

Natural egg mass deposition by the Humboldt squid (*Dosidicus gigas*) in the Gulf of California and characteristics of hatchlings and paralarvae

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*The jumbo or Humboldt squid, *Dosidicus gigas*, is an important fisheries resource and a significant participant in regional ecologies as both predator and prey. It is the largest species in the oceanic squid family Ommastrephidae and has the largest known potential fecundity of any cephalopod, yet little is understood about its reproductive biology. We report the first discovery of a naturally deposited egg mass of *Dosidicus gigas*, as well as the first spawning of eggs in captivity. The egg mass was found in warm water (25–27°C) at a depth of 16 m and was far larger than the egg masses of any squid species previously reported. Eggs were embedded in a watery, gelatinous matrix and were individually surrounded by a unique envelope external to the chorion. This envelope was present in both wild and captive-spawned egg masses, but it was not present in artificially fertilized eggs. The wild egg mass appeared to be resistant to microbial infection, unlike the incomplete and damaged egg masses spawned in captivity, suggesting that the intact egg mass protects the eggs within. Chorion expansion was also more extensive in the wild egg mass. Hatchling behaviours included proboscis extension, chromatophore activity, and a range of swimming speeds that may allow them to exercise some control over their distribution in the wild.*

Keywords: Humboldt squid, jumbo squid, *Dosidicus gigas*, ommastrephid, egg mass, rhynchoteuthion paralarvae

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INTRODUCTION

Squids are broadly divided into the primarily nearshore Myopsida and mostly oceanic Oegopsida. Myopsids are more tractable study organisms and consequently provide most of our knowledge of squid biology, including reproduction and development (Gilbert *et al.*, 1990). Much less is known about the oegopsids. 'Our almost complete ignorance of the eggs and early larval stages of oegopsid squids is the most extraordinary omission in our knowledge of cephalopod biology.' This assertion was made over 40 years ago (Clarke, 1966), and information concerning the spawning and egg masses of these oceanic cephalopods remains sparse (see reviews: Young *et al.*, 1985a; Boletzky, 2003). The few oegopsid egg masses that have been observed are larger than those of myopsids and show greater diversity of form. Some are gelatinous, free-floating, and contain many small (1.2–1.8 mm)

eggs, such as those of *Thysanoteuthis rhombus* Troschel, 1857 (Sabirov *et al.*, 1987) and *Brachioteuthis* sp. Verrill, 1881 (Young *et al.*, 1985b). In other cases, as in *Gonatus onyx* Young, 1972, a few larger (2–3 mm) eggs are brooded by the female (Seibel *et al.*, 2000). This diversity is not surprising, given that oegopsids inhabit all open ocean environments throughout the world, from the surface to great depths.

Of the 23 oegopsid families, the family Ommastrephidae is unique in passing through a post-hatching paralarval stage called the rhynchoteuthion. During this stage, the two tentacles are fused into a proboscis, the use and development of which is not well understood (Shea, 2005). Rhynchoteuthion paralarvae have been produced by artificial fertilization (Arnold & O'Dor, 1990; Sakurai *et al.*, 1995), captured in plankton tows (e.g. Brunetti, 1990), and collected from spawned egg masses. Spawning in captivity has been documented only for *Todarodes pacificus* Steenstrup, 1880 (Bower & Sakurai, 1996) and *Illex illecebrosus* Lesueur, 1821 (O'Dor & Balch, 1985). Wild egg masses have been discovered only for *Sthenoteuthis pteropus* Steenstrup, 1855 (Laptikhovskiy & Murzov, 1990) and *Nototodarus gouldi* McCoy, 1888 (O'Shea *et al.*, 2004).

