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The salt myth revealed: Treatment of gyrodactylid infections on ornamental guppies, *Poecilia reticulata*

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ABSTRACT

Salt is commonly recommended as an inexpensive treatment against many fish parasites in freshwater fish culture; however, few studies have scientifically evaluated its efficacy. Amongst the monogeneans, salt has only been previously tested against *Gyrodactylus salaris* infecting Atlantic salmon (*Salmo salar*) and *G. derjavini* infecting rainbow trout (*Oncorhynchus mykiss*). Here we tested the efficacy of salt treatments against *G. bullatarudis* and *G. turnbulli* on guppies (*Poecilia reticulata*), both commercially important pathogens in the ornamental fish industry. In vitro survival of both parasites was negatively correlated with increasing salinities of 3, 5, 7 and 33 gL^{-1}. Parasite establishment on guppies maintained at 0, 3 and 7 gL^{-1} salinity decreased drastically for *G. turnbulli* from 94% in the control to 78 and 0% on fish in 3 and 7 gL^{-1} salinity, respectively. *G. bullatarudis* establishment was still 100% at 3 gL^{-1} salinity but was reduced to 73% in 7 gL^{-1}. Throughout an infection, parasite populations of both species increased faster on guppies in 3 gL^{-1} salinity compared to dechlorinated water, whereas population growth was severely affected at 7 gL^{-1} salinity. Overall a short duration, high concentration salt bath was most effective at treating gyrodactylid infections: 15 min exposure to 25 gL^{-1} salinity for adults or 5 min for juvenile fish removed 100% of *G. turnbulli* or 72% of *G. bullatarudis*. The results reflect the generalist characteristics of the more tolerant *G. bullatarudis* compared to *G. turnbulli*, but have wider implications for treatment application: clearly one treatment regime does not suit all even within a genus.

1. Introduction

In freshwater aquaculture, salt is used against a range of pathogens from protozoans to helminths (Lio-Po and Lim, 2002). It is less harmful to fish hosts compared to more traditional anti-parasitic treatments, such as formalin or malachite green (reviews by Schelkle et al., 2009; Srivastava et al., 2004) and its low cost and availability make it the recommended treatment against a variety of fish diseases in ornamental fish keeping (e.g. www.fishdoc.com, www.fishkeeping.co.uk). Exposing freshwater organisms to saline conditions disrupts their osmoregulation, resulting in water loss and dehydration (Shephard, 1994). Ectoparasites or free-living parasitic stages are more severely and rapidly affected by such disruption in osmoregulation compared to their fish hosts due to their increased surface area to volume ratio. Nevertheless, despite its wide practical use there have been very few empirical studies to test the efficacy of saline conditions on fish pathogens, and most have focussed on *Flavobacterium columnare*, *Ichthyophthirius multifiliis* and *Saprolegnia* spp. (see Supplementary Material Table 1). These studies indicate that the effectiveness of treatment depends strongly on the fish host, parasite strain, application scheme, temperature and salt concentration. For instance, salt is most effective against White Spot disease when applied at a low dose over several days since it acts against the free-living theront stage (Garcia et al., 2007; Mifsud and Rowland, 2008; Tiemann and Goodwin, 2001). Continuous exposure of White Spot infected fish to saline conditions also ensures that theronts emerging at different times from encysted tomonts are killed, reducing the chances of re-infection (Mifsud and Rowland, 2008).

For helminth ectoparasites salt baths are often the most practicable since a high dose, short duration treatment acts aggressively against the parasites. Salt not only causes direct osmotic problems to the parasites, but also strips the fish of its protective mucus layer which to a certain extent buffers the parasite from the external environment (reviewed by Bakke et al., 2007; Burka et al., 1997). Additionally, short duration chemical treatments forego the problem of increased mucus production in the fish host, a physiological response triggered in response to adverse environmental conditions (reviewed by Shephard, 1994). Salt has only been tested against two helminth species (Supplementary data: Table 1), both from the economically and ecologically important gyrodactylids. Soleng et al. (1998) and Soleng and Bakke (1997) focussed on salt treatments in the Atlantic salmon (*Salmo salar*)–*Gyrodactylus salaris* host–parasite system, whereas Buchmann (1997) investigated salinity against *G. derjavini* infecting rainbow trout (*Oncorhynchus mykiss*). Treatments were 0 to 100% effective with concentration- and/or time-dependent

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effects apparent in all studies. However, both Atlantic salmon and rainbow trout are diadromous fish, migrating between marine and freshwater habitats. Salt treatment against helminths infecting purely freshwater fish has not been previously tested.

The guppy is a small, tropical freshwater fish that originates from Trinidad and Central and South America and is p
opular as an aquarium fish because of its elaborate colours and ease of maintenance (Magurran, 2005). Guppies are natural hosts to *G. bullatarudis* and *G.* (see Harris and Lyles, 1992), which under the confined conditions and the associated stress for fish in the aquarium industry find ideal conditions for increased transmission and population growth. Additionally, global fish transport may enable the parasites to encounter and infect alternative or reservoir hosts, which they usually would not come into contact with in their natural habitat, resulting in host switches (see King and Cable, 2007; King et al., 2009). Gyrodactylids are difficult to control and existing treatments are associated with low efficacy, toxicity to the host, human health concerns and difficulties in application (reviewed by Schelkle et al., 2009). The old adage that 'prevention is better than control' remains, but once a disease outbreak occurs treatment is necessary to avoid economic losses and prevent animal suffering (Ashley, 2007). This can be achieved by chemical control measures which may keep parasite prevalence in ornamental fish populations low and disease epidemics at a minimum.

