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RESEARCH ARTICLE

Observations on the spawning behavior, egg masses and paralarval development of the ommastrephid squid *Todarodes pacificus* in a laboratory mesocosm

Pandey Puneeta§, Dharmamony Vijai*, Hae-Kyun Yoo‡, Hajime Matsui and Yasunori Sakurai

ABSTRACT

The spawning behavior of ommastrephid squids has never been observed under natural conditions. Previous laboratory observations of Japanese flying squid (*Todarodes pacificus*) suggest that pre-spawning females might rest on the continental shelf or slope before they ascend above the pycnocline to spawn, and that the egg masses might settle in the pycnocline. Here, two mesocosm experiments were conducted in a 300 m^3 tank that was 6 m deep to investigate this hypothesis. In the first experiment, a thermocline (2.5–3.5 m) was established in the tank by creating a thermally stratified (17–22°C) water column. In the second experiment, the temperature was uniform (22°C) at all depths. Prior to spawning, females did not rest on the tank floor. In the stratified water column, egg masses remained suspended in the thermocline, but in an unstratified water column, they settled on the tank bottom, collapsed and were infested by microbes, resulting in abnormal or nonviable embryos. Eleven females spawned a total of 18 egg masses (17–80 cm in diameter), indicating that females can spawn more than once when under stress. Paralarvae hatched at stage 30/31 and survived for up to 10 days, allowing us to observe the most advanced stage of paralarvae in captivity. Paralarvae survived after consumption of the inner yolk, suggesting they might have fed in the tank.

KEY WORDS: Spawning behavior, Egg mass, Thermocline, Paralarvae, *Todarodes pacificus*

INTRODUCTION

All ommastrephids have a pelagic lifestyle, with reproductive behavior characterized by the extrusion of fragile, neutrally buoyant egg masses, the release of paralarvae into the surface plankton and the use of large-scale current patterns for larval transport, leading to the assisted migration of populations (Bower and Sakurai, 1996; Boyle and Rodhouse, 2005; Nishikawa et al., 2014). Neutrally buoyant gelatinous egg masses are thought to maintain their location in the water column by floating at the interface between water layers of slightly different densities, known as the pycnocline (Boyle and Rodhouse, 2005; Sakurai et al., 2000).

Todarodes pacificus (Steenstrup 1880) is a squid with second largest mass of commercial landings in the world (351,229 metric tons; Arkhipkin et al., 2015; FAO, 2014) and is an important prey species for a variety of vertebrate predators (Ohizumi et al., 2000; Tamura and Fujise, 2002), yet a full understanding of its life history is hampered because observation is difficult at the depths at which spawning occurs. Naturally spawned egg masses of *T. pacificus* have never been found in the hypothesized spawning ground. All information concerning this critical period of the life cycle of this species has been obtained from laboratory observations of spawned egg masses in captivity (Bower and Sakurai, 1996; Hamabe, 1963; Ikeda et al., 1993a). Spawning observations in captivity have also been documented for the ommastrephids *Illex illecebrosus* (O'Dor and Balch, 1985), *Sthenoteuthis oualaniensis* (Cheslin and Giragosov, 1993) and *Dosidicus gigas* (Staaf et al., 2008). Among ommastrephids, egg masses have been collected from the wild only for *Sthenoteuthis pteropus* (Laptikhovsky and Murzov, 1990), *Nototodarus gouldi* (O'Shea et al., 2004) and *Dosidicus gigas* (Staaf et al., 2008).

For pelagic squids, it is difficult to reconstruct the spawning method from fractionated egg masses or isolated eggs recovered from plankton samples (Von Boletzky, 1998). The spawning of *T. pacificus* is somewhat enigmatic and is assumed to occur above the continental shelf and slope around Japan (Sakurai et al., 2000). One piece of evidence for this hypothesis is that bottom trawls often collect exhausted and spent females on the continental shelf and slope at depths between 100 and 500 m (Hamabe and Shimizu, 1966). During experiments involving two *T. pacificus* females in captivity in a small tank (10 m^3), Bower and Sakurai (1996) found that ∼2 days prior to spawning, the females stopped feeding and instead rested on the tank bottom before producing a total of two egg masses. By combining results from field surveys and captive experiments, Sakurai et al. (2000) proposed the following reproduction hypothesis for *T. pacificus*: after sitting on the bottom at 100–500 m depth, the adult squid swim to an upper layer and spawn in surface waters between 18 and 24°C, preferentially from 19.5 to 23°C, above thermocline. Because of differences in density, the spawned egg masses sink until they reach a neutrally buoyant depth in the thermocline. Once hatched from the egg mass, the hatchlings swim toward the surface (Bower and Sakurai, 1996). Subsequently (Sakurai, 2006; Sakurai et al., 2013), this hypothesis was modified with updated information (Yamamoto et al., 2012) on survival temperatures for paralarvae. Normal embryonic development occurs at temperatures between 14 and 26°C, with the highest survival rates occurring between 14.7 and 22.2°C.

Advancements in artificial fertilization experiments have allowed for detailed studies of embryonic and paralarval development in ommastrephids (Sakai et al., 1998; Vijai et al., 2015a; Watanabe et al., 1996). However, there is a lack of information on the development of *T. pacificus* embryos inside the egg masses in their natural habitat. The most developed paralarvae hatching from a spawned egg mass so far recorded was stage 28 by Watanabe et al.

School of Fisheries Sciences, Hokkaido University, 3-1-1 Minato-cho, Hakodate 041-8611, Japan.
*Present address: Tohoku National Fisheries Research Institute, 25-259 Shimo-mekurakubo, Samemachi, Hachinohe, Aomori 031-0841, Japan. ‡Present address: East Sea Fisheries Research Institute, NFRDI, 1194, Haean-ro, Yeongok-Myeon, Gangneung-si, Gangwon-Do, Korea.

§Author for correspondence (puneeta83@gmail.com)

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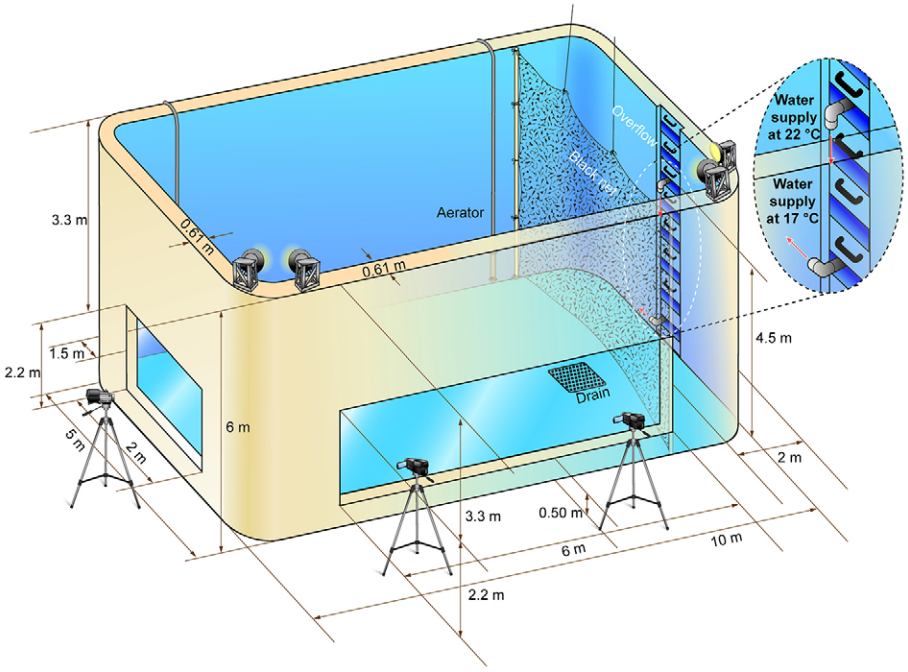

Fig. 1. Experimental tank drawn to scale and highlighting its parameters. Two experiments were conducted: one in the presence of a thermal gradient (created by supplying seawater at 17°C to the bottom and at 22°C to the top) and the second in which a uniform temperature was maintained (only 22°C seawater was supplied).

