Effects of temperature, salinity and dissolved oxygen on excystment of podocysts in the edible jellyfish Rhopilema esculentum Kishinouye, 1891

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ABSTRACT

The scyphozoan jellyfish Rhopilema esculentum Kishinouye, 1891 is a species occurring in the traditional fishery in China. However, the yield of this species in fishery has rapidly declined since 1980s. The podocyst is an important phase during asexual reproduction in Scyphozoa and is considered to be potentially important to the population dynamics of jellyfish. In this study, we investigated the microstructure and effects of environmental factors on the excystment of podocysts. Podocysts of greater than 8 months of age exhibited no morphological differences compared to newly formed podocysts. Following excystment, a smooth-edged hole, probably created by enzymatic digestion was observed. Excystment sharply increased when the water temperature was higher than 18°C, suggesting that temperature ranging from approximately 13-18°C triggers the excystment of podocysts in R. esculentum. Stepwise temperature variation suggested that cooling was much more effective than warming in the induction of R. esculentum podocysts to excyst. The optimal salinity and dissolved oxygen (DO) conditions for excystment were observed to be 20‰ and 6 mg O₂ l⁻¹ respectively. Furthermore, excystment was suppressed by both stepwise increase and decrease in salinity or DO. Though R. esculentum podocysts could survive extreme environmental conditions such as hypoxia and low salinity, excystment rates were markedly reduced compared to those of other bloom jellyfish species when the DO and salinity levels were returned to normal.

Keywords: Environmental factors, Excystment, Podocyst, Rhopilema esculentum

Introduction

The edible jellyfish is a popular seafood in Eastern and Southern Asia, particularly in China, where jellyfish have been commercially exploited for the past 1700 years (Morikawa, 1984; Omori and Nakano, 2001). Recently, the United States, Mexico, Australia and India have also begun to utilise available species of edible jelly fish. This has created a multi-million dollar industry, which is on the increase worldwide (Hsieh et al., 2001; Pitt and Kingsford, 2003; You et al., 2007). It has been reported that more than twelve jellyfish species, belonging to the order Rhizostomeae, were being harvested, processed and sold in international markets (TSID, 2011). In China, the edible jellyfish Rhopilema esculentum Kishinouye, 1891 is a traditional delicacy and is considered good for human health. This has resulted in exploration of the species as an important fishery resource and cultivation for commercial harvest (Dong et al., 2009; Purcell et al., 2013). It has been reported that numbers of wild caught R. esculentum has rapidly declined since the 1980s. Large-scale release of cultured jellyfish was done by the Chinese Government to prevent resource depletion in coastal provinces (Dong et al., 2009).

Jellyfish blooms have become more extensive and frequent worldwide over the past several decades (Mill, 2001; Kawahara et al., 2006; Uye, 2011; Brotz et al., 2012). Along the coastal areas of Eastern Asia where R. esculentum inhabit, other jellyfish species of little or no economic value, such as Nemopilema nomurai, Aurelia aurita and Cyanea nozakii, have formed large blooms since the late 1990s (Dong et al., 2010; Qiu, 2014). It has been hypothesised that man-made climate change is considered to be the main driving influence on jellyfish blooms as well as for the differential bloom population size between R. esculentum and other jellyfish species that lived in the same coastal areas (Mills, 2001; Liu and Diamond, 2005; Purcell et al., 2007; Lo et al., 2008; Dong et al., 2010). However, our understanding of the root causes of the reduction in the R. esculentum population remains limited. Like most scyphozoan species, the life cycle of R. esculentum alternates between a sexual (planktonic) medusa phase...
and an asexual (benthic) polyp phase (Ding and Chen, 1981). With respect to the population dynamics of scyphozoan medusae, the asexual (benthic) polyp phase has been considered an important determinant. Long time dormancy, as well as the tolerance to abnormal environmental conditions of podocysts of N. nomurai and A. aurita showed that their behaviour, maintaining dormancy or mass excystment, might influence medusa population sizes (Thein et al., 2012; Kawhara et al., 2013).

