Efficacy of sea salt, metronidazole and formalin-malachite green baths in treating *Ichthyophthirius multifiliis* infections of mollies (*Poecilia sphenops*)

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Abstract

Ichthyophthirius multifiliis is an obligate fish parasite with devastating consequences for wild and captive fish stocks. In the current study, we compared the efficacy of three treatments, sea salt, metronidazole and a mixture of formalin and malachite green, against an *I. multifiliis* outbreak observed in a stock of mollies (*Poecilia sphenops*) held by an aquarium retailer. The quickest recovery was observed in fish treated with sea salt, with no visible trophonts after 3 days at 27ºC. After 6 days in a continuous bath treatment, the mean prevalence and parasite intensity were also significantly reduced in fish treated with the formalin-malachite green combination compared to metronidazole-treated fish. Our results indicate that metronidazole baths are not effective against *I. multifiliis*, and that a sea salt bath should be the treatment of choice for mollies.

Introduction

The ciliate *Ichthyophthirius multifiliis* is an obligate fish parasite with devastating consequences for wild and captive fish stocks (Dickerson, 2006; Maceda-Veiga et al., 2009). Replication of *I. multifiliis* increases with temperature, ranging from 40 days at 4ºC to 3 days at 26ºC, but above 30ºC is lethal (Dickerson, 2006). The parasite causes white spot disease, which can be easily diagnosed because fish are covered by small white nodules that correspond to the feeding stage of the parasite (trophonts) (Noga, 2010). Large numbers of trophonts severely damages the gills and skin causing fish mass mortalities due to secondary infections, respiratory stress and osmoregulatory dysfunctions (Lom and Dyková, 1992; Matthews, 1994). Many treatments have been developed against *I. multifiliis*, with salt and the formalin-malachite green mixture baths showing the highest efficacy (reviewed by Picón-Camacho et al., 2012). The former, however, is reportedly unsuitable for stenohaline freshwater fish species (Noga, 2010), and a ban in 2000 curbed the use of malachite green in food fish production in North America and Europe as it is a carcinogen retained in fish flesh (EC directive 90/676/EEC; article 14, regulation 2377/90/EEC). However, in non-food fish facilities of research centres and in the aquarium trade, its application is still allowed, although certain fish taxa (e.g. catfish and characids) show a marked sensitivity to malachite green derivatives (Noga, 2010). For those species intolerant to malachite green or salt, metronidazole baths, which are not approved for use in food fish (reviewed by Picón-Camacho et al., 2012), are used quite extensively amongst aquarium hobbyists as a successful treatment against *I.*

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multifiliis (Toni Griñó, *pers. comm.*). The efficacy of metronidazole against *I. multifiliis* has, however, only been demonstrated as an oral treatment in rainbow trout (Tojo-Rodriguez and Santamarina-Fernández, 2001), without any evidence of its efficacy as a bath treatment (Wahli et al., 1993).

In an attempt to reduce the use of aggressive chemical treatments in the aquarium industry, the present study assessed the anti-parasitic efficacy of metronidazole in a stock of the euryhaline mollies (*Poecilia sphenops*) infected with *I. multifiliis* compared to sea salt and the traditional mixture of formalin and malachite green.