(1996). At stage 28, a considerable amount of internal yolk remains within the paralarvae body. In nature, hatching may occur at a later stage, after more inner yolk has been consumed. Under laboratory conditions, paralarvae continue to grow, consuming the inner yolk. However, since the first feeding prey of squid paralarvae have not yet been identified (Bower et al., 1999), after reaching stage 34 and once the inner yolk is completely utilized, the paralarvae starve to death in the laboratory (Watanabe et al., 1996).

Here, we present the experimental results from observations regarding spawning behavior, egg mass characteristics, and paralarval development of *T. pacificus*, and compare our findings with the reproductive hypothesis of Sakurai et al. (2000, 2013). We then assess the effects of the thermal gradient on egg mass distribution and subsequent embryonic development. Implications of utilizing natural seawater supply to enhance the survival and feeding of the paralarvae were also assessed.

MATERIALS AND METHODS

Tank parameters and establishment of thermal gradient

The experiment was conducted from September to October 2014 in a large experimental tank (depth, 6 m; length, 10 m; width, 5 m; volume, 300 m^3; Fig. 1) located in the Hakodate Research Centre for Fisheries and Oceans (HRCFO), Japan (Sakurai and Vijai, 2015). The tank was constructed of reinforced concrete with two acrylic (transparent) viewing windows: one in the front (length, 6 m; height, 2.2 m) and the other on side (length, 2 m; height, 2.2 m). During our experiments, the tank was filled with natural seawater to 4.5 m (225 m^3). The baseline light level was reached by adjusting four LED lamps (each 400 W) fixed over the tank. One pair of lamp lights was filtered by a screen (to reduce intensity) and placed in front, while another pair of lamp lights was turned upward to provide reflected light to the tank, because direct intense light on the tank might disturb the squid as they reach the surface water. During the day, a cloth screen in front of the observation window was used to cover the aquarium in order to block sunlight. The photoperiod was kept constant at 11 h light from 07:00 h to 18:00 h. A small 80 W red light (Toshiba BRF) was used to prevent skin damage resulting from contact with the tank wall during the dark period.

In order to establish a thermally stratified water column, filtered cold seawater (17°C) was first circulated through PVC pipes. Then, natural seawater (~22°C) from the Tsugaru Strait was pumped into the tank. Continuous overflow and drain mechanisms were maintained in the tank, and a thermocline was established at a depth of 2.5–3.5 m (Fig. 2) in approximately 72 h. During the experiments, the depth of the water column was maintained at 4.5 m to sustain an overflow system, which was essential to control the temperature of the tank. Vertical profiles of temperature in the tank were recorded at 1 h intervals using four temperature loggers (Onset HOBO UA-002-64), and a hand held Hydrolab (YSI EC300A).

Capture and maintenance of live squid

Adult *T. pacificus* specimens were collected from set trap nets from the inshore waters of southern Hokkaido, Japan, and by hand jigging onboard the T/S Oshoro Maru during August and September 2014. Live *T. pacificus* were subsequently maintained in a small tank (radius, 2 m; height, 1 m; volume, 10 m^3), and were fed a daily diet of frozen Pacific saury (*Cololabis saira*), walleye-pollock fry (*Gadus chalcogrammus*), Japanese horse mackerel (*Trachurus japonicus*) and Japanese anchovy (*Engraulis japonicus*) until the experiments were undertaken. Twelve previously copulated mature females [mantle length (ML), 232±17.1 mm, mean±s.d.] and one mature male (ML, 218 mm) were tagged and released into the large experimental tank for further experiments. Copulation status

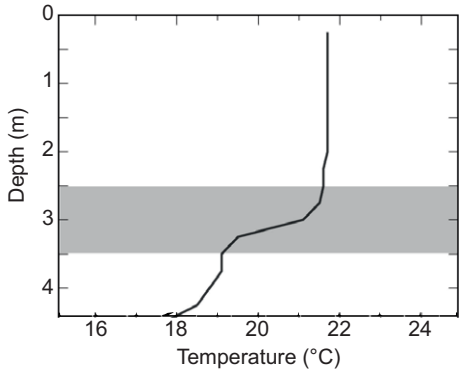

Fig. 2. Temperature profile of the tank while maintaining a thermal gradient. A thermocline (shaded area) is established in the tank between depths of 2.5 and 3.5 m. During experiments, water level was maintained at 4.5 m in the 6 m tank.

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was determined by the presence or absence of spermathecae on the buccal membrane and/or spermatangia on the outer lip of the females, since the males place their spermatophores on the buccal membrane of females; spermatozoa are stored in the female's seminal receptacles for up to several weeks until spawning (Ikeda et al., 1993a,b). Feeding with fillets of frozen Pacific saury continued in the experimental tank at 09:00 h every day. Water circulation and aeration were lowered to prevent any possible damage to egg masses. A black net was installed on the water supply side as a shield to safeguard tenuous egg masses, and also to enhance the visibility of transparent egg masses. Post-mortem examination of dead females was conducted to estimate the residual fecundity (total eggs in the ovary and oviduct after spawning) and nidamental gland condition.

Maintenance of egg masses and video recording

Two experiments were conducted; first, in the presence of a thermal gradient (~17–22°C, bottom to top) in the water column of the tank (September 3–14, 2014) and second, by maintaining uniform temperature (~22°C) in the water column (September 15–26, 2014). During the second experiment, to facilitate the viewing and photographing of the egg masses and the embryos, one of the egg mass was held in a gill net (mesh size, 49 mm) suspended from the surface near an observation window. Egg masses were photographed with a Nikon D700 camera. Live videos from the tank were recorded with a Sony HDR-CX590V (Sony, Minato, Tokyo, Japan) handycam (fixed as well as handheld). Underwater videos were recorded with a GoPro Hero3 camera and Sony handycam in a waterproof case. All video footage was annotated, reviewed and analyzed. Selected sequences from the 30 frames s^{-1} videos were captured with Adobe Premiere and exported as frames into ImageJ (http://imagej.nih.gov/ij/) to observe spawning behavior. The egg mass held within the gill net was observed using a binocular and magnifying lens. Measurements of the egg masses were made using a scale attached to a pole suspended from an onboard inflatable boat, and/or by emitting two parallel laser (Kowa Co. Ltd, Japan) beams of known width (85 mm) through the egg mass, while photographing from the observation window. These scales and widths were then used to estimate the diameter of the egg masses using the calibration function in ImageJ. Distances between eggs and fertilization rates within each mass were determined from the video recordings. The mean inter-egg distance was used to estimate the total number of eggs in each mass (Bower and Sakurai, 1996).

