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EFFECTS OF COPPER SULPHATE ON THE SURVIVAL OF FREE-LIVING STAGE OF *SCHISTOCEPHALUS* CORACIDIA

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Abstract: Heavy metals enter the water ecosystem from a different sources natural and anthropogenic. Among the heavy metals copper is one of the most harmful pollutants of aquatic ecosystems. In this study, the effects of copper concentration (0.2 mg/L, 2 mg/L and 20 mg/L) on a cestode parasite, *Schistocephalus solidus,* were tested. In this experiment, newly-hatched coracidium were added to each 96-well flat bottom microtitre plates containing 100 µL of copper at 0.2 mg/L, 2 mg/L and 20 mg/L. Each coracidium was examined for survival at 1h intervals under a dissecting microscope. Death was confirmed when no swimming movement was visible during a 2 min observation and a change occured from transparent to opaque white colour. copper had a significant effect on the survival of *S. solidus* coracidia. Coracidia survived longer (30 h) when exposed to freshwater than in copper at concentration of 0.2 mg/L (21 h), 2 mg/L (22 h) and 20 mg/L (13 h). In conclusion, copper generally enters from agricultural runoff, through the deliberate use of copper as a pollution influenced reductions in coracidia survival which capable of producing failure in transmission to the next host. Changes in copper in aquatic environments can have implications for the parasite life cycle stages, with potentially complex implications for life cycle dynamics.

Keywords: Host-parasite interaction, *Schistocephalus solidus*, Copper, survival.

Introduction

In aquatic invertebrates, heavy metals such as copper, zinc, Mercury and cadmium change both morphological (Martinez *et al*., 2002) and physiological parameters such as growth, swimming movement, food intake, rate of breathing, productivity, survival and life cycles (Gerhardt, 1993; LaBreche *et al*., 2002). For copper, the main process leading to the mobilization into the ecosystem is extraction from man-made activities including mining, milling and smelting, and also from agriculture industry and waste disposal (Wright & Welbourn, 2002). In aquatic ecosystems, copper generally enters from agriculture runoff, though the deliberate use of copper sulphate to control the growth of microscopic algae and also from direct discharges from industrial processes. Copper is widely used in antifouling paint, treatment for fish diseases and as an algaecide (Gutierrez *et al.,* 2010) and ias an essential element in metabolic processes for both plants and animals (Irwin *et al.*, 1997a), but high concentrations can be toxic to aquatic species such as fish, algae and crustaceans (Sullivan *et al.*, 1983).

In a small amounts copper is not toxic and is required by a living organism for its biological function including the metabolic process. Copper can be defined as a toxic to an organism only if the doses of copper concentration was higher than the volume of physiological detoxification processes. The sensitivity of aquatic invertebrates to copper varies. For example, crustaceans have an LC50 at 48h ranging from 5 to 86 ug/L but for annelids (*Tubifex* sp.), the 48h LC50 ranges from 10 to 890 ug/L/(Hodson *et al.,* 1979). This variation in sensitivity depends in part on the surface area and respiration rates of the animals, which can influence the copper uptake (Hodson et al., 1979). Fish and crustaceans generally are sensitive to copper. For example, the cyprinid

fish *Rutilus kutum* had reduced in body weight and survival rate when exposed to 0.23 mg/L^{-1} of copper sulphate compared to non-exposed fish (Gharedaashi *et al*., 2013). In a freshwater amphipod, copper (100 µg/L^{-1}) significantly affected the osmoregulation process of *G. pulex* by decreasing the haemolymph sodium concentration and sodium influx within 4h. Depending on the species, lower doses of copper (10 µg/L^{-1}) also has the potential to reduce gill Na^+/K^+ ATPase activity (Brooks & Mills, 2003).

Copper, can directly influence the physiology and survival of aquatic organisms through toxic effects (Pietrock & Marcogliese, 2003) and can influence the prevalence, intensity and pathogenicity of parasites. Many diseases in aquatic populations are dependent on complex interactions between hosts, pathogens and environmental factors (Möller, 1987). Aquatic pollution such as copper may affect the incidence and nature of parasitism in many ways, for example, by altering host susceptibility through alteration of host defence mechanisms, by altering the availability of matching intermediate or final host populations, or by reducing the performance or survival of infected hosts (Sures, 2004). The effects of pollutants on parasitism can differ between species and even between developmental stages. The latter effects can be particularly pronounced in parasites of different life stages live in very different environments including both the external and internal environments (i.e. within hosts).

Aim and Objective

Here, the effect of elevated copper concentration on the *S. solidus* parasite at the free-swimming stage (coracidium) was studied. The copper concentrations tested in this study are ecologically relevant to a water inhabited by aquatic organisms including fish three-spined stickleback which is host to *S. solidus* in the UK (Bervoets *et al.*, 2001; Peters *et al.,* 2012). In this study, the following questions were addressed: How is the life-span of the free-living, infective stage of *S. solidus* affected under elevated copper concentration?

