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Seaweed extracts as a natural control against the monogenean ectoparasite, *Neobenedenia* sp., infecting farmed barramundi (*Lates calcarifer*)

Kate S. Hutson *, Leonardo Mata, Nicholas A. Paul, Rocky de Nys

Centre for Sustainable Tropical Fisheries and Aquaculture, School of Marine and Tropical Biology, James Cook University, Townsville, Queensland 4811, Australia

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ABSTRACT

Aqueous extracts from common tropical seaweeds were evaluated for their effect on the life cycle of the commercially important ectoparasite, *Neobenedenia* sp. (Platyhelminthes: Monogenea), through the survival of attached adult parasites, period of embryonic development, hatching success and oncomiracidia (larvae) infection success. There was no significant effect of any extract on the survival of adult parasites attached to fish hosts or infection success by oncomiracidia. However, the extracts of two seaweeds, *Ulva* sp. and *Asparagopsis taxiformis*, delayed embryonic development and inhibited egg hatching. The extract of *A. taxiformis* was most effective, inhibiting embryonic development of *Neobenedenia* sp. and reducing hatching success to 3% compared with 99% for the seawater control. Furthermore, of the 3% of eggs that hatched, time to first and last hatch was delayed (days 14 and 18) compared with the seawater control (days 5 and 7). *Asparagopsis taxiformis* shows the most potential for development as a natural treatment to manage monogenean infections in intensive aquaculture with the greatest impact at the embryo stage.

1. Introduction

Parasite infections are an important animal health and management consideration in aquaculture with persistent disease impacting whole industry sectors. Of the diversity of parasites that impact aquaculture industries, monogeneans (flatworms) of the genus *Neobenedenia* (Platyhelminthes: Monogenea) are known pathogens of tropical and subtropical fishes in marine aquaria and aquaculture (Bondad-Reantaso et al., 1995; Ogawa et al., 2006; Hirayama et al., 2009; Whittington, 2012). This includes the important aquaculture species, Asian sea bass or barramundi (*Lates calcarifer*), in Indonesia (Rückert et al., 2008) and Australia (Deveney et al., 2001) where *Neobenedenia* infections are associated with significant fish losses. For example, estimates of total *L. calcarifer* losses resulting from a single outbreak of *Neobenedenia* in Queensland, Australia, were 200,000 fish (~50 tonnes; worth AUD $500,000) (Deveney et al., 2001).

Neobenedenia are obligate parasites with a direct life cycle. Juvenile and adult parasites attach to the skin, fins and eyes of fish using a sucker-like haptor and graze on host epidermis (Ogawa et al., 1995, 2006). Adult parasites lay eggs into the water column, however filaments on the eggs entangle in net cages and are retained within the culture environment (Kearn et al., 1992). Eggs then hatch into free-swimming, ciliated oncomiracidia that directly re-infect fish. High infection intensities result in damage to the epidermis that facilitates infections by bacteria, fungi or viruses (Thoney and Hargis, 1991).

There are no effective methods to prevent *Neobenedenia* infections in open aquaculture systems and the current parasite management effort only allows temporary respite by removing attached parasite stages (Whittington, 2012). As a treatment, infected fish are typically immersed or 'bathed' in hydrogen peroxide or formalin solutions, or in freshwater (Ernst et al., 2002; Ogawa et al., 2006). These treatments are often ineffective in killing developing embryos within eggs (Sharp et al., 2004; Fajer-Ávila et al., 2007). Treatments are also labour-intensive and stressful to fish, and mortalities may occur due to difficulties in calculating bath solution concentrations, physical damage to fish from crowding or lack of oxygen (Williams et al., 2007). Although bath treatments are effective at killing adult monogeneans, re-infection from untreated eggs and larvae in the culture environment occurs immediately following treatment. In closed systems such as brood stock facilities, land-based nurseries and public aquaria, effective parasite control also requires methods to eliminate viable eggs from tanks and equipment (Ernst et al., 2005).

Natural (or "green") treatments are emerging as an alternative approach to control bacterial, viral and fungal pathogens in aquaculture (Lio-Po et al., 2005; Genovese et al., 2012; Selvin et al., 2011; Sudheer et al., 2011) and within this framework, compounds

* Corresponding author. Tel.: +61 7 47816216; fax: +61 7 47814585.
E-mail address: kate.hutson@jcu.edu.au (K.S. Hutson).

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isolated from marine algae (seaweeds) have potential to control parasites. Seaweeds are a rich source of biologically active natural products with properties ranging from antibacterial to anti-inflammatory and anthelmintic (see Smit, 2004; Blunt et al., 2012 for reviews). We hypothesise that algal natural products can control metazoan parasite infections of fishes as an underlying basis for natural treatments. Furthermore, seaweeds can be cultivated in proximity to, or integrated within, finfish aquaculture systems with benefits for both resources (Chopin et al., 2001; Troell, 2009; Mata et al., 2010). Water-soluble natural products are specifically targeted through selective extraction, as this methodology negates the use of organic solvents and can be directly implemented with basic technologies (McCloud, 2010). However, no studies have examined the potential impact of either live seaweeds, or natural products released from seaweeds, on ectoparasites. Therefore, the aim of this research was to determine the effects of water-soluble natural products (extracts) from a range of tropical seaweeds across egg, larval and adult life stages of the ectoparasite, *Neobenedenia* sp., infecting *L. calcarifer*.