Paralarval collection

Vertically swimming paralarvae in the water column were monitored through the observation window. Paralarvae distributed on the surface water of the tank were observed and collected by scooping the surface water in a jar onboard an inflatable boat. Collected paralarvae were immediately categorized within the scheme of developmental stages given by Watanabe et al. (1996), under a stereomicroscope (Nikon SMZ 1500). After staging, advanced paralarvae (stage 34+, day 7+) were preserved in 4% formalin for later stomach content analysis.

RESULTS

Spawning behavior

Frequent mating in the 'male–female parallel' (Hamabe, 1962) position was observed throughout the experiment. The female squids did not rest on the bottom of the tank prior to spawning at any point, although the occasional normal sitting behavior common to squids (Harrop et al., 2014) was observed both in females and the male throughout the study. First spawning was observed 2 weeks after the squids were introduced into the tank, and all squids displayed normal feeding behavior before and after spawning. Three days after obtaining the first egg mass, 2–3 egg masses were observed in the tank at least every other day. Essentially, continuous spawning was observed, followed by the presence of dead squid in the tank (Fig. 3). A total of 18 egg masses with diameters ranging from 17 to 80 cm (Fig. 3) were produced by 11 squid. Whenever a

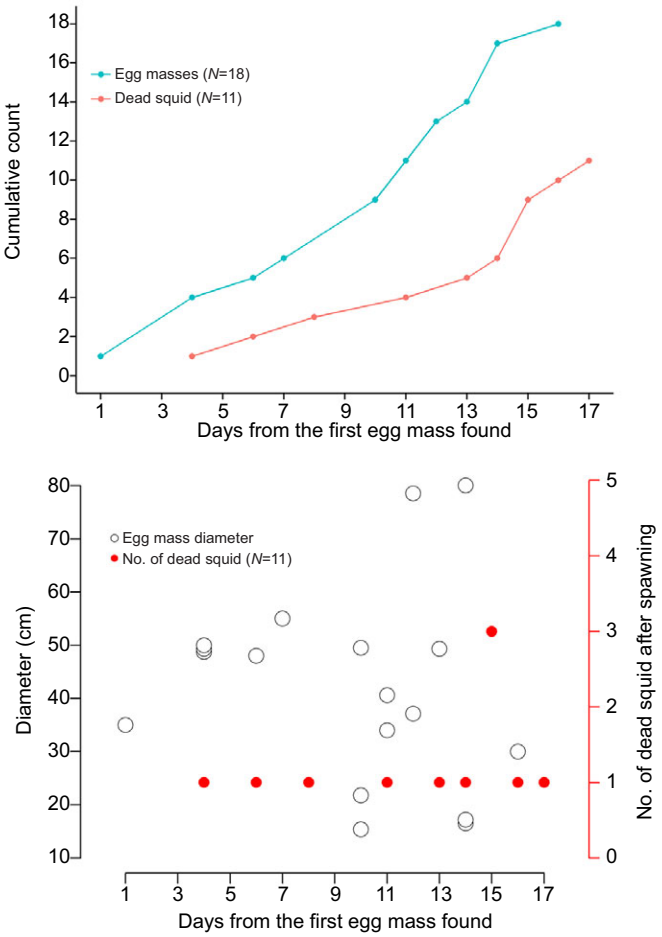

Fig. 3. Post-spawning summary for the ommastrephid squid *Todarodes pacificus* inside the experimental tank. (A) Number of egg masses and dead post-spawn females. (B) Size of the egg masses.

smaller, incomplete egg mass (diameter 15–21 cm) was produced, we also observed a larger (complete) egg mass (45–80 cm diameter) on the same day (Fig. 3B). The smaller egg masses (35–45 cm diameter) contained approximately 38,000 eggs whereas the larger ones contained approximately 200,000 eggs. More than 90% of the eggs within the mass were fertilized, with localized areas of unfertilized eggs evident. At the end of the experiment, 11 post-spawned squid were found dead in the tank. Once spotted they were removed from the tank for post-mortem examination. The last female was manually removed from the tank in order to allow for the continued observation of undisturbed egg masses. It was later determined that this female did not spawn.

Normally the squid school remained in sub-surface waters. Immediately before spawning, a female consistently moved away from the school to a lower depth in the tank. If more than one female was ready to spawn, they moved together away from the school to a lower depth, competed for a spawning area and displayed aggressive behavior. The spawning females flashed chromatophores rapidly over its entire body surface in a consistent pattern. Swimming activity was limited, and only implemented to maintain position in the water column (at approximately 2 m depth). Extrusion of the egg mass began from this position but the squid was unable to maintain its location by swimming (jet propulsion) and slowly sank, ultimately maintaining a fixed position just above the bottom of the tank. The mantle was slightly inclined (Fig. 4A) and since the fin

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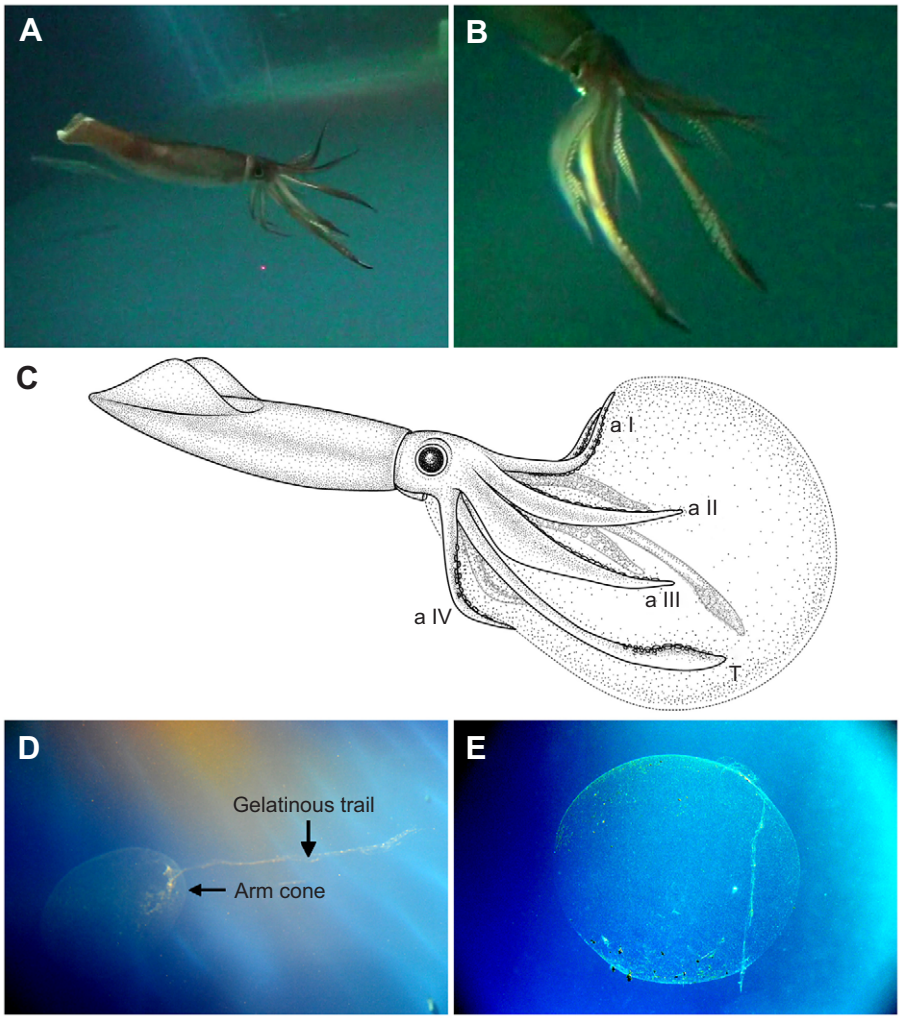

