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Cleaner shrimp remove parasite eggs on fish cages

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ABSTRACT: Benthic stages of cultured fishes' ectoparasites are a major contributor to persistent reinfections in aquaculture. These stages are resistant to chemical therapies and are costly to manage in terms of time and labour. Cleaner shrimp, unlike cleaner fishes, prey on benthic stages, suggesting they have the potential to reduce parasite reinfection pressure without having to be in direct contact with the client fish. Cleaner shrimp have never been used as biocontrols in commercial aquaculture, but offer an advantage over cleaner fishes in that they are not susceptible to the ectoparasites of their clients. We present the first investigation of a cultured cleaner shrimp, *Lysmata vittata*, as a biocontrol agent against the eggs of the economically important cosmopolitan ectoparasite *Neobenedenia girellae* infecting cultured juvenile grouper, *Epinephelus lanceolatus*, under simulated recirculating aquaculture conditions. *L. vittata* removed the eggs of *N. girellae* entangled on the mesh of the culture cages and significantly reduced *N. girellae* recruitment to fish by ~87%. Our results demonstrate the value of cleaner shrimp in addressing ectoparasite problems and highlight the importance of investigating novel biocontrol strategies in aquaculture.

KEY WORDS: Biocontrol · Cleaner shrimp · *Lysmata vittata* · Aquaculture · Ectoparasites

INTRODUCTION

Biocontrols are living organisms used to suppress the density of a pest organism's population or its associated impact, rendering it less abundant and less problematic (Eilenberg et al. 2001). However, where the targeted pest organism is parasitic or pathogenic, it is critical to select appropriate biocontrol agents that are not susceptible and which do not pose a risk of enhancing pathogen virulence (cf. Madhusudana Rao & Lalitha 2015).

Biocontrol use in marine environments remains largely underexplored and is in a current stage of infancy (Atalah et al. 2015). Aquaculture consideration of, and the use of biocontrols against, pathogenic agents has focused largely on the use of microbial control strategies, e.g. probiotics, bacteriophages, and specific predatory bacteria to target economically important bacterial finfish and shellfish diseases (see examples in Verschuere et al. 2000, Cao et al. 2014, Madhusudana Rao & Lalitha 2015). To date, the only biocontrol application against fish ectoparasites in commercial aquaculture has been the use of cleaner fishes such as wrasses, *Centrolabrus exoletus* Linnaeus, 1758, *Ctenolabrus rupestris* (Linnaeus, 1758), *Labrus bergylta* Ascanius, 1767, *Symphodus melops* (Linnaeus, 1758), and lumpfish, *Cyclopterus lumpus* (Linnaeus, 1758) to control sea lice, *Lepeophtheirus salmonis* (Krøyer, 1837) and *Caligus elongatus* von Nordmann, 1832, parasitic on Atlantic salmon *Salmo salar* Linnaeus, 1758, and other salmonids farmed in marine waters in Europe (Deady et al. 1995, Treasurer 2002, Skiftesvik et al. 2013, Leclercq et al. 2014, Blanco Gonzalez & de Boer 2017).

The control of sea lice by cleaner fishes follows an augmentative biocontrol approach, which involves

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the introduction of indigenous natural predators to control pest organisms (see Atalah et al. 2015). This strategy offers clear benefits in salmon farming by reducing numbers of reproductive adult sea lice. However, the success of this type of biocontrol strategy relies primarily on the feeding preferences of the biocontrol agents (Hajek 2004, Atalah et al. 2015). The utility of the cleaner fishes' model, notably wrasses in Europe, had traditionally been supported by the combination of a specific feeding preference of the selected cleaner species for a few problematic sea lice species (see Blanco Gonzalez & de Boer 2017), and little overlap of their known parasite diversity with that of the cultured salmon (Treasurer 2012). Nonetheless, recent evidence suggests that cleaner fishes, including lumpfish, are susceptible to other more generalist pathogens important to salmon and other fishes, including *C. elongatus*, and *Paramoeba perurans* (Young, Crosbie, Adams, Nowak & Morrison, 2007) sensu Feehan et al. (2013), the aetiological agent of amoebic gill disease (Karlsbakk et al. 2013, 2014, Karlsbakk 2015, Haugland et al. 2017, Powell et al. 2017). This demonstrates a clear risk of using a cleaner fish model against the pathogens of other fishes, but also the limited scope for using cleaner fishes against other host–parasite models and in other geographical regions.

