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Efficacy of garlic (*Allium sativum*) extract applied as a therapeutic immersion treatment for *Neobenedenia* sp. management in aquaculture

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Abstract

Garlic, *Allium sativum* L., extract administered as a therapeutic bath was shown to have antiparasitic properties towards *Neobenedenia* sp. (MacCallum) (Platyhelminthes: Monogenea) infecting farmed barramundi, *Lates calcarifer* (Bloch). The effect of garlic extract (active component allicin) immersion on *Neobenedenia* sp. egg development, hatching success, oncomiracidia (larvae) longevity, infection success and juvenile *Neobenedenia* survival was examined and compared with freshwater and formalin immersion. Garlic extract was found to significantly impede hatching success (5% ± 5%) and oncomiracidia longevity (<2 h) at allicin concentrations of 15.2 μL L^{-1}, while eggs in the seawater control had >95% hatching success and mean oncomiracidia longevity of 37 ± 3 h. At much lower allicin concentrations (0.76 and 1.52 μL L^{-1}), garlic extract also significantly reduced *Neobenedenia* infection success of *L. calcarifer* to 25% ± 4% and 11% ± 4%, respectively, compared with 55% ± 7% in the seawater control. Juvenile *Neobenedenia* attached to host fish proved to be highly resistant to allicin with 96% surviving 1-h immersion in 10 mL L^{-1} (15.2 μL L^{-1} allicin) of garlic extract. Allicin-containing garlic extracts show potential for development as a therapy to manage monogenean infections in intensive aquaculture with the greatest impact at the egg and larval stages.

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Introduction

Neobenedenia (MacCallum) (Monogenea: Capsalidae) are marine ectoparasites of finfish known for their ability to cause major epizootics in mariculture (Bondad-Reantaso *et al.* 1995; Deveney, Chisholm & Whittington 2001; Yamamoto *et al.* 2011). *Neobenedenia* graze on host epithelial tissues, causing lesions and haemorrhaging (Hirazawa *et al.* 2010; Whittington 2012). While feeding, adult *Neobenedenia* continuously oviposit eggs that hatch into infectious, free-swimming oncomiracidia (larvae), capable of directly re-infecting the primary fish host. This parasite's direct life cycle, along with short generation times and broad host susceptibility, causes difficulties in managing infections and has severely limited the expansion of mariculture (e.g. Seng 1997; Hirazawa *et al.* 2004; Liao *et al.* 2004).

Management of *Neobenedenia* infections primarily relies on reactive treatments as opposed to proactive preventative management strategies. The established methods of treatment involve recurrent acute bathing of infected stock primarily in either formalin or freshwater solutions (Kaneko *et al.* 1988; Thoney & Hargis 1991; Fajer-Ávila *et al.* 2008). While these treatments kill attached *Neobenedenia* juveniles and adults, eggs are generally resistant and can exist outside the treatment area (Mueller, Watanabe & Head 1992; Ellis & Watanabe 1993). The physiological strain placed on the host during such treatments significantly

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increases susceptibility to re-infection (Ohno, Kawano & Hirazawa 2009). Furthermore, the use of formalin is complicated by the potential for environmental impacts and harm to human health (Masters 2004; Wooster *et al.* 2005). Consequently, the development of alternative control measures to prevent or reduce the intensity of *Neobenedenia* outbreaks is highly advantageous.

Substantial research interest has been directed towards antimicrobial plant-based phytochemicals for preventative parasite management, many of which are without the negative side effects associated with synthetic drugs (Palavesam, Sheeja & Immanuel 2006; Immanuel *et al.* 2009; Hutson *et al.* 2012). Allicin, a phytochemical agent of garlic, *Allium sativum* L., is a promising antiparasitic compound demonstrating selective toxicity towards microbes (Rabinkov *et al.* 2000; Davis 2005). In aquaculture, garlic has been demonstrated to exhibit antibacterial activity against a number of pathogenic bacteria of freshwater fish, but only a few reports describe the use of garlic extracts for management of parasitic diseases in fish (Lee & Gao 2012). Garlic extracts have previously been reported to eradicate ectoparasitic trichodinids where formalin proved ineffective (Madsen, Buchmann & Mellergaard 2000) and has demonstrated *in vitro* toxicity towards aquaculture significant protozoan parasites (e.g. *Ichthyophthirius multifiliis* [Fouquet], see Buchmann, Jensen & Kruse 2003; *Spironucleus vortens* [Poynton, Fraser, Francis-Floyd, Rutledge, Reed & Nerad], see Millet *et al.* 2011; *Neoparamoeba pemaquidensis* [Page], see Peyghan, Powell & Zadkarami 2008). The usefulness of garlic for the management of more complex metazoan parasites has only recently been explored (see Abd El-Galil & Aboelhadid 2012), and further studies assessing prospects of garlic therapy for managing metazoans are needed.

