論文の内容の要旨

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論文題目　Studies for development of integrated parasite management (IPM) system against *Cryptocaryon irritans*, a parasitic ciliate of marine fin fishes
(海産魚に寄生する繊毛虫 *Cryptocaryon irritans* に対する総合的寄生虫管理システムの開発のための研究)

Chapter 1. General Introduction

Rapid growth of aquaculture is a key factor to satisfy the massive volume of demand for aquatic animal and its products, a healthy source of animal protein. However, one of the factors crippling the growth of the aquaculture industry is disease, which is an inevitable result of high stocking density and intensive culture. Apart from bacterial and viral diseases, parasitic diseases contribute a large portion of fish mortality and massive economic losses.

Cryptocaryon irritans is the causative agent of cryptocaryoniasis, one of the major contributors to mass mortalities in tropical and sub-tropical aquaculture farms due to its ubiquitous nature, wide variety of strains, rapid proliferation and complex life-cycle. This ciliate invades the epithelium of the skin and gills of fish, disrupting the osmotic control and respiratory function of the host and causing death in serious infections. Arrays of preventive measures and chemotherapeutic methods with varying efficacies have been developed but the damage caused by cryptocaryoniasis is still very significant. Considering the complex life-cycle, economical impact and frequent occurrence of mass mortalities in cage cultures and tank cultures, researches for development of an integrated parasite system (IPM) by combining several components of different principles and actions is required to cope with this disease especially in intensive culture systems. IPM is widely used in agriculture as the application of different biological, mechanical and chemical approaches against parasites and has been successful. Therefore, a combination of different methods targeting several stages of *C. irritans* may provide a better result in controlling the occurrence and reducing the severity of cryptocaryoniasis. In this study, three different approaches against different life stages of *C. irritans* were studied as potential components of an integrated parasite management (IPM) system against cryptocaryoniasis.

Chapter 2. Application of physical control method with manipulation of movement ability of theronts

The theront stage is often considered the most exposed, unprotected life stage of *C. irritans*, making it a logical target for a preventive measure. In this study, the swimming ability, life-span, and circadian rhythm of the theront stage were investigated and the control of the disease was attempted using a countermeasure developed based on the present results.

Swimming ability, movement, circadian rhythm, and distribution of theronts

In the first experiment, the ratio of active swimming theronts over time was evaluated by enumerating the number of swimming theronts and the number of dead or non-swimming theronts at 0, 1, 2, 3, 6, and 12 h post-collection. Simultaneously, a video analysis of theront movement was carried out by recording the movement of theronts in a square chamber (10 mm L x 10 mm W x 1 mm D) with a video microscope and by analyzing the movement with a motion analyzing software. Circadian rhythm and excystment pattern of theronts were evaluated by enumerating the number of theronts excysted for in petri dishes, and laboratory propagation tanks (50 L) and outdoor fish rearing tanks (1 m^3) containing tomonts every two hours for 24 hours. Vertical distribution of theronts in an outdoor tank (1.5 m^3) was also assessed by siphoning seawater from different depths. Results showed that the number of swimming theronts declined over time with only 46% and 5% of swimming theronts remaining at 6 h post-collection and 12 hours post-collection respectively. The distance travelled by theronts was as low as 1.6 mm/s, which also decreased over time. Theronts were gradually released from late evening to a peak release at 0400-0600 regardless of environment with slight differences depending on the daily illumination time and period. Theronts were found to mostly accumulate at below the 5 cm point from the bottom of the tank. These data suggested low theront swimming. Therefore, theronts seemed to require vertical water current in order to distribute themselves in water columns. This best explains the frequent occurrence of cryptocaryoniasis in cage cultures in Japan during autumn, not during summer, when the disruption of thermocline and typhoons improve water circulation in the sea in autumn. Additionally, the aeration system seems to also act as one of the facilitators of cryptocaryoniasis infections in tank culture.

Trial on the preventive measure of cryptocaryoniasis by modification of rearing tanks

Based on the low swimming ability, circadian rhythm and vertical distribution of theronts, red seabream were reared in outdoor fish rearing tanks (2 m L x 1 m W x 0.6 m D) modified to be able to flush out theronts concentrated at the bottom of the tanks and by setting up physical barriers at 20 cm point from the bottom of the tanks. However, the modification did not show positive results in improving survivability of fish in the tanks. More studies are required to revise better modification that can be done on tank culture systems.

Chapter 3. Toxicity assessment of oral administration of sodium salinomycin in fish and its efficacy against *C. irritans*

Sodium salinomycin has been reported to be effective against cryptocaryoniasis but extensive study on the toxicosis, safety dosage and applicability in different species of fish has yet to be done. In this study, the acceptability, toxicosis and effective dosage against *C. irritans* infection was carried out in 4 domestically important food fish species in Japan to increase the target species.