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Though our knowledge of ommastrephid reproduction and development is limited, ommastrephids are considered monocylic, with adults reaching sexual maturity and reproducing during a single continuous spawning season. This reproductively active time may be a single session in which all of a female's eggs ripen and are spawned during a brief period (simultaneous terminal spawning; Rocha *et al.*, 2001), as occurs with *Doryteuthis* (= *Loligo*) *opalescens* Berry, 1911. Or there may be a more prolonged spawning season, during which eggs ripen and are spawned in sequential batches. In some species that pursue this strategy, mature adults stop eating and divert all their energy into reproduction (intermittent terminal spawning; Rocha *et al.*, 2001), while individuals of other species continue to feed and grow during their reproductively active time (multiple spawning; Rocha *et al.*, 2001).

Nigmatullin & Laptikhovskiy (1994) divided the ommastrephid family into two groups in which reproductive and ecological strategies align. The 'offshore' ommastrephids, such as *Todarodes pacificus* (Ikeda *et al.*, 1993a) and *Illex* spp. (Laptikhovskiy & Nigmatullin, 1993), are intermittent terminal spawners, while the 'oceanic' ommastrephids, such as *Sthenoteuthis oualaniensis* Lesson, 1830 (Harman *et al.*, 1989) and *Dosidicus gigas* d'Orbigny, 1835 (Nigmatullin *et al.*, 2001), are multiple spawners.

Dosidicus gigas attains the greatest size of any ommastrephid and supports the largest invertebrate fishery in the world, which is also the 14th largest of all fisheries, with record landings of 768849 t in 2005 (<ftp://ftp.fao.org/fi/stat/summary/a1e.pdf>). This species also plays a significant and variable role in regional ecologies. In the Gulf of California, adult jumbo squid consume an enormous quantity of small mesopelagic fish, crustaceans and squids (Markaida & Sosa-Nishizaki, 2003), while serving as a prominent prey item for larger fish and marine mammals (Klimley *et al.*, 1993; Abitía-Cárdenas *et al.*, 2002; Rosas-Aloya *et al.*, 2002; Ruiz-Cooley *et al.*, 2004). In the California Current of central California, on the other hand, they feed at a higher trophic level, preying heavily on a variety of larger neritic fish (Field *et al.*, 2007).

The historical range of *D. gigas* is in the tropical and subtropical eastern Pacific from 30°N to 25°S, with episodic appearances as far as 40°N and 47°S (Nigmatullin *et al.*, 2001). This area remains the centre of the species' range, which has recently expanded northward. *Dosidicus gigas* has been resident in the Monterey Bay (37°N) since the 1997–1998 El Niño (Zeidberg & Robison, 2007) and individuals have been reported from as far north as south-east Alaska (59°N) (Cosgrove, 2005; Wing, 2006; unpublished data). Within its range *D. gigas* shows considerable phenotypic plasticity in size at first maturity, with DML ranging from 14–100 cm (Nigmatullin *et al.*, 1999). Jumbo squid that mature at the larger end of this size-range have the largest potential fecundity of any cephalopod, ranging from 5 to 32 million oocytes in a single female (Nigmatullin & Markaida, 2002; Nigmatullin *et al.*, 1999).

Laboratory studies have employed artificial fertilization of *D. gigas* eggs to reveal embryonic development *in vitro* (Yatsu *et al.*, 1999), but natural spawning and early development are poorly understood. Based on an abundance of rhyndoteuthion paralarvae, the Costa Rica Dome is thought to be a major spawning area for either *D. gigas* and/or *Sthenoteuthis oualaniensis* (Vecchione, 1999). Spawning of *D. gigas* has also been identified in the central Gulf of

California, based on the presence of newly hatched paralarvae (genetically identified) and putative mating by adults (Gilly *et al.*, 2006a). In no case have naturally deposited eggs been observed.

To our knowledge, this paper constitutes the first report of naturally deposited eggs of *D. gigas* and of the first spawning in captivity by this species. We present observations of the embryos and hatchlings from these egg masses, and compare them with embryos and hatchlings produced by artificial fertilization.

MATERIALS AND METHODS

Field observations presented in this paper were made on the RV 'New Horizon' in the Gulf of California, from 8–22 June 2006, and from 29 May to 12 June 2007.