The Asia-Pacific region produces the majority of the world's aquaculture products, yet no biocontrol use is employed against the ectoparasites of farmed fishes in this region. Recently, Shinn et al. (2015) estimated aquaculture stock losses in parts of Asia to be between 30 and 50% as a result of parasitic agents, excluding viruses and bacterial pathogens. The diversity of economically important ectoparasites of cultured marine finfish listed by Shinn et al. (2015) for this region is high, represented by many protozoans and metazoans with a direct life cycle, many of which have a wide distribution range and low host specificity (see Shinn et al. 2015). It is therefore unlikely that any cleaner fishes would offer a viable option for ectoparasite biocontrol in tropical finfish aquaculture. A potentially viable alternative may be the use of cleaner shrimp in a similar augmentative biocontrol approach (Vaughan et al. 2018).

There are an estimated 51 cleaner shrimp species known globally (Vaughan et al. 2016) that interact naturally with various client species, of which the majority are marine teleosts. Many cleaner shrimp species directly remove and consume the ectoparasites in a density-dependent manner (e.g. Becker & Grutter 2005) from their clients through repetitive symbiotic cleaning interactions, and some species are also known to prey on the environmental (benthic) stages of the ectoparasites (e.g. Militz & Hutson 2015, Vaughan et al. 2018). In so doing, these shrimp can reduce the reinfection pressure on host fishes (Militz & Hutson 2015). No cleaner shrimp species is known to be susceptible to the ectoparasites of marine fishes, which reflects the co-evolved host specificity of these fish ectoparasites (Poulin 1995), and a subsequent advantage that cleaner shrimp may offer over cleaner fishes in finfish aquaculture. Cleaner shrimp have never been used as a biocontrol agent against fish ectoparasites in commercial aquaculture. However, the gregarious rock shrimp *Rhynchocinetes typus* H. Milne Edwards, 1837, a non-cleaner species, was used successfully to reduce biofouling of suspended scallop cultures by Dumont et al. (2009). This is the only example of a shrimp being used as a biocontrol in aquaculture, and benefits included reduced mortality and increased growth of the farmed scallops (Dumont et al. 2009).

A large contributor to the ectoparasite problems in aquaculture is the resilience and sheer volume of the benthic stages of the different parasite species. These eggs, cocoons, and cysts remain attached to culture cages and other farm infrastructure and ultimately hatch or release their re-infective stages to infect farm stock in high numbers. A prime example is *Neobenedenia girellae* (Hargis, 1955), a cosmopolitan monogenean fluke ectoparasite of serious economic concern throughout the Indo-Pacific region (Brazenor et al. 2018a), which is responsible for morbidity and mortality in a diversity of cultured marine fishes including members of Carangidae Rafinesque, 1815, Cichlidae Bonaparte, 1835 (marine acclimated tilapias), Lateolabracidae V. G. Springer & Raasch, 1965, Latidae Jordan, 1888, Paralichthyidae Regan, 1910, Rachycentridae Gill, 1896, Serranidae Swainson, 1839, and Tetraodontidae Bonaparte, 1831 (Ogawa et al. 1995, Brazenor & Hutson 2015, Shinn et al. 2015, Shirakashi & Hirano 2015, Brazenor et al. 2018b). Acute infections of farmed fish with *N. girellae* result in severe mortality events, with fish subjected to stressful conditions or naïve stock without prior-acquired immunity most at risk (Deveney et al. 2001, Shirakashi & Hirano 2015). The traditional control measure for *N. girellae* eggs on fish cages remains the frequent cyclic replacement of contaminated nets with disinfected nets, which is largely ineffective, and which contributes to labour time and cost (Shirakashi & Hirano 2015).

We recently selected the cleaner shrimp *Lysmata vittata* (Stimpson, 1860) for testing as the first cleaner shrimp biocontrol candidate under aquaculture con-

ditions, based on its superior performance at benthic parasite stage reduction in a series of previous laboratory trials (Vaughan et al. 2018). In the present study, we aimed to test the efficacy of *L. vittata* against the benthic egg stage of *N. girellae* on a farmed grouper, *Epinephelus lanceolatus* (Bloch, 1790) kept in oyster mesh net cages under simulated recirculating aquaculture conditions.

