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Dormancy induced by a hypoxic environment in tomonts of *Cryptocaryon irritans*, a parasitic ciliate of marine teleosts

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ABSTRACT

We incubated tomonts of *Cryptocaryon irritans* in a hypoxic seawater environment (1.4–1.7 mg/L O_2) (low dissolved oxygen; DO) and examined their development using a acetocarmine whole-mount staining method we developed for nuclear staining. They showed little development and stayed in the dormant phase in the hypoxic environment. When transferred into the hypoxic environment after incubation in an oxic environment (air-saturated, 8.7–8.9 mg/L O_2) for 1–4 days, their development stopped in 1 day. However, when dormant tomonts generated in the hypoxic environment were transferred to the oxic environment, they resumed development and released theronts. These results indicate that tomonts can become dormant when exposed to a hypoxic environment, but can resume development when exposed to an oxic environment at any developmental stage. When exposed to the oxic environment, tomonts recovered from 1-month dormancy and released as many theronts as control tomonts constantly incubated in the oxic environment. The infectivity of theronts from the recovered tomonts was similar to the control tomonts. Thermoclines prevent oxygen-rich surface seawater from reaching the bottom of water column and create a hypoxic sea floor environment in summer; these thermoclines are broken down in autumn or after typhoons. The long-term viability of dormant tomonts in hypoxic environments may be a key factor in the autumn outbreaks of cryptocaryoniasis in floating net cages in temperate waters.

1. Introduction

Cryptocaryon irritans Brown 1951 is a parasitic ciliate of marine teleosts, which causes cryptocaryoniasis or “marine white spot disease”. The life cycle of *C. irritans* consists of four developmental stages. Theronts, the free-swimming stage, invade the surface organs of fish, including the skin, fins and gills, and transform into trophonts, the parasitic stage. Trophonts feed and grow in size, without cell division, in the epithelium of the surface organs. Mature trophonts leave the host as protomonts. Protomonts sink, settle on the substrate and become encysted as tomonts. The tomont stage is the cell division phase, in which daughter cells called tomites are produced by fast repetition of binary cell divisions (palintomy). Mature tomites, or theronts, are then released into the seawater (Sikama, 1937; Brown, 1963; Wilkie and Gordin, 1969; Colorni, 1985, 1987).

When fish are severely infected, the epithelial tissues of the skin and gills are damaged, resulting in death from osmotic regulation and respiratory function disturbances (Colorni and Burgess, 1997). Cryptocaryoniasis was originally thought to affect fishes only in closed environments, such as private and public aquaria and land-based aquaculture tanks. However, the disease now frequently occurs in fish cultured in relatively open environments as well, such as in floating net cages. Cryptocaryoniasis repeatedly occurs in Japan and sometimes results in mass mortality in cultured marine fishes, such as red seabream (*Pagurus major*), greater amberjack (*Seriola dumerili*), tiger puffer (*Takifugu rubripes*) and Japanese flounder (*Paralichthys olivaceus*), and causes large economic losses in the aquaculture industry (Yoshinaga and Nakazoe, 1997; Kochi Prefecture, 2005; Katata et al., 2006; Watanabe et al., 2011; Kadohara, 2013).

In Japan, outbreaks of cryptocaryoniasis in floating net cages located in inner bays frequently occur in autumn when water temperatures decrease (Yoshinaga, 2001; Kochi Prefecture, 2005; Katata et al., 2006). Yoshinaga (2001) reported that *C. irritans* tomont development was suppressed by a hypoxic environment, and that development resumed after tomonts were transferred into an oxic environment. This suggests they become dormant in hypoxic environments. It may be presumed from this that tomonts remain in a dormant phase on the seabed around net-cage fish farms in summer, when the bottom layer of the water column becomes hypoxic because of the formation of a thermocline in inner bays. The frequent outbreaks of cryptocaryoniasis

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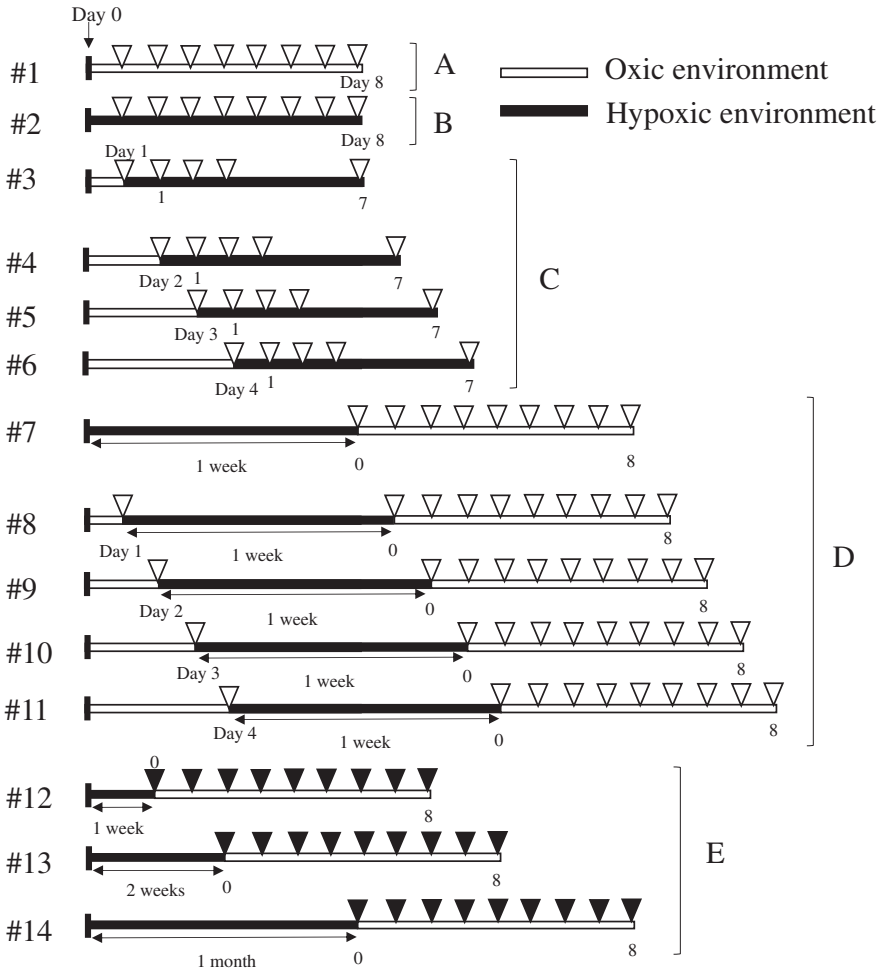