The podocyst that is produced beneath the pedall disc of the polyp is an encapsulated dormant stage in the life cycle of many rhizostome and semaeostome jellyfish species (Chapman, 1968; Arai, 2009). Histological examination reveals that podocysts are chitin-covered cysts which contain stores of organic compounds. This possibly results in a tolerance of the podocyst to unfavourable environmental conditions, including low temperature, hypoxia, infestation by bacteria and fungi, as well as to predatory nudibranchs (Blanquet, 1972; Black, 1981; Kawahara, 2006; Ikeda et al., 2011; Thein et al., 2012). According to present knowledge, a single polyp can produce dozens of podocysts under certain environmental conditions. When cultured artificially, a single polyp of R. esculentum, Chrysaora quinquecirrha and Chrysaora fuscescens formed 10 (Lu et al., 1997), 52 (Cargo and Schultz, 1967) and 53 (Widmer, 2008) podocysts, respectively within a month’s time. However, the excystment rates of podocysts vary. For example, excystment rates of podocysts of R. esculentum were 18-69%, according to different studies (Lu et al., 1997; You et al., 2010). Therefore, it seems that excystment, at least for R. esculentum, ultimately affects the recruitment of polyps relative to the formation of podocyst.

Previous studies have shown that excystment of podocysts are affected by environmental factors such as temperature, salinity and dissolved oxygen (DO) (Arai, 2009; Han and Uye, 2010; Wang et al., 2014; Feng et al., 2015). Moreover, the key induction factors and conditions varied among different jellyfish species, such as lowering temperatures and returning to aerobic conditions from hypoxia in Chrysaora pacifica, C. nozakii and A. aurita and high temperature, low salinity and hypoxia in N. nomurai (Thein, 2012; Kawhara et al., 2013; Thein et al., 2013). Here, we hypothesised that the three environmental factors viz., temperature, salinity and DO influence the excystment of R. esculentum podocysts. In the present study, we investigated the ecophysiological characteristics of R. esculentum podocysts, and studied the effects of different environmental factors on its excystment.

Materials and methods

Podocyst preparation

Podocysts of R. esculentum were produced by polyps used for artificial seed production from a medusa breeding farm in Jiangsu Province, China. Mature wild R. esculentum medusae were caught and induced to spawn at 26°C during mid-August from the coastal area of Jiangsu Province. Hatched planulae were allowed to attach to opaque corrugated plates (40×35 cm) after 28 h. The metamorphosed polyps were fed their own unattached planulae before they developed to 8-tentacled polyps and then with fresh Artemia nauplii. Finally, the polyps were maintained in a refrigerated room (6-8°C) for long term preservation. The following May (9 months post-collection), polyps were transferred into a 20 m³ pool and fed fresh Artemia nauplii daily to induce artificial medusa production at 20±2°C. In addition to medusa production, abundant podocysts were also produced after the breeding season. These podocysts were then kept for 8 months (from August to March) in a 10 m³ pool, where the water temperatures mimicked those of their natural habitat, ranging from 8 to 28°C, with a salinity of 29.5±1‰ and exposure to light was prevented, to the greatest extent possible. The water in the pool was replaced with filtered seawater (DO 5-6 mg l⁻¹) biweekly.

Examination of podocysts by light and electron microscopy

Podocysts were examined under light microscopy and scanning electron microscopy (SEM) to study podocyst microstructures at different developmental stages. Briefly, 200 podocysts (~8 months of age) were maintained at 23°C in the dark in glass culture dishes filled with fresh seawater. Excystment was studied with the assistance of a stereo microscope (Olympus AX and Olympus SZX, Japan). For SEM, podocysts in dormancy and after excystment were fixed in 3% glutaraldehyde prepared in 0.1 M Na-cacodylate buffer (pH 7.2) at 4°C. Following fixation, the podocysts were washed in cacodylate buffer for 15-30 min, then dehydrated in 50% ethanol for 3 min, 100% ethanol for 5 min, 100% acetone for 3 min and dried in Tetramethylsilane. The dried samples were mounted on the specimen holder with double sided adhesive tape, sputter-coated with platinum/palladium and examined in a scanning electron microscope, (JEM-1200EX). The SEM images were transferred to a computer for image analysis.
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Effects of environmental conditions on excystment

Prior to the experiment, corrugated sheets were cut into 10 × 5 cm pieces and unqualified podocysts, including dead (which showed dispersed cell mass or empty in cyst capsule under light microscope) and small ones (diameter less than 200 μm), were removed. Sheets with more podocysts (more than 100 per piece) were selected and reared in 2 l glass beakers. For all environmental experiments on excystment, podocysts were stored in 250 l incubators (Jiangnan SPX). The water in the beakers was replaced every five days.

