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Dynamics and distribution properties of theronts of the parasitic ciliate *Cryptocaryon irritans*

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ABSTRACT

Cryptocaryon irritans is a parasitic ciliate that causes major economic losses in marine fish aquaculture globally. Despite the wide array of treatment methods, control of cryptocaryoniasis is still very challenging, especially in food fish culture. Thus, additional control methods against this parasite might be required to further reduce the occurrence of this disease. In this study, the swimming ability, excystment sequence, and distribution of theronts, the infective stage of *C. irritans*, were investigated in our effort to develop a physical control strategy. A video analysis for assessing the swimming ability of theronts showed diminishing mobility over time. The excystment of theronts primarily occurred during the dark period. Examination of the vertical distribution of theronts showed that they were mostly distributed at the 5 cm sampling point measured from the substrate, indicating that they have low upward swimming ability. From these results, we conclude that theronts possess limited mobility. Theronts also displayed an excystment pattern that might be influenced by photoperiod, since most theronts were released during the dark period of the day. Control strategies can be developed from these properties, such as increasing the water flow in a culture tank during the release period. Further, combined treatment methods against multiple stages of *C. irritans* can help minimize the occurrence of cryptocaryoniasis in culture facilities.

1. Introduction

Cryptocaryon irritans Brown 1951 is a ubiquitous ciliate causing cryptocaryoniasis and is one of the major parasitic threats to marine fishes cultured in temperate and tropical seas globally (Colorni, 1985; Colorni and Diamant, 1992). This parasite was first described in detail in Japan (Sikama, 1937) and has been reported worldwide since. Its lifecycle includes four main phases: the infective theront stage, parasitic trophont stage, external protomont stage, and reproductive tomont stage (Colorni, 1985; Dickerson, 2006). Previous studies have showed that it has a broad range of fish hosts (Burgess and Matthews, 1995). It parasitizes the epithelium of the skin and gills of fish, affecting their functioning and disrupting the host's osmotic control (Colorni, 1985). Hosts experiencing cryptocaryoniasis exhibit clinical signs such as pinhead-sized white nodules on the epithelium, mucus hyperproduction, skin discoloration, anorexia, and respiratory distress. Infected fish also show behavioral alterations such as fin tremors, hyperactivity, flashing, and, in severe cases, hovering at the water surface, cloudy corneas, and opaque fins due to sloughing skin (Colorni and Burgess, 1997). Severe infections can cause mass mortalities, leading to severe financial losses.

To mitigate the negative impact of this parasite on aquaculture, several authors have proposed an array of control measures ranging from the manipulation of its life stages and environment to chemical control and vaccination. Hypersaline and hyposaline conditions were among the first control measures, reported by Huff and Burns (1981) and Colorni (1985), respectively. Many other control methods have been described, albeit with varying efficacies. Chemical treatments such as copper sulfate (Colorni, 1987); formalin (Herwig, 1978); acriflavine, malachite green, and methylene blue (Van Dujin, 1973; Herwig, 1978, Tookwinas, 1990); potassium permanganate, sodium chlorite, sulfathiazole nitrofurazone, and penicillin (Wilkie and Gordin, 1969; Herwig, 1978); medium chain fatty acids (Hirazawa et al., 2001); oral administration of sodium salinomycin (Yoshinaga et al., 2011); and Romet® 30 (Kawano et al., 2012) have been described. Physical and mechanical treatments have also been described. For example, switching tanks every 3 days (Colorni, 1987), exchanging sand filers (Colorni and Burgess, 1997), ultraviolet (UV) irradiation (Spotte, 1979), ozone (Wilkie and Gordin, 1969), storing seawater for at least 24 h before use (Colorni and Burgess, 1997) and temperatures above 34 °C combined with hyperoxic and hypoxic conditions (Yoshinaga, 2001). Additionally, an immobilization antigen cDNA was proposed as a potential DNA vaccine against *C. irritans* (Priya et al., 2012). Mechanical control was described against the freshwater counterpart of

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C. irritans, *Ichthyophthirius multifiliis*, by utilizing suction and low-adhesion polymer surfaces to reduce the encystment and development of tomonts (Shinn et al., 2009). Considering the lifecycle similarities between these two species, a similar mechanical and physical approach against *C. irritans* might be possible in culture systems.

Despite the development of many treatment methods, the complete prevention of cryptocaryoniasis is still not possible. Therefore, application of an integrated control system may have better efficiency in minimizing the occurrence and impact of cryptocaryoniasis. Integrated pest management (IPM) is a pest management system that, in the context of the associated environment and the population dynamics of the pest species, utilizes all suitable techniques and methods in as compatible a manner as possible and maintains the pest population at levels below those causing economic loss (Stern et al., 1959). IPM is difficult to define, as it is an adaptive strategic approach balancing cultural, biological, and chemical measures for a situation (Sandler and Mason, 2010). It has been postulated that a combination of several less effective methods can potentially reduce infection levels significantly, providing decent control of the targeted disease (Thamsborg et al., 1999). IPM strategies have been extensively applied in agriculture with promising results. Although the application of IPM in aquaculture is newer than in agriculture, successful IPM strategies for controlling parasites in oyster culture have been reported (Dumbauld et al., 2006). The development of IPM strategies against sea lice on farmed salmon was also proposed, where an array of existing recommendations for the development of IPM plans against sea lice infections was described (Brooks, 2009). Effective planning based on monitoring programs and regional environments was highlighted as an important component in developing a successful localized IPM system.