MATERIALS AND METHODS

Animal ethics and welfare

Ethical approval was granted prior to commencement of this study under the James Cook University Ethics Committee Permit number A2260, conforming strictly to the national regulations set out by the National Health and Medical Research Council (2013). Fish were subjected to temporary infection by the ectoparasite *Neobenedenia girellae*. As part of the experiment, freshwater bathing for 5 min using dechlorinated tap water was employed to kill and dislodge 100% of these ectoparasites (Kaneko et al. 1988) for recovery and counting, and is a routine method used in aquaculture to control ectoparasites (Hutson et al. 2018).

Animals and experimental design

A total of 480 juvenile *Epinephelus lanceolatus* from a single cohort (~150 mm in total length) were donated for our research by a commercial grouper hatchery in Cairns, Queensland, Australia. All fish were initially given a 5 min freshwater bath with dechlorinated tap water on arrival before being quarantined together in the commercial trials laboratory of the Marine Parasitology Laboratory (MPL), James Cook University (JCU) for 30 d on a dedicated marine recirculating life-support system. A total of 120 commercially produced peppermint cleaner shrimp *Lysmata vittata*, also of a single cohort, were purchased from a commercial producer in Tasmania, Australia, and shipped to us once they had reached adulthood (~30 mm in total length). On arrival, all cleaner shrimp were quarantined for 30 d in a separate, isolated recirculating system. During the quarantine period and the experiment, all fish were fed to satiation daily with Ridley Aquafeed marine float commercial marine fish pellets, and the cleaner shrimp were fed daily with defrosted, commercially available *Mysis* sp. shrimp.

The commercially important monogenean *N. girellae* is continuously cultured in the separate MPL culture facility at JCU (see Hutson et al. 2018). Prior to experimentation, freshly laid *N. girellae* eggs were isolated from the culture and incubated at 24°C in a large glass Petri dish containing fresh, filtered seawater (salinity = 35 ppt). Eggs were monitored daily under a Leica M60 dissection microscope for embryonic development and hatching (see Hutson et al. 2018). Free-swimming larvae (oncomiracidia) hatched on Day 4 (see Brazenor & Hutson 2015) and were collected via pipette and counted before immediately being transferred to a glass beaker of fresh, filtered seawater for the experiment.

All fish were transferred to a circular 500 l tank containing fresh, pre-filtered seawater supplied with continuous aeration through an air diffuser. The glass beaker containing ~10 000 fresh viable oncomiracidia was carefully introduced to the tank of fish, with care to distribute the contents as evenly throughout the tank as possible while maintaining continuous aeration. Fish were cohabited with the oncomiracidia for 1 h. After 1 h, individual fish were netted out using a soft aquarium hand-held net and randomly assigned to 8 identical separate 500 l circular tanks containing an inner plastic oyster mesh cage (1 m diameter), representing 4 treatment and 4 control replicates (i.e. 60 fish cage^{-1}). These 4 treatment and 4 control tanks received constant recirculating aerated and biologically filtered seawater. In addition, seawater was recirculated through an algae scrubber containing live, growing *Caulerpa taxifolia* (M. Vahl) C. Agardh, 1817, for nitrate export. No UV disinfection or foam fractionation was employed, and no seawater exchanges were performed during the experiment. Seawater conditions (Fig. 1) were monitored daily with a Hach hand-held temperature and dissolved oxygen meter, a standard refractometer, a Eutech Scan2 pH meter, and ornamental aquarium nitrogenous waste test kits. Artificial light (cool white fluorescent overhead lighting) was maintained on a 12 h light:12 h dark regime.

