

211 and centrifugation (see above), 10 µL of 2% sodium sulfate (w/v) and 270 µL of

212 chloroform/methanol 1:2 (v/v) were added to 20 µL of supernatant. After 1 h incubation on ice,

213 the samples were centrifuged at 3000x g for 10 min at 4° C. The pellet was used for glycogen

214 analysis (see below). For determination of the lipid content, 100 µL of the supernatant were

215 transferred to clean reaction tubes. The tubes were incubated overnight at room temperature to

216 evaporate the solvent. Subsequently, 200 µL of 95% sulfuric acid were added and heated for 10

217 min at 95° C in a water bath. After cooling for 5 min, 4.8 mL of phospho-vanillin reagent were

218 added and the tubes were vortexed until development of a reddish color. After 10 min incubation

219 at room temperature, 300µL of the solution were pipetted into wells of a 96-well plate and

220 measured within 10 min at 535 nm in a plate reader. All samples were measured in triplicate. The

221 content of total lipid was determined from a series of cholesterol standard solutions in

222 concentrations of 100 - 2000 µg/mL. The measurements were expressed in µg lipid/µg total

223 protein.

224

225 *Heat shock protein 70 analysis*

226 Levels of hsp70 were analyzed by the method described in Frank et al. (2013). Briefly, 20µg of

227 proteins were separated by discontinuous SDS-Page, transferred to a nitrocellulose membrane by

228 Western Blot and visualized using monoclonal anti hsp70 antibodies (mouse anti hsp70, antibodies

229 online), a horseradish peroxidase labeled second antibody (goat-anti mouse, DAKO) and

230 detections by 4-chloro-1-naphthol. Gammarids that died during the experiment were excluded

231 from this analysis. To allow inter-gel comparability, a reference sample (fish liver homogenate)

232   was run on all gels. The hsp70-bands, were scanned and quantified densitometrically with ImageJ

233   (Abràmoff, Magalhães & Ram, 2004). All sample values were divided by the value of the standard

234   of the respective gel and were expressed as relative hsp70 values.

235

236   **Statistical analyses**

237   The weight difference of male and female gammarids was tested with the Welch two sample t-test

238   for unequal variances. A possible effect of the treatment on the mortality rate of gammarids was

239   tested with Kruskal-Wallis-test and Dunn's multiple comparison tests. The effects of gammarid

240   sex, microsporidian and acanthocephalan infection and exposure, as well as the influence of

241   parasitism on host size (separately for females and males) was analyzed by linear models and

242   subsequent ANOVA. Due to the variance heterogeneity, the GLS (generalized least squares fit by

243   restricted maximum likelihood, REML) function of the nlme-library (Pinheiro et al., 2014) was

244   used, which allows to correct for unequal variances. Possible correlations of the biochemical

245   parameters were tested with the cor.test-function (Pearson product moment correlation). The

246   statistical analyses were carried out in R v.3.0.1 (R Core Team, 2013) and graphs were created in

247   GraphPad Prism v5.0.

248

249   **Results**

250   **Prevalence of *P. minutus***

251   After the exposure experiment, a total of 299 living gammarids was recovered. Among these, 88

252   of the 160 males (55.0%) and 55 of the 139 females (39.6%) were infected with *P. minutus*. The

253   overall prevalence of this acanthocephalan in the test population was 48% (supplemental table S1).

254

Metal analyses

Cadmium levels in control water were lower than 0.1 µg/L during the experiment. In cadmium-exposed groups, concentrations were declining gradually after addition of stock-solution and reached a stable concentration of 2.89 µg/L on average after about 5 h (figure 1). The tissue concentrations of uninfected and *P. minutus*-infected control gammarids were below 0.2 µg/g. Cadmium-exposed female gammarids had almost ten-fold higher cadmium concentrations, and the respective males had close to eight-fold higher concentrations than control animals. Cadmium-exposed female gammarids accumulated cadmium to a higher extent than male gammarids (1.5 and 1.1 times higher for uninfected and *P. minutus*-infected group respectively). The cadmium concentrations determined in *P. minutus* cystacanths from cadmium-exposed gammarids were approximately four times higher than those of cystacanths from control gammarids (figure 2).

