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Effects of temperature and salinity on the life cycle of *Neobenedenia* sp. (Monogenea: Capsalidae) infecting farmed barramundi (*Lates calcarifer*)

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Abstract Effective parasite management can be achieved through strategically timed treatments that break the life cycle. We examined the effects of temperature (2 °C increments from 22 to 34 °C) and salinity (0, 11, 22, 35, 40‰) on the life cycle (embryonation period, hatching success, oncomiracidia (larvae) longevity, infection success, and time to sexual maturity) of *Neobenedenia* sp. (Monogenea: Capsalidae), a harmful ectoparasite of farmed marine fishes. Experiments were conducted in controlled conditions in the laboratory. The life cycle was faster in warm, high saline conditions compared to cooler conditions (10–13 days between 26–32 °C, 40‰; 15–16 days between 22–24 °C at 40‰). Warm seawater and high saline conditions (24–32 °C, 35–40‰) improved egg hatching success, reduced time to sexual maturity, and resulted in parasites reaching sexual maturity at a larger size (at 30–32 °C) compared to cooler conditions (22 °C). In contrast, cool, hypersaline conditions (22 °C, 40‰) increased oncomiracidia longevity and infection success. Linear and quantile regression models were used to construct an interactive, online parasite management interface to enable strategic treatment of parasites in aquaculture corresponding to observed temperature and salinity variation on farms in the tropics. It was recommended that farmers treat their stock more frequently during summer (27–31 °C) when parasites can complete their life cycle more quickly. Nevertheless, farmers should be aware of the potential for increased *Neobenedenia* sp. infections during winter months (21–26 °C) due to increased infection success.

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Introduction

Integrated parasite management of farmed stock requires comprehensive and accurate knowledge of parasite life cycles and the influence of environmental parameters to be effective. In agriculture, parasite management may include a combination of chemical treatments, resistant stock breeds and grazing pasture management practices such as fallowing or alternative species grazing (Barger and Southcott 1978; Baker 1996; Barger 1997; Waller 1997; Stromberg and Averbeck 1999). The aquaculture industry is, comparatively, in its infancy and knowledge of parasite/fish biology is not always sufficient to enable efficient and effective treatments. One method of managing ectoparasites in aquaculture is to administer treatments at times that disrupt or break the life cycle of the parasite. The life cycle is interrupted by initially killing parasites on the host. Successive treatments are temporally coordinated to occur after all eggs have hatched but before new parasite recruits reach sexual maturity and contribute new eggs into the system (Tubbs et al. 2005). Essential to this method is the accurate determination of embryonation periods and time to sexual maturity at a range of environmental parameters, allowing for treatments to be tailored to particular localities and farms (Hirayama et al. 2009). Effective administration of this method results in lower re-infection rates, ultimately necessitating fewer treatments (Tubbs et al. 2005). Coordinated treatments designed to improve the efficiency of parasite management are recommended and have been successful in controlling parasites in agriculture including cow and sheep nematodes (Bumgarner et al. 1986; Stuedemann et al. 1989; Kelly et al. 2010) and sea lice outbreaks on salmon farms (Costello 2004),

whereas issues such as a lack of life cycle understanding and improperly coordinated treatments have been implicated in reduced management efficiency (Bravo et al. 2008; Bravo et al. 2013). Management strategies are not routinely implemented for Monogenean infections due to a lack of knowledge of parasite life cycle parameters.

Monogeneans in the family Capsalidae are a threat to the aquaculture industry as some species have been responsible for epizootic events (Deveney et al. 2001; Whittington 2004). Species of the genus *Neobenedenia* Yamaguti 1963, (Monogenea: Capsalidae) are known to be harmful ectoparasites and have been recorded from over 100 teleost species, many of which are important tropical and subtropical fin fish species in marine aquaria and aquaculture (Whittington and Horton 1996; Ogawa and Yokoyama 1998; Hirazawa et al. 2004; Ogawa et al. 2006; Whittington 2012). Their direct life cycle, short generation time and filamentous eggs, which entangle on structure, cause difficulties in managing infections and large numbers of parasites can become present in a system over a short period of time (Jahn and Kuhn 1932; Ogawa et al. 1995; Ogawa et al. 2006). *Neobenedenia* spp. graze on the epithelial surface of their host and feeding habit on host fish causes inflammation, dermal ulceration and allows the ingress of secondary infections (Kaneko et al. 1988).

Current management methods for capsalid Monogeneans involve immersing ('bathing') infected fish in hydrogen peroxide, formalin or freshwater (Ernst et al. 2002; Ogawa et al. 2006), which only temporarily relieves stock of infection by removing attached parasite stages (Whittington 2012). These treatment methods are ineffective in killing embryos within eggs (Sharp et al. 2004; Militz et al. 2013) and re-infection can occur immediately following treatment of stock. Bathing continues to be the most commonly used management practice for Monogenean infections and is widely used in the treatment of *Gyrodactylus* spp. epizootics (Schmahl and Mehlhorn 1988; Schmahl and Taraschewski 1987; Santamarina et al. 1991; Buchmann and Bresciani 2001; Buchmann and Kristensson 2003; Schelkle et al. 2011). Although promising results have been recorded from the administration of praziquantel through intubation, the oral administration of anthelminthics has been hindered by palatability issues (Williams et al. 2007; Hardy-Smith et al. 2012; Forwood et al. 2013) and variable efficacy between species (Hirazawa et al. 2013).

Data on parasite life cycle parameters in a variety of temperature and salinity conditions enables more accurate bathing regimes and can improve the efficacy of current farm practice. The aim of this study was to investigate the effects of temperature and salinity on the life cycle parameters (embryonation period, hatching success, oncomiracidia longevity, infection success and time to sexual maturity) of *Neobenedenia* sp. in order to develop an interactive strategic management tool for the treatment of farmed barramundi (*Lates calcarifer*, Bloch, 1790) (Perciformes: Latidae) (Table 1). This enabled strategically timed management recommendations that can be tailored to temperature and salinity conditions on aquaculture farms.

Materials and methods

Ethics statement

This work was conducted using barramundi, or Asian sea bass (*L. calcarifer*)—*Neobenedenia* sp. model system with all procedures approved by the James Cook University Animal Ethics Committee (A1579). *Neobenedenia* sp. used in experiments were collected from a private land in north Queensland, Australia. Future permissions should be directed to Coral Coast Barramundi Pty Ltd.