Toxicity and effective dosage of sodium salinomycin in fishes

The acceptable and safety dosage of sodium salinomycin medicated feed was tested by feeding 4 fish species (*Pagrus major*, *Takifugu rubripes*, *Paralichthys olivaceus*, *Seriola lalandi*) daily at 5% body weight in various dosages for 7 days. *P. major* and *T. rubripes* showed no apparent signs of toxicosis up to 80 mg/kg BW/day, while *S. lalandi* and *P. olivaceus* showed maximum safety dosage below 8 mg/kg BW/day. In the experiment in which fish were challenged with *C. irritans* and then treated with medicated feed at different dosages for 3 days, sodium salinomycin was effective in reducing the total number of protomont recovered from fish at 20 mg/kg BW/day and higher in *P. major* and *T. rubripes*. Reduction of recovered number of protomonts was also observed in *S. lalandi* at 4 mg/kg BW/day. In the experiment in which challenged fish were treated with medicated feed at different dosages for 7 days, sodium salinomycin increased survivability in *P. major* and *T. rubripes* when compared to the control group. These results suggest that oral administration of sodium salinomycin can reduce the parasitic load and increase tolerance against *C. irritans* within the safety dosage, although the safety dosage of sodium salinomycin differs among fish species. Additionally, in our experiment, sodium salinomycin showed considerably higher toxicity in *P. olivaceus* than that in a previous report, although the experimental water temperature differed between our experiment and the previous one, which may suggest the effect of temperature on the

toxicity of sodium salinomycin. Effectiveness of oral administration of sodium salinomycin was confirmed in some fish species in this study, but careful considerations must be taken to apply this substance in target fish and to avoid cases of lethal toxicosis.

Direct effects of sodium salinomycin in fish blood and against theronts

The effects of sodium salinomycin on hemolysis of fish blood was examined with the blood of 4 fish species (*P. major*, *T. rubripes*, *P. olivaceus*, *S. quinqueradiata*) at the final concentration of 5, 10, 20, 25 and 50 ppm over 9 or 12 h. The level of hemolysis varied according to fish species and hemolysis was observed in as early as 15 minutes with 77.4% hemolysis in *S. quinqueradiata* compared to 10.5% in *P. olivaceus*, suggesting that sodium salinomycin can induce hemolysis very quickly in some fish species. Although *P. olivaceus* showed low resistance to sodium salinomycin medicated feed as described above, the blood of *P. olivaceus* showed low hemolysis. Therefore, sodium salinomycin may affect fish in different ways in addition to hemolysis, considering that it generally affects the nerves and muscles of terrestrial animals. To theronts, sodium salinomycin showed high parasiticidal activity at 25 ppm, with 72% theront mortality at 1 h indicating sodium salinomycin has high parasiticidal activity against theronts within a short period of time as well as against trophont as described in a previous report.

Chapter 4. Production of iAg vaccine derived from *Tetrahymena thermophilla* against Cryptocaryoniasis

Over the years, acquired immunity based on immobilization antigen (iAg) has been used for development of vaccines against ciliates, particularly *I. multifiliis* and *C. irritans*. A recent study has produced a recombinant DNA vaccine against *C. irritans* that provided vaccinated fish with moderate protection. However, codon changing was required to produce this recombinant vaccine due to the unique codon usage in ciliates. Furthermore, difference in post-translational modification of protein between ciliates and other organisms may affect the antigenicity of produced proteins. Therefore, in this study, we expressed the iAg of a *C. irritans* strain in our laboratory in *Tetrahymena thermophilla*, a culturable ciliate of which transformation protocols have been well established. The efficacy of iAg expressed in *T. thermophila* as vaccine was evaluated in this study. The total RNA of *C. irritans* was extracted and subjected to RT PCR followed by touchdown PCR on the 3' and 5' end. The partial and full sequence for this iAg was confirmed in reference to the available Genbank sequences from previous studies. This sequence was transformed in *T. thermophilla* prior to a series of selection and confirmation protocols. Expression of this iAg gene was then induced in the selected tranformant and the transformant was preserved at -80°C. Vaccination was carried out by interperitoneal injection of frozen transformant or the extracted iAg protein expressed in the transformant. The efficacy of iAg vaccine was tested by ELISA, immobilization assay, protomont recovery and cumulative mortality in challenge experiments.

In immobilization assay, sera from fish vaccinated with the transformant and naïve fish immobilized 56.2% and 26.7% of theronts, respectively, after 1 h. No significant difference was detected in number of recovered protomonts between immunized fish and non-immunized fish. In fish vaccinated with iAg protein, ELISA results confirmed the expression of iAg antigen but significant difference was not observed in the cumulative mortality of challenged fish. This may be due to the serotypic variance of *C. irritans* as we used theronts of a strain different from the strain used for development of iAg for challenge experiments. This was due to senescence of the original strain, which is a common occurrence in laboratory propagated *C. irritans*. Additionally, the presence of intraspecific variants of this parasite requires the development of vaccines efficient against all serotypes or variants of *C. irritans*.

Chapter 5. General discussion

Based on the behavioral nature of theronts including low swimming ability, vertical distribution, and circadian rhythm in excystment revealed or confirmed in the present study, fish rearing tanks can be improved to reduce fish mortality by cryptocaryoniasis, although the improvement trial in the present study did not work well. Furthermore, being combined with the suppression of development of tomonts in low dissolved oxygen revealed by a previous study, the information on theronts obtained in this study can be used to forecast the occurrences of cryptocaryoniasis in cage cultures. Sodium salinomycin medicated feed was proven to be partially effective in reducing parasite load and increasing survivability of infected fish,

showing promising usage. However, the toxicity and efficacy of sodium salinomycin need to be clarified to ensure safe application in target fish species. In terms of vaccine production, the presence of multiple serotypes in iAg of *C. irritans* is a major obstacle in the development of vaccines against this parasite. An IPM system with improved tank modification system coupled with sodium salinomycin medicated feed may be a good solution to minimize cryptocaryonaisis until the development of a non-serotype dependent vaccine against this disease.