Blue water diving

Blue-water dives were carried out daily, as conditions permitted, according to the procedures in Haddock & Heine (2005). Thirty dives were conducted during the two research cruises. On 21 June 2006, a dive was conducted at 27°7.1'N 111°16'W, where the sea floor was at a depth of approximately 1800 m (Figure 1). At a depth of 16 m, the divers encountered an egg mass and took video *in situ*. Divers sampled the mass by filling inverted jars with exhaled air and extending them well into the mass. When a jar was turned upright to allow the air to escape, it became filled with a sample that presumably reflected ambient egg concentration in the mass. In this manner, one 1 l collecting vessel (Jar 1) and two 500 ml jars (Jars 2 and 3) were filled with egg mass and returned to the ship. Forty ml of egg mass from each jar were poured into individual 50 ml clear plastic vials, and the eggs in each sample were then counted to assess egg density.

Two vertical water column profiles were measured in close proximity to the site of the egg mass discovery, one on 19 June 2006 at 27°6.06'N 111°14.37'W, and another on 20 June 2006 at 27°5.63'N 111°11.24'W (Figure 1). The instrument used to measure these profiles was a Sea-Bird 911*plus* CTD with sensors for pressure (401K-105), temperature (SBE3*plus*), conductivity (SBE4C) and dissolved oxygen (SBE43).

Capture and maintenance of adult squid

Squid were caught nightly with weighted jigs, usually between 2000 h and 2200 h. Individual live squid were held on the ship in either a 514 l acrylic tank or a 280 l fibreglass tank. The fibreglass tank was oxygenated with constantly running surface seawater (28°C). In 2006, water in this tank was also partially circulated through a chiller to maintain temperature at 23°C. The cooler temperature seemed to have no effect on the squid, so the chiller was not used in 2007. The acrylic tank was filled with surface seawater and aerated with a bubbler. An oxygen probe was used to ensure that oxygen saturation was comparable to ambient levels. Over the course of 5–10 h, this tank was chilled to either 10 or 20°C for other research purposes.

Both tanks were kept dark and covered, with minimal disturbance by observers. Individual squid were maintained on board the ship for 24 h or less. If squid inked, the tank was flushed as quickly as possible with surface seawater until the water was clear. Spawned egg masses were removed from

