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Exposure to a Mixture of Zinc and Copper Decreases Survival and Fecundity of *Discocotyle sagittata* (Leuckart) Parasitizing Juvenile Atlantic Salmon, *Salmo salar* L.

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Abstract We assessed the effects of zinc and copper on freshwater monogenean ectoparasites (*Discocotyle sagittata* Leuckart) infecting juvenile Atlantic salmon (*Salmo salar* L.). Exposure to 47 μg/L zinc and 3 μg/L copper reduced survival and fecundity of adult *D. sagittata*, while egg hatching success was only reduced at high exposure concentrations (2704 μg/L zinc and 164 μg/L copper). Parasitized salmon had decreased plasma chloride, but this was negated in infected fish exposed to metals. No other effects on Atlantic salmon survival and physiology (plasma osmolality, hematocrit) were noted, suggesting that *D. sagittata* may be more susceptible to metal toxicity than its host fish.

Keywords Parasites · Metals · Fish physiology

The susceptibility of most parasite taxa to specific types of pollution remains largely unknown. This complicates the interpretation of field-based studies of the interaction of pollution and parasitism, because it is difficult to conclusively attribute observed changes in parasitism to specific contaminants without subsequent experimental verification (Overstreet 1997; Marcogliese 2005). It also complicates the identification of pollution-susceptible parasite taxa that could be used as indicators in biological assessment (Marcogliese 2005). Several studies have attempted to address this knowledge gap, by exploring the responses of fish parasites to sewage (Escher et al. 1999), hydrocarbon-contaminated sediments (Khan and Payne 2004), and metals (e.g., see Gheorgiu et al. 2007), as well as the survival and infectivity of digenean transmission stages in controlled laboratory experiments (e.g., see Morley et al. 2003). However, more information on the susceptibility of potential indicator species to specific contaminants under controlled conditions is required (Poulin 1992; Lafferty 1997; Marcogliese 2005).

Prior field work in rivers in northeastern New Brunswick, Canada, indicated that *Discocotyle sagittata* (Leuckart), a common monogenean gill parasite of North American salmonids in fresh water, was largely absent on Atlantic salmon (*Salmo salar* L.) at sites downstream from a metal mine site, despite being abundant at upstream and reference sites (Blanar 2008). The absence of this parasite could not be attributed to any biological (Atlantic salmon population structure and condition; fish community composition) or physical (depth, elevation, conductivity, flow regime, substrate size) variables (Blanar 2008). The only factor strongly correlated with decreased parasitism was position downstream of the mine. Although the mine ceased operations in 1999, several metals still approached or exceeded Canadian Water Quality Guideline (CWQG) levels for the protection of aquatic life at sites close to the mine, and concentrations of both zinc and copper exceeded background levels for several kilometres downstream (EcoMetrix 2004). This field data suggested that *D. sagittata* was susceptible to zinc and copper. The present study was designed to verify this experimentally. Our primary

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hypothesis was that *D. sagittata* would be sensitive to metals at certain stages of their life cycle, so we examined the effects of a zinc and copper mixture on parasite survival, fecundity, and hatching success. Our secondary hypothesis was that fish with prior infections of *D. sagittata* would be unable to withstand the additional stress imposed by the metals, resulting in the death of any infected fish downstream of the mine. Therefore, we also examined the effects of the parasite and metal mixture on Atlantic salmon survival, growth, and osmoregulatory function.