Here we tested various salt concentrations on the in vitro survival of *G. turnbulli* and *G. bullatarudis*. Further, the establishment of both parasite species on guppies maintained in saline waters of 3 and 7 gL^{-1} salinity was investigated and the efficacy of salt bath treatments of 15 and 25 gL^{-1} salinity was tested on guppies infected either with *G. turnbulli* or *G. bullatarudis*. The salt concentrations chosen are within the range of typical recommendations for freshwater aquarium owners for disease treatment (1–7 gL^{-1} continuous exposure for 1–2 days and 15–30 gL^{-1} for short duration salt baths) according to a variety of forums and information on websites for freshwater aquarists.

2. Materials and methods

2.1. Source of animals and compounds, and screening methods

Guppies (*Poecilia reticulata*) originating from a mixed pet shop stock were used and fed daily on Aquarian® (API) fish flakes and at least twice weekly with live *Daphnia* or frozen *Tubifex*. All fish were maintained under a 12-h light:12-h dark cycle, at 25 ± 1 °C. For the experiments, fish were screened for parasites at regular intervals using 0.02% MS222 and a cold light (see Schelkle et al., 2009). Highly infected fish from *G. turnbulli* and *G. bullatarudis* laboratory cultures with no chance of survival were euthanized by a prolonged exposure to anaesthetic followed by pithing, and used as donor fish for Experiments 1 and 2. All procedures were carried out according to the UK Home Office licence regulations under project licence 30/1824.

G. turnbulli (*Gt3*) and *G. bullatarudis* populations have been isolated and maintained in laboratory culture on ornamental guppies since November 1997 and November 2008, respectively. Additionally, a small number of replicates ($n = 31$) infected with four different *G. turnbulli* strains originating from different fish stocks and isolated for different time periods were included for the high dose, short duration salt bath studies. Statistical analysis confirmed that isolation year of the parasite strain and the parasite strain itself did not have an effect on the susceptibility of parasites to salt. Aquarium salt (Aquarian® API) was used to make up salt water of 3, 5, 7, 15, 25 and 33 gL^{-1} salinity for all experiments.

2.2. Experiment 1: in vitro parasite survival (0, 3, 5, 7 and 33 gL^{-1} salinity)

Parasites were gently removed from the donor fish using an insect pin and transferred individually in 25 μL water into the wells of a 96 well plate using a Gilson pipette. Transfer was rapid to avoid parasites attaching to the pipette tip. One hour after parasites had been moved to the plates, they were observed for movement under a binocular microscope illuminated with a fibre optic source to ensure that removal from the fish host had not caused damage, potentially impairing parasite survival. At this time point any dead or moribund worms i.e. parasites that only moved after physical stimuli caused by stirring the water slightly in the near vicinity with an entomological pin, were excluded from the experiment (<0.01% of those transferred). Then salt stock solutions were added to make up the required salt concentrations of 3, 5, 7 or 33 gL^{-1} salinity in the wells with a total of 150 μL volume of water per well. Dechlorinated aquarium water was added to the control treatments and the time of addition of treatments was defined as zero. From thereon, parasites ($n = 79$–98 per treatment for each species) were observed hourly for movement until death occurred.

2.3. Experiment 2: in vivo parasite survival (0, 3 and 7 gL^{-1} salinity)

Over a 7-day period, guppies ($n = 100$, SL: 7–23.1 mm) were gradually habituated to their experimental salt concentrations by an incremental increase in salinity levels of 1% starting 7 or 3 days ahead of the experiment for 7 and 3 gL^{-1} treatments, respectively. Guppies were maintained individually in 1-L pots throughout the habituation and experimental periods, and received water changes at least every other day. On day 0 (D0) naive guppies were infected with two parasites each (either *G. turnbulli* or *G. bullatarudis*) by anaesthetizing the recipient fish and bringing the donor fish in close contact to enable parasites to transmit from one fish to the other. Time to infection was recorded and any infection in which the parasite failed to transmit within 120 s was aborted. Anaesthetic was made up in the appropriate salt water concentration in which the fish was maintained. After infection, fish were screened on D1 to check whether parasites had established, i.e. whether at least one parasite was still attached to the host. Thereafter, fish were screened every other day until fish were either parasite free or had succumbed to infection.

2.4. Experiment 3: efficacy of salt baths (15 and 25 gL^{-1} salinity)

Ornamental guppies ($n = 96$, SL: 6.5–26.4 mm) that acquired *G. turnbulli* or *G. bullatarudis* infections in previous experiments were randomly assigned to a 15 or 25 gL^{-1} salt bath treatment or a control treatment of dechlorinated aquarium water. The use of fish with unknown infection age and different initial parasite burdens aimed to simulate natural infections of individual fish within wild or cultured fish populations. Adult guppies (>13 mm) were exposed to their respective treatments for 15 min, whereas juveniles received treatment for 5 min only to reduce osmotic stress. Parasite loads were recorded before and after treatment by screens of the anaesthetized host.

2.5. Statistical analysis

Data for Experiment 1 were analysed with a non-parametric Cox proportional hazard model with an average hazard, time-to-death as independent variable and parasite species and treatment as dependent variables. For Experiment 2, differences in infection trajectory between parasite populations under different saline conditions were assessed with a Generalized Linear Mixed Model (GLMM) using restricted maximum likelihood analysis in ASReml-R and fitted with a Gaussian error structure and an identity link. *Gyrodactylus* species, salinity, day, fish sex and size were used as independent variables (excluding data for *G. turnbulli* infected guppies at 7 gL^{-1} salinity due to 0% establishment) and parasite load at any given day was used as dependent variable. The random model included day and fish ID as independent variables. Data were normalised by a natural log (ln)