

## The Effects of Short Freshwater Bath Treatments on the Susceptibility to Different Stages of *Neobenedeniagirellae* Infecting Barramundi (*Lates calcarifer*)

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### Abstract

*Neobenedeniagirellae* is one of the most pathogenic parasites affecting marine fish in captivity conditions. The use of chemicals for parasite prevention and treatment have several benefits; however, they can cause various negative side-effects. In an effort to discover cost-effective and sustainable practices, our current study was aimed at investigating the efficiency of freshwater treatments on *N. girellae*. A challenge test was conducted to produce infected fish which became materials for the freshwater immersion experiments. The duration and reaction of the parasites at different development stages from eggs to adult parasites were examined. Our findings revealed that 100% of the adults and oncomiracidia of *N. girellae* were killed quickly in freshwater. The eggs of *N. girellae*, however, were highly resistant to freshwater with a hatching success rate of more than 95% in all the freshwater immersion treatments (2, 5, 10 and 30min). The eggs hatched mainly on day 7 and finished hatching on day 8. Thus, the freshwater immersion method can be applied to treat *N. girellae* at most stages excepted for the egg stage. The best practical treatment for this parasite is to perform a replicated immersion recommended 8 days following the first treatment.

### Keywords

*N. girellae*, freshwater, treatment, parasites, marine fish

### Introduction

*Neobenedenia* spp. have a direct life cycle and short generation

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time. Eggs are released into a water environment and tend to attach to nets or substrates in aquaria environments due to their long filamentous threads, then they hatch and reinfect fish at a higher intensity after a week. *Neobenedenia* has been misclassified and there is some disagreement and controversy in discriminating among species (Hoai & Hutson, 2014). However, *N. melleni* and *N. girellae* have reported as the two main species causing disease outbreaks worldwide. While most species of monogeneans are host specific, *N. melleni* has been reported to be highly aberrant with more than 100 teleost species in more than 30 families (Whittington & Horton, 1996; Bullard *et al.*, 2003). *Neobenedenia* appears to have low site-specificity and may infect the skin, fins, eyes, gill cavity, and nasal cavity of the host. Newly recruited parasites tend to attach to the anterodorsal region of the head and corneal surface (Kaneko *et al.*, 1988).

*Neobenedenia* spp. cause disease, production losses, and mortality to teleosts in marine aquaculture (Whittington & Chisholm, 2008; Whittington, 2011). The infection source of this parasite is often unknown as many fish are susceptible to *Neobenedenia* (Kaneko *et al.*, 1988; Deveney *et al.*, 2001; Ogawa *et al.*, 2006). *Neobenedenia* spp. have been reported to limit development, progress, and expansion of sea cage culture of marine finfish in several countries including cobia in Taiwan (Liao *et al.*, 2004), spotted halibut and Japanese flounder in Japan (Hirazawa *et al.*, 2004), and barramundi (*Lates calcarifer*) in Australia (Deveney *et al.*, 2001). A *Neobenedenia* sp. was associated with a disease outbreak of wild barramundi in Australia, causing red and cloudy eyes, skin discoloration, scale loss, skin damage, and lesions (Brazenor & Hutson, 2015). There are several methods used to treat *Neobenedenia* spp. Praziquantel incorporated into feed at 40 mg kg<sup>-1</sup> body weight delivered over 11 days has been reported as having a strong antiparasitic effect against *N. girellae* (Hirazawa *et al.*, 2004). Ohashi *et al.* (2007) also suggested that calcium and magnesium could provide control against the disease because these chemicals could disrupt intercellular junctions in *N. girellae* and result in

decreased survival. However, these methods are not economical and can cause environmental issues. In addition, Kaneko *et al.* (1988) reported that a freshwater dip for 2-2.5min can kill all *N. melleni* effectively and the freshwater treatment was considered as a potential treatment for this disease. However, the problem is that this disease is often recrudescence after being treated with freshwater. Therefore, consideration should be given to experimental work with freshwater treatments for this parasite to clarify reasons for this issue in order to improve freshwater treatments for this disease in the future.