Fig. 1. Timetable of tomont incubation and sampling. Open lines indicate incubation in the oxic environment, and solid black lines indicate incubation in the hypoxic environment. Open triangles indicate when sampling and staining with acetocarmine occurred. Solid black triangles indicate the observation days for theront release.

may then be related to the disappearance of the thermocline in autumn, when oxygen is recirculated to the seabed. Detailed information about the development and dormancy of tomonts in hypoxic environments, and on the dynamics of dissolved oxygen (DO) in seawater around marine net-cage farms, would be useful to help predict outbreaks of cryptocaryoniasis. This would also assist planning the timing of transferring net cages containing fish to the open sea, a method conventionally used and effective in preventing heavy infections and resulting mass mortalities in net-cage farms (Yoshinaga, 2001). However, little is known about the time required for theronts to be released after oxygen is supplied to the seabed, or the length of time for which tomonts remain viable when kept in hypoxic environments. This information is necessary to plan the appropriate time to transfer net cages to the open sea.

In a previous study on the effect of a hypoxic environment on tomont development (Yoshinaga, 2001), the effects were evaluated only by the delay in theront release. There was little research on the development of tomont internal structure, especially regarding the effects of environmental factors, because serial sectioning and staining are necessary to observe the internal structures, but these procedures are both labor and time intensive. We recently developed an acetocarmine whole-body staining method for tomont nuclei, which enables us to easily observe the nuclear development of tomonts (Watanabe et al., 2016).

We conducted this study to obtain detailed information on *C. irritans* tomont dormancy induced by a hypoxic environment, and recovery from dormancy in an oxic environment. We focused on the timing of nuclear development using whole-body staining of the nuclei. In addition, we also examined the release and infectivity of theronts produced from tomonts kept dormant for one month.

2. Materials and methods

2.1. Parasites

C. irritans was isolated from an ornamental blue surgeonfish (*Paracanthurus hepatus*), purchased in a local pet shop. The parasite was passaged and propagated on seawater-adapted black mollies (*Pocillia* sp.) (mean body length 2–3 cm), according to Yoshinaga and Dickerson (1994) with some modifications as previously described in Watanabe et al. (2016). Ten black mollies were exposed to 10,000 theronts (1000 theronts/fish) in 1.5 L of filtered seawater in a 2 L plastic aquarium for 6 h in the dark at 25 °C. Throughout this study, challenge and maintenance of fish were carried out in the dark at 25 °C in an incubator unless otherwise stated. The challenged black mollies were transferred to another 2 L plastic aquarium containing 1.5 L of seawater and maintained with gentle aeration. After 3 days of challenge, infected black mollies were placed in a 1.5 L plastic aquarium containing 1 L of filtered seawater. Protomonts leaving the fish were allowed to settle and transform into encysted tomonts in the dark at 25 °C. Encysted tomonts attached to the bottom of the aquarium were rinsed with filtered seawater and incubated in 50 mL of filter-sterilized seawater supplemented with antibiotics (500 IU/mL penicillin G potassium and 500 μg/mL streptomycin sulfate). The seawater in the aquarium was replaced with fresh filter-sterilized seawater supplemented with the antibiotics every day. Most tomonts released theronts 5–7 days after collection; these were collected and used to challenge fish. This process was repeated to propagate and maintain the *C. irritans* infection.

Protomonts were collected soon after leaving their hosts, and before encystment. They were washed five times with filter-sterilized seawater supplemented by antibiotics, using Pasteur pipettes and glass bowls, before being used in the experiments.

2.2. Seawater for tomont incubation

Seawater (34–35 ppt salinity) recovered from the surface Kuroshio Current around the Izu Islands was purchased from Tokai Kisen Co. Ltd. (Tokyo, Japan) and used for rearing fish and in the experiments. The seawater was filtered with a 1 μm line filter for routine propagation and passaging of *C. irritans* on black mollies, or sterilized with a 0.22 μm membrane filter and supplemented with 500 IU/mL penicillin G potassium and 500 μg/mL streptomycin sulfate for the incubation of tomonts. For incubation experiments, seawater was saturated with air by 30–60 min aeration. The DO concentration in the air-saturated seawater was 8.7–8.9 mg/L O_2 when determined by an optical oxygen monitor (Piccolo 2, BSA Co Ltd., Tokyo, Japan). The air-saturated seawater was used for the incubation of protomonts and tomonts in an oxic environment throughout this study, unless otherwise stated.

2.3. Timing of tomont development in oxic and hypoxic environments

We compared the development of tomonts in the oxic environment (Fig. 1A) and the hypoxic environment (Fig. 1B). We prepared eight Φ 40-mm cell culture dishes each for oxic incubation and hypoxic incubation of tomonts, adding 2 mL of air-saturated seawater to each dish. Fifty protomonts were placed in each dish and transformed into tomonts. For oxic incubation, the eight dishes were placed in a humid chamber in an incubator (LH-30CCFL-8CT; Nippon Medical & Chemical Instruments Co., Ltd., Osaka, Japan). Seawater in the dishes was exchanged for fresh air-saturated seawater every day, and one dish was sampled every day for 8 days. The hypoxic environment was established using an anaerobic culture system for bacteria (AnaeroPack-kenki 5%; Mitsubishi Gas Chemical, Tokyo, Japan), which consisted of a jar and a bag containing both an oxygen absorber and a carbon dioxide generator. The DO concentration in seawater in the jar was 1.4–1.7 mg/L O_2, determined by the Piccolo 2 optical oxygen monitor. The seawater was not changed, to prevent oxygen from entering the dishes. The jar was opened every day to sample one of the eight dishes. When the jar was opened, the bag containing the oxygen absorber and carbon dioxide generator was exchanged for a new one. Tomonts sampled from both oxic and hypoxic environments were stained with the acetocarmine stain (Watanabe et al., 2016), which enabled us to observe their nuclei. The tomonts were covered with the acetocarmine solution (1% acetocarmine solution dissolved in 45% acetic acid solution supplemented with several drops of 4% iron alum solution) in the dishes for 10 min and decolored with 1% hydrochloric acid in 70% ethanol. The stained tomonts were observed under a stereomicroscope (Olympus SZ61, Olympus, Tokyo, Japan) and classified to their developmental stages according to Watanabe et al. (2016) (Fig. 2). These were: Stage 1, tomonts containing numerous acetocarmine-positive granules representing denatured nuclei of host cells; Stage 2, tomonts with a coiled macronucleus; Stage 3, tomonts with a massive nucleus; Stage 4, early stage of cell division; Stage 5, later stage of cell division; empty cyst, empty tomonts after theront release; dead cyst, dead tomonts. The same batch of protomonts collected from the same group of infected black mollies was used for oxic and hypoxic incubations. This experiment was repeated three times using different batches of tomonts collected on different occasions.