To establish parasite egg biofouling on the experimental cages, *N. girellae* were allowed to develop on the fish to sexual maturity (7 d post-infection; Brazenor & Hutson 2015) and an additional 2 d to allow at least 3 consecutive days' egg production according to the biological data of Brazenor & Hutson (2015) at 26°C and 35 ppt salinity. On Day 9 post-infection, all fish were removed from their oyster mesh cages and given a 5 min freshwater bath in dechlorinated tap water to kill and remove adult *N. girellae* (see Kaneko et al. 1988), and therefore cease further egg produc-

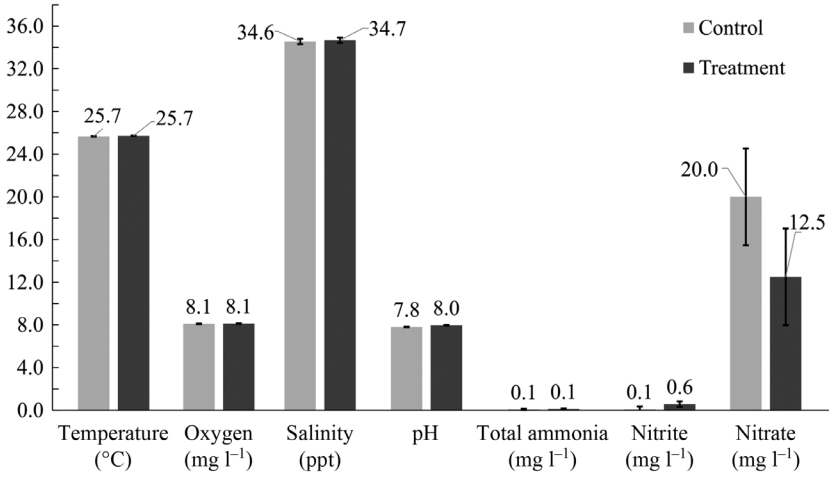

Fig. 1. Mean (±SE) water quality parameters recorded for the duration of the experiment

tion, before being returned to their original cages. A mean (±SE) intensity of 18.7 ± 2 (range: 11 to 32) adult *N. girellae* fish^{-1}, representing ~90% initial infection success, was calculated from a sample of 10 fish and was considered benign for similar-sized hosts by Deveney et al. (2001). Immediately after the fish were returned to their cages, 30 individual adult *L. vittata* were introduced to each of the 4 treatment tanks to patrol the outside of the oyster mesh cages with entangled monogenean eggs (Fig. 2).

A total of 20 fish were sampled haphazardly from each cage on Days 11, 12, and 13 post-infection, corresponding with hatching of the eggs at 26°C and 35 ppt salinity and subsequent recruitment (Brazenor & Hutson 2015), and were individually given a 5 min freshwater bath using separate plastic buckets of dechlorinated tap water. The contents of each bath was filtered through a 23 µm sieve, decanted into separate labelled sample jars and preserved in 70% ethanol for subsequent counting. After their freshwater bath, all fish were introduced to a separate recirculating marine life-support system to recover. There were no fish or shrimp mortalities during the experiment.

Each sample jar was emptied into a large glass Petri dish and its contents inspected under the Leica dissection microscope. All individual *N. girellae* parasites were collected via pipette, manually counted, and preserved in separate, labelled vials of 70% ethanol.

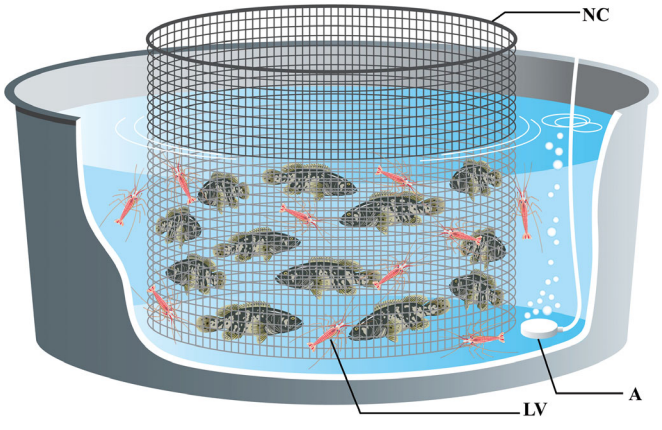

Fig. 2. Graphic representation of a replicate treatment tank containing juvenile *Epinephelus lanceolatus* inside oyster mesh net cages (NC), and *Lysmata vittata* (LV) on the out side of the cages. A: constant aeration