Hypothesis

Therefore, the following hypothesis is proposed:

H1: There is a significant effect of copper concentration on the survival rate of free-living infective stage of *S. Solidus*.

Justification of work

During their off-host, free-living phases, parasites are directly exposed to the environmental conditions in their habitat (Poulin, 1992). Any natural environmental factor or pollutant caused by anthropogenic activities can therefore directly influence the survival and performance of free-living parasite stages in their transition from one host to the next (Pietrock & Marcogliese, 2003). Among taxa and species, the free-living infective stages of parasites display an enormous variety of morphological and physiological properties (Pietrock & Marcogliese, 2003). To date, several digenean parasite species have been used as test organisms to investigate the effects of copper, but so far, there are few data concerning such effects on cestode parasites, including *Schistocephalus solidus*. A number of studies have investigated the effects of chemical substances on the cercarial stage of digenean trematodes (Evans, 1982a; Cross *et al.,* 2001; Pietrock *et al.,* 2002), probably because of their large size and the ease with which cercariae can be produced by infected snails in the laboratory, and also because some (such as *Schistosoma mansoni*) can infect humans and affect their health (Crompton, 1999). Most studies of the effects of pollution on parasites have been performed using miracidia or cercariae. Thus, there is a need for information on how stages of other endohelminth taxa respond to pollutants (Pietrock & Marcogliese, 2003).

It is often assumed that the free-living stages, particularly of endohelminth parasites, are highly sensitive to pollutants (Mecham & Holliman, 1975; Wanas *et al.,* 1998; MacKenzie *et al.,* 1995), but sometimes pollutants can even prolong survival, especially at low concentrations

of metals (Morley *et al.,* 2001b; Morley *et al*., 2002; Pietrock & Marcogliese, 2003). Reduced survival of digenean cercariae in the presence of a toxicant may arise because of binding of the toxicant to active sites of enzymatic molecules and de-activation of enzyme systems during encystment. Conversely, an increase in life-span could arise because of binding of metal ions to specific enzymes involved in glycogen utilisation, reducing metabolic activity (Pietrock and Marcogliese, 2003). There is thus a diversity in the sensitivity of parasites to toxicants according to their species and stages (Pietrock & Marcogliese, 2003).

Materials and Methods

Parasite culture

Three-spined sticklebacks *Gasterosteus aculeatus* from naturally-infected populations or experimentally-infected laboratory stocks were dissected to recover plerocercoids of the pseudophyllidean cestode *S. solidus* present in the body cavity. Infected fish can be readily identified outside the breeding season, since the abdomen of infected fish is distended compared to that of non-infected fish. Plerocercoids recovered from infected fish were cultured immediately, or were kept at 4°C overnight in a RPMI (Roswell Park Memorial Institute) -1640 culture medium (Sigma Aldrich, UK), in a covered, sterile Petri dish. Plerocercoids of *S. solidus* from infected fish were weighed using an analytical balance (to 0.001g) and only those with mass >50mg were selected for culture. Parasites were cultured in specially-designed culture tubes, using published *in vitro* protocols (Smyth, 1946; Wedekind, 1997). Screw-top boiling tubes (volume: 70 ml) were filled with a 50:50 mixture of RPMI-1640 medium and heat-inactivated horse serum (H1138, Sigma Aldrich, UK) with 500 µg of Penicillin. Horse serum acts as buffer against the toxic effect of acidic metabolic products that are produced during the cultivation process (Smyth, 1946) and Penicillin acts as an antibiotic to reduce fungal infection of the culture (Barber & Scharsack, 2010). The RPMI-1640 medium was prepared by adding 4.16g of RPMI medium powder to 400ml of double distilled water (ddH_2O). To ensure the culture was sterile, the RPMI medium was autoclaved before being used.

In the tubes, worms were suspended in narrow dialysis tubing (Medicell Membranes Ltd) to compress the worm, to promote insemination and fertilization and stimulate egg production (Smyth, 1946). In this study, two plerocercoids were placed together in the dialysis tube, to permit cross-fertilisation (Wedekind, 1997). The culture vessel containing the parasites was kept in a water bath at 40°C for 7d and was shaken gently to help the distribution of metabolic products from worms (Barber & Scharsack, 2010). The plerocercoids were monitored daily to check for worm survival and medium condition. Any dead plerocercoids were removed immediately from the dialysis tubing, to avoid a build-up of waste products that otherwise made it hard to clean and collect the eggs and which could have affected their development and hatching. The *in vitro* plerocercoid culturing period typically lasted 7d.