Microsporidian infections in gammarids

After the exposure experiment, 212 *G. fossarum* individuals were tested for microsporidian infections. Gammarids used for metal analyses could not be tested by PCR, so their microsporidian-infection status remains unclear. By sequencing, three different microsporidian isolates were obtained: (1) 1300 bp (GenBank accession no. KR871381), 99% similarity to *Microsporidium* sp. 505 (FN434085); (2) 433 bp (KR871382), undefined *Microsporidium* sp. MH with 93% identity to *Cystosporogenes* sp. (GQ379704) and (3) 680 bp (KR871380), 98% similarity to *Dictyocoela duebenum* (JQ673483). Due to the sequence divergence between previous isolates of *D. duebenum* and our *Dictyocoela* species, we can only make a putative identification here. The prevalences of the different microsporidians in the population are shown

277   in supplemental figure S1. Individuals infected only with *D. duebenum* were considered in

278   biochemical analyses, as this species was the most abundant.

279   In total, 65% of the gammarids were infected with either one or several microsporidian species

280   simultaneously. The prevalence of microsporidians was higher in males (77.7%) than in females

281   (48.4%). Infections with *D. duebenum* only were recorded in 70.8% of females and 62.3% of

282   males. In total 26.4% of gammarids were infected simultaneously with microsporidians and *P.*

283   *minutus* cystacanths, with a higher percentage of males (38.0%) compared to females (11.0%)

284   (supplemental table S1).

285

286   **Effects of infection on gammarid weight**

287   The mean weight of females (19.96 mg) and males (25.63 mg) differed significantly (Welch two

288   sample t-test; t = -5.18, p < 0.001; (figure 3). Additionally, acanthocephalan infected gammarids

289   had a significantly lower weight than their uninfected conspecifics (females: infected 18.84 mg vs

290   20.47 mg for uninfected gammarids, F = 5.90, p < 0.05; males: infected 22.94 mg vs uninfected

291   28.70 mg for uninfected gammarids, F = 11.07, p < 0.01). In contrast, microsporidian infection

292   was not correlated with the weight of gammarids (females: F = 0.01, p = 0.92; males: F = 0.27, p

293   = 0.61; figure 3).

294

295   **Mortality rate of gammarids**

296   In the control groups, mortality was slightly higher in the *P. minutus*-infected group (20.0%)

297   compared to the group of uninfected gammarids (17.0%, see figure 4). The mortality in cadmium-

298   exposed groups was overall higher, with a slightly higher death rate (27.0%) in uninfected

299   gammarids compared to *P. minutus*-infected ones (26.1%). On average, the mortality in the control

300 group was 18.4%, whereas 26.6% of the cadmium exposed gammarids died. No significant

301 differences between the groups were detected (figure 4).

302

303 **Biochemical analyses**

304 Uninfected, *P. minutus* infected, *D. duebenum* infected and gammarids infected with both parasites

305 were used for further analyses. At all a total of 54 female and 60 male gammarids was available

306 for the biochemical analysis. The output of the GLS model is shown in supplemental table S2.

307

308 *Glycogen*

309 Overall, cadmium exposure decreased glycogen levels, but the analyses indicated a significant

310 increasing effect of cadmium-exposure on glycogen levels in microsporidian infected female

311 gammarids. A similar increase was found in unexposed males (table 2, figure 5). Also, a significant

312 interaction was present for microsporidian and acanthocephalan infection showing increased

313 glycogen levels (table 2, figure 5, supplemental table S2). According to the GLS, increased

314 glycogen levels are also present in females compared to males, independent of exposure and

315 parasites (table 2, figure 5, supplemental table S2).

316

317 *Lipids*

318 Similar to glycogen, total lipids were significantly decreased by cadmium exposure, but this effect

319 was modulated by parasite infection. Also, the total lipid content was highly dependent of host

320 sex, with lower concentrations in males than in females (figure 6, table 2). The acanthocephalan

321 infection significantly decreased the total lipid level in all groups, except for cadmium-exposed

322 males, which is reflected by the significant interactive effect of host sex and *P. minutus* infection

323   (table 2, supplemental table S2). Microsporidians caused a significant increase of total lipids

324   especially in females. This increase was less pronounced in cadmium exposed individuals (figure

325   6, table 2).

326

327   *hsp70 levels*

328   For 3 samples (3 exposed, uninfected females), no hsp70 values were obtained (no visible band)

329   and 2 (1 exposed and 1 unexposed, uninfected female) dropped out as outliers (> 3 fold above

330   group average). The combination of cadmium exposure and microsporidian infection had a

331   significant increasing effect on hsp70 levels, while microsporidians alone had no effect on hsp70

332   (table 2, figure 7, supplemental table S2). Cadmium exposure also increased the hsp70 level in the

333   other infected and uninfected groups. Infection with *P. minutus* did not have a significant influence

334   on the hsp70 levels (table 2, supplemental table S2). Nevertheless, a slight increase in the hsp70

335   level can be observe for unexposed and acanthocephalan infected females according to figure 7.