Parasite collection, temperature and salinity treatments

A laboratory infection of *Neobenedenia* sp. (Fig. 1) sourced from farmed marine *L. calcarifer* was established and

Table 1 Life cycle parameters of *Neobenedenia* sp. infecting *Lates calcarifer* in various temperature/salinity combinations

Temperature (°C)	Salinity (‰)	F/LH (days)	OL (hours)	SM (days)	LC (days)
22	22	6–8	2±0.0	–	–
	35	6–7	37±3.3	12	18
	40	7–8	49±1.5	9	16
24	22	–	5±0.5	–	–
	35	4–5	22±0.8	11	15
	40	4–5	31±1.1	12	15
26	22	–	2±0.0	12	–
	35	4–5	19±1.4	7	11
	40	4–5	28±2.6	6	10
28	22	–	3±0.5	–	–
	35	4–5	11±0.7	9	13
	40	4–5	10±1.0	9	13
30	22	4–9	3±0.5	8	12
	35	4–6	8±0.8	6	10
	40	4–6	7±0.4	6	10
32	22	4	2±0.3	–	–
	35	4–8	8±0.8	6	10
	40	4–8	6±0.3	8	12
34	22	9	2±0.0	–	–
	35	–	7±1.2	12	–
	40	6–9	6±0.9	8	14

F/LH time to first and last hatch, *OL* average oncomiracidia longevity± SE (OL), *SM* minimum time to sexual maturity; *LC* minimum time to completion of life cycle (LC)

maintained to ensure a continuous source of parasites for experimentation (see Militz et al. 2013). Experimental *L. calcarifer* were sourced from a freshwater hatchery and acclimated to 35‰ seawater for 24 h prior to experimentation. Fish were not previously exposed to *Neobenedenia* sp. infection. The species of *Neobenedenia* sp. investigated in this study is presently unidentified to species level due to the absence of diagnostic criteria to differentiate between geographical/host isolates and species (Whittington 2004, 2012). Phylogenetic analysis of approximately 40 *Neobenedenia* sp. isolates collected from multiple fish hosts in northern Australia is ongoing and may provide species-level clarification (Brazenor et al. unpublished data). Meanwhile, representative specimens mounted on slides were accessioned in the South Australian Museum, Australia (SAMA), in the Australian Helminth Collection (AHC); SAMA AHC 35461 (see Hutson et al. 2012). Temperature (22, 24, 26, 28, 30, 32 and 34 °C) and salinity (0, 11, 22, 35 and 40‰) treatments were selected to represent seasonal temperature and salinity variation experienced in tropical monsoonal climates in Queensland, Australia. Two-degree increments were chosen from the minimum average winter sea surface temperature (22 °C) to the average summer ocean temperature (34 °C) predicted by the year 2050 at Lucinda, Queensland (18° 31′ 41.271′ S: 146° 19′ 53.04″ E; Australian Institute of Marine Science (AIMS 2008)) under the Intergovernmental Panel on Climate Change (IPCC) (Allali et al. 2007; Solomon et al. 2007) scenario of 'business as usual' emission levels. Selected increments included the current average summer ocean temperature (30 °C) and maximum summer ocean temperature (32 °C) (AIMS 2008). Temperatures were described as 'cool' (22 °C), 'mild' (24–28 °C), 'warm' (30–32 °C) and 'hot' (34 °C) (see Results below). A range of salinities including freshwater (0‰), hyposaline (11 and 22 ‰), seawater (35‰), and hypersaline solutions (40‰) were chosen to represent extreme salinity fluctuations observed in open, semi-closed and closed aquaculture systems used to farm *L. calcarifer* in tropical climates. Hyposaline solutions (11 and 22‰) were prepared by mixing distilled water and filtered seawater to the desired concentration in a sterile container. The hypersaline solution was prepared through the addition of Mermaid marine salt to 35‰ filtered seawater. Salinities were determined using a refractometer.

Fig. 1 Live adult *Neobenedenia* sp. (ventral view) collected from *Lates calcarifer* epithelium. *Scale*=500 μm

Embryonation period and hatching success

The effect of temperature and salinity on the embryonation period and hatching success of *Neobenedenia* sp. eggs was examined in the laboratory. *Neobenedenia* sp. eggs were collected from infected laboratory fish as per Militz et al. (2013). Eggs were divided into groups of ten using fine-tipped forceps and placed into individual glass cavity blocks (40 mm^2). Six replicates were made for each temperature/salinity combination. The blocks were filled to the brim and a glass cover placed on top in order to reduce the occurrence of oncomiracidia lysing or becoming trapped in the surface tension (Ernst and Whittington 1996). Eggs were incubated (Sanyo: ML-351 Versatile Environmental Incubation Chamber) with fluorescent lighting for a 12:12 light/dark (LD) cycle. One third of the solution was changed each day with minimal disturbance to the eggs. Blocks were monitored every 24 h at 1100. When hatching had occurred, oncomiracidia were removed with a pipette and the day of hatching was recorded. Experiments were continued until 48 h passed without hatching in any treatment, at which time, any unhatched eggs were not considered viable. Embryonation period was defined as the time taken to first and last hatching of eggs. Hatching success of *Neobenedenia* sp. eggs was measured as the number of oncomiracidia removed from each cavity block divided by the total number of eggs.

Oncomiracidia longevity

The effect of temperature and salinity on the life span of *Neobenedenia* sp. oncomiracidia was examined in the laboratory. *Neobenedenia* sp. eggs did not hatch in 0 and 11 ‰ treatments, and consequently, these salinities could not be investigated in any further experiments. Approximately 100 eggs were incubated in a petri dish in each salinity (22, 35 and 40‰) at each temperature (22, 24, 26, 28, 30, 32 and 34 °C) (Model UP150 refrigerated incubator) with fluorescent lighting for a 12:12 LD cycle. One third of the solution in each petri dish was changed every 24 h. In order to obtain newly hatched oncomiracidia, each egg

mass was placed into fresh solution on the day hatching was first observed. Oncomiracidia that hatched over the next 2 h were used for experimentation. Individual oncomiracidia were gently removed from petri dishes using a plastic pipette and placed into separate glass cavity blocks in the salinity in which they were incubated. Eggs incubated in 22‰ solution at 24, 26 and 28 °C did not yield enough oncomiracidia for sufficient experimental replication. Consequently, oncomiracidia used in 22‰ treatments were sourced from 35‰ at the corresponding temperature. The solution in each cavity block was filled to the brim. Water was not changed throughout the experiment in order to minimise disturbance to oncomiracidia. Six replicates, containing a single individual oncomiracidium per replicate, were made for each temperature/salinity treatment. Each oncomiracidium was monitored every 2 h to assess survival. Oncomiracidia were considered dead once they showed no signs of motion and failed to respond to a gentle stream of water from a plastic pipette. Once determined to be dead, oncomiracidia were examined in the subsequent monitoring periods to confirm death. Oncomiracidia longevity was expressed as the elapsed time from initial immersion in the treatment to death.