Materials and methods

In July 2014, an outbreak of *I. multifiliis* occurred in a stock of 72 black mollies (*Poecilia sphenops*) imported from a European wholesaler by an aquarium retailer in Barcelona, Spain. Fish (Total length: 40 mm, commercial size) were feed on Sera Vipan® fish flakes, and maintained under a 12h light: 12h dark cycle at 27±1ºC in aquaria with air supply in the quarantine room of the aquarium retailer during the experiment. For the experiment, the whole surface of each fish was screened by eye for *I. multifiliis* trophonts using gentle immobilisation (~30 s) in a 20 ml glass tube. Parasite identity was confirmed by screening mucus scrapings (n=5 fish) using an Olympus CH2 microscope at x400 magnification (Lom and Dyková, 1992). Anaesthesia was not employed to avoid possible interactions with the treatments. Fish were then distributed in batches of 8 individuals in three 38 L aquaria per treatment (n=24 fish per treatment), balancing the trophont intensity per aquaria (mean±sd=15±1). Treatment doses were applied based on information provided on freshwater aquarist websites and the personal experience of the aquarium retailer: salt (Instant Ocean® sea salt, 10 g/L), metronidazole (Flagyl®, 10 mgL^{-1}) and a mixture of malachite green and formalin (25 mg/L formalin + 0.1 mg/L malachite green, Leteux and Meyer, 1972). An untreated control group was not feasible due to commercial restrictions but infected tropical fish, if left untreated, die after 6 days at 27ºC due to the rapid replication and pathologic nature of *I. multifiliis* (see Dickerson, 2006). Half water volume was changed every other day and then a new dose of the drugs was added or the salinity was adjusted. Fish were screened for *I. multifiliis* trophonts on Day 3 and 6 post-treatment, and fish recovery was confirmed by screening mucus scrapings of some of these fish (n=5) as detailed above. The mean trophont intensity and prevalence were compared between treatments over time using a generalised linear mixed model (GLMM), where aquaria ID was assigned as a random variable in order to account for the pseudoreplication present in the study design, and time and treatment incorporated as fixed effects. Poisson and binomial error distributions were used in the GLMM for trophont intensity after squared root transformation and prevalence, respectively. Poisson and binomial errors are suitable for count data and proportions, respectively (Thomas et al., 2013). Residual diagnostic plots from the Poisson model were used to verify the assumptions of normality and homoscedasticity of model residuals, and to test for unduly influential observations (Zuur et al., 2010). The residual deviance was smaller than the residual degrees of freedom in all models so overdisperson did not occur (Thomas et al., 2013). All analyses were performed in R version 2.14.2 (R Development Core Team, 2012), and significance of explanatory variables (factor's effects) in GLMs was assessed using the χ^2-statistic (Thomas et al., 2013). Significance was reached at $p \leq 0.05$.

Results and discussion

The application of the most effective treatment against *Ichthyophthirius multifiliis* is required in fish facilities as it causes mass mortalities and high economic losses in the industry (Dickerson, 2006; Shinn et al., 2009). In the current study, fish mortality (n=72) was not observed in any of the tanks over 6 days, indicating all three treatments effectively stopped the disease spreading at 27°C, and that the concentrations used were within the safety threshold for mollies (*Poecilia sphenops*). The mean prevalence and intensity of *I. multifiliis* trophonts were significantly reduced in the fish treated with formalin-malachite combination or sea salt (GLMM, All $P<0.01$; Figure1), supporting previous data (reviewed by Picón-Camacho et al., 2012). However, the prevalence and mean intensity of *I. multifiliis* trophonts remained unaltered following a 6 day metronidazole bath. This contrast with the high efficacy of metronidazole against *I. multifiliis* reported in aquarists' websites, but confirms the results from 24h *in-vitro* assays (Wahli et al., 1993). In aquarium circles, the success of metronidazole baths against *I. multifiliis* could be explained by the fact that this antibiotic is usually combined with elevated temperature (≥30ºC), which kills the parasite (Dickerson, 2006; Noga, 2010). Such high temperatures may, however, not be tolerated by many tropical fish species (Noga, 2010). After the experiment, infected fish from the metronidazole treatment were treated with sea salt due to commercial and kept under the same temperature, and 25% mortality was recorded in this batch during the next week. This indicates that application of a salt bath is only effective in minimising fish mortality if applied in the early stages of a whitespot outbreak.

In the current study, the sea salt bath was most effective against *I. multifiliis*, with all fish observably free of trophonts by Day 3, in contrast to those fish treated with the formalin-malachite green mixture (GLMM, All $P<0.001$), (Figure 1). The response of *I. multifiliis* to formalin-malachite green combination was, however, slower than expected at 27±1ºC since the life cycle of *I. multifiliis* is completed in 3 days at 26ºC (Lom and Dyková, 1992; Matthews, 1994), and 0.1 mgL^{-1} malachite green is reported to be effective against the free-living stages of the parasite (reviewed by Picón-Camacho et al., 2012). Our findings could be explained by the presence of multiple *I. multifiliis* strains in the aquarium trade that may differ in sensitivity to drugs (Nigrelli et al., 1976), and/or the additional benefits of salt baths for fish as they not only kill parasites but aid host recovery with regard to osmotic imbalance and the loss of salts caused by exiting trophonts (Tiemann and Goodwin, 2001; Mifsud and Rowland, 2008). In conclusion, our results indicate that metronidazole baths are not effective against *I. multifiliis*, and that a sea salt bath should be the treatment of choice for mollies.