We also examined the development of tomonts in the oxic environment without exchanging the seawater to learn of any effects from the lack of exchange, as we also did not exchange seawater in the hypoxic incubation. We carried out the same experimental steps as in the oxic environment, but did not exchange seawater at all. This experiment was repeated three times using batches of tomonts collected on different occasions.

2.4. Effect of hypoxic environment on tomonts at different developmental stages

We generated tomonts at various developmental stages by incubating them in the oxic environment and then in the hypoxic environment (Fig. 1C). We incubated 20 dishes containing 50 tomonts in the oxic environment, exchanging the seawater daily. Five dishes were sampled each day from 1 to 4 days after incubation, to obtain tomonts at different developmental stages. Tomonts in one of the five dishes were immediately stained with acetocarmine. The remaining four dishes were transferred to the hypoxic environment where they were incubated without seawater exchange. Tomonts in one dish were stained with acetocarmine every day from 1 to 3 days and 7 days after transfer to the hypoxic environment. As a control, protomonts from the same batch were incubated in the oxic environment with daily exchange of seawater. Eight dishes containing 50 protomonts were prepared and tomonts developed from the protomonts in one of the dishes were stained with acetocarmine every day for 8 days. This experiment was repeated twice using protomonts collected on different occasions.

2.5. Recovery of tomonts from dormancy, induced by hypoxic incubation, by oxic incubation

We induced dormancy in tomonts by hypoxic incubation and then incubated them in the oxic environment to observe their recovery from dormancy (Fig. 1D, #7). Nine dishes containing 50 tomonts were incubated in the hypoxic environment without seawater exchange. After 7 days, all of the dishes were transferred into the oxic environment for incubation. Seawater was then exchanged daily with air-saturated seawater. One dish was sampled daily to day 8, and tomonts in the dish were stained with acetocarmine and observed under a stereomicroscope. This experiment was repeated twice using tomonts collected on different days.

We also incubated tomonts in the oxic environment for 1–4 days to obtain specimens at various developmental stages. These were then incubated in the hypoxic environment for 1 week to induce dormancy at various developmental stages. Finally, we incubated the dormant tomonts in the oxic environment to observe their recovery from dormancy (Fig. 1D, #8–11). Forty dishes containing 50 tomonts were incubated in the oxic environment. Ten dishes were sampled every day after incubation for 1–4 days. Tomonts in one of the ten dishes were immediately stained with acetocarmine. The remaining nine dishes were transferred and incubated in the hypoxic environment for a further 7 days. Subsequently, one of the nine dishes was sampled and tomonts in the dish were stained with acetocarmine. The other eight dishes were transferred and incubated in the oxic environment with daily seawater exchange. One of the eight dishes incubated in the oxic environment was sampled every day for 8 days and the tomonts in the dish were stained with acetocarmine. This experiment was repeated twice using protomonts collected on different occasions.

2.6. Release and infectivity of theronts after tomont dormancy in the hypoxic environment

We examined the release of theronts from dormant tomonts kept in the hypoxic environment after a longer dormancy. We prepared four dishes containing 50 protomonts from the same batch. One dish was incubated in the oxic environment and the remaining three were incubated in the hypoxic environment. After 1 week, 2 weeks and 1 month (31 days), a dish incubated in the hypoxic environment was transferred and incubated in the oxic environment with daily seawater exchange (Fig. 1E). Tomonts releasing theronts or having completed theront release were counted under a stereomicroscope every day, when seawater was exchanged, for 8 days. We also counted theronts