Materials and Methods

Atlantic salmon parr (age 1+ and 2+) were captured by electrofishing from the Northwest Miramichi River, New Brunswick, Canada (47°11′13.04″ N, 65°53′39.71″W), transported live under aeration to the University of New Brunswick at Saint John, Saint John, New Brunswick, Canada. They were held in aerated 200 L tanks supplied with 4 L/min carbon-filtered dechlorinated municipal tap water and gradually transferred to a pelleted feed diet (1.5–2.0 mm, Corey Feed Mills, Fredericton, NB). Three weeks prior to the beginning of the experiment, fish were anesthetized (buffered tricaine methane sulfonate or TMS, 0.1 g/L, Syndel, Vancouver, BC), weighed (wet weight, ± 0.1 g), measured (fork length, ± 1 mm), and their gills examined for the presence of *D. sagittata*. Infected and uninfected fish of similar sizes were allocated among three possible treatments: uninfected fish were placed in the 'control' group; *D. sagittata*- infected fish were randomly divided into the 'parasite' group, which received no additional treatment, and the 'combined' group, which were additionally exposed to water contaminated by zinc and copper as outlined below. Three replicate tanks of five fish were allocated to each treatment. There were no initial differences in fish length or weight among tanks and among treatments (GLM; length: $F = 0.916, \mathrm{DF} = 2, p = 0.41$; weight: $F = 2.339; \mathrm{DF} = 2$, $p = 0.11$; GLM, $p > 0.05$ for all treatment × tank interactions).

Individual tanks were supplied with 0.5 L/min flow-through filtered dechlorinated tap water. The tanks in the 'combined' treatment were also individually supplied with 10 mL/min of a stock solution of dechlorinated tap water, zinc chloride, and copper chloride (ZnCl2 and $CuCl_2{\cdot}2H_2O$, Anachemia, Lachine QC), such that the final exposure concentration in the 'combined' treatment was 47 µg/L zinc and 3 µg/L copper. These exposure levels exceeded Environment Canada CWQG for the protection of aquatic life, and approximated peak discharge levels at the field sites where *D. sagittata* was absent (see EcoMetrix 2004; Blanar 2008). Continuous delivery of the stock solution was provided via a Masterflex L/S 77300-50 peristaltic pump equipped with nalgene tubing. Water quality was excellent throughout the experiment (pH: 6.6 ± 0.2; hardness: 14 ± 4 mg/L; sodium 4 mg/L; chloride 4.3 mg/L; total organic carbon 3 mg/L; selenium, total nitrate, ammonia and phosphate below detection limits; oxygen at saturation as temperature gradually increased from 9 to 12°C). Fish were fed once daily ad libitum, and excess feed and feces removed daily. No mortalities were observed during the test period, and fish in all treatments were observed to feed normally throughout the experiment. Photoperiod was set to a 12 h light: 12 h dark cycle.

After 32 days, fish were anesthetized in TMS, weighed and measured. Caudal blood samples were collected using a 1 mL heparinized syringe. Hematocrit was assessed from duplicate samples of whole blood drawn into microcapillary tubes and centrifuged for 5 min at 10,000×g. Remaining blood was centrifuged in 1.5 mL Eppendorf tubes for 4 min at 10,000×g. Plasma osmolality was measured directly on duplicate 20 µL samples using a Model 3300 advanced micro-osmometer (Advanced Scientific Instruments), and chloride concentration of the supernatant plasma was assayed using a digital choridometer (Model 442–5000, Labonco Instruments).

Immediately following the collection of blood, fish were killed by cervical dislocation, carefully decapitated to avoid damaging gill tissue, and the heads placed in 10% buffered formalin. Subsequently, gill arches were individually removed and examined for the presence of *D. sagittata*. All *D. sagittata* were enumerated, measured using a stage micrometer under ×2–×10 magnification (±10 µm), and their fecundity estimated by counting the number of eggs visible in the uterus. The presence of juvenile *D. sagittata* was also noted; immature worms could be discerned based on their small size, the absence of at least one pair of clamps or the reduced size of the most-recently formed clamps, and the absence of eggs. Parasite infection prevalence (% of fish infected with at least one parasite) and intensity (number of parasites per infected fish) were calculated as per Bush et al. (1997).