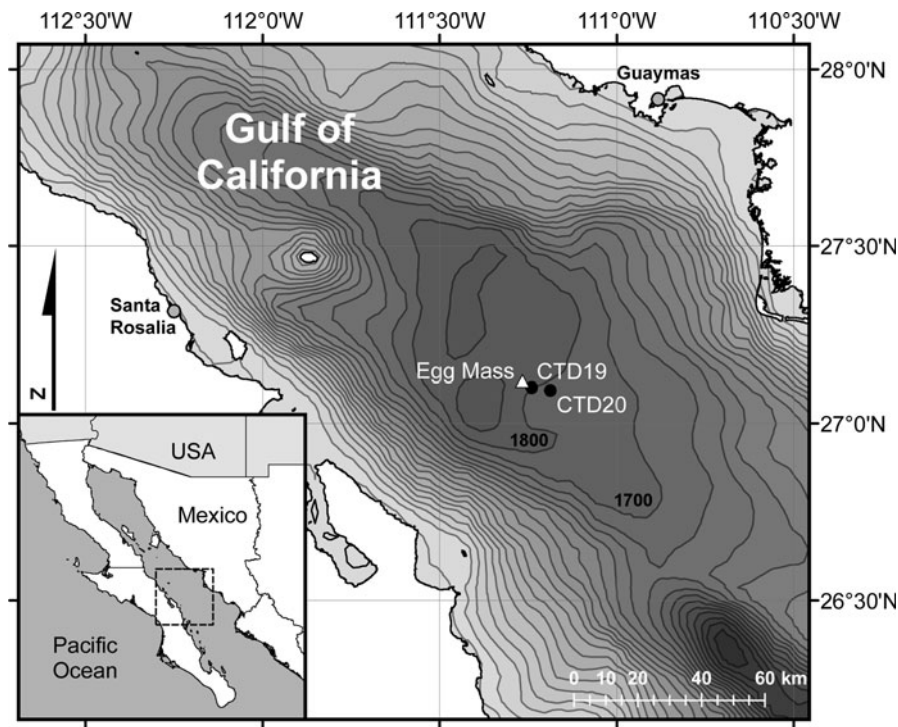


Fig. 1. Location of CTD casts and egg mass in the Guaymas Basin. Inset locates the region in the Gulf of California. Depth contours are every 100 m; 1700 m and 1800 m contours are labelled. Bathymetry data from US Department of Commerce, National Oceanic and Atmospheric Administration, National Geophysical Data Center, 2006. *2-minute Gridded Global Relief Data (ETOPO2v2)* <http://www.ngdc.noaa.gov/mgg/fliers/o6mggo1.html>

the tanks upon discovery, which was always in the early to mid-morning. Moribund post-spawning females were removed from tanks and euthanized by rapid decapitation (Boyle, 1991; Moltchanivskyj *et al.*, 2007). They were then weighed, measured and dissected for examination of the reproductive organs. Maturity stage was assigned following the procedure of Lipiński & Underhill (1995).

Artificial fertilization

Arnold & O'Dor (1990) obtained the first success from artificial fertilization to hatching of an ommastrephid with *Sthenoteuthis oualaniensis*. The technique was refined and modified by Sakurai & Ikeda (1994) for *Todarodes pacificus*, and later applied to *Sthenoteuthis oualaniensis* and *Ommastrephes bartramii* by Sakurai *et al.* (1995) with some further modifications. Yatsu *et al.* (1999) successfully applied the technique to *Dosidicus gigas*.

The protocol used in the current study was based on techniques in Sakurai *et al.* (1995) and Yatsu *et al.* (1999) with two modifications. First, lyophilized oviducal gland material was isolated from *Dosidicus gigas* rather than *Ommastrephes bartramii*. Second, antibiotics were used. After filtering seawater with a 0.2 µm filter, 25 mg/l each of ampicillin and streptomycin was added, resulting in noticeable improvement in the survival of embryos cultured in this treated water.

Maintenance of eggs and paralarvae

Developing eggs from artificial fertilization, captive-spawned egg masses, and the wild egg mass were all kept in filtered, antibiotic-treated seawater at room temperature (18–20°C). This temperature range was between the previously reported

culture temperature of 18°C (Yatsu *et al.*, 1999) and the temperature at which the natural egg mass was found (25–27°C; see Figure 2). Water was changed daily and the developing embryos were photographed periodically. Artificially fertilized eggs and portions of the captive-spawned masses were kept in sterile Petri dishes, while the wild embryos were kept in the jars in which they had been collected.

Approximately 24 h after collection, eight hatchlings from the wild egg mass were moved from Jar 1 into a 650 ml flask. All of the remaining hatchlings in Jar 1 were preserved in ethanol for DNA analysis. Both living and preserved samples were transported back to CIBNOR, Guaymas, and ultimately to Hopkins Marine Station, where the live paralarvae were maintained in a rectangular 1 l aquarium at room temperature (~20°C). Attempts were made to feed these hatchlings with rotifers, brine shrimp nauplii, and various algae that were cultured at the Monterey Bay Aquarium, as well as wild zooplankton from surface net tows in Monterey Bay. Standard measurements (dorsal mantle length, mantle width, head width and proboscis length) were made from video clips (see next section) of paralarvae on 3 and 6 days post-hatching. Jar 2 was taken to the CIBNOR laboratory in Guaymas, and the hatchlings were all counted and preserved in 80% ethanol approximately 2 days after hatching.

Individual embryos and paralarvae fixed in ethanol and formalin for analyses reported in this paper were provided by C. Salinas, CIBNOR, Guaymas, to be archived at Hopkins Marine Station.