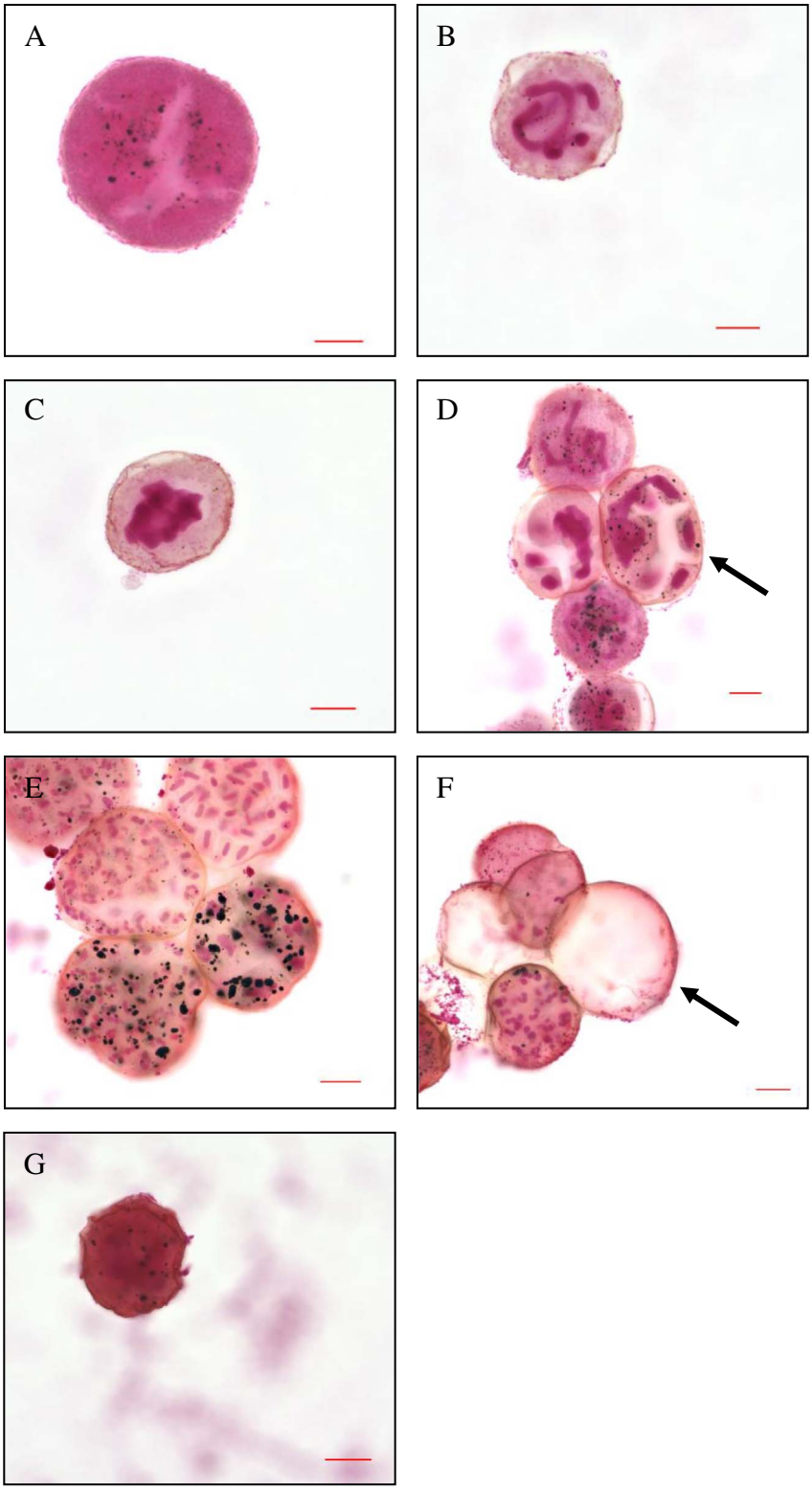

Fig. 2. Tomonts stained with acetocarmine. A: tomont containing numerous acetocarmine-positive granules (Stage 1); B: tomont with a coiled macronucleus (Stage 2); C: tomont with a massive nucleus (Stage 3); D: early stage of cell division (arrow) (Stage 4); E: later stage of cell division (Stage 5) with tomites containing the lobed macronucleus; F: empty cyst after theront release (arrow) (Empty); G: dead cyst stained evenly and losing internal structures(Dead). Scale bars = 50 μm.

released from tomonts between 4 and 5 days after transfer to the oxic environment. Theronts released after 4 and 5 days from tomonts incubated in the oxic environment only were counted as a control. This experiment was repeated three times using protomonts collected on different days.

We also examined the effect of dormancy in the hypoxic environment on the infectivity of theronts released from dormant tomonts, and on the development of tomonts obtained from fish challenged with theronts released from dormant tomonts. To examine infectivity, black mollies were infected with theronts released from tomonts incubated for 1 month in the hypoxic environment and subsequently incubated in the oxic environment. The experimental challenge was carried out according to the protocol of Yoshinaga et al. (2007) with some modification. Three black mollies were exposed to theronts (200 theronts/fish), within 3 h of their excystment from tomonts, in 300 mL of seawater without antibiotics in a 1 L plastic aquarium. After the challenge,

the mollies were kept in 1.5 L of seawater in a 2 L plastic aquarium. Two days after the challenge, the fish were kept individually in 1 L of seawater in a 1.5 L plastic aquarium with gentle aeration; seawater was changed daily without antibiotics. Protomonts leaving the fish 3–5 days after the challenge were collected and counted. As a control, black mollies were challenged with theronts released from tomonts incubated in the oxic environment only, and protomonts leaving the fish were counted. Protomonts of the same batch were used for both the experiment and the control.

We collected protomonts from the black mollies challenged with theronts released from tomonts after 1-month dormancy and examined the release of theronts. Three dishes containing 50 protomonts from the challenged black mollies were incubated in the oxic environment. Theronts were counted on day 5, when their release peaked. As a control, three dishes containing 50 tomonts from the fish challenged with theronts from tomonts incubated in the oxic environment only (the previous control experiment) were incubated in the oxic environment. Theronts released 4 and 5 days after the beginning of incubation were counted. Protomonts of the same batch were used for both the experiment and the control.

2.7. Statistical analysis

Statistical analysis was performed on the long dormancy experiments (Section 2.6). The percentages of tomonts kept in the hypoxic environment for different periods and released theronts were analyzed using one-factor repeated measure ANOVA after arcsine transformation. The numbers of theronts released and obtained from Petri dishes containing 50 tomonts were analyzed using one-factor repeated measure ANOVA.

The percentages of protomonts obtained from fish challenged with theronts released from tomonts after 1-month dormancy and control tomonts were compared using an F-test and Student's *t*-testafter arcsine transformation. The numbers of theronts released from tomonts obtained from the challenged fish were compared using an F-test and Student's t-test. Values $P < 0.05$ were regarded as statistically significant.

3. Results

3.1. Timing of tomont development in oxic and hypoxic environments

The timing of tomont development in the oxic environment with daily seawater exchange is shown in Fig. 3 (upper chart). The mean percentage of each stage from three repetitions, carried out on different occasions, is shown in the figure. On the first day after encystment, the elongation of macronuclei (Stage 1) commenced. On day 2, many tomonts had a coiled macronucleus. Tomonts at Stage 3 showed a massive macronucleus, and Stage 4 (days 3 and 4) showed several cells in the early stage of cell division, but their percentages were relatively lower than other stages. Most tomonts entered Stage 5 on day 4, showing numerous tomites with a lobed macronucleus, representing the late stage of cell division, and empty cysts representing completed release of theronts began to appear. The percentage of empty cysts increased considerably on days 5 and 6, reaching almost 50%, but increased little thereafter. Even on day 8, 40% of tomonts were still at Stage 5, in which many tomites were left in the cysts and unreleased. When tomonts were kept in the oxic environment, but without seawater exchange, they showed a similar development to those incubated with seawater exchange (Fig. 3, lower chart). The percentage of empty cysts was higher in the oxic environment without seawater exchange. However, a precise comparison was not achieved, as the batches of protomonts used in this experiment differed from those used in the oxic environment with seawater exchange.

Tomonts incubated in the hypoxic environment showed little development (Fig. 4). Most stayed at Stage 1, displaying numerous acetocarmine granules, which represented the denatured nuclei of host cells.

3.2. Effect of the hypoxic environment on tomonts at different developmental stages

When tomonts at different stages generated by oxic incubation for 1–3 days were transferred and incubated in the hypoxic environment, development soon stopped. Development did proceed slightly, even under hypoxic incubation, but depended on the initial period of oxic incubation (Fig. 5). Tomonts incubated in the oxic environment for 1 day developed to some extent, and there was an increase in the percentage reaching Stage 2. However, the development thereafter changed little even after hypoxic incubation for 7 days (Fig. 5A). The percentages in different developmental stages changed little in tomonts incubated in the oxic environment for 2 days (Fig. 5B). Although tomonts incubated in the oxic environment for 3 days proceeded to develop and some released theronts in the hypoxic environment, almost 70% of them did not release theronts (Fig. 5C). After hypoxic incubation for 4 days, tomonts began to release theronts after 1 day in the hypoxic environment. Around 70% released theronts (Fig. 5D); the final percentage of tomonts that released theronts did not differ much from tomonts incubated only in the oxic environment for 7 days (Fig. 5E).