Infection success, time to sexual maturity and size at sexual maturity

The effect of temperature and salinity on *Neobenedenia* sp. oncomiracidia infection success, time to sexual maturity and size at sexual maturity was examined in vivo. Approximately 200 eggs were incubated in each temperature (22, 24, 26, 28, 30, 32 and 34 °C) and salinity (22, 35 and 40‰) treatment to provide a source of oncomiracidia for the infection experiments. Eggs incubated at 32 and 34 °C and in 22‰ solution did not yield enough oncomiracidia for sufficient replication. Oncomiracidia used in 22‰ treatments were sourced from 35‰ and oncomiracidia used in 32 and 34 °C treatments were sourced from 30 °C.

Immediately following hatching, ten vigorously swimming oncomiracidia were gently aspirated with a glass pipette and slowly ejected from the pipette into a 10-L aquarium containing in 6 L of UV-filtered seawater and an individual *L. calcarifer* (mean 180 L_T (150–200 mm)). Five replicates were made for each temperature/salinity treatment. The aquaria were placed into an incubator (Sanyo: ML-351 Versatile Environmental Incubation Chamber) with fluorescent lighting for a 12:12 LD cycle and aerated using battery powered aerators. Pieces of large gauge (1 mm) netting (20×10 cm) were placed into each of the aquaria and inspected under a stereomicroscope at×2 magnification daily at 10 a.m. for tangled eggs, indicating the presence of at least one sexually mature parasite. A pilot study showed that eggs always entangled on the netting on the first day that they were laid (AKB, unpublished data). Accordingly, time to sexual maturity was considered to be the day that eggs were first observed on netting.

The following day, fish infected with sexually mature parasites were bathed twice in dechlorinated freshwater for 5 min and the epithelial surface gently rubbed in order to dislodge attached parasites (see Militz et al. 2013). The solution was filtered through a 60-μm mesh to collect dislodged individuals which were counted under a stereo-microscope. Infection success was determined as the proportion of parasites collected divided by the number of oncomiracidia introduced to the fish. *Neobenedenia* sp. were fixed in 70 % EtOH and then stained with haematoxylin, dehydrated through an alcohol series, and mounted on microscope slides in Canada balsam. Total length, total width, anterior hamuli, and accessory sclerites were measured using a micrometre and ImageJ 1.44p (Java 1.6.0_20) (Table 2). Measurements are given as: mean (minimum–maximum range) and follow Lackenby et al. (2007).

Life cycle

The life cycle, or minimum time to re-infection by sexually mature parasites, was calculated as the sum of time taken for eggs to begin hatching and minimum time to sexual maturity. Oncomiracidia were considered to be able to infect fish immediately following hatching (A. T. Gonzalez unpublished data).

Theoretical strategic management tool

A theoretical model was developed (from data obtained in above paragraphs) to determine when fish should be bathed in order to maximise treatment efficiency in varied temperature and salinity scenarios. The time between the initial and subsequent treatment(s) was determined to occur within a period that allowed eggs deposited by adult parasites pre-treatment to hatch, but before new parasite recruits matured on fish. Assumptions were as follows: (1) an initial bath treatment kills 100 % of attached parasite stages on stock on day one, (2) that oncomiracidia can reinfect fish immediately following treatment and (3) that oncomiracidia survive no longer than 24 h more than the observed life span. The assumptions were made given that the efficacy of freshwater bath treatments against *Neobenedenia melleni* is 100 % with treatments as short as 2 min (Kaneko et al. 1988), while oncomiracidia can infect fish within 15 min in laboratory conditions (A.T. Gonzalez, unpublished data). To account for variable oncomiracidia longevity observed between studies

Table 2 Comparative measurements of *Neobenedenia* sp. infecting *Lates calcarifer*

Temperature (°C)	Salinity (‰)	Sample size (*n*)	Length (μm)	Width (μm)	Anterior hamulus length (μm)	Accessory sclerite length (μm)
22	22	–	–	–	–	–
	35	6	834 (464–2056)	312 (133–964)	95 (55–198)	49 (23–136)
	40	17	1081 (699–1788)	425 (187–754)	113 (69–167)	56 (41–98)
24	22	–	–	–	–	–
	35	6	1834 (1087–2099)	905 (502–1082)	185 (130–234)	78 (45–97)
	40	23	1899 (1358–2586)	956 (605–1445)	174 (119–233)	81 (53–114)
26	22	2	2804 (2413–3194)	1224 (1188–1260)	181 (155–206)	112 (100–124)
	35	6	1461 (1228–1676)	647 (490–847)	133 (128–142)	65 (47–84)
	40	13	1739 (1278–2170)	777 (553–1028)	162 (118–217)	67 (40–102)
28	22	–	–	–	–	–
	35	9	1591 (1228–1850)	707 (558–847)	147 (126–189)	69 (47–101)
	40	26	1167 (611–1794)	592 (258–876)	122 (53–194)	59 (19–94)
30	22	4	2952 (2793–3277)	1738 (1622–1836)	249 (219–264)	139 (125–160)
	35	18	1715 (1498–2858)	855 (170–1545)	161 (65–266)	101 (39–177)
	40	25	1956 (1065–2625)	917 (397–1587)	174 (115–258)	110 (69–156)
32	22	–	–	–	–	–
	35	16	2013 (999–2672)	1049 (427–1631)	187 (111–254)	116 (46–163)
	40	38	2078 (1079–3124)	1029 (424–2347)	188 (111–340)	114 (59–184)
34	22	–	–	–	–	–
	35	1	1865	914	157	107
	40	1	2147	948	180	126

Measurements in micrometres (μm); mean followed by range in parentheses

– indicates no measurement given as no infecting parasites were observed

(Militz et al. 2013) and to convert longevity into whole days for use in a calendar-day program, longevity values were rounded up to the nearest day (Fig. 2b; Table 1).