Fig. 4. Spawning posture of female squid and the spawned egg mass inside the experimental tank. (A,B) Frames from standard 30 frames s^{-1} underwater video clips showing the distinct arm postures of a spawning squid. The egg mass is completely transparent (visible only with light from a flashlight). (C) Schematic view of a spawning squid, with inclined mantle position, extruding the gelatinous egg mass, which is held by the arms and tentacles. A, arm; T, tentacle. (D) Egg mass immediately after spawning with an ellipsoid arm cone and gelatinous trail (formed as the arms and tentacle withdraw from the egg mass). (E) Egg mass once it has attained a spherical shape.

movement was the only source of locomotion, the fins moved continuously and faster (144 cycles min^{-1}) than was observed during normal swimming behavior (~100 cycles min^{-1}). The mantle pumped seawater constantly, introducing seawater into the jelly secreted by the nidamental glands. The siphon was directed toward the buccal area, blowing the jelly mixture, which was mixed with large amounts of water by the mantle pumping, extruding the large gelatinous egg mass that was then held by the arms and tentacles. The arms and tentacles were continuously stretched and oriented in the same position and the mantle was inclined upward (on the posterior side) throughout spawning. While spawning, the distal half of arms I were stretched outward and distal half of arms IV were stretched inward, whereas, in arms II and III, the proximal half was stretched outward similar to the petals of a flower and the distal half was in a straight position (Fig. 4A–C). The proximal quarter of the tentacle was extended outward and the remainder was stretched parallel to the body. The spawning process continued from the bottom of the tank until the entire egg mass was extruded, which took approximately 7 min.

The egg mass was not perfectly spherical immediately after spawning as there was an ellipsoid arm cone and gelatinous trail (formed as the arms and tentacle withdrew from the egg mass, Fig. 4D,E), which resulted in a bulb shape. After 20–30 min, however, the egg mass gradually became spherical or nearly spherical in shape as a result of changes in density of the surrounding seawater. All of the egg masses were observed in the early to mid-morning and at noon.

Post-mortem examination of the oviducts revealed only females that produced the larger egg masses spawned the majority of their eggs. Only one squid (ML, 239 mm) had 0 eggs in the oviduct and granular ovary after death. All other squid had >25,000 eggs in the oviduct and a residual fecundity of >150,000. The anterior ends of the nidamental glands from post-spawned females were attenuated and slightly translucent. We could not analyze the relationship between mortality of the squid after spawning and size of the egg mass spawned because, in some cases, we were not able to assign the egg mass to the squid that spawned it.

Effects of the thermal gradient

In the first experiment, a thermal gradient was formed, causing a density gradient. Because of these changes in density, the egg masses became buoyant and were lifted off the bottom of the tank. Once suspended, all of the egg masses (N=4) maintained this position and continued to float in the thermocline (Fig. 5A,B). In the second experiment, the thermocline was removed and the water was maintained at a uniform temperature throughout the water column. Fourteen egg masses were spawned during this experiment, all of which settled on the bottom of the tank (Fig. 5C,D). After 2–3 days, all egg masses gradually collapsed and during this process were infested by bacteria, protozoa and other zooplankton and

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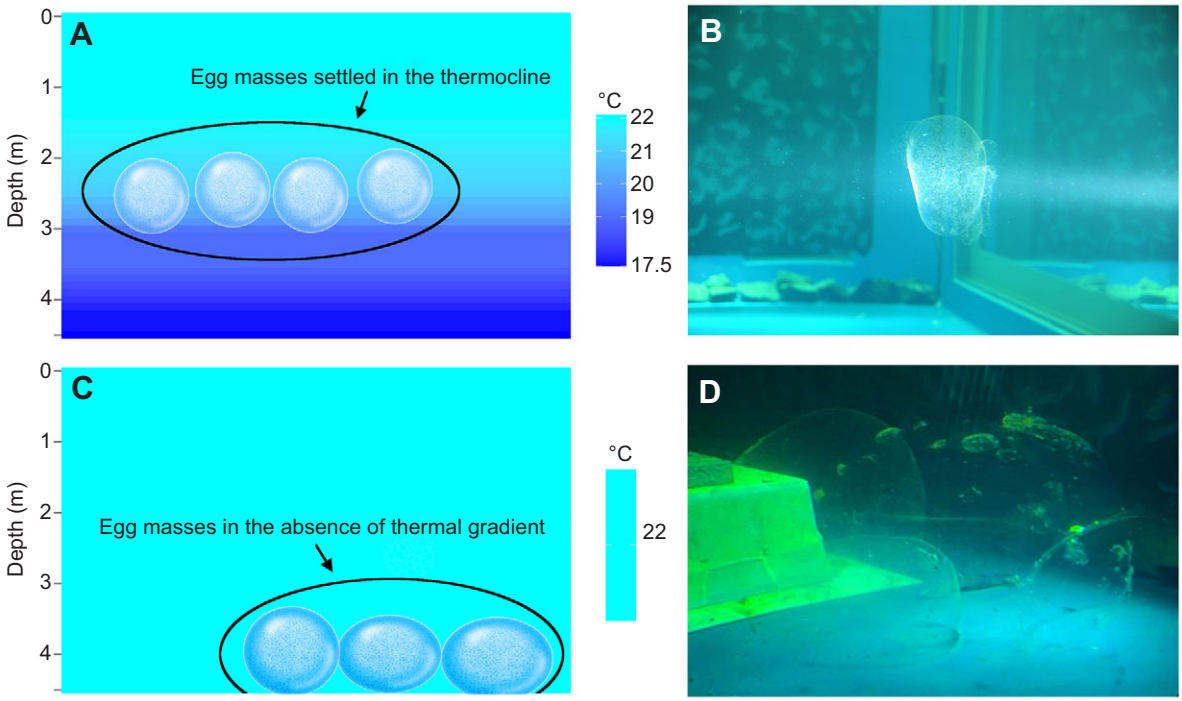

Fig. 5. Effects of thermal gradient on the depth of egg mass settlement. (A,B) In the first experiment with thermal gradient, egg masses are suspended in the thermocline layer. (C,D) In the second experiment, the thermocline was removed, uniform water temperature is maintained and egg masses settle on the bottom of the tank.

microorganisms, before finally disintegrating on the floor of the tank (Fig. S1). As a result, all of the embryos within these egg masses were found to be abnormal or nonviable.

Embryonic development inside the egg mass

Eggs were positioned 0.4–2.0 cm apart throughout the inner mass. Arrangement of eggs inside the egg mass was dependent on the total egg mass size. Many eggs within the smaller masses (15–22 cm diameter) formed a clump, which was presumably due to the outer membranes of the eggs sticking them together (Fig. 6A,B). Additionally, the eggs were not distributed homogeneously in the smaller masses, in which substantial areas were devoid of eggs, whereas within the large egg masses, individual eggs were distributed homogeneously (Fig. 6C). We were not able to determine whether the same squid spawned both the small and large egg masses.