336

337   Correlations

338   Correlation analyses revealed significant negative correlation between lipids and hsp70 (n = 109,

339   p < 0.001, ρ = - 0.32), and weak positive correlations between lipid and glycogen (n = 109, p <

340   0.01, ρ = 0.28). No significant correlation was found for hsp70 and glycogen.

341

342   **Discussion**

343   The present study provides data on combined and single effects of parasite infections

344   (Acanthocephala and Microsporidia) and cadmium-exposure on mortality, energy reserves of the

345   gammarid host and its stress response quantified by the hsp70 levels. Three microsporidian isolates

346   were detected in the naturally infected *Gammarus* population, including one novel isolate. For the

347   biochemical analyses, only infections with *D. duebenum* (species assignment putative) were

348   considered. The latter parasite was found frequently in different amphipod species and is

349   characterized by a vertical mode of transmission and is known to feminize the host population by

350   sexual conversion (Terry et al., 2004). Virulence of this microsporidian is considered to be low,

351   but sub-lethal effects on the host were not well studied until now. Surprisingly, we did not observe

352   a female bias among *Dictyocoela*-infected *G. fossarum* in the present study. Nevertheless, this

353   parasite might influence host metabolism in the attempt to elicit feminization. This might have

354   influenced the outcome of our analyses, but this effect remains speculative here.

355   According to our results, the weight of *G. fossarum* was lower in acanthocephalan infected male

356   and female hosts, while microsporidian infection showed no significant effect. This indicates that

357   either smaller individuals were more likely to be infected with *P. minutus*, or that this parasite

358   influences host feeding behavior and its growth rates (Agatz & Brown, 2014).

359   The cadmium concentration used in the present study was about factor 100 below the 96h LC50

360   values reported for *G. fossarum* in previous studies (Alonso, De Lange & Peeters, 2010; Boets et

361   al., 2012) therefore no severe mortality was expected. Nevertheless, besides a baseline mortality

362   of about 20% we observed an additional mortality of 6% - 10% in the cadmium exposed groups.

363   Our results showed also no negative effect of *P. minutus* on host mortality, while mortality was

364   slightly higher (although not significant) in cadmium exposed *G. fossarum*. Gismondi, Cossu-

365   Leguille & Beisel (2012b) found an increased cadmium tolerance in male *G. roeseli* infected with

366   *P. minutus*, while infected females became more susceptible to combined stressors. Such sex

367   dependent difference in susceptibility to cadmium was also reported for *G. fossarum*, where

368   females were less tolerant to the exposure than males (Schill, Görlitz & Köhler, 2003).

369   The energy reserves of *G. fossarum* were influenced by both cadmium-exposure and parasite

370   infection. Glycogen content increased in *D. duebenum* infected individuals with a heterogeneous

371   effect of host sex. Gismondi et al. (2012a) also reported an increase in glycogen in *Dictyocoela-*

372   infected *G. roeseli*, but a decrease after cadmium exposure. In another study (Gismondi et al.,

373   2012b), an increase of glycogen levels was detected depending on both microsporidian infection

374   and cadmium exposure, similar to the present study. Only females infected with microsporidians

375   were used in the latter study, therefore effects of host sex cannot be compared. In our study, slightly

376   elevated glycogen levels in acanthocephalan infected female amphipods were found. This effect

377   due to acanthocephalan infection was also described previously (Plaistow, Troussard & Cézilly,

378   2001), but the sex effect was dependent on the season when amphipods were sampled (Gismondi,

379   Beisel & Cossu-Leguille, 2012b). An increase in glycogen levels in acanthocephalan infected

380   individuals seems to be a common phenomenon. It might be explained by the increased uptake of

381   nutrients due to extended energy requirements of the infected host or due to a quick mobilization

382   of energy storage during the growth phase of the parasite (Sparkes, Keogh & Pary, 1996). In

383   contrast, the need for detoxification of pollutants might cause an increased metabolic activity that

384   depletes glycogen storages. Furthermore, presence of both acanthocephalan and microsporidian

385   infection caused significantly increased glycogen concentrations. This is in contrast to Gismondi

386   et al. (2012b) who found depleted glycogen levels in *G. roeseli* infected with microsporidians and

387   acanthocephalans at the same time.