To examine the effects of environmental conditions on excystment, the cumulative excystment rates and mortality of podocysts were observed under different temperature, salinity and DO (Table 1). Numbers of podocyst excystments were counted every five days for two months. The experimental design was as follows:

**Temperature:** Three treatments (Treatment I used constant temperature, Treatment II used stepwise increase in temperature and Treatment III used stepwise decrease in temperature). Treatment I: Twelve sheets with more than 100 dormant podocysts in each were randomly divided into four groups and transferred directly to four temperature levels of 13, 18, 23 and 28°C. Treatment II: twelve sheets were randomly divided into four groups (A	extsubscript{td}, B	extsubscript{td}, C	extsubscript{td} and D	extsubscript{td}). The temperature of the A	extsubscript{td} group was maintained at 13°C. Temperature of B	extsubscript{td} group was increased from 13 to 18°C at a rate of 1°C per day and then maintained at 18°C. Similarly, the temperatures of C	extsubscript{td} and D	extsubscript{td} groups were increased from 13 to 23 and 28°C respectively. Treatment III: twelve sheets were randomly divided into four groups (A	extsubscript{td}, B	extsubscript{td}, C	extsubscript{td} and D	extsubscript{td}). The temperature of A	extsubscript{td} was maintained at 28°C. The temperature of B	extsubscript{td} group was decreased from 28 to 23°C at a rate of 1°C per day and then maintained at 23°C. Similarly, the temperatures of the C	extsubscript{td} and D	extsubscript{td} groups were decreased from 28 to 18 and 13°C, respectively, at a rate of 1°C per day.

**Salinity:** For the salinity experiments, the environmental salinity concentrations were achieved by diluting seawater to calculated percentages in purified freshwater. Three separate conditions were assessed. In treatment I, podocysts were transferred directly to different salinity levels, either 15, 20, 25 or 30‰. In treatment II, the salinity of four groups were all increased stepwise from 15 to 20, 25 and 30‰ by gradually elevating the salinity 1‰ per day; In treatment III, the salinity of four groups were all decreased from 30 to 25, 20 and 15‰ by gradually lowering the salinity 1‰ per day.

**Dissolved oxygen:** DO experiments were conducted in 3 l enclosed conical flasks. For the hypoxic group, the DO concentration was controlled by bubbling 99.9% nitrogen through the water. For the oxygen-enriched groups, DO concentration was adjusted by bubbling of 95% oxygen through the water. DO concentrations in each flask were monitored daily to verify the stability of the experimental conditions, using HQ30d multi-parameter meter (HACH, Beijing). Moreover, in order to maintain DO stability while monitoring the excystments and dead podocysts, podocysts and the experimental seawater were quickly transferred into a petridish, checked with an inverted microscope (Nikon, 80i), and then returned to the flask. In the DO experiments, three treatments were assessed. In treatment I, podocysts were transferred directly to DO levels of 1, 3, 6 and 9 mg O	extsubscript{2} l	extsuperscript{-1}. In treatment II, the DO of four groups were all increased stepwise from 1 mg O	extsubscript{2} l	extsuperscript{-1} to 1, 3, 6 and 9 mg O	extsubscript{2} l	extsuperscript{-1}, respectively, at the rate of 0.4, 0.6 and 0.6 mg O	extsubscript{2} l	extsuperscript{-1} per day. In treatment III, the DO of four groups were decreased stepwise from 9 mg O	extsubscript{2} l	extsuperscript{-1} to 6, 3 and 1 mg O	extsubscript{2} l	extsuperscript{-1}, respectively, at the rate of 0.6, 0.6 and 0.4 mg O	extsubscript{2} l	extsuperscript{-1} per day.