The chorion surrounding each egg expanded to a diameter of 1.9–2.3 mm before hatching (Fig. S2). The embryos within the egg mass were often oriented vertically, with the head facing down (Fig. 7A). This position was maintained by a continuous or rhythmic jetting and contraction of the mantle. When the embryo stopped jetting it became horizontally oriented, after which it would begin to jet again and return to a vertical head down position. In some instances, embryos were oriented with the head facing up, where it was inclined left and right while in this position. While the embryo shifted inside the chorion, it tended to return to the head down position after mantle contraction (Fig. 7B–E). The embryos hatched in the same head-down position with the posterior side up.

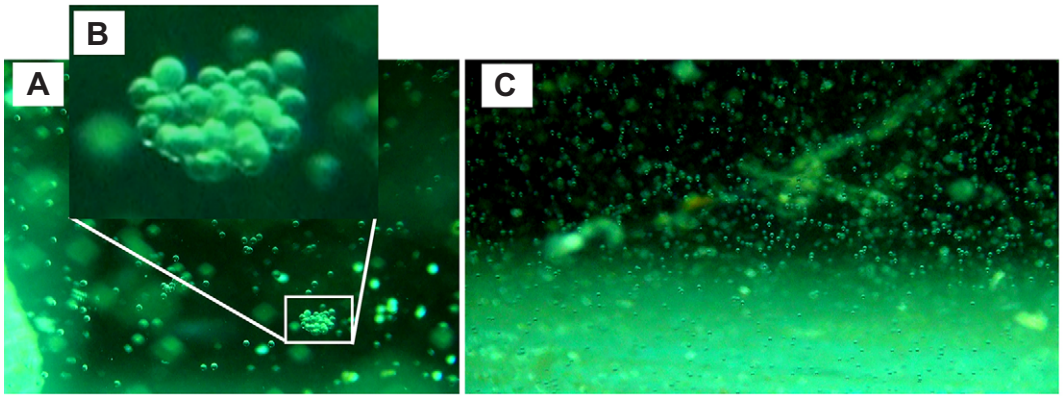

Fig. 6. Egg distribution within small and large egg masses. (A,B) Many eggs within the smaller (15~22 cm diameter) egg masses clump together. Furthermore, the eggs are not distributed homogeneously and substantial areas are devoid of eggs. (B) Enlarged view of a clump, where the outer membranes of eggs presumably stick them together. (C) Within the large egg masses, the eggs are homogeneously and individually distributed.

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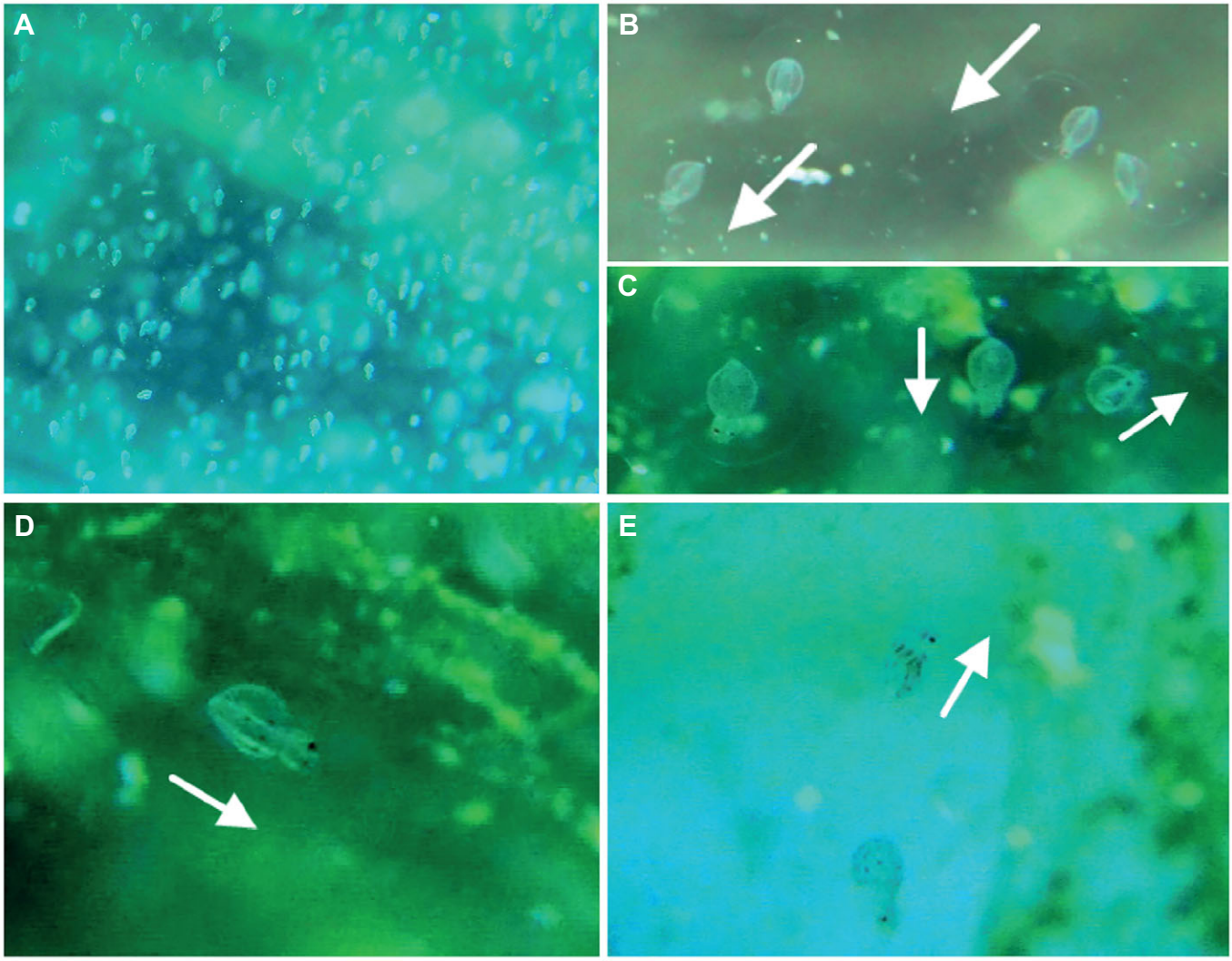

Fig. 7. Orientation patterns of the developing embryos within the egg masses. Representative images from standard 30 frames s^{-1} underwater video clips of egg masses. (A) Most of the embryos are oriented with their heads down. (B) Embryos with head facing left. (C) Two embryos oriented vertically with the heads down and one turned to the right. (D,E) Head oriented right and up, respectively. Arrows indicate the direction that the head is facing.