Statistical approach

We used mixed effects random-intercept models to analyse the parasite count data over the 3 sampling days, providing the resolution to optimise data modelling to density-dependent predation of the cleaner shrimp, while accounting for repeated sampling from experimental tanks. Generalised linear regression was not required because parasite count data, which consisted of predominantly high counts and no zeros, when log transformed produced normally distributed residuals (see the Supplement at www.int-res.com/articles/suppl/q010p429_supp.pdf). In addition, the mixed effects random-intercept models were more applicable to account for different levels of residual variation in the response variable (log of parasite counts) after log transformation (e.g. between days and between treatment; see the Supplement). Water quality data were separately analysed over the entire experiment using a standard linear regression. All analyses were performed in R v.3.4.0 (R Development Core Team 2017). Mixed effects random-intercept models were produced using the package 'nlme' (Pinheiro et al. 2018). All models passed diagnostic scrutiny. We constructed 3 mixed effects random-intercept models; 2 with correlation of variance structures for variance differences in treatment, or treatment–day combination groups, and 1 without a correlation of variance structure (see the Supplement). These models were then compared using the anova() function, and the model accounting for correlation of variance structures for the treatment–day

combination was considered the most improved model for our data (see the Supplement). The improved model tested the log of parasite counts (the response variable) as a function of treatment (with or without shrimp) and day (the fixed effects), using the interaction terms treatment × day, and tank as the random effect (see the Supplement).

RESULTS

Lysmata vittata consumed *Neobenedenia girellae* eggs entangled on the oyster mesh fish cages (Fig. 3) and subsequently reduced *N. girellae* recruitment by ~87% (ANOVA: $F_{1,6}$ = 173.36, p < 0.0001; Fig. 4). A mean (±SE) of 964.2 ± 77.4 (range: 101 to 9851), and 123.4 ± 3.5 (12 to 350) *N. girellae* post-larvae were recovered from fish in the control and treatment groups, respectively. Numbers of *N. girellae* on fish across the experiment decreased with time by ~37% by Day 13 (ANOVA: $F_{2,468}$ = 31.20, p < 0.0001; Fig. 4). The regression results for the fixed effects are presented in Table 1. Water quality parameters were not statistically different between treatment groups (ANOVA: $F_{1,24}$ = 1.27, p = 0.27) and remained stable for the duration of the experiment (see Fig. 1). Temperature and salinity remained at ~26°C and ~35 ppt.

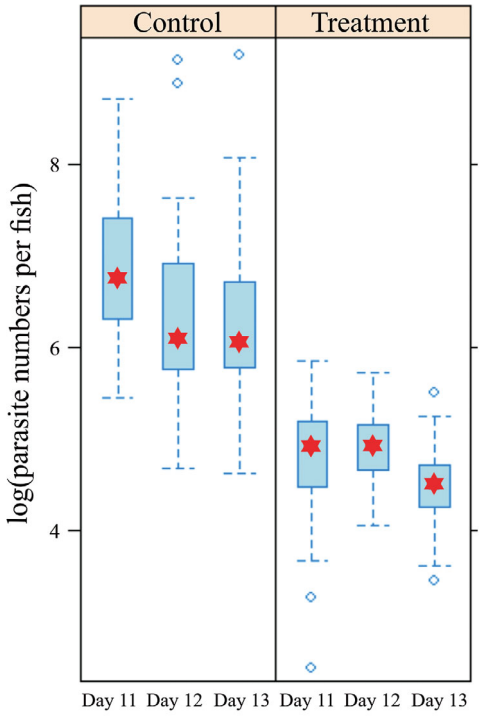

Fig. 4. Effect of *Lysmata vittata* on the number of *Neobenedenia girellae* infecting juvenile *Epinephelus lanceolatus*. Red star: median; boxes are standard 50% interquartile range; clear circles: outliers generated by the analysis; day: day post-infection