One-sample Kolmogorov–Smirnov test (SYSTAT 2004) was used to verify assumptions of parametric tests for final fish length, weight, growth, plasma osmolality, plasma chloride, and hematocrit. All data met these assumptions except for hematocrit, which required arcsine square root transformation prior to analysis. Treatment effects were tested using GLM with treatment as a main factor and tank as a nested factor in SYSTAT. Effects on the length-weight relationship were examined by performing linear regression on log-transformed data, and comparing the slopes of the lines using the ANCOVA procedure outlined in Zar (1996); values >5 standard errors from the mean were removed as outliers. Parasite mortality was inferred by comparing initial and final *D. sagittata* infection

prevalence (percentage of fish infected with at least one worm), and the percentage of fish carrying immature parasites was calculated. The effects of metal exposure on the prevalence of adult and juvenile parasites were each assessed using a Chi-square (χ^2) test (Zar 1996). The effects of metal exposure on infection intensity (the number of worms carried by an individual infected fish) and parasite length (in mm) were determined using a Mann–Whitney test (Zar 1996), while effects on fecundity (as number of eggs visible in uterus) were assessed using GLM in SYSTAT, with treatment as a main factor and tank as a nested factor. There were no significant treatment × tank interactions for any analyses.

In a second experiment, *D. sagittata* eggs were collected using the protocol outlined by Gannicott and Tinsley (1997). Eggs were siphoned from the bottom of a 200-L tank holding naturally-infected fish. To ensure that all eggs were at similar developmental stages, egg collections were initiated following transfer of fish to a new tank that was washed and rinsed with hot water before use. Eggs were collected over 2 days by siphoning water from the bottom of the tank through 105 μm mesh screen. The screens were rinsed into glass jars and allowed to settle. Subsamples of the settled particles (including a minimum of 300 eggs) were pipetted into twelve (12) mason jars containing 250 mL of aerated dechlorinated, filtered municipal tap water ('controls') or water containing a solution of zinc chloride and copper chloride such that the final concentrations were 26 μg/L zinc and 1 μg/L copper; 271 μg/L zinc and 16 μg/L copper; and 2704 μg/L zinc and 164 μg/L copper. The lowest dose level was selected because it represents typical levels at sites adjacent to the mine, while the middle and highest doses represent historical maximal values recorded in the area. Each of the four treatments was replicated three times. Jars were aerated and covered with parawax to limit evaporation; solution levels were topped up to 250 mL as necessary. All jars were housed in a flow-through water bath to stabilize and homogenize temperature, which varied between 12 and 14°C during the exposure period. Beginning on day 20, subsamples of eggs were pipetted from each jar onto glass slides. To ensure that each subsample represented a random sample, each jar was gently agitated by hand (using a rotating motion) between subsamples. The first 30 eggs were examined on each slide, and the percentage that had hatched was recorded; hatched egg shells were clearly distinguishable from unhatched eggs. The presence of larval *D. sagittata* was also noted.

Hatching percentage was compared among treatments using repeated measures GLM in Systat 11, with metal exposure level as a fixed effect factor and time as a repeated factor (SYSTAT 2004), on arcsine square root transformed data. The model statement tested the main treatment effects (control + 3 metal exposure levels) over time; treatment and time interaction, and differences among replicates (nested factor within treatment). Post-hoc multiple comparison tests were conducted using the Bonferroni procedure, as outlined in Zar (1996).

Exposure zinc and copper concentrations were determined by ICP-AES (Optima 5300 DV, Perkin Elmer, Shelton, CT USA), based on three replicate samples per treatment, filtered at 0.45 μm, and acidified with 2 mL/L nitric acid. Copper and zinc concentrations were monitored at wavelengths of 324.752 and 213.857 nm; detection limits were 0.2 μg/L for zinc and 0.4 μg/L for copper.