3.3. Recovery of tomonts from dormancy induced by hypoxic incubation through oxic incubation

The vast majority of tomonts incubated in the hypoxic environment just after collection from fish (99%) were still at Stage 1 after incubation for 1 week, indicating that they developed little in the hypoxic environment. When the tomonts were transferred and incubated in the oxic environment, development resumed and as much as 80% released theronts in 8 days. Release of theronts peaked after 5 days of oxic incubation (Fig. 6A), similar to the tomonts incubated in the oxic environment (Fig. 3, upper and lower charts).

Tomonts at various developmental stages incubated in the oxic environment for 1–4 days and then incubated in the hypoxic environment for 1 week developed a little. Development was considerable after transfer to the oxic environment and theronts were released; as much as 80% in any group finally released theronts (Fig. 6 B–D). Tomonts incubated in the oxic environment for 0, 1 and 2 days showed peak theront release at 5, 4 and 3 days after transfer to the oxic environment, respectively. The total oxic incubation period (before and after hypoxic incubation) needed for tomonts to reach peak theront release was 5 days, except for tomonts incubated in the oxic environment for 3 and 4 days before the incubation in the hypoxic environtment. Roughly 70% of those tomonts incubated in the oxic environment for 3 and 4 days had released theronts when hypoxic incubation ceased.

3.4. Long-term viability of tomonts in the hypoxic environment and infectivity of theronts released from dormant tomonts

All tomont groups with different hypoxic incubation periods released theronts, with most released 5 days after tomonts were transferred into the oxic environment. The cumulative percentage of tomonts that had released theronts was > 70% over the 8-day oxic incubation period without significant differences among the groups (one-factor repeated measure ANOVA after arcsine transformation, $P > 0.05$) (Fig. 7). The number of theronts released from 50 tomonts between 4 and 5 days after transfer into the oxic environment was: 6210 ± 550 (mean ± standard deviation) at 0 days (control) incubated in the hypoxic environment before oxic incubation, 6506 ± 292 at 1 week, 7221 ± 312 at 2 weeks and 6016 ± 140 at 1 month. No significant differences were observed in the number of theronts among the groups (one-factor repeated measure ANOVA analysis, $P > 0.05$).

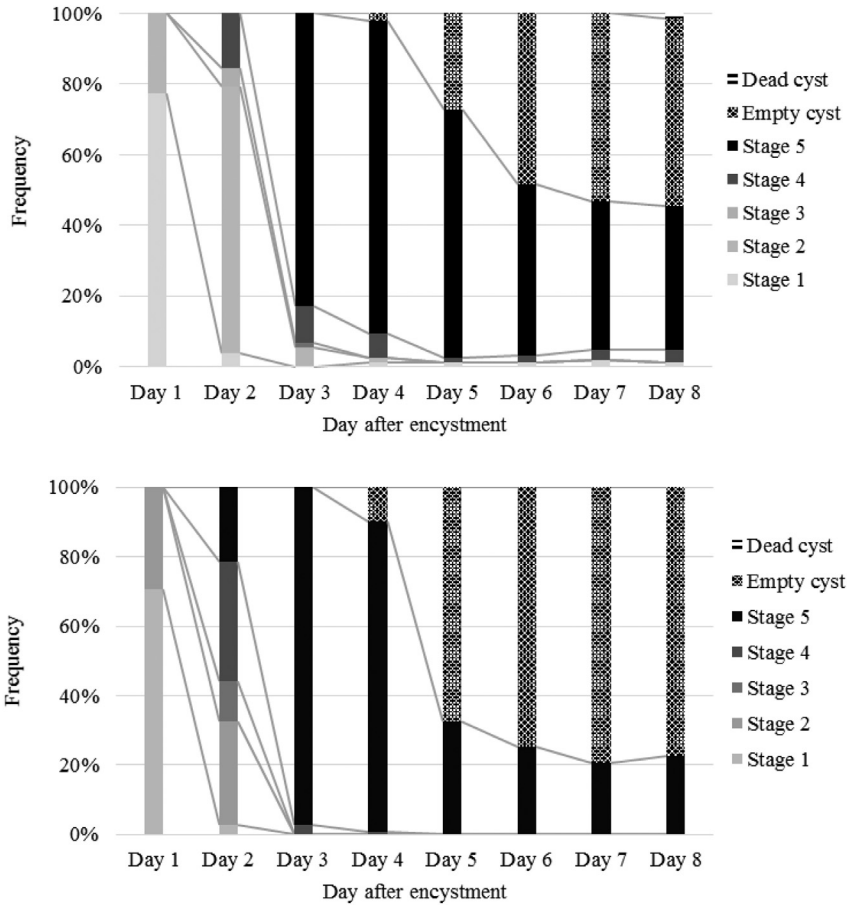

Fig. 3. Development of tomonts incubated in the oxic environment with water exchange (upper chart; Fig. 1A) and no water exchange (lower chart). See caption in Fig. 2 for stage definition. This experiment was repeated three times using different batches. Each column represent the mean of the repetitions.

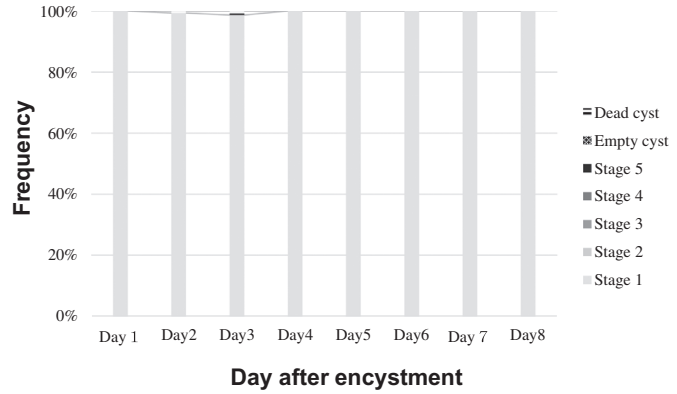

Fig. 4. Development of tomonts in the hypoxic environment (corresponding to Fig. 1B). This experiment was repeated three times using the tomont from the experiment of tomonts incubated in the oxic environment with water exchange (Fig. 3, upperchart).Each column represents the mean of the repetitions.