388   Similar to glycogen, an increase of total lipids was observed in males and females of *G. fossarum*

389   infected with the microsporidium *D. duebenum*, while cadmium-exposure had a decreasing effect.

390   Previously, a similar increase in lipid levels due to microsporidians compared to the uninfected

391   control was found for *G. roeseli* exposed to a nominal cadmium concentration of 8 µg/L (Gismondi

392   et al., 2012b), but not at 2 µg/L or in unexposed control groups. Therefore, the nominal cadmium

393   concentration of 4 µg/L (2.9 µg/L measured) used in the present study might be the threshold that

394   caused an effect in the exposed amphipods. Interestingly, the opposite was observed in *G. roeseli*

395   infected with *P. minutus*, where decreased lipid levels were reported after exposure to 2 µg/L

396   cadmium concentration, but not to 8 µg/L (Gismondi et al., 2012b; Gismondi, Beisel & Cossu-

397   Leguille, 2012b). In the present study, the acanthocephalan infected group showed slightly

398   decreased lipid levels, which was most pronounced in females independent of exposure condition.

399   Similar to glycogen storage, the parasite might drive host metabolism to utilize the lipid storage

400   depending on its own requirements. The variable results observed for the lipid content in different

401   studies could be due to seasonal variation in reserves substances like lipids or glycogen (Gismondi,

402   Beisel & Cossu-Leguille, 2012a,b). Additionally, they could indicate different response patterns

403   to cadmium and parasites by the two host species *G. fossarum* and *G. roeseli*.

404   To summarize, the response of *G. fossarum* and *G. roeseli* to cadmium and parasites on the level

405   of energy reserves is essentially similar although slight species-specific differences might be

406   present. Due to the positive correlation between glycogen and lipid levels in the present study, it

407   can be assumed that they respond similarly to parasite infection. Especially lipids represent

408   nutrient reserves to prevent starvation and to provide energy for reproduction; therefore changes

409   of lipid levels represent long-term physiological effects in the host that might impair its survival

410   and that can have an effect on the population development under stressed conditions (Cargill et

411   al., 1985).

412   Increased levels of hsp70 indicate a general stress response (protein damage) of an organism, e.g.

413   to chemicals or effects of parasite infections (Frank et al., 2013). In the present study, a clear stress-

414   effect was detected in microsporidian-infected *G. fossarum* exposed to cadmium, while no

415   significant effect of infection with *P. minutus* was observed. A similar increase of hsp70 due to

416   multiple microsporidian infection was already observed previously in *G. pulex* (Grabner,

417   Schertzinger & Sures, 2014). In the study of Gismondi et al. (2012b), malondialdehyde as a marker

418   for lipid oxidation and therefore cellular damage, similar to hsp70, was increased in *G. roeseli*

419   after cadmium-exposure. This effect was even more pronounced, if the amphipods were

420   additionally infected with microsporidians, acanthocephalans or with both together. These findings

421   provide evidence for the idea that intracellular replication of microsporidians causes a

422   destabilization of cell metabolism leading to protein damage (measured by hsp70 or

423   malondialdehyde), if additional stressors (here cadmium) are present.

424   An irregular pattern of the hsp70 response was observed in previous studies, caused by the

425   combined effect of *P. minutus* infection and thermal or chemical stress on gammarids. No

426   increased levels of hsp70 were detected in infected *G. roeseli* after exposure to either heat or

427   palladium (Sures & Radszuweit, 2007). Similar results were reported for *G. fossarum* infected

428   with *P. minutus,* comparing uninfected and cadmium-exposed animals (Frank et al., 2013). Such

429   response patterns were not observed in the present study, as *P. minutus* induced no considerable

430   hsp70 response. Again differences between the amphipod species might provide an explanation.

431   Peschke et al. (2014) found remarkable differences in the hsp70 response of uninfected *G. pulex*

432   and *G. roeseli* populations from sampling sites with different pollutant burdens. The response of

433   hsp70 in *G. pulex* showed variations, while no effect was seen in *G. roeseli* from the same sites.

434   Similar differences might exist between *G. roeseli* and *G. fossarum*. Nevertheless the different

435   findings of Frank et al. (2013) and the present study, both using *G. fossarum*, remain to be

436   elucidated. Interestingly, a negative correlation was found between hsp70 and lipids, which

437   indicates that individuals with depleted energy reserves are more prone to stress effects.