Video observations

Videos were taken of the hatchlings from the wild egg mass, both in the 1 l aquarium and in a smaller, thin acrylic

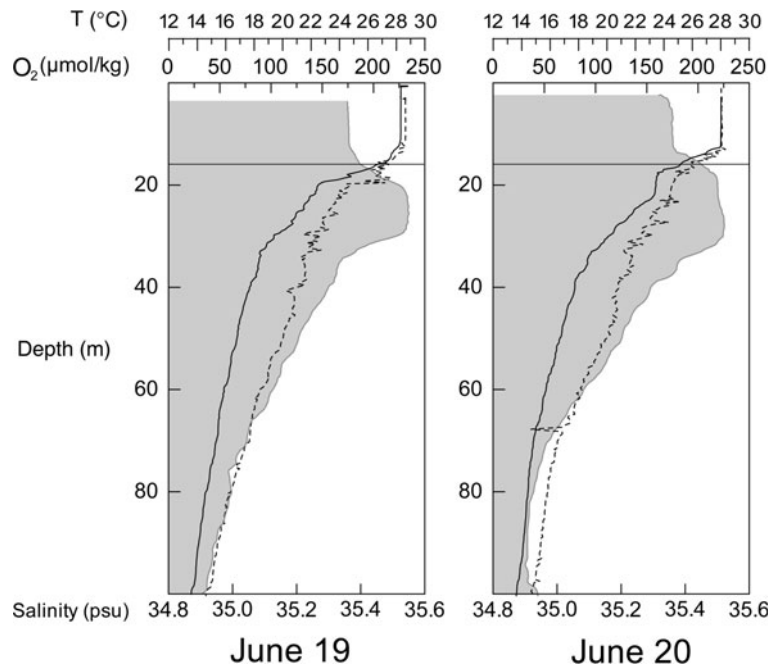


Fig. 2. Oceanographic features revealed by CTD casts (salinity, dotted line; temperature, solid line; oxygen, filled grey) near the site where the egg mass was found. A line is drawn across both casts at 16 m, the depth of the egg mass discovery.

chamber ($72 \times 47 \times 7$ mm), with a Sony DCR-TRV70 mini-DV camcorder. Selected sequences that covered a range of swimming movements were captured with Adobe Premier 5.0 (Adobe Systems Incorporated) and exported as frames into ImageJ (NIH, <http://rsb.info.nih.gov/ij/>) to analyse the dynamics of mantle contraction and speed of swimming. Rate of mantle contraction was calculated as the number of mantle contractions per second. Magnitude of mantle contraction was calculated as a fraction, by dividing the ratio of mantle width (MW) to dorsal mantle length (DML) (MW:DML) in each frame by the maximum MW:DML measured in that clip. Sequences that contained proboscis extensions were also captured and exported as frames. Proboscis extension was measured as the ratio of proboscis length to mantle length (PL:DML) in a given frame divided by the resting PL:DML in that sequence. Swimming speed was measured as the absolute distance travelled per second based on a scale included in the video field and a 29.97 Hz frame rate.

Chromatophore activity also occurred frequently. A representative 10 minute video segment was selected for analysis of the duration and degree of chromatophore expansion and of responsiveness to stimuli.

RESULTS

Wild egg mass

CTD casts near the site of the egg mass discovery show that temperature, oxygen and salinity all changed rapidly at or near the 16 m depth at which the egg mass was discovered (Figure 2). Temperature and salinity began to drop, while oxygen began to rise to a subsurface peak at 20–30 m before dropping off again. Temperature at 16 m was 25°C in the 19 June cast and 27°C in the 20 June cast.

The egg mass resembled a semi-transparent grey cloud (Figure 3). Its shape was an ellipsoid, with the broad axis parallel to the sea surface. Divers' estimates of the dimensions ranged from 2–3 m for the minor equatorial diameter, from 3–4 m for the major equatorial diameter and from 2–3 m for the polar diameter. The consistency of the mass was loosely gelatinous but not firm enough to offer tangible resistance to an ungloved hand. Embedded within this gelatinous matrix were individual eggs, each containing a single developing embryo, distributed diffusely enough that divers were clearly visible through the entire thickness of the mass. The distribution of eggs appeared homogeneous.