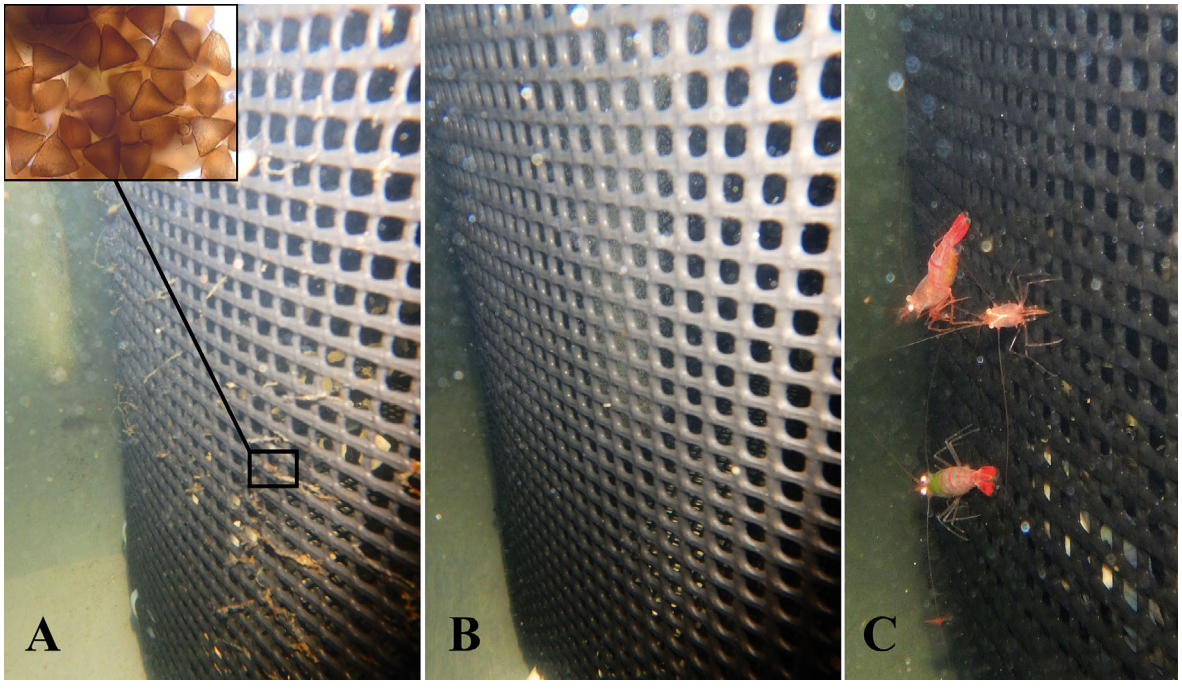

Fig. 3. Underwater photographs of net cages during experimentation; (A) control cage (absence of *Lysmata vittata*) with accumulation of *Neobenedenia girellae* egg masses; (B) treatment cage (presence of *L. vittata*); (C) *L. vittata* feeding on the external surface of a net cage

Table 1. Fixed effects regression results

Fixed effects	β	95% CI
Intercept	6.88	[6.65, 7.10]
Treatment (shrimp)	−2.11	[−2.50, −1.73]
Day (Day 12)	−0.62	[−0.86, −0.37]
Day (Day 13)	−0.60	[−0.83, −0.38]
Treatment (shrimp): Day (Day 12)	0.75	[0.47, 1.03]
Treatment (shrimp): Day (Day 13)	0.32	[0.06, 0.60]

DISCUSSION

Our results demonstrate for the first time the potential of the cleaner shrimp *Lysmata vittata* as an effective biocontrol agent under simulated recirculating marine aquaculture conditions, and its potential for use on fish farms. The ability of *L. vittata* to consume *Neobenedenia girellae* eggs on cage netting is significant because the eggs constitute the main source of reinfection to fishes in aquaculture (Shirakashi & Hirano 2015) and are resistant to chemical treatments used to control adult parasites on the host fishes (Whittington & Kearn 2011). The traditional method to control *N. melleni* egg accumulation on farms is the manual replacement of nets; however, the most efficient timing of net changes is unknown, and the daily accumulation of *N. girellae* eggs exacerbates an already labour-intensive and time-consuming farm practice (Shirakashi & Hirano 2015). Recently, Shirakashi & Hirano (2015) evaluated some of the distribution dynamics of *N. girellae* eggs in a culture cage in support of the development of novel future egg removal methods. We believe that *L. vittata* (and possibly other shrimp species) may offer this novel solution, particularly for land-based operations, broodstock facilities, hatcheries, and nurseries, because shrimp are capable of locating and consuming these eggs, which offer a rich source of protein and lipids, including saturated, monounsaturated, and polyunsaturated fatty acids (Brazenor et al. 2017).