This study investigates the use of an allicin-containing garlic extract as a therapeutic immersion or 'bathing' treatment against all life stages of *Neobenedenia* sp. using the commercial host barramundi, *Lates calcarifer* (Bloch), as a model to determine the potential of garlic in ectoparasite management.

Materials and methods

Garlic extract

Garlic (variety Glen Large) grown in Queensland, Australia, was freshly peeled and homogenized with H_2O at a ratio of 5 mL per 1 g for 60 s. The homogenate was allowed to stand for 5 min without agitation before being filtered through a 90-μm screen and Whatman no. 1 filter in succession to produce the garlic extract. Stock solutions of the garlic extract were stored at 4 °C until use and used within 1 month. Prior to each experiment, a sample of the extract was removed and filtered to 0.2 μm, and the allicin content was determined by high-performance liquid chromatography (HPLC).

Determination of the garlic extract's allicin content was carried out using an Applied Biosystems SPERI-5 C18 250 mm × 4.6 mm column (5 μm bead size) with a methanol and water (50:50) mobile phase run at a flow rate of 0.5 mL min^{-1}. An injection volume of 100 μL was used, and peaks were detected at 254 nm and recorded by a Varian Pro Star UV-visible recorder. An aqueous solution of pure (>98%) allicin was created using previous methods (Lawson & Wang 2001) and used as the HPLC standard for allicin quantification. The concentration of allicin present in the garlic extract was determined to be 0.76 ± 0.03 μL mL^{-1} across all experiments. The four concentrations of the garlic extract examined for their therapeutic effect against *Neobenedenia* sp. are shown in Table 1.

Source of fish and *Neobenedenia*

The experimental host *L. calcarifer* were juvenile (100–200 mm) freshwater hatchery-reared fish obtained from a local nursery in Queensland, Australia. Naivety to parasitic infections was confirmed through hatchery records and by visually inspecting 10% of the experimental stock, anaesthetized with AQUI-S, under a stereomicroscope prior to experimentation.

Embryonated *Neobenedenia* sp. eggs were initially collected from an inland, marine *L. calcarifer* farm in Queensland, Australia. Filamentous algae containing *Neobenedenia* sp. eggs were removed

Table 1 The four garlic, *Allium sativum,* extract concentrations assessed for their therapeutic effect on *Neobenedenia*

Garlic extract concentration mL L^{-1}	Allicin content μL L^{-1}
1	0.76 ± 0.03
2	1.52 ± 0.06
10	7.60 ± 0.30
20	15.20 ± 0.60

from infected raceways and maintained in sea water (35 g L^{-1}) until the onset of hatching. Newly hatched (<6 h post-hatch) oncomiracidia were introduced to naive *L. calcarifer* maintained in 20-L non-renewal aquaria (35 g L^{-1}, 25 °C).

The species of *Neobenedenia* investigated in this study (hereafter as *Neobenedenia*) is presently unidentified given the absence of diagnostic criteria to differentiate between geographical/host isolates and species pending a revision of the genus (Whittington 2012). Representative adult specimens were accessioned in the Australian Helminth Collection, South Australian Museum, Australia (SAMA AHC 35461).

Neobenedenia eggs

The efficacy of acute and continuous garlic extract immersion therapy for the prevention of *Neobenedenia* embryo development and hatching success was investigated. These effects were compared against embryo development and hatching in commercially applied treatment (freshwater and formalin) regimes and a seawater control (35 g L^{-1}). Bundles of oviposited *Neobenedenia* eggs were collected by isolating infected barramundi in a 5-L aquarium with UV-filtered sea water (35 g L^{-1}) for 12 h. Eggs were collected by filtering the sea water through a 60-μm filter screen and backwashing the filtrate into a collection dish. Eggs were carefully separated, and between 15 and 20 eggs were allocated to an individual replicate glass cavity block (40 mm^2).

Each cavity block was randomly allocated to one of two experiments: acute 1-h immersion or continuous immersion. Within each experiment, cavity blocks were randomly allocated one of seven treatment solutions including the seawater control, formalin, freshwater, or 1, 2, 10 and 20 mL L^{-1} concentrations of garlic extract. Formalin concentration differed between the two experiments following the general guidelines for acute (100 μL L^{-1}) and continuous (20 μL L^{-1}) formalin treatment of ectoparasites (Noga 2010). Eleven replicates were made for each treatment in acute and continuous experiments. Cavity blocks were incubated at 22 °C in an environmental incubator on a 12:12 LD cycle (Illuminance: 5000 l×). For the acute immersion experiment, eggs were transferred from their treatment solutions to a new cavity block containing sea water (35 g L^{-1}) following 1 h and returned to the incubator.

Eggs were examined 24 h post-treatment and every 24 h thereafter under a stereomicroscope. Embryo development was scored according to four developmental stages: stage I: not embryonated and/or damaged, where eggs were distinctively translucent or visibly damaged; stage II: embryonated, where eggs were opaque and dark brown in colour; stage III: developing, eyespots present; and stage IV: hatched, translucent with open operculum (see Hutson *et al.* 2012). Hatched oncomiracidia were removed and discarded to prevent water fouling.