438   In the present study cadmium-concentrations were slightly lower in males of *G. fossarum* than in

439   females. No significant differences were detected if *P. minutus*-infected and uninfected amphipods

440   were compared. In contrast, significant lower cadmium-concentrations were detected in *G. roeseli*

441   (Gismondi, Cossu-Leguille & Beisel, 2012b) or *G. fossarum* (Frank et al., 2013) infected with *P.*

442   *minutus* although both studies used similar sublethal concentrations of cadmium. This might be

443   explained by the low number of individuals analyzed in the present study that did not allow

444   detection of such differences. Cystacanths showed a lower cadmium accumulation potential

445   compared to host tissues, but this finding is based only on one replicate. Similar findings were

446   described before for other host-parasite systems, e.g. *Asellus aquaticus* - *Acanthocephalus lucii*

447   (Sures & Taraschewski, 1995), *G. pulex* - *Pomphorhynchus laevis* (Siddall & Sures, 1998) as well

448   as for *G. fossarum* - *P. minutus* (Frank et al., 2013).

449

450   **Conclusion**

451   Microsporidian infections were found to influence the metabolism and the stress response of *G.*

452   *fossarum* exposed to sub-lethal cadmium concentrations. In contrast, the effect of *P. minutus*

453   cystacanths on energy reserves and stress response of the host was less pronounced. Our data

454   together with data from previous studies indicates slight species dependent differences in the

455   response to multiple stressors (e.g. parasitism and metal pollution). Furthermore, we highly

456   recommend that parasitism should be taken into account in future ecotoxicological surveys, as

457   various aquatic organisms are commonly infected and monitoring data could be misinterpreted if

458   their infection status is not considered.

459

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463

464   **References**

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Primers

Table 2. Specific primers used to detect microsporidians.

Primer	Sequence (5' - 3')	Amplicon length	Source
Mspec 505	F: CAT CAA CTA ACT TTG GGA AAC TAA G R: TGG CCT CCC ACA CAT TCC GAG TG	1300 bp	Grabner et al., 2014
Micro MH	F: GTA GAA CTG CGA TGA TTT AGT CTG R: GCT ATA CCA TGT TCC CCA TTG	433 bp	this study
Dict	F: GGG CGA TTT ATT TGT TCT CCT GT R: TGA TTT CTC TTC CGC AAT ACC AAA TC	680 bp	this study

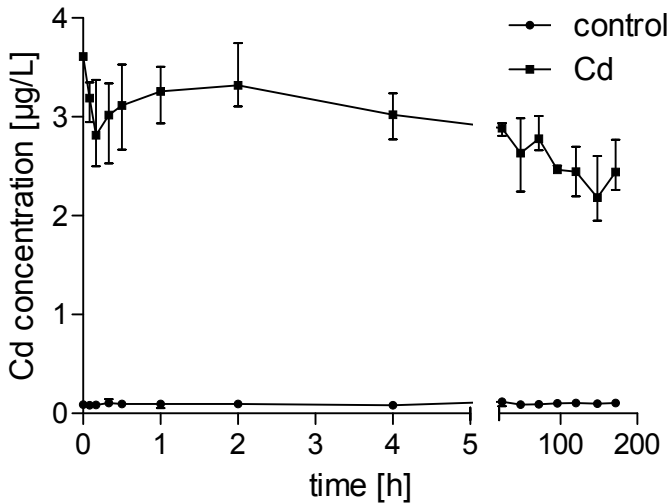
Analysis

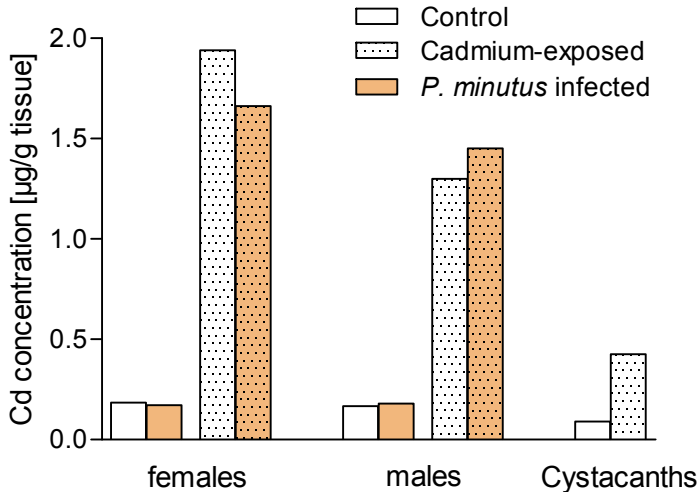
Table 4. Results of the ANOVA of the different groups on biochemical parameters