With its wide success in agriculture and increasing application in aquaculture, it might be worth considering the development of an IPM system against cryptocaryoniasis. However, the complex, quadriphasic lifecycle of this parasite may lead to inefficiencies if the control strategy is only applied against a single life stage. Therefore, in order to design a robust IPM system against this disease, the components of the system should pose a negative impact against all four life stages of this parasite. Most previous studies have focused on a wide array of chemotherapeutants to disrupt the theront and tomont stages in the culture environment. Hence, in this study, the dynamics of theronts was assessed in our attempt to develop an additional approach against *C. irritans* infections via physical control. Specifically, the swimming ability and vertical distribution of theronts were investigated. Possible countermeasures based on the data acquired in this study and considerations in developing an IPM against cryptocaryoniasis were also discussed.

2. Materials and methods

2.1. Propagation and collection of C. irritans

Several ornamental fishes infected with *C. irritans* were obtained from a local pet store as sources for laboratory *C. irritans* propagation. The parasite was maintained and propagated on *Poecilia* sp. (black molly, 2–3 cm standard length) as hosts in a 60 l seawater aquarium with an overhead biological filter at 25 °C. To obtain theronts, protomonts were collected from infected black mollies following Yoshinaga and Dickerson (1994) with slight modifications. Briefly, five to ten fish were added into the aquarium every 3–4 days to maintain the *C. irritans* lifecycle, and dead individuals were removed whenever possible. When visible white spots appeared on the infected fish, they were removed from the aquarium, placed in a 2 l plastic aquarium, and incubated at 25 °C. On the following day, protomonts that detached from the fish were collected, washed five times, and incubated at 25 °C in 10-cm^2 Petri dishes containing 4 ml of filter-sterilized seawater. The seawater was replaced on a daily basis. A few theronts were obtained 5 days post-encystment, and the release rate peaked 6–7 days post-encystment.

2.2. Video footage analysis of theront movement

Freshly excysted theronts were collected in a 15 ml tube within 30 min post-excystment. A theront suspension of 100 μl containing about 50 theronts was placed in a square chamber (10 mm × 10 mm × 1 mm) with an opening (10 mm × 10 mm) on a silicon plate (1 mm thickness) pressed between two glass plates. To improve contrast and aid video observation, a black silicon plate was attached to the back of the chamber. The chamber was diagonally illuminated with a cold light tube. The video of the movement of theronts was recorded with a video microscope (Olympus® PV10) for at least 5 min at the intervals mentioned above. Video footage of theronts was recorded at 0, 1, 2, 3, 6, and 12 h post-excystment. These videos were analyzed for theront movement with the motion plotting program Undoukun™ (http://www.rikakoubou.com/undoh.html) by plotting the original location of a theront and its final location after 1 s (24 frames/s) in the video. A total of 30 theronts were randomly selected and tracked for 1 s for their relocation distance and direction. The magnitude of relocation was interpreted in a graph in terms of X-axis and Y-axis movement to show the direction and potential travelling distance in 1 s.

2.3. Excystment pattern of theronts in a laboratory propagation tank

Seawater samples of 45 ml were collected from a circulating laboratory propagation tank in which the *C. irritans* source was maintained as described above. Samples were collected every 2 h from 8 am for 24 h and fixed with formalin at 1%. Then, the seawater samples were centrifuged in 50 ml centrifuge tubes at 800 rpm for 5 min to concentrate the contents. Following centrifugation, most of the supernatant was discarded, leaving about 1 ml for the resuspension of contents. The suspension was then transferred into a Sedgewick–Rafter chamber and stained with a fluorescent dye, HOECHST 33342, in a final concentration of 50 ng/ml for 20 min. Finally, all of the stained theronts were enumerated under a florescent microscope (BX 60, Olympus). Staining with HOECHST 33342 resulted in distinct identification of theronts because the four nucleus lobes emitted bright blue luminance by UV excitation under the florescent microscope.

2.4. Excystment pattern and vertical distribution of theronts in outdoor tanks

One standard large (1.5 t) rectangular tank and one standard small (1 t) rectangular tank with an overflow pipe outlet at one end were prepared in the Laboratory of Fisheries, University of Tokyo, Hamamatsu City, Japan. Seawater was pumped from Lake Hamana, where the occurrence of cryptocaryoniasis was observed when this assessment was carried out. Seawater was supplied via an inlet across from the outlet. The water exchange rate was set at 7 l/min and aeration was provided at 5 l/min. In each tank, *C. irritans* was maintained and propagated on *Pagrus major* (red sea bream, average weight 61.8 g) by routinely replacing dead fish with uninfected healthy fishes.

2.4.1. Excystment pattern of theronts in outdoor tanks

Every 2 h from 8 pm for 24 h, 500 ml water samples were taken near the outlets of both tanks and then fixed with formalin at 1% (v/v) and stored at 4 °C until use. The ambient luminance was also measured during sampling with a light meter. Four 50 ml aliquots were taken from each sample, processed, stained, and the theronts were enumerated as described above.