The interactive strategic treatment tool can be accessed via internet connection (including smart phone), which provides farmers easy and flexible access to the program. The model was developed using the linear and quantile regression equations. Equations and decision functions were entered manually into JavaScript 1.8.5. The program was then exported to http://marineparasites.com/paratreatmentcal.html and hosted on this URL. The program can be accessed using either Firefox or Google Chrome web browsers. The website interface presents hatching success, infection success and recommended timed treatments for each whole degree Celsius and salinity (‰) increment between 22–34 °C and 22–40‰. This range encompasses temperatures and salinities experienced throughout a year in the tropics.

Statistical analyses

Hatching success, oncomiracidium longevity and infection success data did not fulfil the conditions of normality and homogeneity of variance and were analysed by permutational analysis of variance in the PERMANOVA function of PRIMER 6.0. PERMANOVA compares the observed value of a test statistic (F ratio) against a recalculated test statistic generated from random permutation of the data. PERMANOVAs with 9999 permutations based on Euclidean distance were used to statistically evaluate experimental treatments (hatching success, oncomiracidia longevity and infection success), followed by pairwise comparisons. Significance was accepted at $p<0.05$.

A two-way analysis of variance (ANOVA) was used to determine whether temperature and/or salinity had significant effects on parasite size. A one-way ANOVA was used to assess the effects of temperature on parasite size (as salinity was found to have no significant effect). Analyses were performed using the S-Plus 8.0 software package from Spotfire®. Significance was accepted at $p<0.05$. Post hoc comparisons of group means were performed using Tukey's honestly significant difference (HSD) test.

Linear models were used to describe the relationship between treatment (temperature and salinity) and

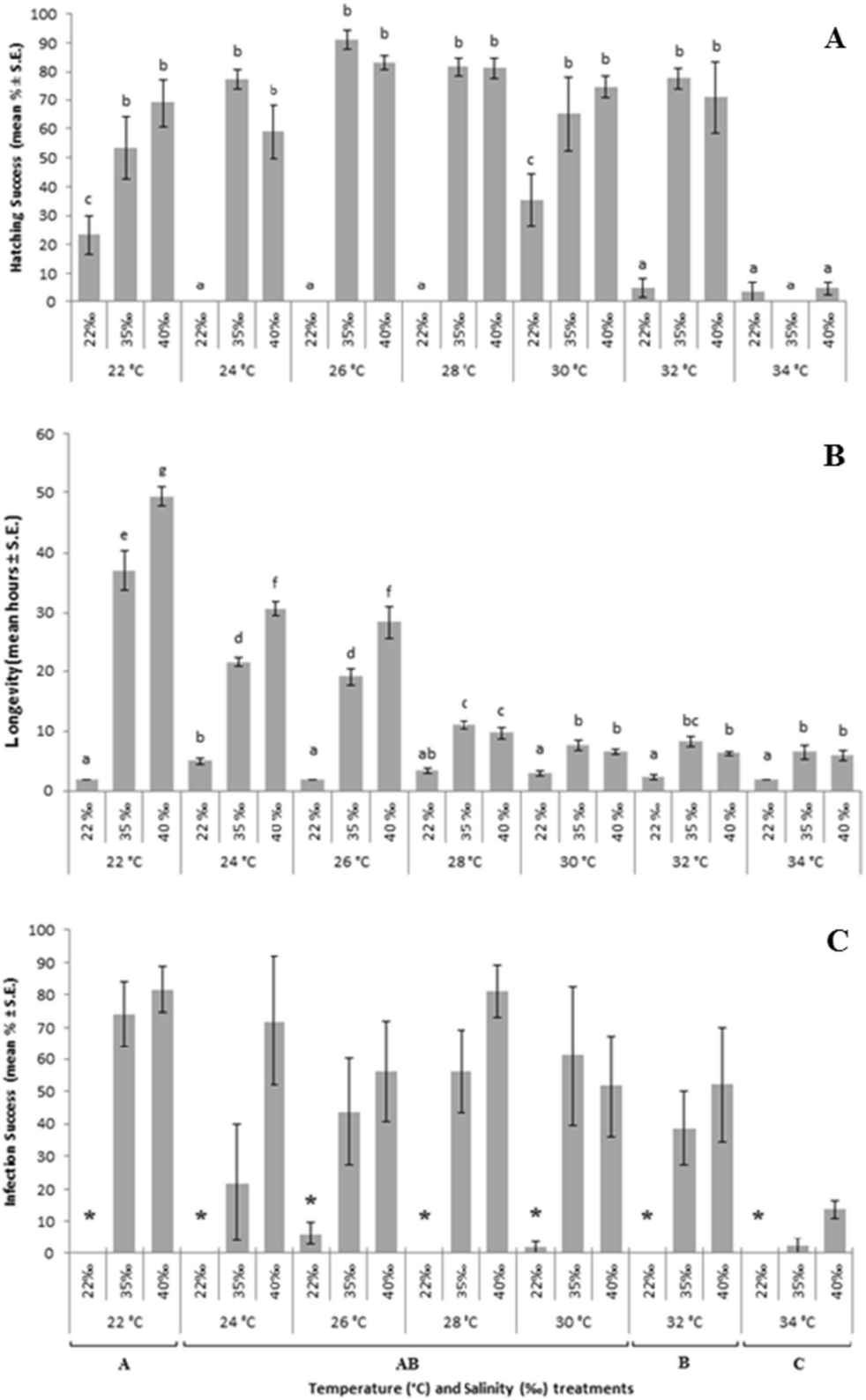

Fig. 2 **a** *Neobenedenia* sp. hatching success in temperature and salinity treatments (0 and 11 ‰ not shown; no hatching observed). *a*, *b* and *c* differences between pairs of means determined using PERMANOVA pairwise comparison test, $p<0.05$. **b** *Neobenedenia* sp. oncomiracidia longevity (in hours) in temperature and salinity treatments. *Letters above columns* differences between pairs of means determined using PERM ANOVA pairwise comparison test, $p<0.05$. **c** *Neobenedenia* sp. infection success in temperature and salinity treatments; *A*, *AB*, *B* and *C* differences between temperatures. *Differences between pairs of means for salinity determined using PERM ANOVA pairwise comparison test, $p<0.05$

response variables (hatching and infection success, time to last hatch and time to sexual maturity). Quantile regression models were created for 'time to last hatch' (tau=0.95) and 'time to earliest sexual maturity' (tau=0.05) data. Equations used to generate each relationship are outlined in Table 3. Linear and quantile regression models were created using the statistical program R 3.0.0. using the 'quantreg' package (Koenker 2013).