The only variability in the homogeneous nature of the mass was in a small area near the periphery, where the gelatinous matrix formed slightly denser, whitish strands (bottom of Figure 3). The divers observed two dead fish attached to this area and tentatively identified them as myctophids (lantern-fish). One of these fish was relatively intact, while the other appeared to be partially decayed.

Egg densities in the 40 ml samples were 0.427 (Jar 1), 0.600 (Jar 2) and 0.650 (Jar 3) eggs/ml. In addition to these sample counts, the total number of eggs in Jar 2 yielded a density of 0.192 eggs/ml. Assuming an ellipsoid shape for the egg mass and using the most conservative estimate of its dimensions, its volume was calculated to be 3.13 m³, or 3130 l. With an egg density of 192 to 650 eggs/l, the potential number of eggs in the entire mass ranges from 0.6 to 2 million.

Over 99% of the collected eggs were fertilized, and all of these were classified as developmental stage 25 (Watanabe *et al.*, 1996) based on the presence of distinct chromatophores, lens primordia, and a gutter (apical groove) in the inner yolk sac. In *Todarodes pacificus*, hatching from artificially fertilized eggs occurs at stage 26, but may be induced in stage 25 by external stimulation (Watanabe *et al.*, 1996).

Each egg was surrounded by a discrete spherical envelope, slightly larger than the chorion (Figure 4A). The chorion was