Paralarvae

Hatching usually occurred 5–6 days after spawning at approximately 21.5°C. Hatching of paralarvae occurred at stage 30 or 31 (~1.22 mm ML) (Fig. 8). Soon after hatching, paralarvae demonstrated a hop and sink swimming behavior within the egg mass, which is common in ommastrephid paralarvae (Vijai et al., 2015b). Paralarvae spent approximately 2 h within the egg mass before swimming out vertically. They ascended slowly from the mid-water in the tank until they reached the surface, where they swam in circular or random patterns. In the surface waters, they demonstrated a wide variety of behavior, including the ball posture (withdrawing the head inside mantle) typical of ommastrephid paralarvae (Vijai et al., 2015b). They developed beyond stage 34 (Fig. 8C–E), the most advanced stage observed for *T. pacificus* in laboratory conditions. Seven days after hatching, the inner yolk was completely utilized by the paralarvae, after which they survived for an additional 3 days. Although there was no considerable increase in ML after stage 34 (1.3 mm), there was a slight increase in the proboscis length, eye width and body width (Fig. 9), with prominent arm IV primordia clearly visible in day 10 paralarvae. Stomach content analysis of the 10-day-old paralarvae (1.34 mm) revealed no identifiable food.

DISCUSSION

Spawning

The results from our study suggest that there are two major differences between the true reproductive behavior of *T. pacificus* and the hypothesis proposed by Sakurai et al. (2000, 2013). First, the theorized sitting behavior of pre-spawning females was never observed, and second, there was no change in the feeding patterns of females after spawning in comparison to normal squid. Sitting behavior observed in previous experiments (Bower and Sakurai, 1996) was probably due to the stress of being in an environment of limited space (15 m^3) compared with our more generous volume (225 m^3). Moreover, all squids (females and male) exhibited occasional sitting behavior throughout the present study, indicating that this behavior may not only associate with spawning. Some aspects of squid biology also preclude pre-spawning sitting behavior, especially if there is a second spawning event; emaciation following the first spawning could limit locomotion and render squids unfit for swimming in the water column. These new observations challenge traditional theories on the spawning areas of *T. pacificus*, originally based on the supposed sitting behavior of females. Although *T. pacificus* is a

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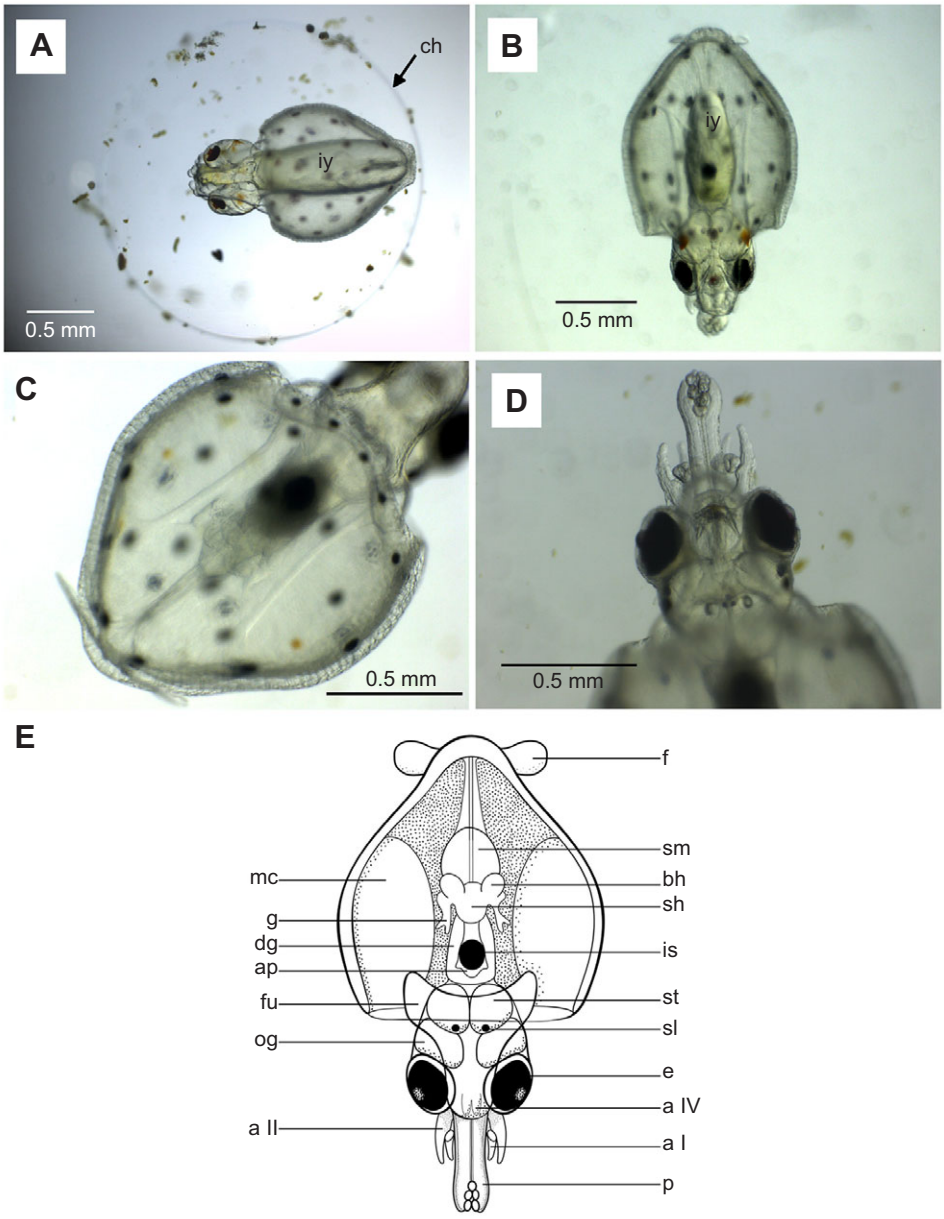

Fig. 8. Paralarval squid at the hatching stage and most advanced stage of development observed. Classification of stages after Watanabe et al. (1996). (A) At previously reported hatching stage (stage 28), embryos can be seen still inside the chorion. (B) Paralarvae hatched at stage 31. Internal organs (C), head portion (D) and diagrammatic representation (E) of 10-day-old paralarvae. a, arm (a IV is primordia of arm IV); ap, anal papilla; bh, branchial heart; ch, chorion; dg, digestive gland; e, eye; f, fin; fu, funnel; g, gill; is, ink sac; iy, inner yolk; mc, mantle cavity; og, optic ganglion; p, proboscis; sh, systemic heart; sl, statolith; sm, stomach; st, statocyst.

mesopelagic spawner, sitting behavior demands a shallow depth for the females to rest before spawning. Thus, the ideal spawning area was believed to be at a mid-surface depth above thermocline, which is found where the sea bottom depth is 100–500 m (Sakurai et al., 2000, 2013). These areas are above the continental shelf and slope around Japan, and restricted between 21 and 41°N and 121 and 142°E, with an absolute depth of 100–500 m, located along the Kuroshio axis, and more specifically near the Tsushima Strait (Kidokoro and Sakurai, 2008; Rosa et al., 2011). Our results provide an opportunity to assume that since *T. pacificus* is a pelagic spawner, its spawning area may extend to pelagic areas where the water depths are beyond 500 m. However, the survival of paralarvae may depend on prevailing oceanographic features, including sea surface temperature and currents.