Results and Discussion

The primary objective of this study was to examine the effect of a metal mixture on survival and fecundity in *D. sagittata* infecting juvenile Atlantic salmon. *D. sagittata* were clearly negatively affected by exposure to zinc and copper. Parasite prevalence decreased from 100% (15 of 15) among unexposed infected fish to 73% (11 of 15) in infected fish exposed to zinc and copper, with at least one fish being completely free of parasites in each replicate tank within that treatment (Table 1). However, there was no statistically significant effect of metal exposure on mean intensity of infection, or on the incidence of new infections as indicated by the presence of juvenile worms. Parasite mean length was similar in both treatments. The parameter most affected by zinc and copper exposure was fecundity, which decreased by 46%, from 6.0 ± 1.2 eggs per individual in the 'infected' treatment to 3.3 ± 1.6 eggs in the 'combined' treatment. This decrease in fecundity was observed in all metal- exposed tanks (treatment by tank interaction $p > 0.05$).

A second experiment monitored in vitro egg hatching success at several exposure levels of zinc and copper. Egg hatching was first noted after 20 days of incubation. The percentage of eggs hatched varied over time (Fig. 1; RM ANOVA, $F = 413.38$, DF = 3, Greenhouse-Geisser adjusted $p < 0.01$), increasing significantly between day 25 and 30 (Bonferroni adjusted $p \leq 0.02$ for all treatments), and stabilized between day 30 and 35 (Bonferroni adjusted $p \geq 0.23$ for all treatments). Live, actively swimming larvae were observed as of day 20 in all treatments, and there was no apparent effect of treatment on their short-term survival. Reduced hatching success was only observed in the group exposed to the highest metal concentrations, with egg hatching success significantly inhibited compared to controls as of day 25 (RM ANOVA, $F = 102.63$, DF = 3, Greenhouse-Geisser adjusted $p = 0.01$).

To our knowledge, this is the first experimental test of the susceptibility of *D. sagittata*, a monogenetic ectoparasite, to metals. The most significant result of this study is

Table 1 *Discocotyle sagittata* infection rates and fecundity (mean ± SD) in dechlorinated municipal tap water (Parasite) and water laced with a mixture of zinc and copper (Combined); statistically significant differences are in bold text

	Parasite	Combined	Test used	Test statistic		DF	*p*
Prevalence (% infected)	100	**73**	Chi-square	χ^2	4.62	1	**0.03**
Intensity (#/fish)	1.9 ± 1.1	1.3 ± 1.0	Mann–Whitney	U	85.0	1	0.23
Length (mm)	6.9 ± 1.3	7.1 ± 1.1	Mann–Whitney	U	55.0	1	0.17
Fecundity (eggs/parasite)	6.0 ± 1.2	**3.3 ± 1.6**	GLM	F	5.512	1	**<0.01**
Immature worms	3	0	Chi-square	χ^2	3.33	1	0.07

DF degrees of freedom; please refer to "Materials and Methods" for further details on the statistical tests used

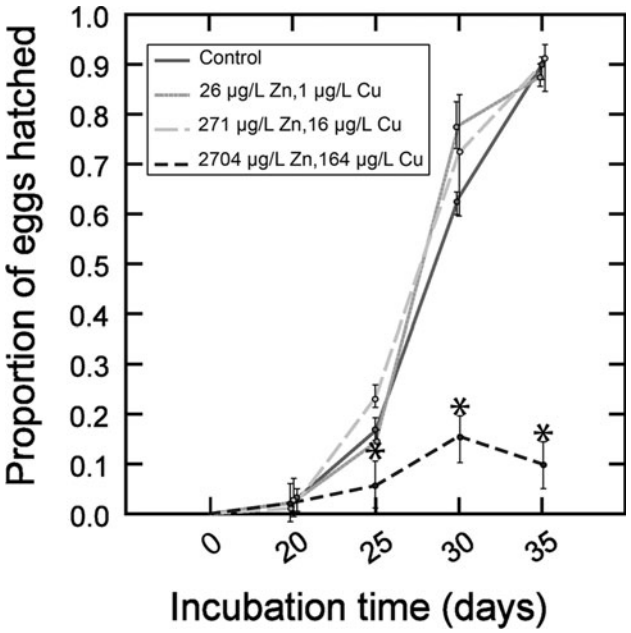

Fig. 1 Proportion (±SD) of *Discocotyle sagittata* eggs hatched over time in filtered tap water (control), and water laced with increasing concentrations of zinc and copper. Egg hatching success was significantly lower relative to controls only in the highest treatment level, as of day 25 (indicated by *asterisks*)