Following examination of the eggs, 2 mL of treatment solution or sea water was exchanged in each cavity block daily. Care was taken to ensure eggs remained submerged at all times. The experiment was terminated as per Mueller *et al.* (1992) where embryo development and hatching were considered complete if there was no increase in the number of stage II or III cases across all treatments for 120 h. Egg hatching success was expressed as the proportion of hatched eggs to the total number of eggs in each replicate.

Oncomiracidia longevity

The four concentrations of garlic extract (1, 2, 10 and 20 mL L^{-1}), acute formalin (100 μL L^{-1}) and freshwater immersion were assessed for their effect on *Neobenedenia* oncomiracidia longevity *in vitro*. A total of fourteen replicate wells split evenly across two 96-well plates were randomly assigned a treatment and a seawater control by row. Each well was filled with 300 μL of the respective treatment solution.

Newly hatched oncomiracidia were obtained by incubating *Neobenedenia* eggs in sea water (35 g L^{-1}) until hatching occurred. One oncomiracidium (<6 h post-hatch) was individually pipetted into each replicate well. Each oncomiracidium was monitored for up to 1 min every 2 h to assess survival. Dead oncomiracidia were characterized by individuals showing no signs of motion and failed to respond to flashing light (a tactile stimulus for even moribund oncomiracidia). Once determined to be dead, oncomiracidia were still examined in the subsequent monitoring period to confirm death. Oncomiracidia longevity was expressed as the time period from treatment immersion to death.

Oncomiracidia infection success

The four concentrations of garlic extract (1, 2, 10 and 20 mL L^{-1}) were assessed for their effect on *Neobenedenia* oncomiracidia infection success in a static non-renewal system. *Lates calcarifer* (48.6 ± 2.8 g) were individually allocated to fifty 10-L aquaria containing 5 L of UV-filtered sea water (35 g L^{-1}) receiving gentle aeration. Following a 24-h acclimation period to the new environment, garlic extract at a concentration corresponding to one of five treatments (1, 2, 10 and 20 mL L^{-1} garlic extract and seawater control) was added to the aquaria. Treatments were randomly assigned to 10 replicate aquaria. Fifteen newly hatched *Neobenedenia* oncomiracidia were introduced to each replicate aquarium, and aeration was suspended during and for 1 h following oncomiracidia introduction to facilitate infection as per Hirazawa *et al.* (2010). Fish were maintained in their respective treatment aquaria for 5 days to allow oncomiracidia to attach to their host and commence development. Fish were not fed during the trial, and temperature and salinity were maintained at 24.6 ± 0.6 °C and 35.6 ± 0.2 g L^{-1}, respectively. Fish were monitored every 6 h for mortalities, and total ammonia nitrogen (TAN) was measured in each replicate aquaria at the conclusion of the experiment using a commercial sodium salicylate/sodium hydroxide test kit (API: LR8600). Where mortalities occurred, TAN was assessed at the time of death.

On day six post-infection, fish were gently removed from their treatment aquaria using a fine mesh net and double bathed in separate containers of freshwater for five minutes each. The freshwater was roughly agitated during the bathing period to assist in the detachment of dead *Neobenedenia* from the fish's epithelial surfaces. Live *Neobenedenia* are transparent; hence, the only method to accurately quantify the total number infecting fish is to kill the parasites, which renders them opaque. The freshwater solution was filtered through a 60-μm mesh, backwashed into a Petri dish and examined for juvenile *Neobenedenia* under a stereomicroscope with incident and transmitted light. The net, freshwater container and 60-μm mesh were examined for any retained *Neobenedenia* for each replicate fish.

Infection success was expressed as the number of *Neobenedenia* recruits collected from fish on day six post-challenge to the number of oncomiracidia initially introduced to each aquaria. Mean infection intensity and the prevalence of infection were determined as per Bush *et al.* (1997).

Survival of juvenile *Neobenedenia*

Three concentrations of garlic extract (1, 2 and 10 mL L^{-1}) were assessed for their effect on juvenile *Neobenedenia* survival following 1-h acute immersion. The 20 mL L^{-1} concentration was omitted due to host mortality observed for the oncomiracidia infection experiment. Twenty-eight *L. calcarifer* were held in individual 10-L aquaria containing 5 L of UV-filtered sea water (35 g L^{-1}) receiving gentle aeration. Fish were given 24 h to acclimate to the new environment before the introduction of 10 oncomiracidia (<6 h old) to each aquarium as previously described. Oncomiracidia were given 5 days to attach and commence development on the *L. calcarifer* hosts. During this period, temperature and salinity were maintained at 23.3 ± 0.4 °C and 35.2 ± 0.4 g L^{-1}, respectively.