## Materials and Methods

### Source of fish and parasites

Hatchery reared freshwater barramundi (*Lates calcarifer*; mean size 125 ± 23mm) were purchased from the Good Fortune Bay Hatchery, Queensland, Australia for use in the infection experiments. The fish were naïve to *N. girellae* infection, which is restricted to saltwater environments. The fish were transported to the laboratory in 50L tanks with air supplied through battery-powered aerators and held in freshwater in 100L aquaria until required. The fish were fed pellets (Ridley Corporation Limited, Melbourne, Australia) formulated for barramundi until their use in the experiments. The fish were not fed during the experiments to maintain the water quality in the small 10L aquaria. The fish were acclimated to seawater 48h prior to the experiments by increasing the salinity to 5, 10, 20, 30, and 35ppt over 6h intervals as described by Hoai & Hutson (2014). Seawater used in the experiments was UV 10µm filtered, 35ppt, unless stated otherwise.

Embryonated *N. girellae* eggs collected from a land-based marine *L. calcarifer* farm in Queensland, Australia were introduced to *L. calcarifer* (size range was 110-220mm) and held in 100L aquaria to maintain a continuous source of parasites. The eggs were collected in the first three hours of the day that the adult *N. girellae* laid eggs. Subsequently, the eggs were incubated in cavity blocks and their development observed prior to being selected and used for further experiments.

### Effect of acute freshwater immersion on adult *N. girellae*

To determine how freshwater can treat adult *N. girellae*, five naïve fish were maintained in five separate aquaria (containing 5L saltwater 35ppt) in laboratory conditions, and groups of embryonated eggs ( $n = 50$ ) were then used to infect each naïve fish in the aquaria. Because the eggs of *N. girellae* always get entangled in nettings on the day of sexual maturity, a piece of netting was placed into each aquarium and checked daily under a stereomicroscope to determine the onset of egg production. The day that eggs were observed on the netting was recorded as the time to sexual maturity (adult parasites) and the experimental infection process was terminated. The infected fish were used to perform the treatment of freshwater immersion. Each fish was carefully moved to a bottle containing 1L freshwater. The times of death of the adult *N. girellae* were recorded, including the initial appearance of the parasites on the fish bodies after immersion, the time of initial and completed precipitation, and the number of parasites observed on each fish.

### Effect of acute freshwater immersion on the oncomiracidia of *N. girellae*

Bundles of *N. girellae* eggs produced from infected fish were collected from aquaria using fine forceps and carefully separated in seawater using needles under a dissecting microscope. The cavity blocks were maintained at 25°C in culture chambers on a 12:12h day:night cycle. A third of the fresh seawater (2mL) was exchanged daily. Three replicates were made for the freshwater treatments and seawater control. The oncomiracidia in this experiment were removed from the seawater to other cavity blocks containing freshwater for the treatment group and seawater for the control group within the first hour of hatching by using a glass pipette. Twenty oncomiracidia were allocated to an individual glass cavity block containing freshwater and then the oncomiracidia activities were recorded continuously until all the oncomiracidia were killed in the treatment groups. For the control

group, the oncomiracidia statuses were recorded in 15min intervals.

### Effect of acute freshwater immersion on *N. girellae* egg viability

There is currently a paucity of knowledge in the prevention of *N. girellae* infection and current practices of parasite management rely on temporarily removing parasite stages attached to fish (Whittington, 2011). As a treatment, infected fish are typically immersed or ‘bathed’ in freshwater for brief periods. We examined the effects of acute freshwater treatments on the eggs and compared the hatching success of eggs treated with freshwater for different periods of time (2, 5, 10, and 30min) and no treatment (maintained in seawater). Bundles of *N. girellae* eggs produced from infected fish were collected from aquaria using fine forceps and carefully separated in seawater using needles under a dissecting microscope. Fifty eggs were allocated to an individual glass cavity block. Five replicates were made for all four freshwater treatment groups and the seawater treatment for the control group. Eggs were bathed in distilled fresh water for 2, 5, 10, or 30min before being immediately filled with seawater to the brim by gentle pipetting (**Figure 1A**). Cavity blocks were maintained at 25°C in culture chambers on a 12:12h day:night cycle (light and dark phases) (**Figure 1B**). A third of the fresh seawater (2mL) was exchanged daily.

Eggs were then monitored daily at 9 a.m. under a stereomicroscope with both incident and transmitted light. Egg development was noted according to four development stages: stage I (non-embryonated): transparent eggs; stage II (embryonated): eggs with dark brown color and exhibiting evidence of cellular division; stage III (developing): egg with the presence of eyespots; and stage IV (hatched): eggs with opened operculum (**Figure 2**).