Schistocephalus solidus egg collection

Most parasite eggs were collected after 7d of the culturing period. At this time, the plerocercoids had mostly died or were senescent. The dialysis tubing was then rinsed by continuously pipetting distilled water into it until all the eggs released from the plerocercoids had been washed into a sterile Petri dish. Each Petri dish was then sealed using Parafilm™ and covered with aluminium foil to keep it dark and resistant to evaporation, and then incubated at 20°C in the dark for 3 weeks.

Preparation of hard water for Copper exposure experiments

Hard water was prepared by dissolving a calcium chloride ($CaCl_2$) and magnesium chloride hexahydrate in distilled water and making up to 1 L (WHO, 1989). This provided water with a hardness of 342 mg/L calculated as calcium carbonate.

Stock solutions of copper were prepared by dissolving copper sulphate in distilled water (200 ml) to give a Copper concentration of 200 mg/L. The test solution having a range of metal concentrations from 0.2 to 20 mg/L was obtained by diluting stock solutions in synthetic hard freshwater media. All stock solutions were prepared fresh daily.

Free-living stage experiment

Schistocephalus solidus plerocercoids were recovered from three-spined sticklebacks (*Gasterosteus aculeatus*) from the Llyn Frongoch population that had been bred and experimentally infected in the laboratory. Two worms were used as parents in paired culture and were matured in an *in vitro* culture system. On Day 7, the eggs of the plerocercoids were collected and kept in sealed Petri dishes in an incubator at 20°C for 3 weeks to embryonate.

After 3 weeks, the eggs were removed from the incubator and exposed to natural daylight to stimulate hatching. In this experiment, the wells of microtitre plates were each filled with 100 µL of copper solution at 0.2 mg/L, 2 mg/L and 20 mg/L. A single, newly-hatched coracidium was taken from the pool of hatching eggs using a glass Pasteur pipette, and added to one of the wells. The 96-well microtitre plate was covered with a lid and kept in a 12:12 zinc light:dark incubator at 15°C until examination for survival. Each coracidium was examined at 1h intervals under a dissecting microscope (Leica S6E, USA) and the survival of coracidia was recorded. Death was confirmed when no swimming movement was visible during the 2 min observation (repeated 3 times), and there was a change from transparent to opaque white colour.

Statistical analysis

For the free-living stage survival experiment a Non-parametric Kaplan-Meier analysis with log-rank (Mantel-Cox) tests were used to detect overall survival distributions between the groups. Any significant differences resulting from the tests were then followed by a Kaplan-Meier analysis with Wilcoxon (Gehan) tests (pairwise comparisons) to compare survival distributions among groups, with the test statistic based on differences in group means. These pairwise comparisons show which groups are significantly different in survival curves. Statistical significance was set at a value of $P < 0.05$.

Results and Discussion

The mean survival time of coracidia was influenced by the concentration of Copper to which the coracidia were exposed (Figure 1: Log rank test: $\chi^2_2 = 31.164$, $P = 0.0001$). Wilcoxon (Gehan) tests (pairwise comparisons) were then used to compare survival distribution among the copper concentration, with the test statistic based on differences in copper concentration mean. The coracidia survival distribution in control group (0 mg/L) was significantly different from all other groups (0.2 mg/L, $\chi^2_2 = 8.493$, $P < 0.004$, 2 mg/L, $\chi^2_2 = 11.415$, $P < 0.0001$ and 20 mg/L, $\chi^2_2 = 14.627$, $P < 0.0001$), with mean survival time of 30 h followed by; 0.2 mg/L (21 h) > 2 mg/L (22 h) > and 20 mg/L (13 h). These finding were support a previous study (Cross, 2001), in which the survival time of *Cryptocotyle lingua* cercariae from the trematode parasite in copper only survived less than 10 h. Even though the miracidia and coracidia were not from the same parasite species, miracidia are a motile, free-living stage that hatched from the eggs of trematode parasite. This suggested that even the parasites in equivalent, free-living stage respond differently toward the same metal pollutant (Sures, 2008).

The survival distribution of coracidia exposed to 0.2 mg/L Copper concentration was also different from those of 20 mg/L ($\chi^2_2 = 4.381$, $P < 0.036$). The differences in survival distribution were also documented between copper concentration of 2 mg/L with those from the 20 mg/L ($\chi^2_2 = 4.698$, $P < 0.030$). There are no significant differences ($\chi^2_2 = 0.210$, $P < 0.647$) in survival distribution of coracidia exposed in 0.2 mg/L with those exposed in 2 mg/L of copper. Overall, coracidia in non-polluted water