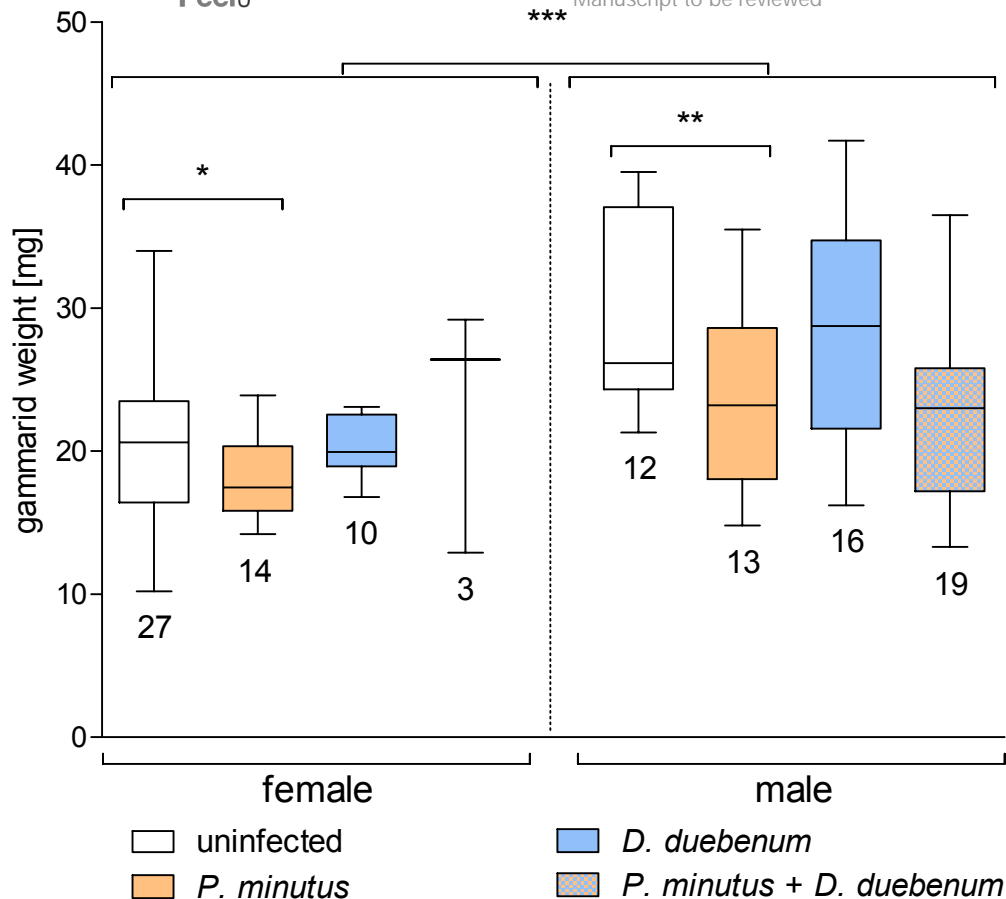
Group	DF	Glycogen F-value	p-value		DF	Lipid F-value	p-value		DF	hsp70 F-value	p-value	
(Intercept)	1	10475360,20	0,000		1	20852,74	0,000		1	772,39	0,000	
sex	1	52,96	0,000	***	1	82,41	0,000	***	1	2,63	0,108	
expo	1	19,58	0,000	***	1	6,30	0,014	*	1	13,40	0,000	***
micro	1	39,65	0,000	***	1	23,05	0,000	***	1	2,18	0,143	
acantho	1	0,39	0,535		1	10,31	0,002	**	1	0,98	0,325	
sex:expo	1	0,09	0,768		1	0,00	0,986		1	6,78	0,011	*
sex:micro	1	0,00	0,996		1	4,14	0,045	*	1	0,01	0,938	
expo:micro	1	5,90	0,017	*	1	3,39	0,068		1	13,06	0,000	***
sex:acantho	1	4,40	0,039	*	1	10,92	0,001	**	1	2,48	0,119	
expo:acantho	1	0,16	0,690		1	4,31	0,041	*	1	3,76	0,055	
micro:acantho	1	4,07	0,046	*	1	2,38	0,126		1	0,58	0,448	
sex:expo:micro	1	8,56	0,004	**	1	0,00	0,987		1	0,03	0,855	
sex:expo:acantho	1	1,47	0,228		1	0,35	0,556		1	0,79	0,376	
sex:micro:acantho	1	1,21	0,273		1	0,24	0,624		1	1,19	0,277	
expo:micro:acantho	1	0,15	0,699		1	0,41	0,523		1	2,28	0,134	
sex:expo:micro:acantho	1	0,62	0,434		1	5,28	0,024	*	1	0,48	0,489	