### Data analysis

The proportion of eggs that hatched after the freshwater immersion treatments for different time periods during the *N. girellae* egg viability

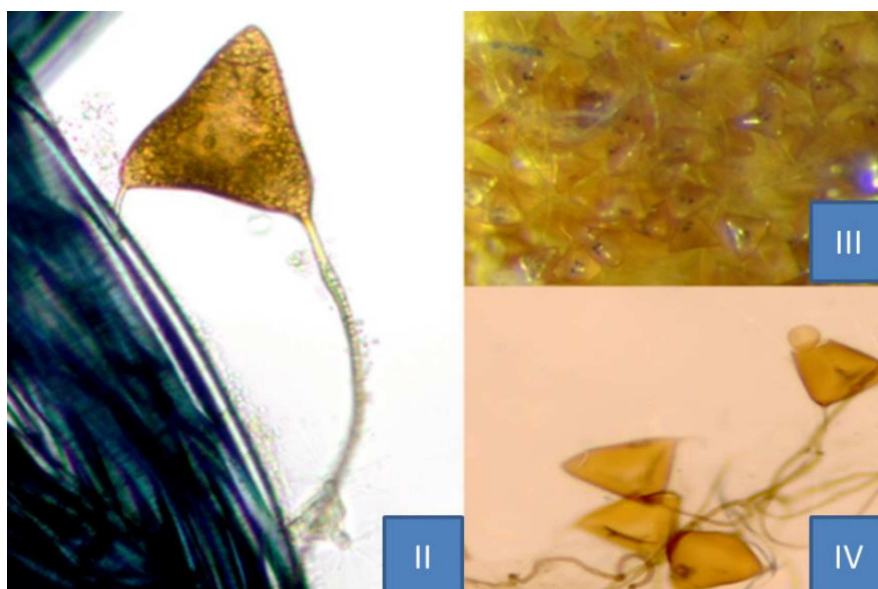


(A)



(B)

**Figure 1.** Experimental design of the acute freshwater immersion for *N. girellae* egg viability (A) and incubation conditions (B)



**Figure 2.** *Neobenedenia girellae* egg development at different stages

experiments was analyzed using IBM SPSS statistics 24 software (IBM 2016). The chi-square test showed the accepted significance level at  $P < 0.05$ .

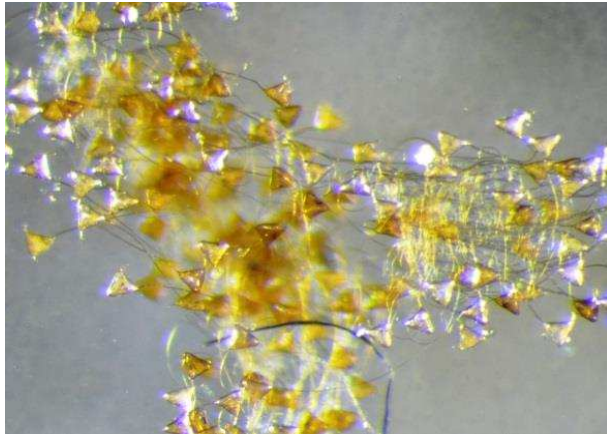
## Results and Discussion

### Effects of acute freshwater immersion on adult *N. girellae*

The infection process was successful with the presence of *N. girellae* on the nettings immersed in the cultured tanks (Figure 3).

However, the adult parasites on the bodies of the infected fish in the culture tanks could not be observed by the unaided eye (Figure 4). The fish were consequently bathed in a separated bottle of 1L freshwater. The parasites were killed quickly and appeared on the bodies of the fish within 1-2min (Figure 5). The adult parasites started to precipitate after 3-4min and finished the precipitation after 10min (Figure 6). The fish were then carefully transferred to other bottles of 1L freshwater containing an anaesthetic solution (Aqui-S 1:1000) and parasites on the external





**Figure 3.** Eggs of *N. girellae* in the experimental tanks



**Figure 4.** Infected fish in seawater (36ppt) before the freshwater treatment



**Figure 5.** *N. girellae* appeared on the fish bodies after applying the freshwater treatment



**Figure 6.** Precipitation of *N. girellae* after the treatment

**Table 1.** Treatment observations from the fish experimentally infected by *N. girellae*

Replicates	Initial appearance after immersion (min)	Initial precipitation	Completed precipitation	N° parasites observed/fish	Percentage of infection (%)
1	1min 13s	3min 18s	9min 5s	48	96
2	1min 24s	3min 25s	9min 4s	46	92
3	1min 27s	3min 46s	8min 6s	46	92
4	1min 34s	3min 39s	9min 7s	49	98
5	1min 35s	3min 43s	10min 5s	47	94
<b>Average</b>	<b>1min 27s</b>	<b>3min 26s</b>	<b>9min 5s</b>	<b>47.2</b>	<b>94.4</b>