To the best of our knowledge, our data also demonstrate for the first time the sudden increase in intensity, and the intensity range, of *N. girellae* post-larvae for an entire susceptible captive host population within days of an initial benign infection. A sudden outbreak of *N. girellae* (sec. Brazenor et al. 2018b) was considered the reason for the acute mortality of 200 000 farmed barramundi *Lates calcarifer* (Bloch, 1790) in the Hinchinbrook Channel in Northern Queensland, Australia (Deveney et al. 2001). A contributing factor was thought to be a precluding period of unfavourable environmental conditions for the fish. Following this initial mass mortality event, and the return to optimal environmental conditions, the surviving fish appeared to make a rapid recovery (Deveney et al. 2001).

Cleaner shrimp in both tropical and temperate environments are known to prey on the ectoparasites of fishes (see Vaughan et al. 2016 for species), but it is likely that only the gregarious species, like *L. vittata*, would offer any meaningful benefit to aquaculture, as individuals of these species naturally occur together in groups, unlike the pair-forming species which are intolerant of additional conspecifics (Wong & Michiels 2011). *L. vittata* has a natural distribution extending throughout the Indo-Pacific (Palomares & Pauly 2018), which includes the major marine aquaculture producing nations. It is currently cultured commercially in Australia for the ornamental trade and has the potential for large-scale development. *L. vittata* may also be effective against other fish ectoparasites in aquaculture. In our recent laboratory trials (Vaughan et al. 2018), *L. vittata* was effective at reducing and consuming the benthic stages of the ciliophoran ectoparasite *Cryptocaryon irritans* Brown, 1951, and the cocoons of the marine leech *Zeylanicobdella arugamensis* de Silva, 1963. Its efficiency against these and other ectoparasites remains to be tested under farm conditions. However, cleaner shrimp biocontrol models may offer a solution to sympatric infestations, which are often a reality in aquaculture. Cleaner shrimp and other shrimp species should therefore continue to be explored for a future role in aquaculture biocontrol, as originally proposed by Becker & Grutter (2004). Hints of the success of using shrimp in biocontrol already exist in the literature; *Rhynchocinetes typus* has been used to reduce net biofouling of scallop cages in Chile (Dumont et al. 2009), while the experimental field trial use of the native freshwater shrimp *Macrobrachium vollenhoveni* (Herklots, 1857) in parts of the Senegal River reduced the prevalence of human schistosomiasis by predation on the snail intermediate host (Sokolow et al. 2015).

Historically, the cleaner fishes biocontrol model has contributed significantly to the reduction of sea lice in European salmon farming (Blanco Gonzalez & de Boer 2017) and reduced reliance on drugs and chemical treatments to control sea lice outbreaks (Treasurer 2002, Powell et al. 2017), thereby reducing the impact of disease and mandatory drug withdrawal periods prior to harvesting. The development and application of cleaner shrimp biocontrols could have a similar result in aquaculture, particularly in sub-tropical and

tropical regions where stock losses from ectoparasites are high (Shinn et al. 2015), and where cleaner fishes are an unlikely option. The global financial loss from pathogens in aquaculture is estimated to be approximately 20% of the total production value (Sitjá-Bobadilla & Oidtmann 2017). Financial losses are linked to livestock mortalities, the impact of non-lethal infections on livestock growth performance, the market rejection of diseased livestock (e.g. Ogawa 1994, Moran et al. 1999), and the associated costs of mitigating diseases (Lafferty et al. 2015). Diseases in general are considered the most significant constraint to future global aquaculture expansion (Stentiford et al. 2017) and will undoubtedly be influenced by the increasing incidence of pathogen resistance to treatments (cf. Conly & Johnston 2005, Done et al. 2015, Watts et al. 2017). Indeed, the development of the cleaner fishes model was driven largely by the increase in resistance of sea lice to chemical therapies (Costello et al. 2001, Costello 2006, Aaen et al. 2015). It is therefore likely that alternative controls against ectoparasites in finfish aquaculture will continue to attract increasing interest and support globally. Biocontrols offer considerable potential in this regard, particularly if included as part of a holistic integrated pest management strategy (Mordue & Pike 2002, Brooks 2009, Sitjá-Bobadilla & Oidtmann 2017) to combine multiple dynamic approaches to disease challenges (Aaen et al. 2015).

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