On day six, seven replicate 2-L aquaria were allocated to each of the four treatments (1, 2 and 10 mL L^{-1} garlic extract dilutions and the seawater control) and filled with the respective treatment solution. Individual *L. calcarifer* previously challenged with oncomiracidia were randomly transferred by hand to one of the replicate treatment aquaria. After 1-h immersion in the treatment the aquaria, the solution was roughly agitated to detach dead *Neobenedenia* from the host. Individual fish were gently transferred by hand to a separate container of a dechlorinated freshwater bath for 5–10 min to kill any surviving parasites. The contents of the treatment and freshwater baths for each *L. calcarifer* were independently examined for juvenile *Neobenedenia*. Survival of attached *Neobenedenia* in response to treatment was expressed as the number of *Neobenedenia* detected in only the freshwater bath compared with the total number of *Neobenedenia* detected in both the treatment and freshwater baths.

Statistical analysis

Values for each parameter measured were expressed as arithmetic mean ± standard error (SE). *Neobenedenia* oncomiracidia infection success and the proportion of eggs at each embryonic stage following acute and continuous immersion treatments

were expressed as percentages and independently analysed using permutational multivariate analysis of variance (PERMANOVA) based on Euclidean distances with the PRIMER 6 (version 6.1.13, PRIMER-E Ltd.) and PERMANOVA+ (version 1.0.3) statistical package. Oncomiracidia longevity was nominally expressed in hours and also analysed using PERMANOVA (PRIMER-E Ltd.).

Results

Egg immersion

Acute immersion in allicin-containing garlic extract significantly reduced hatching success at the highest concentration examined (20 mL L^{-1}). Hatching success in the 20 mL L^{-1} garlic treatment was 94% ± 2% compared with 98% ± 1% in the seawater control (Fig. 1a; $F_{\text{pseudo }(6,\ 70)} = 521.7$, $P < 0.001$). The lower concentrations of garlic extract (1, 2, 10 mL L^{-1}) all demonstrated similar levels of hatching success to the control (Fig. 1a). Embryo development and the embryonation period in all garlic treatments matched the seawater control with hatching commencing on either day four or day five and the majority of oncomiracidia larvae hatching on days six and seven (Fig. 2a).

Acute freshwater immersion was equally effective in reducing hatching success (91% ± 3%) as the 20 mL L^{-1} garlic treatment (Fig. 1a) and likewise demonstrated a similar egg development and hatching pattern to the garlic and control treatments (Fig. 2a). Acute formalin (100 μL L^{-1}) immersion was the only treatment to reduce hatching success below 90% (4% ± 1%; Fig. 1a). The low hatching success observed in the formalin treatment coincided with the majority of embryonated *Neobenedenia* eggs failing to develop (63% ± 4%) and a large portion of developed eggs failing to hatch (33% ± 3%; Fig. 1a). A delay in the onset of first

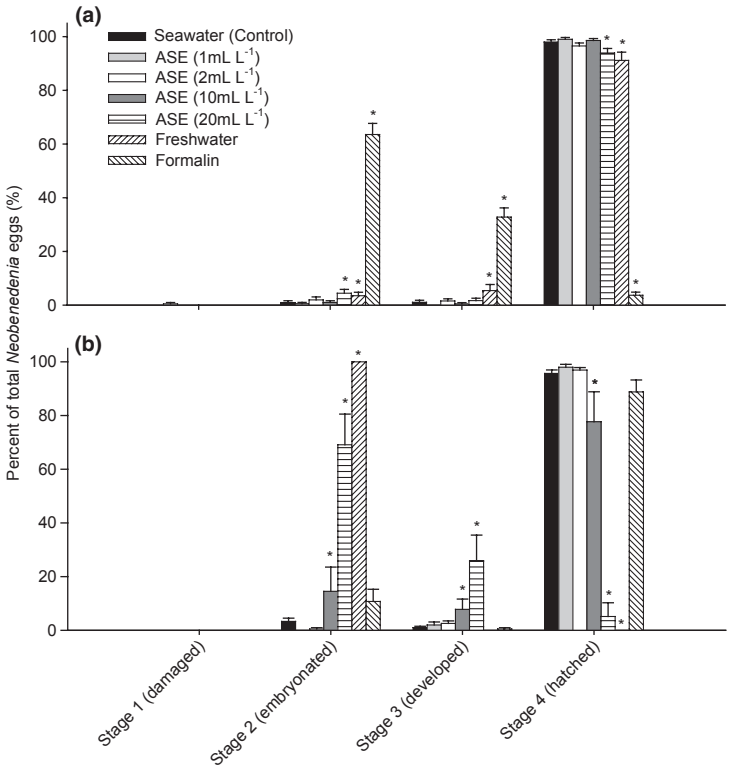

Figure 1 Mean (±SE) per cent of *Neobenedenia* eggs at the four developmental stages following: (a) acute (1-h) immersion of *Neobenedenia* eggs in four *Allium sativum* extract (ASE) dilutions, freshwater, formalin (100 μL L^{-1}) and seawater solutions; (b) continuous immersion of *Neobenedenia* eggs in four ASE dilutions, freshwater, formalin (20 μL L^{-1}) and seawater solutions. *Significantly different from control in each stage.