| Environmental factor tested | Experimental conditions | Temperature (°C) | DO (mg l	extsuperscript{-1}) | Salinity |
|-----------------------------|------------------------|------------------|-------------------------------|----------|
| Temperature                 |                        |                  |                               |          |
| Different temperature levels |                        | 13, 18, 23, 28±0.5| 5-6                           | 30       |
| Stepwise temperature increase|                       | 13 to 18, 23 or 28| 5-6                           | 30       |
| Stepwise temperature decrease|                     | 28 to 23, 18 or 13| 5-6                           | 30       |
| DO                          |                        |                  |                               |          |
| Different DO levels         |                        | 23± 0.5          | 1, 3, 6, 9±0.4                 | 30       |
| Stepwise DO increase        |                        | 23± 0.5          | 1 to 3, 6, or 9               | 30       |
| Stepwise DO decrease        |                        | 23± 0.5          | 9 to 6, 3, or 1               | 30       |
| Salinity                    |                        |                  |                               |          |
| Different salinity levels   |                        | 23± 0.5          | 5-6                           | 15, 20, 25, 30±1 |
| Stepwise salinity increase  |                        | 23± 0.5          | 5-6                           | 15 to 20, 25 or 30 |
| Stepwise salinity decrease  |                        | 23± 0.5          | 5-6                           | 30 to 25, 20 or 15 |
Statistical analysis
Cumulative excystment and mortality rates of podocysts in different groups were calculated as the average rates in three replicates. Statistical analysis (p<0.05) were carried out using one-way ANOVA (SPSS 15.0 software).

Results
Podocyst morphology and excystment
Podocysts of *R. esculentum* were produced beneath the pedal disc of polyps, having a diameter of about 124-400 μm (average 278 μm) in our experiment (Fig. 1a). As for podocysts produced by a same polyp, the diameters of newly formed podocysts were longer than those that were previously formed due to the growth of the polyps’ bodies. Newly formed podocysts contained a milky white cell mass and soft yellowish cyst capsule (Fig. 1a). Moreover, podocysts appeared as dome-like capsules with smooth roof and roughly concave base (Fig. 2a, b). A clear space was also observed between the capsule and cell mass (Fig. 2b).

When spontaneous excystment occurred under laboratory conditions, increased cell mass was first observed under light microscope, the evidence of which was a darkened centre of podocyst, relative to the surrounding area (Fig. 1b). Thereafter, the cell mass extruded through an opening at the roof of the capsule (Fig. 2c) and developed into a four-tentacle polyp (Fig. 1c). For podocysts aged 8-9 months, the duration from cell mass increase to four-tentacle polyp was 30-42 h at 23°C.

In the roof of excysted podocyst, a hole with smooth edge, which seemed to be enzymatically dissolved, was observed (Fig. 1c and Fig. 2d). This feature allowed for the differentiation of living podocysts from those that were dormant or dead (Fig. 1d).

Effect of temperature on excystment
Temperature variations had a pronounced effect on excystment of *R. esculentum* podocysts. After two months, the cumulative excystment reached 40, 46 and 45% at 18, 23 and 28°C, respectively. By comparison, the cumulative excystment was only 5% at 13°C (Fig. 3A). There were significant differences in cumulative excystment rates at 13°C compared with the higher temperatures tested (p<0.05). Moreover, the cumulative excystment rates at 18°C were significantly lower than those observed at 23 and 28°C at days 5-35 of the experiment (p<0.05) (Fig. 3A) (cumulative excystment percentages indicated between vertical dashed lines). However, the differences disappeared when the induction of excystment was carried out for longer than 40 days at the three elevated temperatures tested (p>0.05). When podocysts were transferred from 13 to 18, 23 or 28°C, the cumulative excystment rates were 41, 44 and 46%, respectively and no significant difference was observed among the constant temperature groups (Fig. 3b). However, the cumulative excystment rates reached 57 and 54% when podocysts were transferred from 28°C to 23 and 18 respectively,
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exhibiting substantially higher excystment rates when compared with the constant temperature treatments and stepwise temperature increase conditions (Fig. 3c). These results clearly indicated that cooling induced excystment of *R. esculentum* much more effectively than constant or increasing temperature.