Table 3 Equation values for linear models: 'hatching success', 'infection success' and quantile regression models: 'time to last hatch' and 'time to earliest sexual maturity data'

Terms	Hatching success (%)	Infection success (%)	Time to last hatch (days)	Time to sexual maturity (days)
Intercept	−5.016	$1.660e^{4}$	$3.218e^{2}$	$1.348e^{2}$
a	$2.891e^{-1}$	$-2.292e^{3}$	$-4.574e^{1}$	−6.014
b	$2.120e^{1}$	$-1.184e^{1}$	$1.839e^{1}$	−5.167
ab	−1.633	$5.388e^{-1}$	−1.632	$2.859e^{-2}$
a^2	$-4.862e^{-3}$	$1.172e^{2}$	2.403	–
b^2	−1.207	$1.796e^{-1}$	$-2.120e^{-1}$	–
ab^2	$9.572e^{-2}$	–	$1.073e^{-2}$	–
a^2b	$2.983e^{-2}$	–	$3.975e^{-2}$	–
a^2b^2	$-1.761e^{-3}$	$-2.258e^{-4}$	–	$-9.287e^{-5}$
a^3	–	−2.637	$-5.527e^{-2}$	–
b^3	–	–	$2.823e^{-3}$	–
a^3b^2	–	–	$-2.436e^{-6}$	–
a^2b^3	–	–	$-4.647e^{-6}$	–
a^4	–	$2.198e^{-2}$	$4.702e^{-4}$	$2.522e^{-5}$
b^4	–	–	$-4.473e^{-5}$	$1.118e^{-5}$
a^4b	–	–	$-6.875e^{-6}$	–
ab^4	–	–	$1.173e^{-6}$	–
a^4b^4	–	–	$2.965e^{-11}$	–
a^b	–	–	–	$2.120e^{-62}$
b^a	–	–	–	$-1.136e^{-53}$

– denotes no value

a temperature data, *b* salinity data

Results

Embryonation period and hatching success

Egg embryonation periods were shorter in mild temperatures and in seawater and high salinity (4–5 days at 24–28 °C, 35–40‰) while the embryonation period was longer at the temperature extremes (6–8 days at 22 °C, 22‰; 4–9 days at 30–34 °C, 22–40‰; Fig. 3; Table 1). Time to first hatch was shorter between 24–32 °C, with eggs hatching on day 4, whilst cool, hypersaline conditions (22 °C, 40‰; Fig. 3; Table 1) delayed hatching to day 7.

Mild to warm hypersaline water provided optimal conditions for *Neobenedenia* sp. egg hatching success. More than 80 % of eggs hatched between 26–32 °C, 40‰ (Figs. 2a and 3a). No *Neobenedenia* sp. eggs hatched in low salinities (0–11‰). Hatching success was low in 22‰ saline (<40 % hatching success) and in hot, hypersaline conditions (<5 % hatching success at 34 °C, 35–40‰; Fig. 2a). Egg hatching success was significantly influenced by temperature (PERMANOVA, pseudo-$F_{6,\ 175}$=39.08, p<0.0001) and salinity (PERMANOVA, pseudo-$F_{4,\ 175}$=424.87, p<0.0001) (Figs. 2a and 3a). There was a significant interaction between temperature and salinity on egg hatching success (PERMANOVA, pseudo-$F_{24,\ 175}$= 15.92, p<0.0001).

Oncomiracidia longevity

Oncomiracidia survived for longer periods of time in cool, seawater and hypersaline water. Oncomiracidia longevity was significantly reduced in warm and hot water (8±0.8 h at 30–34 °C) and in low saline (5±0.5 h in 22‰) conditions. Conversely, cool, hypersaline water (22 °C, 40‰) prolonged the longevity of larvae (49±1.5 h; Fig. 2b; Table 1). Longevity was significantly influenced by temperature (PERMANOVA, pseudo-$F_{6,\ 105}$=186.24, p<0.0001) and salinity (PERMANOVA, pseudo-$F_{2,\ 105}$=391.35, p<0.0001) (Fig. 2b). The interaction between temperature and salinity on the longevity of *Neobenedenia* sp. oncomiracidia was also significant (PERMANOVA, pseudo-$F_{12,\ 105}$=53.57, p<0.0001).

Infection success, time to sexual maturity and size at sexual maturity

Cool, hypersaline water provided optimal conditions for *Neobenedenia* sp. infection success (>81 % success at 22 °C, 40‰; Figs. 2c and 4b). Infection success significantly decreased with increasing temperature (<15 % success at 34 °C) and was reduced in low saline solutions (<10 % at 22‰; Figs. 2c and 4b). Infection success was significantly influenced by temperature (PERMANOVA, pseudo-$F_{6,\ 64}$= 4.61, p=0.0006) and salinity (PERMANOVA, pseudo-$F_{2,\ 64}$=41.34, p<0.0001). There was no significant interaction between temperature and salinity on *Neobenedenia* sp. infection success (PERMANOVA, pseudo-$F_{6,\ 64}$=1.66, p=0.103).

Neobenedenia sp. reached sexual maturity rapidly in mild to warm seawater (6 days at 26, 30, and 32 °C, at 35‰; Fig. 5d; Table 1). Parasites took twice the amount of time to reach sexual maturity in cool temperatures (12 days at 22 °C, 35‰; Fig. 4d). Time taken to reach sexual maturity was longer in hyposaline conditions (12 days at 26 °C, 22‰ and 8 days at 30 °C, 22‰) compared to high salinities at the same temperatures (7 days at 26 °C, 40‰ and 6 days at 30 °C, 40 ‰).