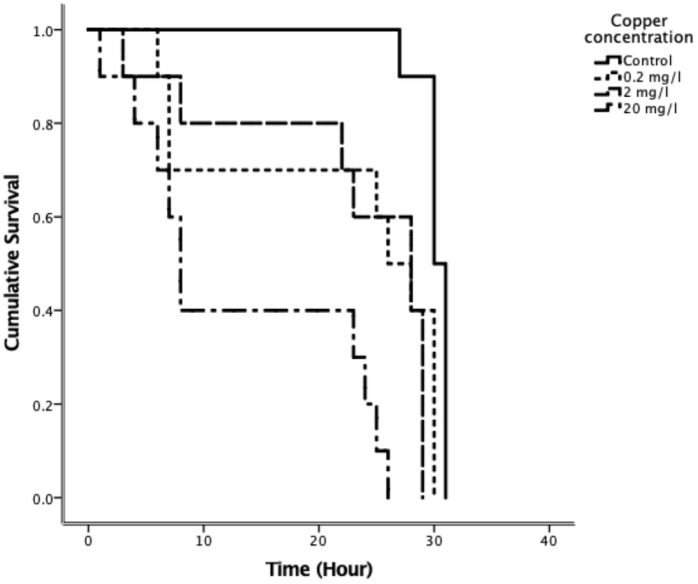

Figure 1: Comparisons of the cumulative survival of *Schistocephalus solidus* coracidia exposed to 0 mg/L, 0.2 mg/L, 2 mg/L and 20 mg/L

(control) survived longer than the coracidia of the shortest-lived copper group.

The decrease in survival of coracidia stages at copper concentration (0.2 to 20 mg/L) might be related to the increased activity level in coracidia, in terms of their swimming and movements (Koprivnikar *et al.,* 2010) which would lead to them consuming energy resources more rapidly (Pechenik & Fried, 1995), eventually causing death. Free-swimming stages of parasites are lecithotrophic and known to contain a limited amount of energy to sustain life and fuel locomotion (Barber *et al.*, 2016). In cestodes, that energy exists in the form of fat and glycogen droplets located in the ciliated embryonal shell of free-living stage (Dubinina, 1980). Based on personal observations, the coracidia swam actively and moved randomly at the bottom and surface in water containing Copper compared to freshwater where the coracidia generally moved slowly at the edge of the well. Increased activity and shortened lifespans have also been observed in different free-swimming stage of other aquatic parasite species, particularly the trematode cercariae (Rea & Irwin, 1992; Pechenik & Fried, 1995; Ford *et al.,* 1998; Koprivnikar & Poulin, 2009; Koprivnikar *et al.,* 2010; Berkhout *et al.,* 2014).

Higher mean survival time of *S. solidus* coracidia at non-polluted water in this study might arise through a number of different mechanisms. Prolonged coracidia survival at 0 mg/L concentrations might arise because of the activation process of the oncosphere. In nature, the circumambient chemical environment where the coracidia are located will stimulate the oncosphere located in the embryophore through the ciliated epithelial coat (Jakobsen *et al*., 2012). This activation process helps the oncosphere to break from their embryophore when the copepod break the coracidia shell. But in this study, copepods were not introduced to coracidia during the survival time experiment, therefore, less energy consume by the oncosphere during break process and uptake of nutrients from the surrounding environment might supply the oncosphere with sufficient nutrients.

Conclusion

This study shows that copper solution does influence the survival of the free-living stage. The results show that copper concentration

(20 mg/L) reduced the survival of the free-living stage. More work is needed to assess the aggregation of Copper in various organs and tissues free-living stage (coracidium) to seek mechanisms underlying mortality of the coracidia. This finding could contribute to the monitoring of increasing Copper pollution in the aquatic ecosystem. Cestode can be a great tool for heavy metal pollution indicator due to their ability to accumulate the pollutant.

The longevity of infective free-swimming stages of parasites is likely to be critical, as this stage needs to locate (or be located by) and invade the susceptible hosts either by penetrating the gut wall after ingestion, such as in cestodes and some nematodes (Dubinina, 1980) or by penetrating the external host tegument from the environment (as in the case of trematode cercariae) to proceed with the life cycles. Therefore, effects on the longevity and activity levels of infective free-living parasite stages by copper concentration are is likely to influence the probability of host encounters as their habitat selection and ability to use effective host-finding behaviours were also affected (Barber *et al.,* 2016). As the copper concentration can affect the free-living stage, it would also effect the intermediate host availability and suitability to act as a host to the parasite in a way that it can affect the probability that the infective parasites and susceptible hosts coexist spatiotemporally or by effect the ability of hosts to resist infection after being exposed to infectious agents (Barber *et al.*, 2016). Therefore, it would also be interesting to work on the other components of the *S. solidus* life cycle regarding the copper effect or other heavy metal, including their infectivity and growth of procercoids in copepod hosts. This is because the effect of environmental heavy metal on the rate at which larval stages of multi-host parasites develop within the bodies of their hosts is a potentially important factor in determining the dynamics of life cycles (Barber *et al*., 2016).

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