**Effect of salinity on excystment**

Salinity was also observed to have an effect on the excystment of *R. esculentum*. The highest excystment rates were 54.98% at 20‰ salinity, followed by 15, 25 and 30‰ (Fig. 4a). Variation of salinity, however, did not benefit excystment. The excystment rates of all stepwise salinity increases or decreases (except one treatment in which salinity increased from 15 to 30) were substantially reduced in comparison to constant conditions. To be precise, the cumulative excystment rates were 28, 36 and 46%, respectively, when podocysts were transferred from 15 to 20, 25 and 30‰ (Fig. 4b). Similarly, the cumulative excystment rates were 30, 44 and 39%, respectively, when podocysts were transferred from 30 to 25, 20 and 15‰ (Fig. 4c).

**Effect of DO on excystment**

The excystment rate of podocysts were significantly different among the four DO treatments tested (Fig. 5a). The highest excystment rate observed was at a DO of 6 mg O$_2$ l$^{-1}$ (52%), followed by 3 and 9 mg O$_2$ l$^{-1}$ (31 and 30%, respectively) and 1 mg O$_2$ l$^{-1}$ (17%). Significant differences were found between DO of 6 mg O$_2$ l$^{-1}$ versus DO of 3, 9 and 1 mg O$_2$ l$^{-1}$ (p<0.05). When podocysts were transferred from 1 mg O$_2$ l$^{-1}$ to 3, 6 and 9 mg O$_2$ l$^{-1}$ in a stepwise manner, the rates of excystment were all lower than those observed under constant DO conditions (Fig. 5b). A similar result was observed when podocysts were transferred from 9 mg O$_2$ l$^{-1}$ to lower DO conditions. The only exception observed was when decreasing DO from 9 to 1 mg O$_2$ l$^{-1}$ (21%) (Fig. 5c).

**Discussion**

**Morphological features of podocysts**

The podocyst is an encapsulated, asexual stage in the life cycle of many rhizostome and semaeostome jellyfish species (Chapman, 1968; Arai, 2009). In our
experiment, the podocysts of *R. esculentum* were the only form of asexual reproduction by polyps observed, which was consistent with observations reported by others in *Rhombilema nomadica*, *Lychnorhiza lucerna*, *N. nomurai*, *Stomolophus meleagris* and *C. pacifica* (Calder 1982; Kawahara et al., 2006; You et al., 2007; Schiariti et al., 2008; Thein et al., 2013). For most jellyfish species, including *R. esculentum*, podocyst formation increased under favourable environmental conditions for polyps, such as increased temperature and abundant food supplies (Lu et al., 1997; Thein et al., 2013; Feng et al., 2015). Following formation of podocysts, the jellyfish became tolerant of long-term, severe changes in the environment, such as hypoxia, low salinity and colonisation by bacteria and fungi (Blanquet, 1972; Thein et al., 2012; Kawahara et al., 2013). Similarly, in our experiment, differences among flattened cell masses could be clearly observed using light microscopy and SEM through the thin transparent capsule of *R. esculentum* podocysts that had been dormant for 8–10 months. However, we were unable to observe any obvious differences in newly formed podocysts. It has been reported that the maximum longevities of podocysts in *A. aurita* and *N. nomurai* were 3 and 6 years, respectively (Thein et al., 2012; Kawahara et al., 2013). These results suggest that encapsulated podocysts are capable of living much longer than other life stages of jellyfish species.

In our observation of spontaneous excystment, the cell mass of podocysts increased before the capsule ruptured. Following excystment, a hole with smooth edges in the middle of the capsule wall was observed, indicating that a mild force, such as enzymatic degradation, as opposed to a strong and sudden force, perforated the capsule. These observations were inconsistent with those of Ikeda et al. (2011), who suggested that tear strength and enzymes contributed to the rupture of inner and outer layers of the capsule, respectively. According to our observations, we inferred that the process probably transmitted a signal inducing enzyme to degrade the capsule during the early stages of excystment.