The infectivity of theronts obtained from tomonts recovered after 1-month dormancy, represented by the percentage of protomonts recovered from challenged fish from the theronts used for challenge, was equivalent to that of the control. No significant difference was found based on the F-test and Student's *t*-test after arcsine transformation ($P > 0.05$). Moreover, 6864 ± 630 theronts were obtained from tomonts recovered from the infected fish, and both the F-test and Student's t-test demonstrated no significant difference ($P > 0.05$) compared with the control (7256 ± 401).

4. Discussion

It is well known that tomont development is influenced by various factors, including water temperature (Wilkie and Gordin, 1969; Yoshinaga, 2001), DO (Yoshinaga, 2001) and salinity (Cheung et al., 1979; Colorni, 1985; Yoshinaga, 2001). To predict and prevent outbreaks of cryptocaryoniasis in marine net cages and land-based tanks, it is crucial to clarify how environmental factors affect the development of tomonts.

In this study, tomonts became dormant at any developmental stage when exposed to the hypoxic environment and resumed development when exposed to the oxic environment. When tomonts were transferred from the oxic environment to the hypoxic environment, they developed only during the first 1 day in the hypoxic environment. According to the manufacturer's instructions accompanying the hypoxic incubation jar, the oxygen concentration in the atmosphere in the jars declines to below 1% within 1 h, but it obviously takes a little longer for gas in the atmosphere to be dissolved in water exposed to that atmosphere. The DO concentration in seawater in the Petri dishes apparently took longer to decrease during tomont incubation. The progress in development probably occurred when the DO was still high enough in the seawater. The incubation periods needed for peak theront release was almost the same (5 days) regardless of the oxic incubation period prior to hypoxic incubation. This also indicates that tomonts were dormant in the hypoxic environment.

In the study by Yoshinaga (2001), tomont development was suppressed and no theront excystment was observed in a hypoxic environment (25% saturation DO, roughly 2 mg/L O_2), and development resumed when tomonts were transferred to an oxic environment. Similarly, Standing et al. (2017) also reported that no theront release was observed from tomonts in a hypoxic environment, but theront release

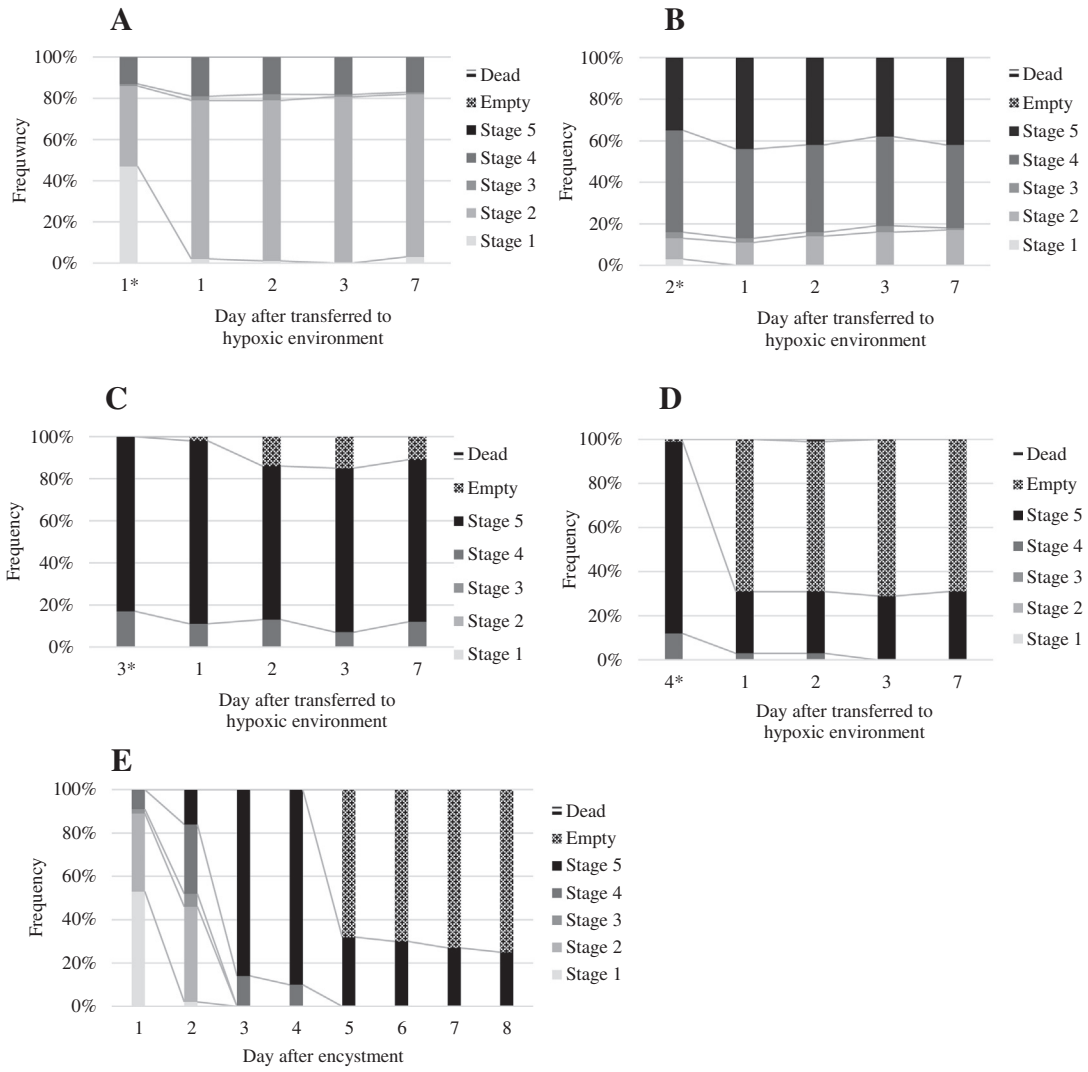

Fig. 5. Development of tomonts at different stages in the hypoxic environment. Asterisks indicate the days in the oxic environment (before transfer to the hypoxic environment). Tomonts transferred to the hypoxic environment after: A, 1-day oxic incubation (Fig. 1C, #3); B, 2-day oxic incubation (Fig. 1C, #4); C, 3-day oxic incubation (Fig. 1C, #5); D, 4-day oxic incubation (Fig. 1C, #6). E: tomonts incubated constantly in the oxic environment (control). This experiment was repeated twice using protomonts collected on different occasions. Each column represents the mean of the repetitions.

was observed after the tomonts were transferred to an oxic environment. Although our results are consistent with these two other studies, our study is the first to demonstrate the ability of tomonts to become dormant in a hypoxic environment and to resume development in an oxic environment at any developmental stage. However, our study was carried out in a hypoxic environment generated by an anaerobic jar (1.4–1.7 mg/L O_2), and the relationship between DO concentrations in seawater and the dormancy of tomonts is still unknown. Moreover, the dormancy mechanisms are not known at all; thus, further study is still needed on tomont dormancy in *C. irritans*.