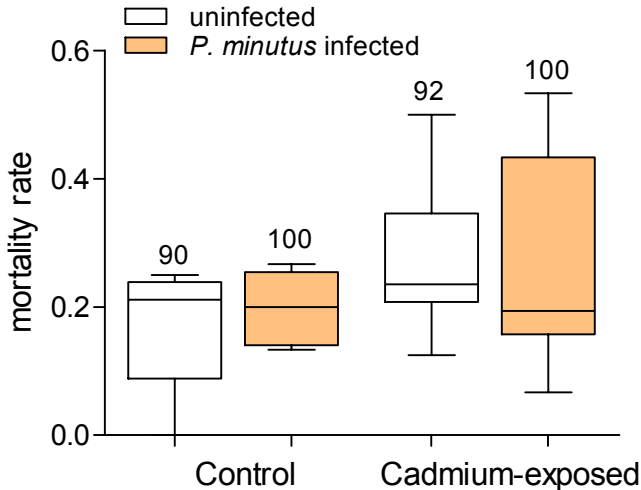
sex: host sex; expo: exposure condition; micro: microsporidian infection; acantho: acanthocephalan infection; DF: degrees of freedom

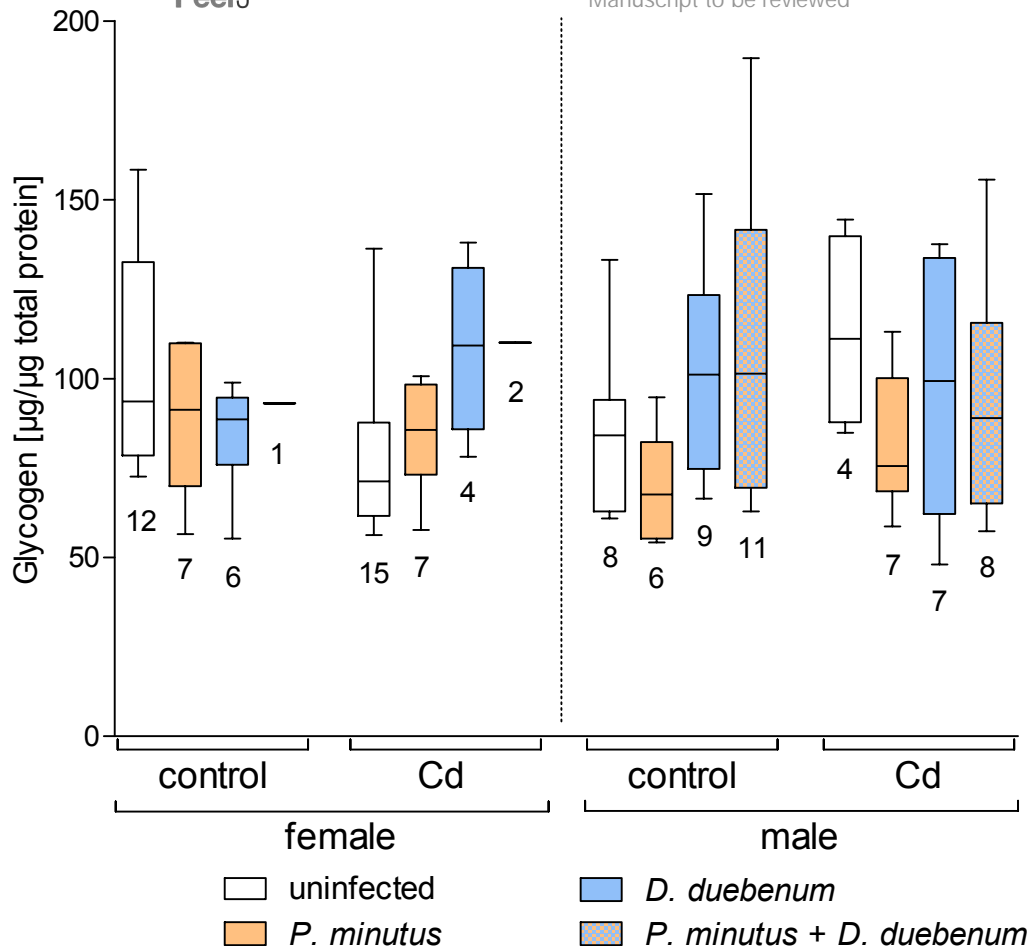
Water concentrations

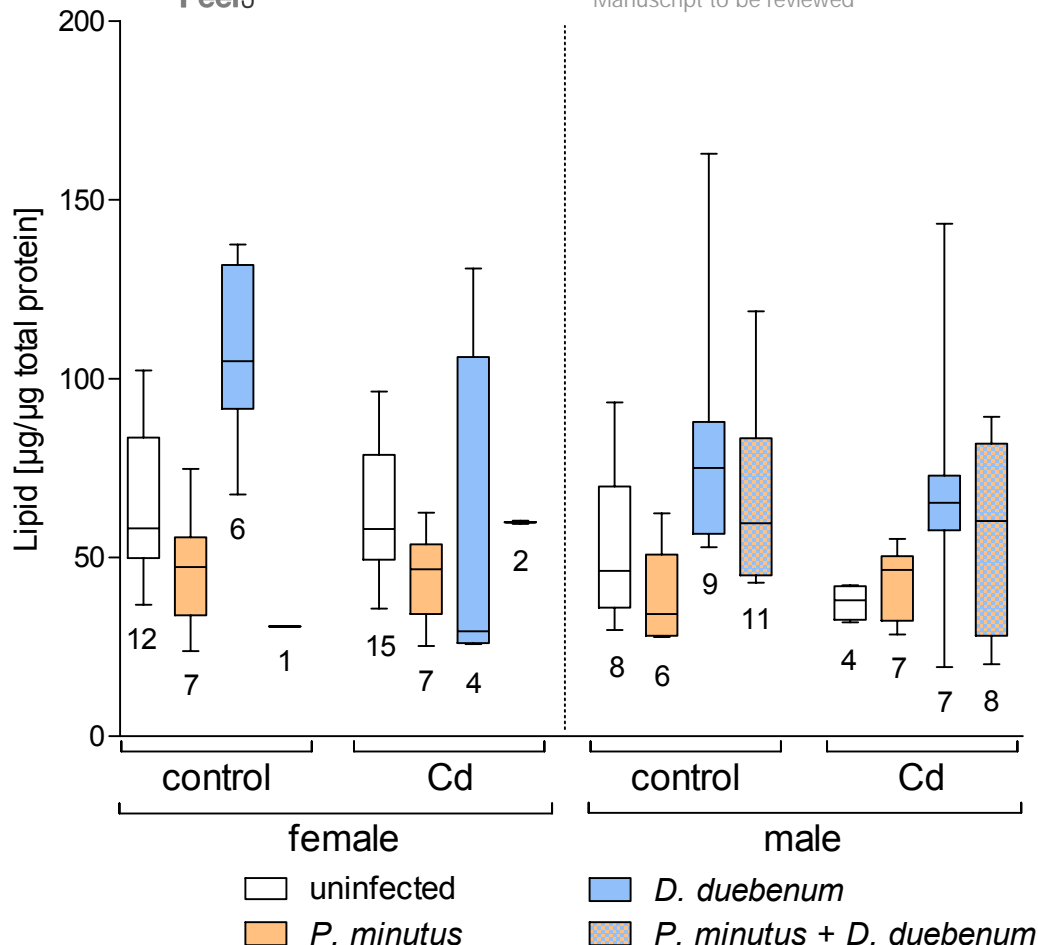
Water concentrations of cadmium in control and exposure tanks over time. n = 3.

Cadmium concentrations in tissues

Cadmium concentrations in pools of *P. minutus* infected and uninfected *G. fossarum* (n=2) and *P. minutus* cystacanths (n=1).

Gammarid weight

Box plot of gammarid weight depending on infection and sex. Numbers below boxes show number of gammarids. Asterisks indicate significant differences.

Mortality rate of *G. fossarum*

Mortality rate of *G. fossarum* in the test groups (n=6). No significant differences between the groups were detected. Numbers above bars indicate number of gammarids.

Glycogen content

Glycogen content in infected and uninfected *G. fossarum* females and males after cadmium exposure. Numbers below boxes show number of gammarids. For significant differences see table 4.

Lipid content

Lipid content in infected and uninfected *G. fossarum* females and males after cadmium exposure. Numbers below boxes show number of gammarids. For significant differences see table 4.

Relative hsp70 content

Relative hsp70 content in infected and uninfected *G. fossarum* females and males after cadmium exposure. Numbers below boxes show number of gammarids. For significant differences see table 4.