### Environmental factors and excystment of podocyst

There were strong relationships observed between excystment of jellyfish and environmental factors, the key inducing factors varying among different jellyfish species (Thein, 2012; Kawhara et al., 2013; Thein et al., 2013). In our examination of excystment at different temperatures, the excystment rate was much lower at 13°C (only 4.77%) than higher temperatures tested over a two months period, suggesting that there was a critical temperature (13-18°C) for the induction of excystment of *R. esculentum*. This is consistent with the finding of Lu et al. (1997), who reported that podocysts did not excyst below 10°C. Moreover, the cumulative rate of excystment did not remarkably increase when temperatures increased gradually over a period of ≥40 days, suggesting that the excystment potential of dormant podocysts remained the same at excystment-triggering or higher temperatures. Temperature-trigger mechanism has been reported in other life stages such as polyp strobilation of *A. aurita* and *R. esculentum* (Dong et al., 2009; Fuchs et al., 2014).

The excystment rates were significantly higher (an increase of 12-13%) under warming conditions than in warming conditions, suggesting that autumn is the major excystment season for podocysts in *R. esculentum*. Similar findings were also reported in *C. pacifica*, *C. nozakii* and *A. aurita* (Thein et al., 2012; Thein et al., 2013). However, warming was observed as a trigger for excystment in other jellyfish species, such as *C. quinquecirrha* in the Chesapeake Bay and *N. nomurai* in East Asian marginal seas (Cargo and Schultz, 1967; Cargo and Rabenold, 1980; Kawahara et al., 2013).

Salinity has been implicated as a key factor in jellyfish dynamics (Willcox, 2006). In *N. nomurai*, low salinities...
Excystment of podocysts in the jellyfish *Rhoplema esculentum* (8-24) benefited excystment (Kawahara et al., 2013). In our experiment, the optimal salinity for excystment in *R. esculentum* was 20‰, resulting in an excystment rate of 54.98%. However, no significant differences were found among the 15-30‰ groups. These results indicated that salinity was not a key factor in the excystment stage of *R. esculentum*. Our study has shown that salinity fluctuation (increase or decrease) reduced the excystment of *R. esculentum*, suggesting fluctuation of supplemental freshwater affected the excystment of podocysts in the natural marine environment, as well as jellyfish population recruitment.

The excystment promoting effect of hypoxia has been observed in many jellyfish species (Thein et al., 2013). For example, excystment in *N. nomurai* increased significantly when subjected to hypoxic conditions (1.0 mg O$_2$ l$^{-1}$). This phenomenon was also observed in *A. aurita* (Thein et al., 2012). However, for *R. esculentum*, which has the same geographical distribution in the coastal region of East Asia, hypoxia markedly reduced excystment in comparison to normal DO concentration. Furthermore, it is perplexing that the excystment of podocysts was induced at higher DO (9 mg O$_2$ l$^{-1}$), although it appears to be decreased in comparison to those under normal DO conditions.

In conclusion, though the podocysts of *R. esculentum* are capable of survival in extreme environmental conditions, such as hypoxic (1 mg O$_2$ l$^{-1}$) and low salinity (15), the excystment rates were lower when transferred to normal environmental conditions in comparison to those continually residing in a normal and constant marine environment. In contrast, other blooming jellyfish species, such as *N. nomurai* and *A. aurita*, exhibited significant increases in excystment when transferred form hypoxic to well-oxygenated seawater (Thein et al., 2012; Kawahara et al., 2013). These phenomena suggest that the podocysts of *R. esculentum* possess less adaptation mechanisms compared to other jellyfish species, which might be one of the important causes for its population decrease.

In artificial breeding of *R. esculentum*, the quantity of polyps is the most important factor that affects seed production. Excystment induction is considered to be an important method for recruitment of polyp population. However, this technique has not been established yet. On the basis of already available information (Guo, 1990; Lu et al., 1997; You et al., 2007; Liu et al., 2015) and the formation and excystment patterns of podocysts under various environmental conditions examined in this study, there are several methods that can be used to enhance polyp recruitment by excystment. Firstly, elevated temperature (but not warmer than 30°C), appropriate salinity (16-26‰) and enough food supplementation benefit podocyst formation at the early polyp stage. Second, rearing podocysts under reduced temperature conditions (≤13°C) would aid in long-term preservation. Third, a gradual temperature reduction, from 28 to 23°C, at a rate of 1°C per day can efficiently induce excystment. Finally, maintenance of constant salinity and DO levels is important for increased excystment efficiency.

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