Dormant tomonts incubated in the hypoxic environment for 1 month, as well as those incubated for 1 and 2 weeks, released theronts when transferred to the oxic environment, similar to those incubated in the oxic environment only. Theronts from tomonts that had experienced dormancy for 1 month could infect fish. Therefore, it is clear that dormant tomonts can retain their viability and infectivity for at least 1 month.

Several dinoflagellates are also known to form dormant (resting) cysts. The cysts are formed when the surrounding environment becomes unfavorable, such as when nitrogen and phosphorus are depleted. The dormant cyst then germinates when the environment becomes favorable for the growth of vegetative cells (Pfiester and Anderson, 1987). Some factors that control germination of the dormant cysts are water temperature, light irradiation and DO. It is thus possible that the dormancy of tomonts of *C. irritans* may be a reaction to an unfavorable environment.

Burgess and Matthews (1994a) explained the photoperiod-sensitive nature of tomonts and theronts base on an assumption that *C. irritans* had evolved in coral reefs. How et al. (2015) demonstrated that theronts swam little and had a tendency to stay close to the bottom of the water column. When the nature of the tomonts and theronts is considered, it is probable that the parasite evolved in coastal waters, even if not in coral reefs.

As hypoxic environments are often formed in bottom waters where vertical exchange is poor, tomonts of *C. irritans* are thought to experience hypoxia. Fish avoid hypoxic environments in general and, therefore, tomonts that settle on substrate would have limited opportunity to invade fish hosts if they only developed and released infective theronts in hypoxic environments. The nature of tomonts (their dormancy in hypoxic environments, retaining viability during a long dormancy and recovering from dormancy in an oxic environment) must be advantageous in an evolutionary context and allow *C. irritans* to encounter and invade fish. The DO concentrations may affect the survival of a theront as well as tomont development, but this subject requires further

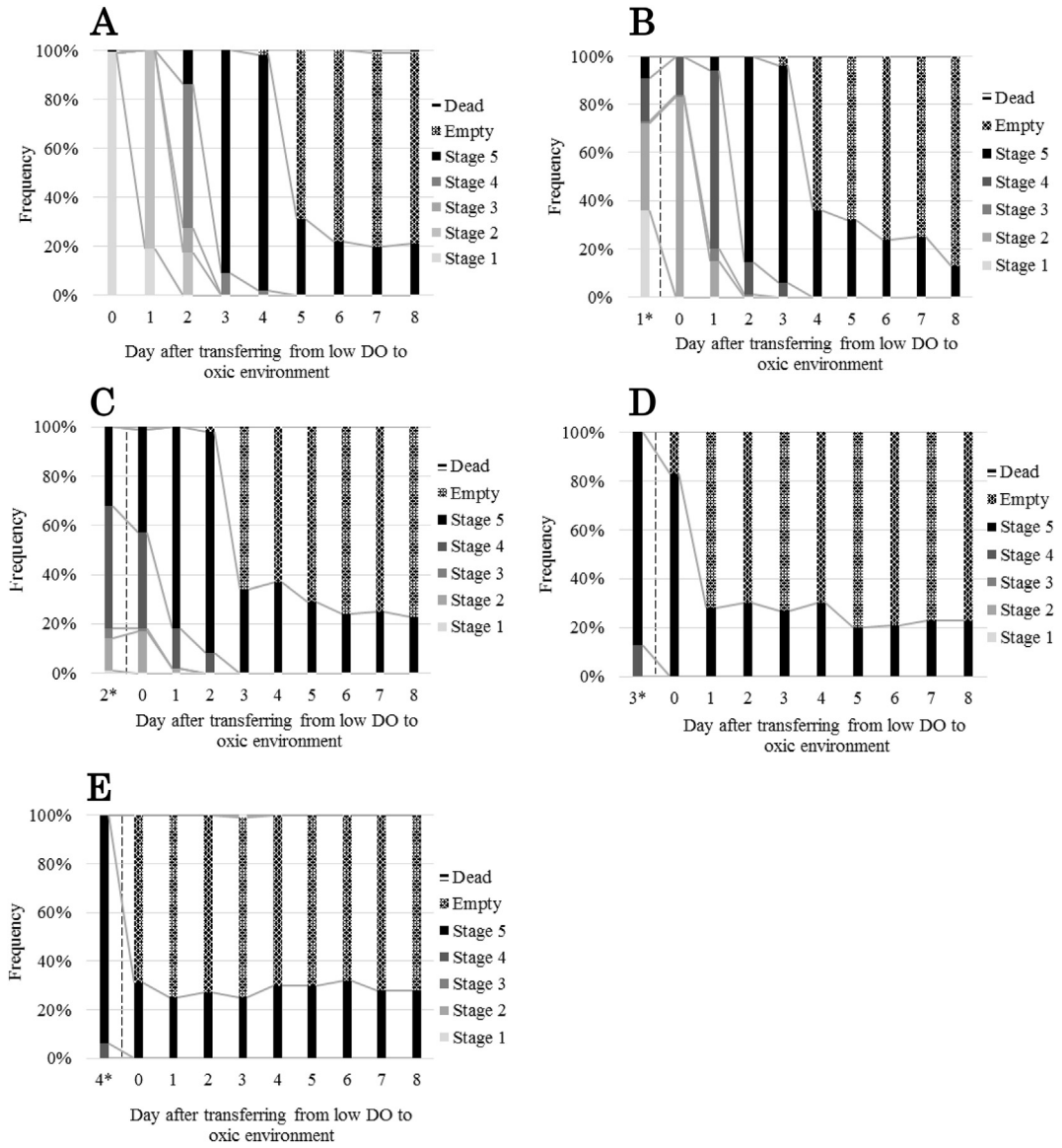

Fig. 6. Development of tomonts incubated in the hypoxic environment after various oxic incubation periods and subsequently transferred to the oxic environment. Asterisks indicate the days in the oxic environment before incubation in hypoxic environment. A: tomonts incubated for 1 week in the hypoxic environment after leaving hosts and then incubated in oxic environment (Fig. 1D, #7); B: tomonts incubated for 1 day in the oxic environment, 1 week in the hypoxic environment and then transferred to the oxic environment (Fig. 1D, #8); C: tomonts incubated for 2 days in the oxic environment, then 1 week in the hypoxic environment and transferred to the oxic environment (Fig. 1D, #9); D: tomonts incubated for 3 days in the oxic environment, 1 week in the hypoxic environment and then transferred to the oxic environment (Fig. 1D, #10); E: tomonts incubated for 4 days in the oxic environment, 1 week in the hypoxic environment and then transferred to the oxic environment (Fig. 1D, #11). This experiment was repeated twice using protomonts collected on different days. Each column represents the mean of the repetitions.

analysis and is outside the scope of this study.