2. Materials and methods

2.1. Source and identification of monogenean parasites

Neobenedenia sp. used in experiments were collected from two separate localities. The first collection was from hatchery-reared juvenile barramundi, *L. calcarifer*, maintained in seawater (35‰) in the Marine Aquaculture Research Facility Unit (MARFU) at James Cook University, Australia (19°19′42″S 146°45′40″E). The original source of infection in hatchery-reared fish is unknown. Parasites from this collection were used in experiments described in Sections 2.3 and 2.4. A laboratory infection was also established from embryonated *Neobenedenia* sp. eggs collected from a land-based marine *L. calcarifer* farm, Good Fortune Bay Fisheries Ltd., Queensland, Australia (19°56′24″S 147°55′54″E), in June 2011. Eggs were introduced to laboratory fish (supplied by Mainstream Aquaculture Ltd., Australia) held in 100 L aquaria in the laboratory and maintained at 35‰. Parasites from the laboratory infection were used in experiments described in Sections 2.5 and 2.6.

The species of *Neobenedenia* investigated in this study is unidentified. Although *Neobenedenia melleni* was previously identified from Queensland (Deveney et al., 2001) it is now considered to be a species complex due to its geographic distribution, host associations, biology and taxonomy (Whittington, 2004). *Neobenedenia girellae* and *Neobenedenia parguerae*nsis are also considered to be synonyms of *N. melleni* (see Dyer et al., 1992; Whittington and Horton, 1996). Representative specimens from the two populations sampled were removed from fish by immersing them in dechlorinated freshwater for 5 min. Parasites were collected from the solution and fixed in 100% absolute ethanol. Fixed parasites were placed in distilled water before being stained in Mayer's haematoxylin and then destained in 1% HCl in 70% ethanol. Specimens were dehydrated in an ethanol series before being cleared in cedar wood oil and mounted on a slide in Canada balsam. Parasites collected from the two localities were morphologically similar and identified as *Neobenedenia* sp. (hereafter as *Neobenedenia*) by Ian D. Whittington, South Australian Museum, Australia (SAMA), and accessioned in the Australian Helminth Collection (AHC); SAMA AHC 35240–41 ex MARFU, Townsville and SAMA AHC 35461 ex Good Fortune Bay, Bowen, Australia.

2.2. Seaweed extracts

Eight seaweed species were selected to test the activity of extracts on stages of the *Neobenedenia* life cycle based on their potential to be cultivated in tanks, raceways and ponds, and to be integrated with fish aquaculture systems. These are the red seaweeds, *Asparagopsis taxiformis*, *Gracilaria edulis* and *Hypnea musciformis*; the green seaweeds, *Caulerpa taxifolia*, *Derbesia tenuissima* and *Ulva* sp.; and the brown seaweeds, *Cystoseira trinodis* and *Dictyopteris delicatula*. *Cystoseira trinodis* extract was toxic to fish (see Section 2.3) and not used in further experimentation, while *H. musciformis* was unavailable during experiments with eggs and replaced by the red seaweed *Halymenia floresii* (see Section 2.4). Each seaweed species was held or cultured in individual tanks in a recirculation aquaculture system at MARFU prior to extraction. Fresh seaweed was spun dry in a fine mesh bag before weighing. Water-soluble natural products were extracted by blending (Abode Appliances, model No. YD-2198) each species in filtered seawater for 2 min at a wet weight to volume ratio of 0.1 g/ml. Each solution was then poured into a Schott bottle and placed on a shaking table at room temperature for 4 h to allow mixing. Each solution was sieved through a fine mesh cloth (60 μm) into centrifuge tubes, each spun at 5,300*g* for 5 min. The supernatant was then transferred to a clean Schott bottle and stored at 4 °C for no longer than 24 h before use. Extracts were freshly made for each experiment before use and discarded at the end of each experiment (maximum 21 days) to avoid any degradation or loss of natural products through storage.

2.3. Effect of seaweed extracts on in vivo Neobenedenia survival

The effect of seaweed extracts on the survival of attached adult *Neobenedenia* was tested in vivo. Ten replicate *L. calcarifer* infected with *Neobenedenia* were exposed to extracts of each seaweed species (*A. taxiformis*, *G. edulis*, *H. musciformis*, *C. taxifolia*, *D. tenuissima*, *Ulva* sp., *C. trinodis* and *D. delicatula*) at a concentration of 1 mL of extract to 100 mL of seawater, in addition to a UV filtered seawater control. This concentration was selected based on results of a pilot study that showed that adult parasites did not survive >24 h in diluted 1:10 mL or 1:100 mL concentrations of *A. taxiformis* extract in vitro (unpublished data). Experiments using attached adult parasites in this study were conducted in vivo. The effect of extracts on parasite survival was examined following 24 h exposure. *Lates calcarifer* were <1 year-old hatchery-reared juvenile barramundi maintained in a 2,500 L circular tank containing seawater in MARFU. Eighty barramundi were removed from the tank using a net and placed into individual 10 L containers with constant aeration. Ten replicates were used for extracts of each of the eight seaweed species plus 10 replicate controls in UV-filtered seawater. Each fish was maintained in 6 L of either treatment or control solution for 24 h at room temperature. Fish were not fed during this period and mortalities were noted and excluded from data analysis. *Cystoseira trinodis* extract was not included in the analyses due to 100% mortality of experimental fish.

Dead *Neobenedenia* were recovered from the treatment and control solutions by filtering through a 60 μm mesh. Dead parasites usually detach from the host surface and sink to the bottom of the container. In addition, to ensure all dead parasites had detached from the body of the fish, the external surface of each fish was gently rubbed by hand prior to filtering (Chambers and Ernst, 2005). Parasite specimens were collected in 250 mL jars and fixed in 70% ethanol. Fish were removed from treatment containers by hand and placed individually into 6 L of dechlorinated freshwater for 5 min to kill the remaining live *Neobenedenia* attached to fish. 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 and therefore visible to the naked eye. Fish were again gently rubbed by hand to ensure that all dead *Neobenedenia* had detached prior to returning the fish to a seawater recovery tank. The dechlorinated freshwater solution