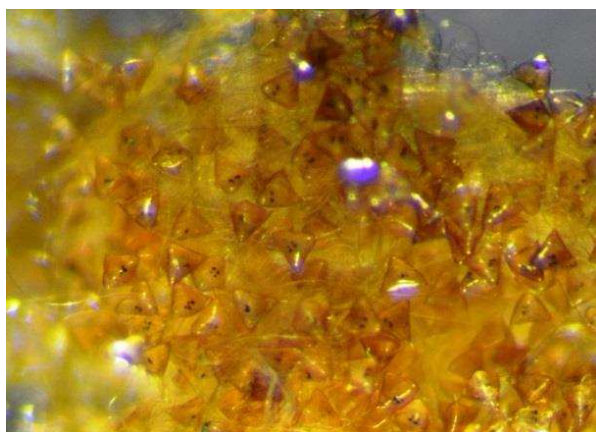
surface were sought out to ensure no parasites remained on their bodies. The results showed that no parasites remained on the fish bodies after being immersed for 10min in freshwater. The experimental infection was highly

successful with a total of 94.4% parasites presented on fish bodies (**Table 1**). The freshwater treatment was highly effective in killing all the infected parasites attached to the fish bodies (100%).

### Effects of the acute freshwater immersion on oncomiracidia

After incubation, the eggs were well embryonated (**Figure 7**). The majority of eggs hatched during the first 3h of the light phase (**Figure 8**). The oncomiracidia were then carefully transferred to new glass cavity blocks containing 5mL freshwater using a glass pipette. The behaviors of the oncomiracidia were immediately observed in terms of swimming status and precipitation. The results of the treatment showed that the oncomiracidia quickly slowed their swimming and all of the oncomiracidia settled down to the bottom of the glass cavity blocks containing the freshwater

within 1min (**Table 2**). Further observations showed that the oncomiracidia were killed and precipitated quickly in freshwater, no mobility was observed after 1min. Although they still exhibited a normal appearance and kept their shape within the first 5min (**Figure 9**), they started to become dehydrated and convulsive after 15min (**Figure 10**). Oncomiracidia in the control group, in contrast, remained alive and showed active swimming after 90min. Several oncomiracidia were observed to run out of energy, became less active and started to die after 2.5h. All the oncomiracidia died after 3h 45min in seawater, as they probably ran out of energy under no host conditions (**Table 2**).



**Figure 7.** Embryonated eggs with eyespots



**Figure 8.** Oncomiracidia hatching and swimming in saltwater



**Figure 9.** Larvae swimming lethargically and sank down to the bottom of the glass cavity block containing fresh water



**Figure 10.** Dehydrated and convulsive larvae in freshwater

**Table 2.** Observations of acute freshwater immersion on oncomiracidia of *N. girellae*

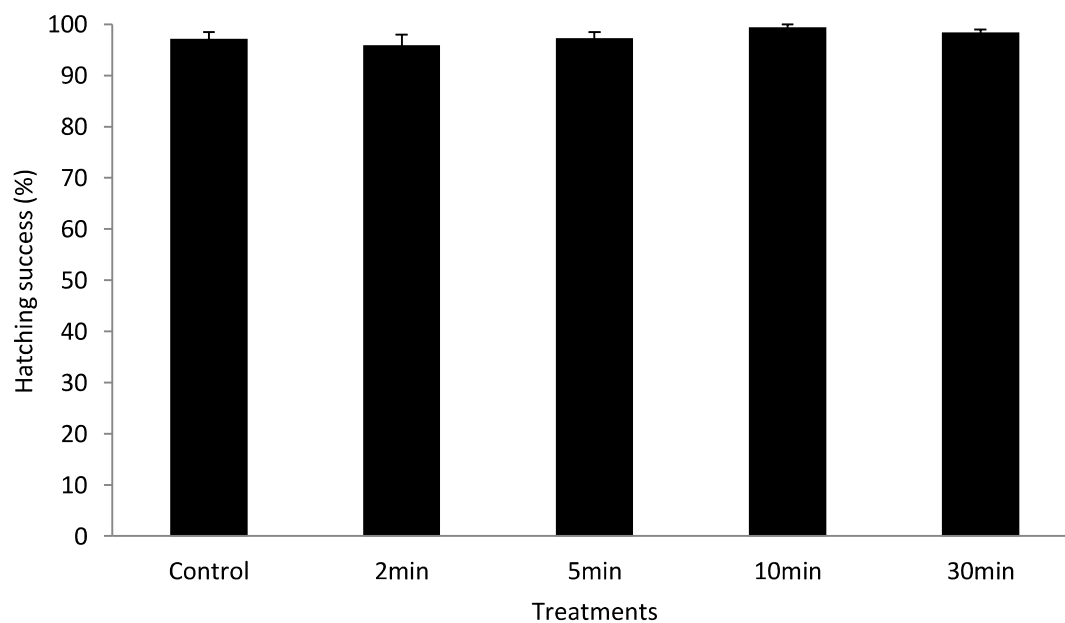
Group	Number of oncomiracidia observed	Time of first death	Time of last death	Time of dehydration and convulsions
Treatment groups (freshwater)	60	21s	42s	10-20min
Control group (seawater)	20	2h 30min	3h 45min	-