Parasites were significantly larger at sexual maturity in warm water. Sexually mature parasites exhibited a total body length of 1.8±0.6 and 2.0±0.5 mm at 30 and 32 °C, respectively, while parasites in cool conditions (22 °C) were 1.0±0.3 mm in length (Fig. 5; Table 2). All morphological features were proportional in their measurements with respect to temperature and showed the same trend of generally increasing in size with increasing temperature. Temperature had a significant effect on all morphological characters measured including total length (ANOVA, $F_{5,\ 199}$=5.119, p<0.0001), total width (ANOVA, $F_{5,\ 199}$=21.091, p<0.0001), anterior

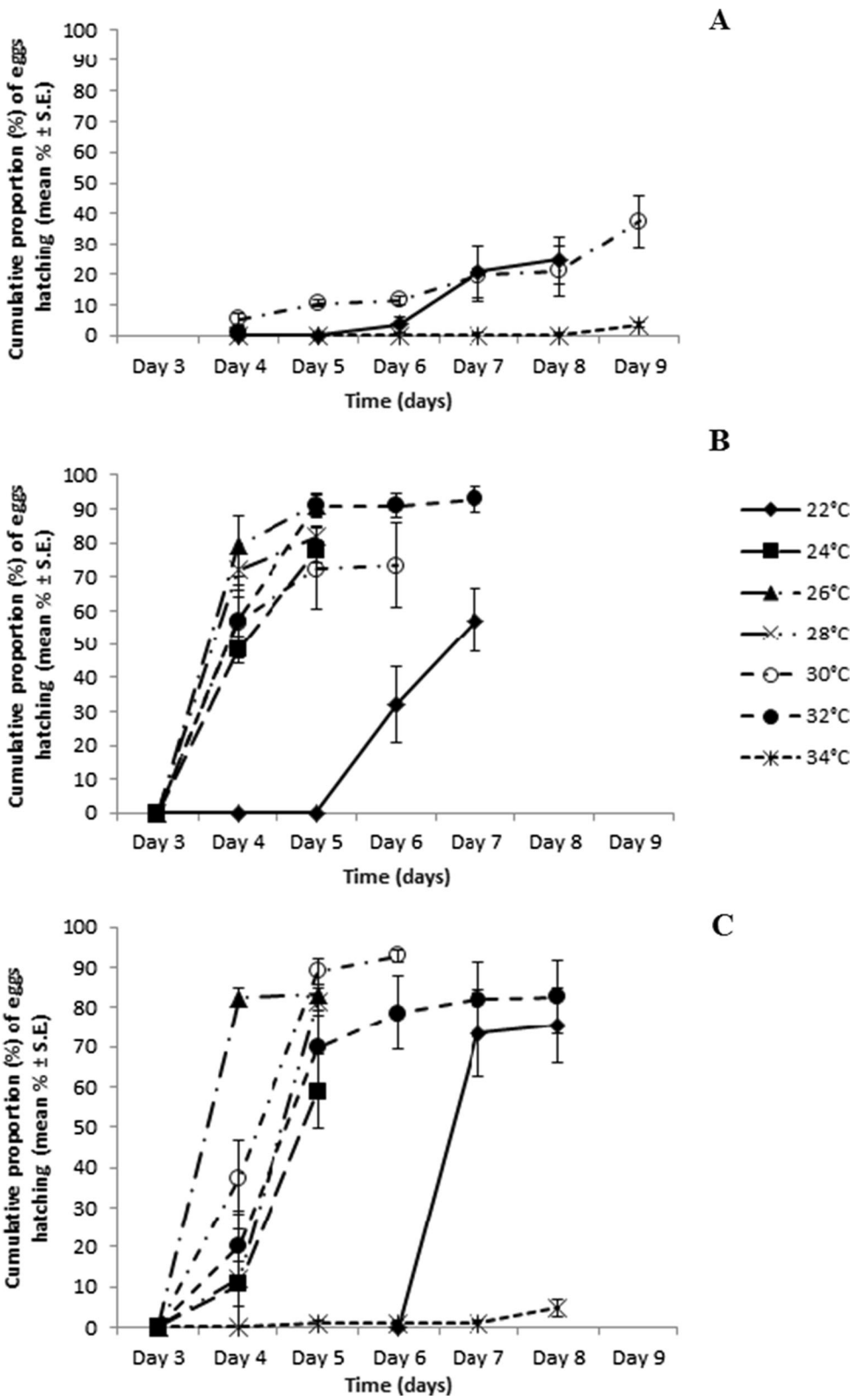

Fig. 3 Cumulative proportion of hatched *Neobenedenia* sp. at 22 ‰ (**a**), 35‰ (**b**), and 40‰ (**c**). Day of last hatch is indicated where data points finish. *Error bars* indicate SE

hamulus length (ANOVA, $F_{5,\ 199}=18.358$, $p<0.0001$) and accessory sclerite length (ANOVA, $F_{5,\ 199}=35.847$, $p<0.0001$) (Fig. 4a, b). Salinity did not significantly influence size at maturity. There was no significant interaction between temperature and salinity on morphological measurements.