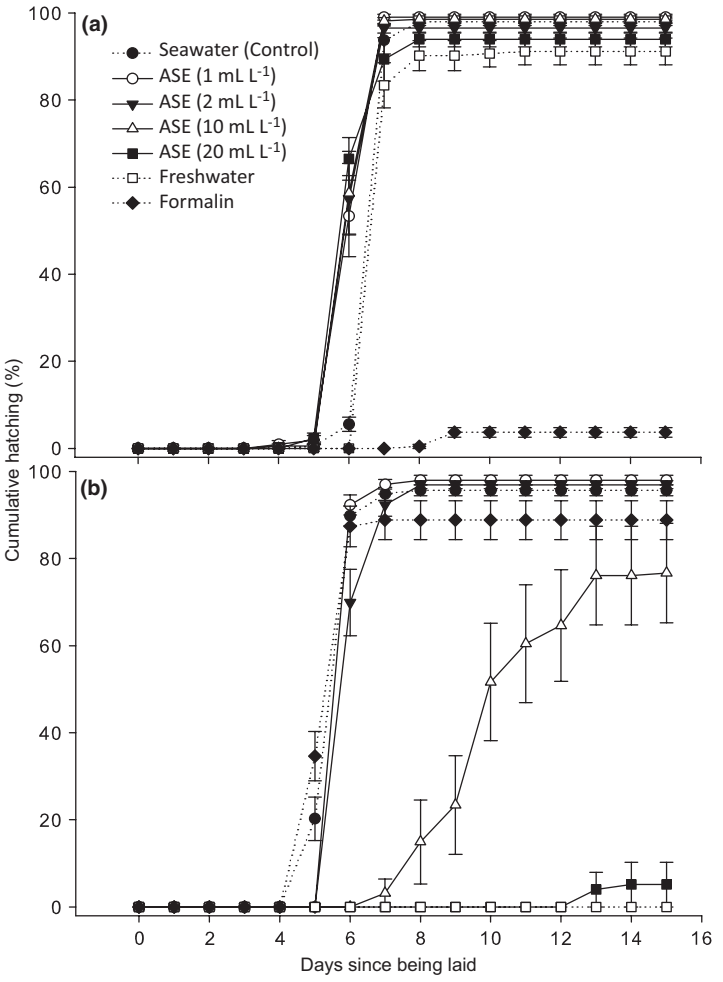

Figure 2 Cumulative daily percentage (±SE) of successful *Neobenedenia* hatching following: (a) acute (1-h) immersion of *Neobenedenia* eggs in four *Allium sativum* extract (ASE) dilutions, freshwater, formalin (100 μL L^{-1}) and seawater solutions on day zero; (b) continuous immersion of *Neobenedenia* eggs in four ASE dilutions, freshwater, formalin (20 μL L^{-1}) and seawater solutions.

hatching was also observed in eggs immersed in formalin with hatching beginning on day eight (Fig. 2a).

Continuous immersion in garlic extract reduced *Neobenedenia* egg hatching success. High concentrations of garlic extract (10 and 20 mL L^{-1}) significantly lowered hatching success to 78% ± 11% and 5% ± 5%, respectively, compared with eggs maintained in the seawater control (96% ± 1%; Fig. 1b; $F_{pseudo(6,70)}$=92.9, P = 0.001). The reduction in hatching success in 10 and 20 mL L^{-1} garlic extract was coupled with a delay in the onset of hatching that did not occur until days six and 12, respectively (Fig. 2b). Eggs began hatching in the control on day four, with the majority (>90%) having hatched within 48 h from the onset. The lower concentrations of garlic extract (1 and 2 mL L^{-1}) exhibited comparable hatching success and a similar embryonation period to the control (Figs 1b & 2b). In contrast to acute formalin immersion, continuous immersion in a therapeutic level of formalin (20 μL L^{-1}) was unsuccessful in prohibiting hatching (89% ± 5% hatching success; Fig. 1b). Eggs in the continuous formalin treatment also demonstrated a similar embryonation period to eggs maintained in the seawater control (Fig. 2b). No eggs hatched in the continuous freshwater immersion (Fig. 1b).

Oncomiracidia longevity

Garlic extract reduced oncomiracidia longevity. All garlic treatments, with the exception of the 1 mL L^{-1} dilution, significantly reduced oncomiracidia longevity compared with the seawater control (Fig. 3; $F_{psuedo\ (6,91)}$=106.6, $P < 0.001$). In the 20 mL L^{-1} treatment, all oncomiracidia died