The squid undergoes continuous stress while extruding an egg mass since only fin movement is used for locomotion, while the mantle continuously pumps water into the new egg mass. The arms and tentacles are also stretched in the same position throughout the process as they hold the egg mass. The total spawning duration reported here for an egg mass of 80 cm diameter was 7 min, which is similar to values recorded in previous experiments. This is longer than spawning in *I. illecebrosus*, which takes 2 min for an egg mass diameter of 30–40 cm (O'Dor and Balch, 1985). The maximum size of the egg mass might be limited by the squid's capacity for pumping water by continuous mantle contraction and length of time that it can maintain the arms and tentacles stretched in the same position.

Except for one squid, which completely extruded all of the eggs in the oviduct, the other post-spawn females died with a considerable amount of eggs (>25,000) in the oviducts. The remaining eggs could have been extruded in a second spawning event. In the present experiment, we obtained more egg masses (N=18), than the number of females that spawned (N=11). This suggests that females do not necessarily spawn all of their eggs in one event. This observation is in accordance with the results found by Ikeda et al. (1993a), who found that some females spawned twice

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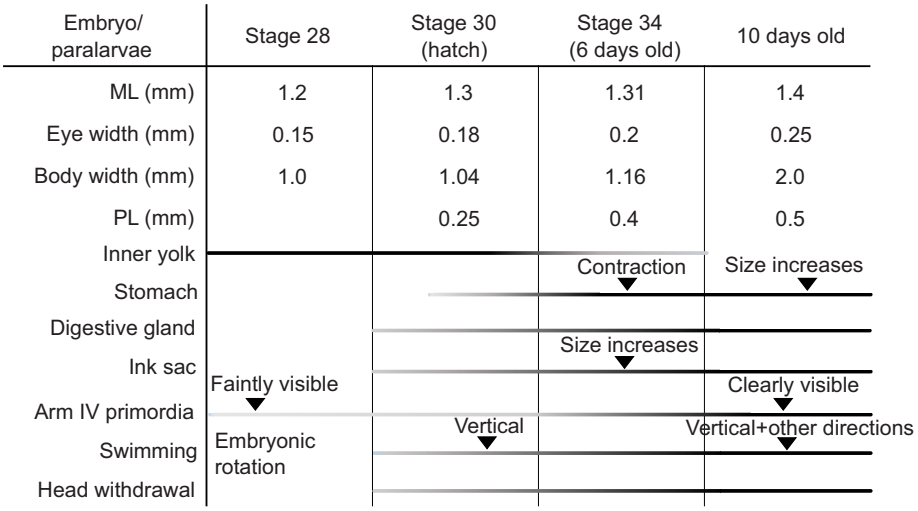

Fig. 9. Ontogenetic changes of *Todarodes pacificus* from stage 28 embryo to 10-day-old paralarvae. Previous studies reported stage 28 as hatching stage and obtained paralarvae until stage 34 (6/7 days after hatching). In this study, the hatching stage was stage 30–31 and the survival period after hatching was 10 days. Mean values of lengths and widths are shown. Bars show presence of factors indicated on a scale from low (white) to high (black). Arrowheads indicate timing of specified events. ML, mantle length; PL, proboscis length.

in a week when their spawning was disturbed. The post-spawn squid with 0 eggs in the oviduct might have already spawned two or three egg masses prior to collection. The range of egg mass sizes (17–80 cm diameter) suggests that the number of spawning events depends on the size of the egg mass previously spawned. In nature, under standard conditions, a female could possibly spawn all of the eggs in the oviduct in one event, producing a single large egg mass. However, if a female is interrupted while spawning, she may abruptly end the process, resulting in the production of a smaller egg mass and might later spawn again in a second event. The interval between these spawning events is probably not long because food availability in the spawning ground of *T. pacificus* is limited (Kishi et al., 2009). The small egg masses obtained in the present study could be due to the constraint imposed by the tank depth on the amount of time available to the squid to inflate the egg mass if the squid is sinking throughout the process. Sinking through the epipelagic zone (as much as 200 m) would allow plenty of time, whereas sinking in the 4.5 m of our experimental tank does not.

The largest egg mass recorded here as well as in a previous study (Bower and Sakurai, 1996) was 80 cm in diameter and both contained approximately 200,000 fertilized eggs. The residual fecundity of all the post-spawned females (except for the squid with an empty oviduct) was always greater than 150,000. This implies that the females observed had additional eggs that could be used for more spawning events. They may be able to spawn multiple egg masses of different sizes until most of the eggs are extruded. Based on the number of eggs found in one egg mass and the potential fecundity, Staaf et al. (2008) predicted the reproductive output of a single *D. gigas* female was between 3 and 20 egg masses. For *T. pacificus*, taking into account the egg count in the largest egg mass (~200,000 eggs), the residual fecundity (>150,000) and the potential fecundity of this species (320,000–470,000; Soeda, 1956), there could be at least two spawning events. The small egg masses also support the 'offshore reproductive strategy' of the family Ommastrephidae proposed by Nigmatullin and Laptikhovsky (1994), where the spawning is intermittent with decreasing intensity: after the end of the first period of egg accumulation in oviducts, the first egg mass or several egg masses are laid (these comprise 30–50% of actual fecundity; subsequent masses comprise 15–5%).

Egg mass

When the oviducts are filled with ripe eggs, the squid is ready to spawn. At spawning, the eggs are first coated with oviducal gland secretion and are then released along with the nidamental gland secretion, through the funnel into the buccal region, where they are fertilized with the sperm stored in the spermathecae. The fertilized eggs are then extruded into the seawater. While extruding eggs, the female continuously contracts its mantle and the egg mass swells gradually. The reaction between mucosubstance in the nidamental gland and surrounding seawater results in the formation of an outer water-soluble layer, which is devoid of eggs and an interior water-insoluble jelly, where the fertilized eggs are distributed homogenously (Kimura et al., 2004). The interior jelly of the egg mass consists of a fibril matrix where the fertilized eggs are arranged in a viscous watery substance (Bower and Sakurai, 1996; Kimura et al., 2004). This network of fibrils might act as a scaffold facilitating the homogenous and discrete distribution of eggs and maintaining the overall shape of the egg mass.

The egg mass was initially bulb shaped, with an arm cone formed in the location where squid held the egg mass, similar to what was reported for *I. illecebrosus* (O'Dor and Balch, 1985). However, as a result of surface tension, the shape of the egg mass slowly changed to become spherical, which provides the smallest ratio of surface area to volume. Thus far, the ommastrephid egg masses observed in the wild and in captivity have all been characterized by their spherical shape, which has unique advantages in the water column. For example, O'Dor and Balch (1985) reported that the egg mass of *I. illecebrosus* behaves hydrodynamically like a rigid sphere. The velocity of a sinking egg mass depends on its density and is determined by its specific gravity and a drag coefficient. The drag coefficient is relatively constant for rigid spheres over a certain range of Reynold's numbers (Blake, 1983). This could be an essential adaptation for egg survival within the mass because it would facilitate greater planktonic dispersal of eggs along the Kuroshio Current, which leads the hatched paralarvae to nutrient-rich waters (Okutani, 1968, 1983; Watanabe, 1965).