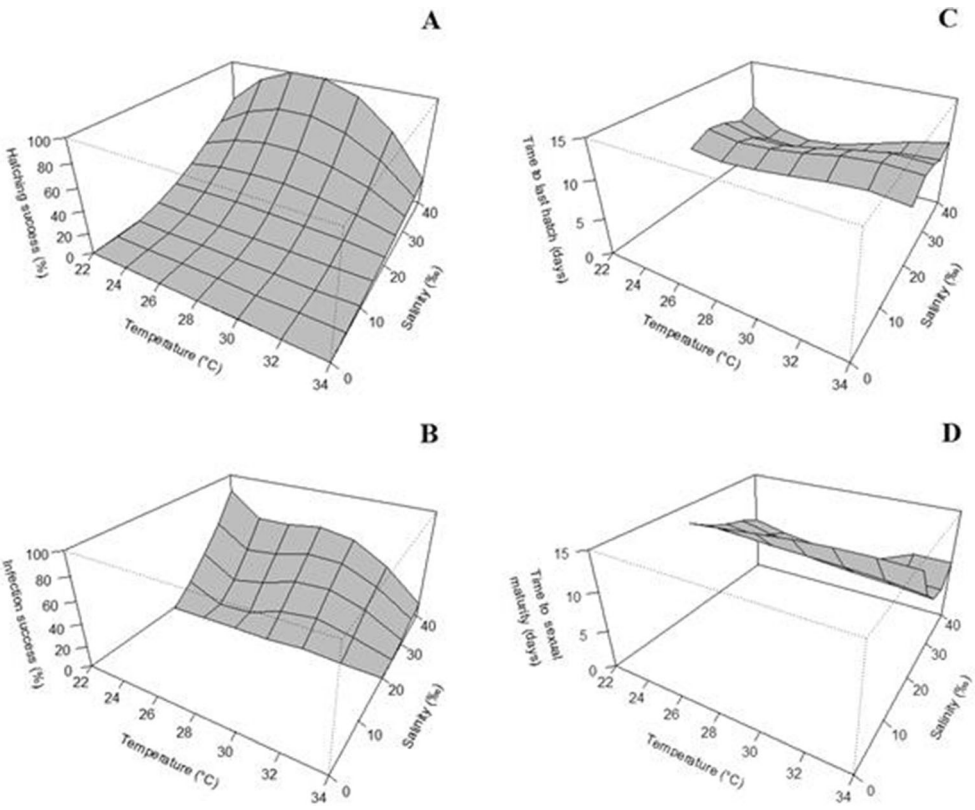

Fig. 4 General linear models describing the relationship between temperature, salinity and **a** hatching success, **b** infection success, **c** time to last hatch and **d** time to sexual maturity of *Neobenedenia* sp.

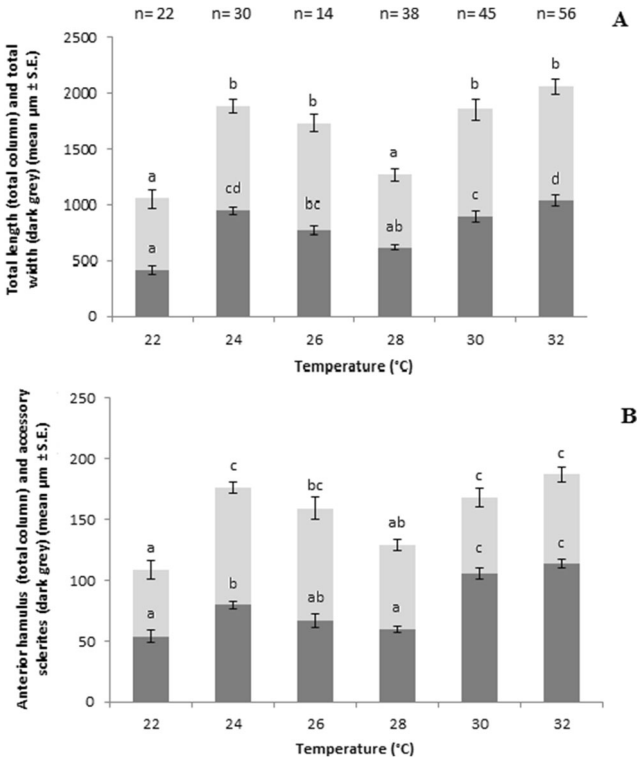

Fig. 5 **a** *Neobenedenia* sp. total length (*total column height*) and total width (*dark grey column*) and **b** anterior hamulus (*total column height*) and accessory sclerite length (*dark grey column*) with respect to temperature. *a*, *b* and *c* differences between pairs of means determined using Tukey's HSD test, $p<0.05$. Each parameter was independently statistically analysed

Life cycle

Parasites completed their life cycle faster in mild to warm, high saline conditions. Parasites took 10–12 days to complete the life cycle in mild and warm (26, 30 and 32 °C), high saline (35–40 ‰) conditions, compared to 18 days in cool seawater (22 °C, 35‰; Table 1). In cool to mild conditions (22–24 °C) and in seawater and high salinity (35–40‰), a second generation of *Neobenedenia* sp. oncomiracidia can emerge between 15–18 days, compared to 10–14 days at warm temperatures (26–34 °C) in comparable salinities. In warm, high saline conditions, *Neobenedenia* sp. has the capacity to produce three consecutive generations within one month (30 days), whereas only two consecutive generations could be achieved within one month in cooler conditions (Fig. 4c, d; Table 1).

Theoretical strategic management tool

The strategic management tool showed that treatments should occur earlier and more often in mild to warm seawater and hypersaline environments (26–34 °C, 35–40‰) compared to cooler conditions (22–24 °C, 35–40‰). The online interface permits the user to determine the appropriate time for subsequent treatments, following an initial treatment of stock (see http://marineparasites.com/paratreatmentcal.html). Generally, only a single subsequent treatment (occurring between days 7–14) is needed to break the parasite's life cycle in cool

conditions in all salinities (22–24 °C). In contrast, warm to hot, high saline conditions (26–34 °C) require two subsequent treatments between days 5–12 (http://marineparasites.com/paratreatmentcal.html).

Discussion

Warm, high saline conditions enabled rapid completion of the life cycle of the ectoparasitic Monogenean, *Neobenedenia* sp.. Short life cycles make strategic parasite management more challenging, as a greater number of treatments are required to effectively break the life cycle and minimise re-infection. Parasites took only 10 days to complete the life cycle in mild to warm conditions, compared to 18 days in cool conditions (Table 1). Rapid life cycle completion at warmer temperatures results in a greater frequency of treatments being required in order to manage infections (http://marineparasites.com/paratreatmentcal.html).

Several Monogenean species exhibit short life cycles in warm temperatures (Buchmann 1988; Roubal and Diggles 1993; Bondad-Reantaso et al. 1995; Ernst and Whittington 1996; Yoshinaga et al. 2000; Tubbs et al. 2005; Lackenby et al. 2007). Accelerated life cycles can be attributed to the increased metabolic and development rate associated with warm conditions (Poulin et al. 1989; Müeller et al. 1992; Conley and Curtis 1993; Ellis and Watanabe 1993). Hirazawa et al. (2010) and Bondad-Reantaso et al. (1995) observed similar life cycle completion times to this study for *Neobenedenia girellae*. The life cycle of *N. girellae* took only 10 days to complete at 30 °C as opposed to 20 days at 20 °C in seawater (33‰) (Hirazawa et al. 2010) and 15 days at 25 °C (Bondad-Reantaso et al. 1995). Time to sexual maturity was influenced by temperature, taking longer at cool temperatures (Table 1; Bondad-Reantaso et al. 1995; Hirazawa et al. 2010). Although maturation times were longer, sexual maturity by *Neobenedenia* sp. was reached at a smaller size at cool temperatures compared to warm conditions (Fig. 5a, b; Table 2; Hirazawa et al. 2010). Larger Monogenean individuals lay more eggs than those of a smaller size (Roubal and Diggles 1993; Whittington 1997), indicating that more eggs per parasite are contributed to a system in warm conditions.