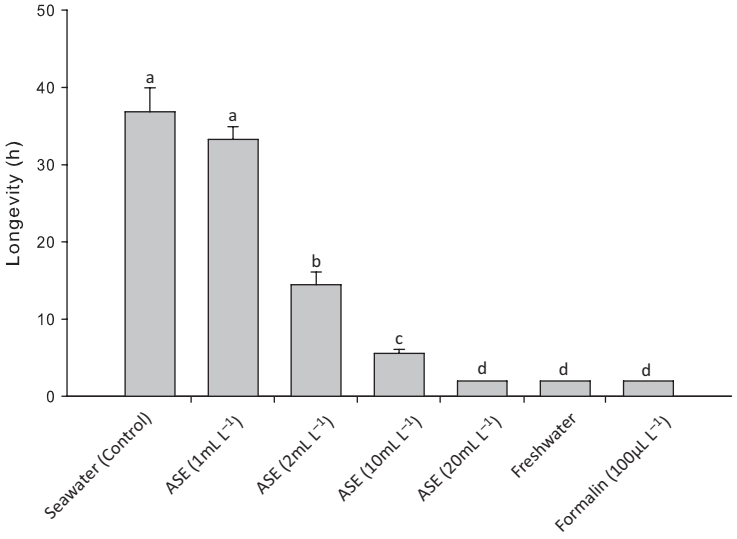

Figure 3 Mean (±SE) longevity of *Neobenedenia* oncomiracidia exposed to four *Allium sativum* extract (ASE) dilutions, seawater control, 100 µL L^{-1} formalin and freshwater *in vitro*. Bars with different superscripts are statistically significant ($P < 0.05$).

prior to the first observational period (2 h). Oncomiracidia survived for 37 ± 3 h in the seawater control and 33 ± 2, 14 ± 2, 6 ± 1 h in the 1, 2 and 10 mL L^{-1} dilutions of garlic extract, respectively (Fig. 3). Oncomiracidia longevity in the freshwater and 100 mL L^{-1} formalin treatments was comparable to the highest concentration of garlic extract (20 mL L^{-1}) with all oncomiracidia killed within 2 h (Fig. 3).

Infection success

Garlic extract significantly reduced oncomiracidia infection success. Oncomiracidia were half as successful (25% ± 4%) and five times less successful (11% ± 4%) at infecting *L. calcarifer* in the 1 and 2 mL L^{-1} garlic extract dilutions, respectively, compared with fish in sea water (55% ± 7%; Fig. 4; $F_{\text{pseudo }(2,39)} = 19.1$, $P < 0.001$). Furthermore, the prevalence of infection was lower in the 1 mL L^{-1} (90%) and 2 mL L^{-1} (70%) dilutions of garlic extract than in the seawater control (100%; Table 2). The mean infection intensity of 1 and 2 mL L^{-1} *A. sativum* extract treatments (4.2 and 2.4, respectively) was significantly lower than the mean intensity of *Neobenedenia* infection in the control (8.2; Table 2; $F_{\text{pseudo }(2,23)} = 13.8$, $P < 0.001$).

Host mortalities were observed within 48 h of exposing *L. calcarifer* to the 10 mL L^{-1} (20% mortality) and 20 mL L^{-1} (70% mortality) dilutions of garlic extract. These trials were aborted in the interests of host welfare. Total ammonia nitrogen (TAN) measures taken at time of death showed all mortalities occurred in solutions with TAN exceeding 8.0 mg L^{-1}. No mortalities were observed in the seawater control or 1 and 2 mg L^{-1} garlic extract dilutions for the duration of the study, and TAN remained below 2.0 mg L^{-1}.

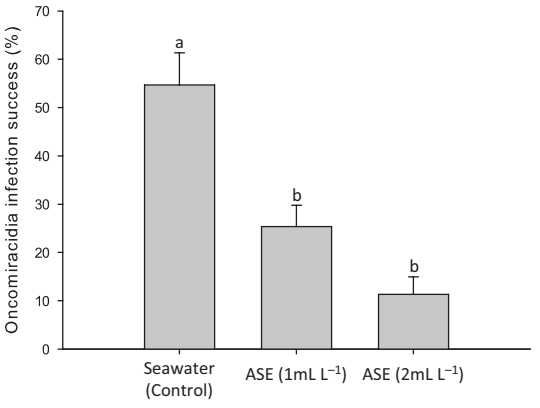

Figure 4 Mean (±SE) *Neobenedenia* oncomiracidia infection success of *Lates calcarifer* held in sea water and *Allium sativum* extract (ASE) dilutions. Bars with different superscripts are statistically significant ($P < 0.05$).

Attached *Neobenedenia* survival

Garlic extract did not kill juvenile *Neobenedenia* attached to fish hosts. All *L. calcarifer* were successfully infected with *Neobenedenia*, and 100% of *Neobenedenia* survived in the seawater control, 1 and 2 mL L^{-1} garlic treatment

Table 2 Prevalence and intensity of *Neobenedenia* infecting *Lates calcarifer* held in different *Allium sativum* treatment solutions following challenge with 15 oncomiracidia

Treatment	*N*	Prevalence (%)	Intensity (range)
Seawater control	10	100	8.2 (2–13)
A. sativum extract 1 mL L^{-1}	10	90	4.2[a] (0–7)
A. sativum extract 2 mL L^{-1}	10	70	2.4[a] (0–5)

[a]Significantly different from control.