that exposure to a mixture of zinc and copper at environmentally realistic concentrations led to 27% mortality and a 48% decrease in fecundity of adult *D. sagittata*. It is not known whether this would be sufficient to extirpate *D. sagitatta* from sites downstream of a metal mine. Most parasites require a minimum density of infected hosts for successful transmission (e.g., see Poulin 2007), but the dynamics of natural populations of *D. sagittata* remain unknown. For instance, there is no information on instantaneous death rates of eggs and larvae, or larval transmission rates (see Poulin 2007 for parasite population model requirements). However, we theorize that a long-lived parasite with relatively low fecundity, such as *D. sagittata*, would be particularly prone to extirpation under conditions of increased mortality and drastically reduced fecundity.

The second experiment indicated that the hatchability of *D. sagittata* eggs was only reduced relative to controls at the highest exposure level. Although such concentrations are unlikely to be observed at our field sites, they could occur near mines that are active and/or have fewer mitigation measures in place, and *D. sagittata* egg survival could conceivably be reduced at such locations. That said, the results of this experiment suggest that the egg stage of *D. sagittata* is relatively resistant to metal exposure when compared with the adults. Furthermore, free-swimming larvae were observed in all treatments (data not shown), suggesting that initial short-term survival of the larvae was not affected. However, as pointed out by Gannicott and Tinsley (1998), larvae that survive to hatch are not necessarily infective. Future experiments should examine the infectivity of *D. sagittata* larvae exposed to metals, as toxic effects have been noted in other parasites (Morley et al. 2003).

Taken together, the information generated from field surveys of infection rates in relation to the mine, combined with the results of the present study, suggest that *D. sagittata* is susceptible to metal contamination. Many parasites have poorly-developed detoxification pathways and insufficient or absent production of metallothionein (Morley et al. 2003). However, the physiological mechanisms of metal toxicity in *D. sagittata* remain unknown, as is the case with most parasite taxa. Regardless of the mechanisms, it is clear that many parasites are sensitive to metals, often more than their hosts. For instance, Soucek and Noblet (1998) found that the digenean *Posthodiplostomum minimum* was more sensitive to copper than its mollusc or fish hosts. At least in some cases, parasites may be the most sensitive species in a given system. Certainly, this seems to be the case for *D. sagittata*, which was absent on juvenile salmon downstream of the mine, whereas benthic invertebrates and fish community had returned to near normal levels following initiation of mitigation measures at the mine (EcoMetrix 2004; Blanar 2008). Several environmental factors may have influenced monogenean parasite abundance at these sites, including biotic factors (e.g., availability and density of potential host fish) and physical

parameters (e.g., temperature, water flow, water chemistry); however, a related field-based project found that these factors did not significantly influence abundance of *D. sagittata* (Blanar 2008). Furthermore, a recent meta-analysis of parasite toxicity studies found that ectoparasites in general- and monogeneans in particular- are sensitive to metal pollution (Blanar et al. 2009). This raises the possibility of using monogenean ectoparasites such as *D. sagittata* as indicators of metal pollution. Indicators that are sensitive to exposure levels at or near regulatory endpoints can serve as early warning signs that a site is exposed, allowing for mitigation measures to be initiated before more 'desirable' species, such as fish, are affected. At the least, they can red flag a potentially problematic area for further study.