Note: (-): no observation.

### Egg hatching success following exposure to the acute freshwater treatments

Embryonated eggs were exposed to acute freshwater treatments (2, 5, 10, and 30min). The results of the experiments showed that the eggs of *N. girellae* were highly resistant to acute freshwater immersion. Eggs began hatching on day 6, with the majority of eggs hatching on day 7 (84%). Egg hatching ceased on day 8 and the experiment was terminated on day 10. Hatching success was more than 95% in all the freshwater treatments and the seawater control (**Figure 11**). There were no significant differences in egg hatching success in the freshwater treatments compared to the seawater control ( $P = 0.5848$ ).

The freshwater bath treatment was demonstrated to effectively remove adult *Neobenedenia* spp. from several fish species such as amberjack (*Seriola dumerili*), yellowtail (*S. quinquerradiata*), and bullseye pufferfish (*Sphoeroides annulatus*) (Fajer-Ávila *et al.*, 2008; Ohno *et al.*, 2009). In this study, we demonstrated that exposing Asian Sea bass to freshwater for 10min is a viable treatment for larval and adult *N. girellae* infections (*Lates calcarifer*). According to Ohno *et al.* (2009), a 2 to 5min freshwater dip is often practiced to prevent *N. girellae* infections of amberjack (*Seriola dumerili*). While Fajer-Ávila *et al.* (2008) reported that infected fish exposed to



Note: Error bars indicate standard errors.

**Figure 11.** *N. girellae* egg hatching success (%) following immersion of eggs in freshwater for different periods of time and seawater

20min of freshwater showed a significant reduction (95%) of adult *Neobenedenia* sp. from bullseye pufferfish. Thus, the immersion duration for the practical treatment of each fish species and/or parasite species may be varied and need to be evaluated. This study also revealed that the short term bath treatment in freshwater was not effective in killing the eggs. According to Hoai & Hutson (2014), *Neobenedenia* species were fecund and could attach widely in ponds and aquaria. Monogenean eggs are protected by a strong, sclerotized protein shell. Chemicals such as trichlorfon, praziquantel, copper sulfate, potassium permanganate, and benzocaine have been reported to kill larvae and adult parasites (Thoney & Hargis, 1991; Buchmann *et al.*, 1992; Diggles *et al.*, 1993; Thoney, 1990; Yoshinaga *et al.*, 2000), however, the eggs are highly resistant to these chemicals (Thoney, 1990). Exposing *N. melleni* eggs to hyposaline solutions (6ppt) over a 7 day period was sufficient to prevent development (Ellis & Watanabe, 1993). Thus, the best practical treatment for this parasite is replicate immersions in which the second treatment should be conducted 8 days after the first treatment in cases where the temperature is around 25°C and salinity 35ppt. This will ensure that all the adults and oncomiracidia will be killed in the first treatment and any eggs remaining on the body or gills will hatch and be killed by the second treatment. The egg hatching times for *Neobenedenia* species depend on several factors such as temperature (Hirazawa *et al.*, 2010), fish host (Hirazawa *et al.*, 2010; Hoai & Hutson, 2014), and salinity (Brazenor & Hutson, 2015).

## Conclusions

The results of this study revealed that adult *N. girellae* and their larvae could be killed quickly in freshwater. Therefore, a short bath treatment can be applied to control infections of this parasite. However, eggs of *N. girellae* are highly resistant to freshwater, and a second bath treatment needs to be applied to improve the resistance towards this parasite species. It is suggested that the life cycle and the hatching time of the parasite's eggs should be tested prior

to applying the second bath immersion to obtain a thoroughly effective treatment.

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