Table 3 Prevalence and intensity of *Neobenedenia* infecting *Lates calcarifer* prior to immersion in *Allium sativum* treatment solutions and percentage of *Neobenedenia* surviving treatment immersion. *Lates calcarifer* were each initially challenged with 10 oncomiracidia

Treatment	*n*	Prevalence (%)	Intensity (range)	Survival (%)
Seawater control	7	100	6.3 (3–9)	100
A. sativum extract 1 mL L^{-1}	7	100	6.9 (5–8)	100
A. sativum extract 2 mL L^{-1}	7	100	6.1 (3–8)	100
A. sativum extract 10 mL L^{-1}	7	100	4.3 (2–6)	96

solutions (Table 3). *Neobenedenia* survival in the 10 mL L^{-1} garlic treatment was 96% with only a single individual *Neobenedenia* observed in the treatment solutions (Table 3). No host mortalities were recorded during the experiment.

Discussion

This study demonstrates that garlic extract can significantly suppress embryo development, reduce hatching success, oncomiracidia longevity and infection success of *Neobenedenia*. Identifying therapeutants capable of reducing hatching and oncomiracidia infection success for *Neobenedenia* management is of paramount significance given the high viability of eggs (98% hatching success) and infection success of oncomiracidia (>50%) in sea water. The observed antiparasitic capacity of the garlic extract is most likely attributed to the presence of allicin, the primary antiparasitic property in garlic (Lee & Gao 2012). This study suggests allicin concentrations as low as 1.5 μL L^{-1} could aid in the prevention of *Neobenedenia* infection. However, delivery of allicin via a crude garlic extract, as trialled in this study, presented a number of limitations to commercial application. These limitations included garlic extract proving ineffective against parasites already infecting fish and potential toxicity to juvenile *L. calcarifer*.

The primary mechanism of action associated with allicin's antiparasitic activity is believed to be the inhibition of SH-containing enzymes critical to parasite cell function (Ankri *et al.* 1997; Anthony, Fyfe & Smith 2005). The high level of tolerance demonstrated by *Neobenedenia* embryos following acute exposure to allicin is likely attributed to the sclerotized protein egg shell protecting the developing embryo as a physical barrier (Kearn 1986). Several studies have shown monogenean egg shells to be virtually impenetrable, being resistant to freshwater (Mueller *et al.* 1992; Ellis & Watanabe 1993), physical and chemical treatments (Diggles, Roubal & Lester 1993; Yoshinaga *et al.* 2000; Sharp *et al.* 2004; Sitjà-Bobadilla, Conde de Felipe & Alvarez-Pellitero 2006). Continuous freshwater immersion (this study) and immersion in hyposaline ($\leq$15 g L^{-1}) solutions (Mueller *et al.* 1992; Ellis & Watanabe 1993) remain the only methods known to prohibit *Neobenedenia* species hatching entirely. Immersion in formalin has not previously been investigated for *Neobenedenia* species' eggs, and this study found acute immersion in 100 μL L^{-1} formalin greatly reduced hatching (<4% success) and was significantly more effective than acute freshwater and garlic treatments. The use of formalin to reduce hatching success for *Neobenedenia* species has previously been discounted (see Whittington 2012) as the eggs of the closely related capsalid monogenean *Benedenia seriolae* (Yamaguti), which has similar egg structure to *Neobenedenia* spp., remained viable following 1-h immersion in 250 and 400 μL L^{-1} formalin (Sharp *et al.* 2004).

In the absence of the sclerotized protein shell protecting *Neobenedenia* embryos, allicin could interact directly with the free-swimming oncomiracidia. Allicin concentrations as low as 1.52 μL L^{-1} demonstrated significant antiparasitic capacity towards oncomiracidia with an apparent negative relationship between allicin concentration and oncomiracidia longevity. By reducing oncomiracidia longevity, as demonstrated with allicin, the opportunity for oncomiracidia to successfully infect a host also diminishes.

In relation to the most effective chemicals used to kill oncomiracidia, Umeda *et al.* (2006) demonstrated potassium permanganate concentrations of 20 mg L^{-1} effectively immobilized all

oncomiracidia of the monogenean *Pseudodactylogyrus* spp. Gusev. Oncomiracidia of *Sparicotyle chrysophrii* (van Beneden & Hesse) were completely immobilized by 300 μL L^{-1} formalin immersion for 30 min (Sitjà-Bobadilla *et al.* 2006). Similarly, this study showed immersion in 100 μL L^{-1} formalin or freshwater immobilized all *Neobenedenia* oncomiracidia in <2 h. Furthermore, the allicin concentration (15.2 μL L^{-1}) needed to immobilize all *Neobenedenia* oncomiracidia within a 2-h period is lower than the concentration of any chemical previously reported to immobilize monogenean oncomiracidia.