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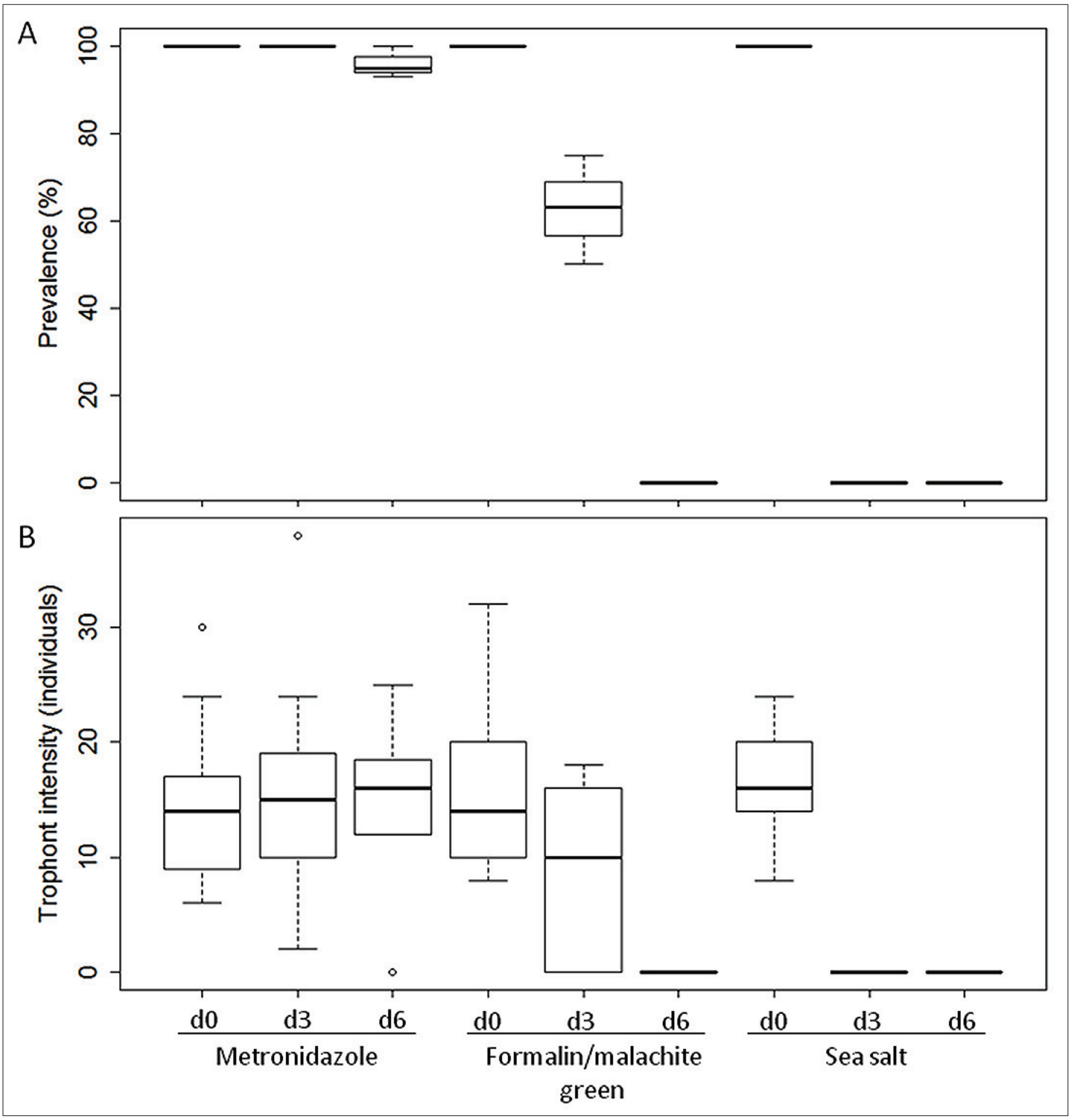

Figure 1. Mean prevalence (A) and intensity of *Ichthyophthirius multifiliis* trophonts (B) on mollies (*Poecilia sphenops*) throughout the exposure period (0, 3 and 6 days) to metronidazole (10 mg/L), formalin/malachite green combination (25 mg/L formalin + 0.1 mg/L malachite green) and sea salt (10 g/L).

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