Cryptocaryoniasis frequently occurs in red seabream and greater amberjack cultured in net cages in autumn and after typhoons (Yoshinaga, 2001; Kochi Prefecture, 2005; Katata et al., 2006). Yoshinaga (2001) suggested that the dormancy of tomont development in hypoxic environments and induction of theront release in oxic environments is a key factor for disease occurrence. This is because oxic water flows across and into the sea bed in fish farms because of the breakdown of the thermocline, caused by cooling surface water (autumn) or turbulence in the water column (typhoons).

Some anecdotes and monitoring of cryptocaryoniasis in fish farms suggest a temporal relationship between the outbreaks and typhoons, although there are few documented reports. An outbreak started in red seabream cultured in Nomi Bay, Kochi Prefecture on 30 September 1997, 2 weeks after a typhoon hit the area on 16 September. Of the red seabream cultured in the bay, 30% died of the disease during the following month. Another outbreak occurred in greater amberjack in the same bay on 4 November 2004, 2 weeks after a typhoon on 20 October. In this incident, ~50% of cultured fish died of the disease during the following week (Kochi Prefecture, 2005). Monitoring of cryptocaryoniasis and gill fluke infection in red seabream in Nomi Bay in 2009 and 2011 showed that the prevalence and intensity of *C. irritans* increased for several days and up to 1 week after a typhoon passed close to the area (Watanabe et al., 2011; Kadohara, 2013). These reports and monitoring results suggest that cryptocaryoniasis often occurs within 2 weeks of a typhoon's passage. *C. irritans* needs almost 7–10 days to complete its life cycle at 25 °C: 3 days as a trophont and 5–7 days as a tomont. The parasite cannot complete its life cycle twice in 2 weeks. Two weeks also seems too short a time for the parasite to gradually propagate from unrecognizable infection intensities to intensities high enough to cause mass fish mortality. It is reasonable to think that a certain number of tomonts had accumulated on the seabed before the

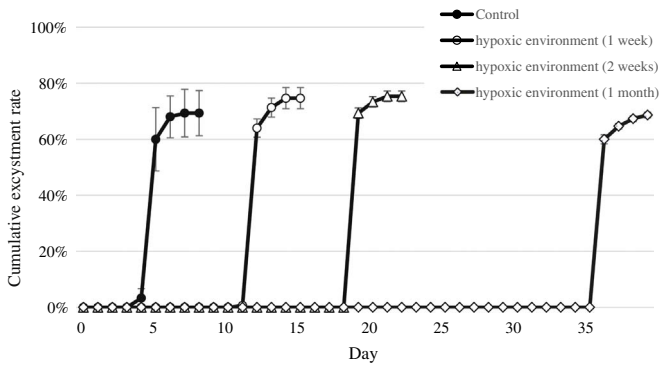

Fig. 7. Percentages of tomonts releasing theronts after incubation for 1 week, 2 weeks and 1 month in the hypoxic environment. Tomonts were transferred to the oxic environment after hypoxic incubation (Fig. 1E). Mean values of the cumulative percentages of tomonts that released theronts are plotted with the standard deviation (vertical bars).

outbreaks. Given that tomonts accumulate on the seabed during summer, their nature, that dormant tomonts at any developmental stage can resume their development when exposed to an oxic environment, may be a good explanation for the sudden outbreak of the disease in net-cage farms. However, it is still unclear whether tomonts can retain viability, even on the seabed under net cages, where bacteria and protozoa flourish on organic substances, such as fish feces and residual feed, generated by fish aquaculture. Further studies, such as an examination of tomont viability in microbe-rich environments and monitoring of their densities on the seabed, are still needed to learn more about the accumulation of tomonts on the seabed in cage-culture farms and the involvement of accumulated tomonts in outbreaks of cryptocaryoniasis.

Recently, How et al. (2015) demonstrated that theronts of *C. irritans* swam little and had a tendency to stay close to the bottom of the water column. The frequent occurrences of cryptocaryoniasis in fish in suspended net cages in autumn and after typhoons is likely associated with the low swimming ability of theronts as well as the resumption of tomont development in oxic environments. The breakdown of the thermocline in autumn and water turbulence during typhoons probably suspends theronts in the water column up to the height of the net cages in the surface water. Stress caused by upwelling of hypoxic water after the thermocline breakdown and turbulence during typhoons is also likely involved.

Propagation of *C. irritans* has been conducted by passing the parasite on live fish (Colorni, 1985; Burgess and Matthews, 1994b; Yoshinaga and Dickerson, 1994; Dan et al., 2006), because no in vitro culture method for their propagation is available, although trophonts of the parasite can be grown in vitro to an extent (Yoshinaga et al., 2007). However, serial passaging on fish requires a great deal of labor and considerable time, and the process cannot be interrupted, even when parasites are not needed for experiments (Dan et al., 2009). In addition, the viability of *C. irritans* is gradually weakened as laboratory propagation cycles continue (Burgess and Matthews, 1994b). This phenomenon hinders the progress of studies on *C. irritans*. If the parasite could be preserved for a long period, this would help considerably. According to Dan et al. (2009), tomonts can be preserved at low temperatures (12 °C) for 4 months, but the percentage of successful theront release drops. In our study, tomonts were preserved in a hypoxic environment using anaerobic chambers for at least 1 month without losing their viability or the infectivity of released theronts. Further study to determine how long dormant tomonts maintain viability in a hypoxic environment would be valuable. By combining the present preservation method in a hypoxic environment with low-temperature methods, we may preserve tomonts for considerably longer periods, maintaining their viability and the infectivity of theronts released from them.

5. Conclusions

This study has demonstrated the ability of tomonts to become dormant in a hypoxic environment and to resume development in an oxic environment at any developmental stage; it has also demonstrated the potential long-term viability of dormant tomonts in hypoxic environments. These results indicate that tomonts can accumulate on the seabed during the summer when it becomes hypoxic. This accumulation of tomonts may be a key factor for the autumn outbreaks of cryptocaryoniasis in floating net cages in temperate waters.

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