This study and others (Hirazawa *et al.* 2004, 2010) have demonstrated that *Neobenedenia* spp. oncomiracidia are highly efficient in recruiting to a host under experimental and aquaculture conditions. Allicin significantly reduced oncomiracidia infection success of *L. calcarifer* at host tolerable concentrations. The efficacy of 0.76 μL L^{-1} allicin (>50% reduction in oncomiracidia success) suggests that while this allicin concentration failed to significantly shorten the longevity of oncomiracidia *in vitro*, the infectivity of those oncomiracidia was still compromised. This could be due to allicin inhibiting an oncomiracidium's ability to locate or successfully recruit to a host at a sublethal level, such as sensory system impairment. Such factors were not addressed in the *in vitro* studies and indicate caution should be used before formulating conclusions on the efficacy of garlic extracts for parasite management based solely on *in vitro* toxicity (i.e. Buchmann *et al.* 2003; Millet *et al.* 2011).

Juvenile *Neobenedenia* attached to host fish were highly resistant to garlic extract immersion compared with oncomiracidia and other finfish parasites previously examined. Abd El-Galil & Aboelhadid (2012) demonstrated 300 mg L^{-1} crushed garlic effectively eliminated the monogenean *Gyrodactylus* sp. von Nordmann infecting tilapia, *Oreochromis niloticus* L., following baths of 24 h and 7 days in commercial ponds. However, the 10 mL L^{-1} concentrations of garlic extract in this study, roughly equivalent to 2 g L^{-1} crushed garlic, failed to eradicate *Neobenedenia* sp. from the *L. calcarifer* hosts over the 1-h exposure period. While the discrepancy in results may arise from the necessity of a longer exposure period, adult *Gyrodactylus* spp. are substantially smaller (0.3–1.1 mm) than adult *Neobenedenia* (2–5 mm), and the resistance exemplified by *Neobenedenia* may simply relate to the increased body volume to surface area ratio (Cone 1995). In such a scenario, allicin may only be able to interact with the parasite's outmost layer of cells proving insufficient to cause mortality. The high efficacy of garlic extracts against smaller protozoan parasites supports this argument (Madsen *et al.* 2000; Peyghan *et al.* 2008; Abd El-Galil & Aboelhadid 2012).

Freshwater and formalin have been used extensively for the treatment of monogenean infections (Kaneko *et al.* 1988; Thoney & Hargis 1991; Fajer-Ávila *et al.* 2008). This study confirmed the efficacy of these treatments in reducing *Neobenedenia* egg viability and oncomiracidia longevity, while previous studies confirm their use to eradicate adult stages (Kaneko *et al.* 1988; Fajer-Ávila *et al.* 2008). The necessary long-term immersion in hyposaline water to eradicate both eggs and adults is feasible for euryhaline species such as *L. calcarifer*, but is likely to be osmotically stressful to stenohaline marine species. Treatment of large, open systems would require substantial freshwater resources that are not always available. Formalin, the primary alternative, is highly toxic creating occupational health concerns (Wooster *et al.* 2005), and toxicity can be extended to plants and animals in the surrounding environment (Masters 2004). Preference should be given to treatment methods without adverse environmental impacts or negative implications for human or fish welfare.

It has been suggested that allicin extracts would be an ideal replacement to freshwater and chemical bathing as allicin biodegrades rapidly, and the selective toxicity of allicin makes garlic relatively harmless to humans and aquaculture stock (Ankri & Mirelman 1999; Lee & Gao 2012). However, this study shows crude garlic extracts appear unsuited to replace the current freshwater and formalin immersion regimes for immediate treatment of *Neobenedenia* infection as garlic extract was ineffective in removing *Neobenedenia* already parasitizing fish. The toxicity of the garlic extract towards *L. calcarifer* also questions the generalized suitability of garlic extracts towards aquaculture finfish, and case-by-case toxicity studies are needed before commercial application. While Abd El-Galil & Aboelhadid (2012) demonstrated that *O. niloticus* was capable of surviving prolonged exposure to garlic extract in earthen ponds, the presence of a natural biological filter may have mitigated the rapid increase in ammonia following extract administration observed in this study. This would suggest some promise for use of garlic

extracts in intensified recirculating systems, although the implications a biological filter could have on allicin availability need to be explored.

In contrast to treatment, proactive management focuses on prohibiting initial sources of infection (Bondad-Reantaso *et al.* 2005). Allicin-containing garlic extracts show promise for further development into proactive management tool to eradicate or weaken *Neobenedenia* eggs or oncomiracidia to prevent the initial infection of aquaculture stock. Only minute quantities of allicin were present (<16 μL L^{-1}) in effective garlic treatments suggesting a purified allicin extract may improve commercial feasibility and should be the direction of future studies.

In conclusion, this study demonstrated that minute quantities of allicin (<16 μL L^{-1}) in garlic extracts enabled significant reductions in *Neobenedenia* egg hatching success (5% success) and dramatically reduced oncomiracidia survival (100% mortality in 2 h) and infection success (11% success). Management of *Neobenedenia* and other monogenean pathogens will benefit from future development of a purified allicin therapeutant.

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