The first experiment also examined the effects of parasite infection, and combined exposure to parasite infection and a metal mixture, on growth and physiological parameters in juvenile Atlantic salmon. All fish survived to the conclusion of this experiment. There were no significant treatment effects on final length, final weight, or growth in terms of weight gain (Table 2). The relationship between length and weight (i.e., fish condition) was not affected, as there was no difference among treatments in slope (ANCOVA, $F_{2,33} = 0.303$; $p = 0.74$) or intercepts ($F_{2,35} = 1.088$; $p = 0.348$). There was no effect on hematocrit or plasma osmolality, but plasma chloride was significantly reduced in the 'parasite' treatment group (Table 2). There were no other effects of parasite infection alone, or in combination with metal exposure. We therefore conclude that the absence of *D. sagitatta* at sites downstream of the metal mine is probably not due to increased mortality of infected fish via the combined stresses of parasites and metals.

Although we did not examine the effects of metal exposure alone due to logistic constraints, the physiological effects of waterborne zinc and copper on salmonid gill physiology have been well-examined. Both metals are ionoregulatory toxicants. Zinc inhibits active uptake of Ca^{2+} across the gill surface, while copper inhibits Na^+K^+ATPase, which in turn inhibits influx of Na^+ and Cl^- and disrupts acid–base regulation (Wood 2001). However, these effects are noted at exposure levels that are higher than those used in the present study. For instance, the worst effects of zinc do not occur below 600–800 μg/L Zn, and salmonids are known to acclimate to chronic sublethal zinc exposures of 150 μg/L (vs. 47 μg/L Zn in present study; Wood 2001). Similarly, gill ionoregulation has been observed at 12 μg/L in freshwater, and fish coughing frequency increases at copper exposures as low as 6–15 μg/L; however there is no clear evidence that these exposure levels cause any toxic effects (contrast with the 3 μg/L Cu used in present study; Wood 2001).

Indeed, the only physiological effect noted in the present study was a slight decrease in plasma chloride in salmon parasitized by *D. sagittata*. The biological significance of this result is difficult to interpret, given that no concurrent change was observed in overall plasma osmolality. Variation in plasma osmolality may have been offset by changes in plasma sodium, lactate, and glucose, which were not measured. Furthermore, this decrease in plasma chloride was ameliorated when infected fish were exposed to the metal mixture. Given the negative effects of metals on *D. sagittata*, it is possible that zinc and copper mitigated any negative impacts of *D. sagittata* on salmon performance and physiology via direct toxic effects on the parasite.

The possibility remains that the observed effects were indirect, due to metal-induced alterations of host physiology affecting attachment and survival of the parasite, rather than via direct toxicity to the parasite. We consider this unlikely, for several reasons. First, unpublished preliminary work did not detect any physiological changes at these exposure levels. Second, published accounts of zinc and copper toxicity indicate that our exposure levels were probably too low to cause any lasting effects on salmon gill physiology. Third, we noted pronounced effects of the metals on *D. sagittata* survival and fecundity. Lastly, this finding is in agreement with a prior meta-analysis of the effects of metals on similar parasites (Blanar et al. 2009), and with the long-standing use of metals (particularly copper) in the prophylactic treatment of ectoparasites in aquaculture (including a member of the genus *Discocotyle*; see Laird and Embody 1931).

Table 2 Effects (mean ± SD) of infection by *Discocotyle sagittata* (Parasite), and combined exposure to *D. sagittata* and a zinc/copper mixture (Combined), on growth and physiological parameters in Atlantic salmon; statistically significant differences are in bold

	Control	Parasite	Combined	*F*	DF	*p*
Length (mm)	146 ± 14	144 ± 11	141 ± 13	0.916	2	0.41
Weight (g)	31.7 ± 6.4	31.5 ± 7.3	27.2 ± 4.8	2.339	2	0.11
Growth in g	9.9 ± 3.1	8.0 ± 5.2	7.7 ± 4.1	1.206	2	0.31
Hematocrit (%)	35.4 ± 7.6	35.8 ± 6.3	35.7 ± 8.5	0.254	2	0.78
Osmolality (mosm)	299 ± 8	297 ± 12	297 ± 10	0.213	2	0.81
Chloride (mEq/L)	120 ± 9	**113 ± 8**	124 ± 8	4.845	2	**<0.01**

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