Smaller size at sexual maturity in cool conditions has also been recorded for *Benedenia seriolae* infecting *Seriola lalandi* (Tubbs et al. 2005; Lackenby et al. 2007). However, the time *B. seriolae* took to reach sexual maturity was considerably longer than recorded for *Neobenedenia* sp. at all temperatures. This finding, combined with the observation that *B. seriolae* has markedly lower infection success than that of *N. girellae* (Hirazawa et al. 2013), indicates that populations of *Neobenedenia* spp. can build more rapidly than those of *B. seriolae* on aquaculture farms at similar temperatures. *B. seriolae* and *N. girellae* co-infect farmed *Seriola quinqueradiata* and *Seriola dumerili* in Japan and differences in population growth and proliferation is crucial to understanding the threat that each species poses to aquaculture stock. *Neobenedenia* spp. are capable of producing three generations per month in warm, high saline conditions which are typical of coastal marine aquaculture facilities in the tropics (Table 1). Presently, the cause for the unpredictable and sporadic nature of *Neobenedenia* sp. infection events is unknown and more vigilant and frequent stock monitoring may be required in warm, high saline conditions as infections can build more rapidly (Marcogliese 2001).

Although warmer temperatures result in an accelerated life cycle, cooler temperatures may facilitate the increase of *Neobenedenia* sp. infection on farmed fish. Oncomiracidia longevity and infection success were inversely correlated to increasing water temperature (Fig. 2b, c). Hirazawa et al. (2010) also observed that infections of *N. girellae* on *S. dumerili* were significantly reduced at 30 °C compared to cooler temperatures (20 and 25 °C) in seawater. Furthermore, cool conditions can compromise the immune system of fish (see Bly and Clem 1992 for review), inhibiting their capacity to withstand parasite infection (Jackson and Tinsely 2002). Deveney et al. (2001) hypothesise that a prolonged period of unseasonably low water temperatures was the cause for a *N. melleni* outbreak on sea caged *L. calcarifer* in Queensland, Australia, during winter. Although the causes behind epizootics of *Neobenedenia* spp. are unknown, increased oncomiracidia longevity and infection success observed at cool temperatures as demonstrated in this study (Fig. 2b, c) may, together with immune-suppressed hosts, contribute to epidemics on farms during cooler months.

Neobenedenia sp. cannot complete its life cycle in low salinity environments. *Neobenedenia* sp. eggs did not hatch in 0 and 11‰ and hatching success was significantly reduced in 22‰ (Fig. 2a). Accordingly, Müeller et al. (1992) observed that the hatching success of *N. melleni* eggs was <12 % when incubated at salinities of ≤18‰ for 4 days. Ellis and Watanabe (1993) found hatching of *N. melleni* was inhibited entirely when eggs were exposed to salinities ≤18‰ for 7 days. Oncomiracidia longevity (Fig. 2b) and infection success (Fig. 2c; Ellis and Watanabe 1993) similarly decreased in hyposaline conditions. Ellis and Watanabe (1993) observed a 100 % reduction in *N. melleni* infection after a 4-day treatment of *Oreochromis* sp. in a range of hyposaline solutions (≤18 ‰). The use of freshwater to treat marine ectoparasites is not a new concept for the mariculture industry and findings from this study and previous studies confirm that both acute and long-term decreases in salinity represent viable treatment options (Fig. 2a–c; Müeller et al. 1992; Ellis and Watanabe 1993).

Freshwater and low salinity solutions represent effective therapeutant options when managing *Neobenedenia* sp.

infections on fish. Many marine fish can tolerate short-term immersion in freshwater and baths of 2–3 min are effective in killing adult Monogeneans (Hoshina 1968; Kaneko et al. 1988; Seng et al. 2006; Ohno et al. 2009). These treatments cause little stress to euryhaline fish and are harmless to the culturist, environment, and consumer (Hoshina 1968; Kaneko et al. 1988; Müeller et al. 1992). Prolonged exposure to hyposaline solutions could be used to reduce egg hatching of *Neobenedenia* sp. on farms (Fig 2a; Müeller et al. 1992; Ellis and Watanabe 1993; Umeda and Hirazawa 2004). This management strategy is highly suited to hatcheries or sea cage farms that can be towed into estuarine environments. However, long-term treatment of stenohaline stock with hyposaline water may be impractical in some situations.

The strategic treatment tool presented in this study is unique in that it offers a method to help break the life cycle of a harmful aquatic parasite while accounting for environmental variables that influence parasite life cycle parameters. The tool enables aquaculturists to adapt timed treatments to specific environmental variables (temperature and salinity) experienced in aquaria or on a farm. Similar to this model, Tubbs et al. (2005) developed treatment timetables for *B. seriolae* and *Zeuxapta seriolae* infecting yellowtail kingfish, *S. lalandi* that account for parasite development time for three separate temperatures. In contrast to *B. seriolae* and *Z. seriolae* which infect a limited number of *Seriola* spp., *Neobenedenia* spp. are known to have exceptionally low host specificity and have been recorded from more than 100 ornamental and aquaculture host fishes (Ogawa et al. 1995; Whittington and Horton 1996). Nevertheless, the proposed tool needs to be tested in the field in order to determine its applicability to multiple *Neobenedenia* isolates, populations and/or species. Accurate treatment timetables reduce the cost incurred by parasite management including labour, stress to stock and cost of chemicals/treatment apparatus, by minimising the number of subsequent treatments required on farm. The proposed treatment tool can be used for *Neobenedenia* sp. infections in tropical commercial operations where similar temperatures and salinities ranges are experienced.

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Conflict of interest The authors declare that there was no conflict of interest that biased the reporting of results in this manuscript.

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