Effects of thermal gradient

According to the reproductive hypothesis previously proposed (Sakurai et al., 2013) and observed egg mass properties (O'Dor and Balch, 1985; O'Dor et al., 1982), in nature, the ommastrephid egg mass is retained in the pycnocline. It is possible that the pycnocline shifts closer to the sea bottom during certain seasons and the egg mass could sink to the bottom. The present experiment was designed to understand how the egg mass is retained in a thermally stratified water column and fate of the embryos within an egg mass if

it sinks and settles on the tank bottom. Distribution of egg masses was highly dependent on the presence or absence of a thermal gradient. The physical properties of egg masses are thought to control their distribution at various depths and the difference in density between the egg mass and the surrounding water depends primarily on the temperature of the water within the mass (O'Dor and Balch, 1985). Variation of changes in the egg mass density depends on the surrounding water temperature and is determined by a phenomenon known as 'thermal diffusivity'. Essentially, if the temperature of the surrounding water shifts, the average temperature in the mass will be 90% equilibrated within approximately 10 h (O'Dor and Balch, 1985).

In the current experiment, floating egg masses (spawned in the presence of a thermal gradient) maintain a spherical shape and outer protective nidamental coat, supporting the successful development of the embryo. However, the egg masses that settled at the bottom of the tank (spawned in the absence of thermal gradient) collapsed, and the outer nidamental gland coat was broken. This resulted in an infestation of crustaceans, protozoans and bacteria, and all the embryos either developed abnormally or died. Examination of the egg mass surface layer by Bower and Sakurai (1996) revealed that the outer nidamental-gland jelly was effective in preventing crustaceans, protozoans and bacteria present in the tank from infesting the egg masses. This highlights the importance of the thermal gradient in the water column, which keeps the egg mass suspended and maintains the spherical outer jelly layer (from nidamental gland secretion).

When the egg mass freely moves along the current, the embryo could be oriented in a head down position by a jetting motion since the chorion is in fixed position within the jelly fibrils (Kimura et al., 2004); the embryo inside the chorion moves and tends to stay in the vertical head down position. A previous study on *T. pacificus* (Bower and Sakurai, 1996) demonstrated that the embryo undergoes development in a vertical position with its head facing down. In this study, however, we observed, that when an embryo achieves mantle contraction on day 3–4, it moves in multiple directions before ultimately returning to a position with its head facing down. The head facing down position of the embryo within the egg mass was also observed in *D. gigas* (Staaf et al., 2008). Development of the embryo with downward head orientation might allow for the hop and sink swimming mechanism after hatching, as it already harbors the ability for a similar mantle contraction beforehand. This position might be an adaptive advantage to ommastrephids in their early life stages.

Paralarval recruitment

The paralarvae from egg masses obtained by Bower and Sakurai (1996) hatched at stage 28 (Fig. 8A). The advanced hatching stage (30/31) (Fig. 8B) observed in our study suggests that hatching time depends on the external conditions of the eggs. It is possible that presence of nidamental and oviducal gland jelly in the undisturbed egg mass could delay hatching. A longer developmental time within the egg mass also provides an enhanced opportunity for developing embryos to absorb organic nutrients from the oviducal gland jelly (Bower and Sakurai, 1996). Vertical swimming experiments conducted by Yoo et al. (2014) with *T. pacificus* revealed that the paralarvae consistently have a preference for surface layers. Field surveys also collected hatchling-sized paralarvae at the surface waters (Yamamoto et al., 2002). The internal yolk remaining at the hatching stage might be utilized during their ascent through the water column before they are able to feed on the food particles on surface water. The yolk is utilized within 3–4 days for somatic growth, development of the digestive system, and locomotion. In this context, the hop-and-sink behavior provides the necessary movement while conserving energy (Staaf et al., 2008). There was no significant increase in the ML of paralarvae at hatching or after 10 days, which is similar to what was observed for the paralarvae of *Illex coindetii* (Villanueva et al., 2011).

Watanabe et al. (1996) were able to describe paralarval development up to stage 34 (7 days old). In nature, the mortality of *T. pacificus* paralarvae is attributable to translocation and starvation (Okutani, 1983; Vidal et al., 2002). All paralarvae in this study died approximately 8–10 days after growth past stage 34. The 10-day-old paralarvae we obtained could be classified as stage 35 in accordance with the stage 34 of Watanabe et al. (1996) or 'paralarval stage II' according to Shigeno et al. (2001). Paralarvae survival might have been prolonged to 10 days by the ingestion of food particles in the natural seawater supplied in the tank. The initial food consumed by any ommastrephid paralarvae have not yet been confidently identified. Furthermore, feeding experiments attempted in ommastrephids, including *Illex illecebrosus* (Balch et al., 1985), *Ommastrephes bartramii* (Sakurai et al., 1995; Vijai, 2015), *Sthenoteuthis oualaniensis* (Sakurai et al., 1995) and *D. gigas* (Yatsu et al., 1999; Staaf et al., 2008), have not been successful. In this study, *T. pacificus* was maintained in a tank for 10 days, which was three additional days after the complete consumption of the inner yolk. Starvation resulting from a failure to feed successfully after absorption of the yolk reserves has been proposed to be a major source of pre-recruitment mortality in squid (Vecchione, 1991).

Little is known concerning the prey of cephalopod paralarvae in the wild due to the small size of this life history stage (Roura et al., 2012), therefore the identification of a suitable initial food source for paralarvae remains an area of much needed research (Villanueva et al., 2014). The use of the proboscis for suspension feeding has been suggested to be the initial mechanism of paralarval feeding (O'Dor and Balch, 1985; Vidal and Haimovici, 1998). Weak musculature in the stomach (smaller than 8.1 mm ML) suggests that paralarvae smaller than this size must consume easily digestible prey (Bower, 1997). In addition to stomach musculature, the rostra of both beaks of the closely related ommastrephid *O. bartramii* paralarvae, smaller than 3 mm ML (<13 days old) are embedded in membrane-like tissue (Uchikawa et al., 2009), also the rostral tip are yet to form in the 10 days old paralarvae (wild) of *T. pacificus* (Bower, 1997). This suggests that the newly hatched paralarvae might not use their beak for biting prey. The present study indicates that paralarvae might have fed on the dissolved organic matter (DOM) from natural seawater. In the field, the Kuroshio front, a plankton rich area, has been suggested to be an optimum zone for the initial feeding of paralarvae (Bower et al., 1999), where they could graze on marine snow and DOM. Water rich in DOM would support the paralarval transition from endogenous to exogenous food sources, which is an important factor leading to paralarval recruitment success. Some ommastrephid paralarvae (*Illex argentinus*) are known to ingest mucus containing micro flagellates, ciliates and bacteria (Vidal and Haimovici, 1998). The head withdrawing behavior is also assumed to be a mechanism for feeding on detritus (O'Dor et al., 1985). The underdeveloped digestive system, arms, and tentacles do not permit for any type of raptorial feeding by the paralarvae. It is possible that the paralarvae in this experiment ingested DOM in the tank to survive after completely consuming the inner yolk. We possibly were not able to identify any stomach content on post-mortem examination due to a rapid digestion rate and resulting short residence time of nutrients, typical in cephalopods (Andrews and Tansey, 1983; Nixon, 1985).

Future studies utilizing molecular methods to examine stomach contents (e.g. Roura et al., 2012) may prove more effective in identifying the initial prey of paralarvae.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
P.P. conceived and designed the study, collected and analyzed the data, and drafted and revised the article. D.V. and Y.S. helped conceive and design the study, offered guidance on data collection and analysis and made substantial improvements to the article. H.-K.Y. and H.M. also helped with design, data collection and analysis.

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