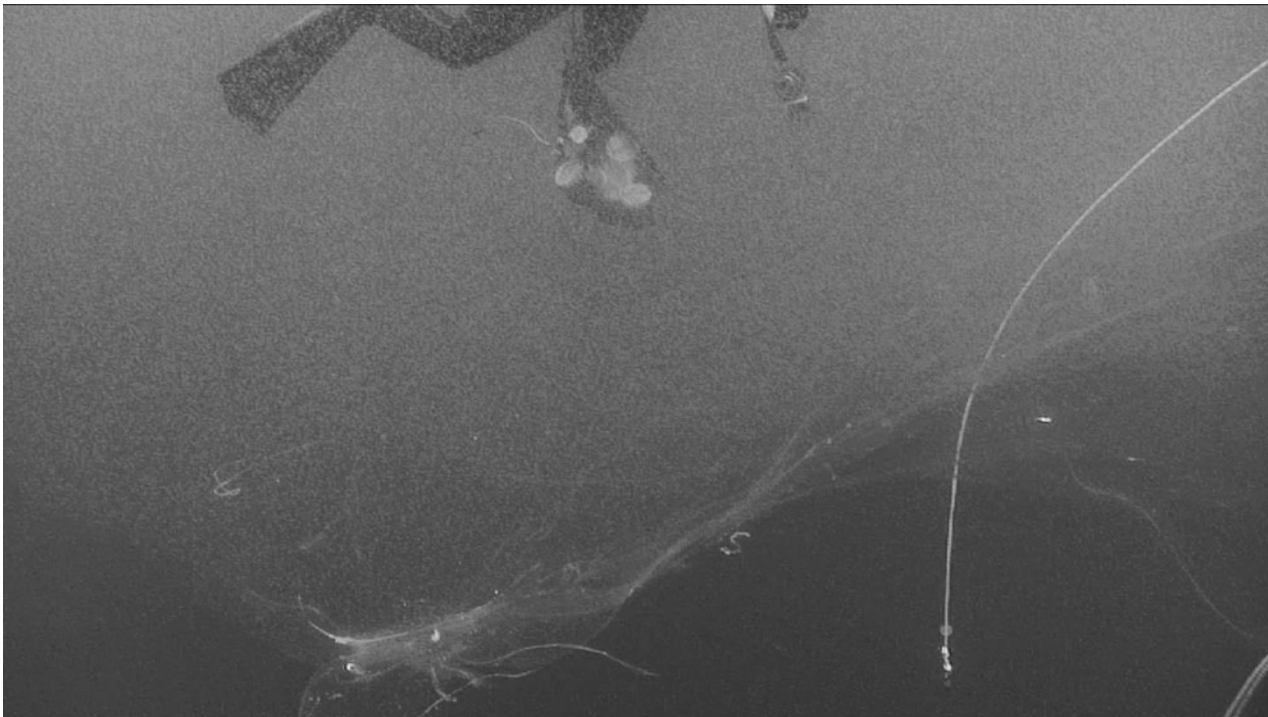


Fig. 3. Part of the naturally deposited egg mass with a portion of a diver and the blue-water safety-line visible. The diver is behind the semi-transparent egg mass. This was a frame grab from video taken at a 29.97 Hz frame rate.

expanded to more than twice the length of the embryo within (Table 1). The embryos were always observed in a vertical position, with the head and arms hanging down, as reported for *Todarodes pacificus* (Bower & Sakurai, 1996), and they remained stationary in this position when the eggs were suspended in a jar. However, when eggs were placed with a small amount of jelly in a culture dish for photography, the embryos swam in circles in the horizontal plane around the inside of their eggs.

No developmental abnormalities were visible. Paralarvae began hatching in the evening of the day the egg mass was discovered and continued hatching throughout the night. Analysis of cytochrome *c* oxidase I sequences from embryos taken back to Hopkins Marine Station confirmed their identity as *Dosidicus gigas* (Gilly *et al.*, 2006a, GenBank Accession No. DQ191367).

Egg masses spawned in captivity

Four squid spawned egg masses on the 2007 cruise, all within 12 h of capture. The first two egg masses were discovered on the morning of 30 May 2007—one in the chilled acrylic tank at 0500 h (DML: 43 cm, mass: 3.1 kg) and one in the fibreglass tank at 0730 h (not measured). Both of these masses contained fertilized eggs. The third and fourth egg masses were discovered at 0930 h on 31 May 2007 (DML: 42 cm, mass: 2.6 kg), and at 0500 h on 3 June 2007 (DML: 35.5 cm, mass: 1.76 kg), both in the chilled acrylic tank. Eggs in these two masses were not fertilized. One squid (DML: 57 cm, mass: 5.1 kg) spawned an egg mass containing fertilized eggs on the 2006 cruise. It was discovered around 0100 h on 15 June 2006. All squid survived for some hours after spawning events, but to our knowledge none spawned more than once in captivity.

Four of the five moribund females were sacrificed and dissected after they deposited eggs. The oviducts were extended and flaccid, but empty of eggs, and the ovary appeared granular with yellowish oocytes. The nidamental and oviducal glands were large, white, and well developed. Had these squid not been known to spawn in captivity, they would have been classified as stage IV in the scheme of Lipiński & Underhill (1995). Stage IV is defined as a maturing female that does not yet have ripe eggs in the oviducts, whereas stage V females are fully mature, with ripe eggs in the oviducts.

The spawned egg masses were transparent and gelatinous, with no apparent internal structure. Their consistency was similar to that of the wild egg mass but denser and less watery. They were fragmented, and while some parts of each mass contained eggs, other substantial areas were devoid of eggs. In addition, many more eggs were generally found loose on the bottom of the tank, perhaps having been separated from the mass by aeration, or perhaps never incorporated into it. This indicates that the eggs themselves, when independent of the gelatinous mass, are negatively buoyant. Although the fragmented nature of the masses prevented them from being completely extracted from the tanks, the volume that could be successfully retrieved was in the range of 1–2 l for each mass.

Chorion expansion in the captive-spawned eggs was slightly greater than that of artificially fertilized eggs but considerably less than that of the wild eggs when measured at a comparable stage of development (Table 1). Some, but not all, of the captive-spawned eggs appeared to be surrounded by a spherical outer envelope similar to that described above for wild-spawned eggs. This envelope was often present even around unfertilized eggs (Figure 4B). Many of the developing embryos suffered mortality, apparently due to bacterial

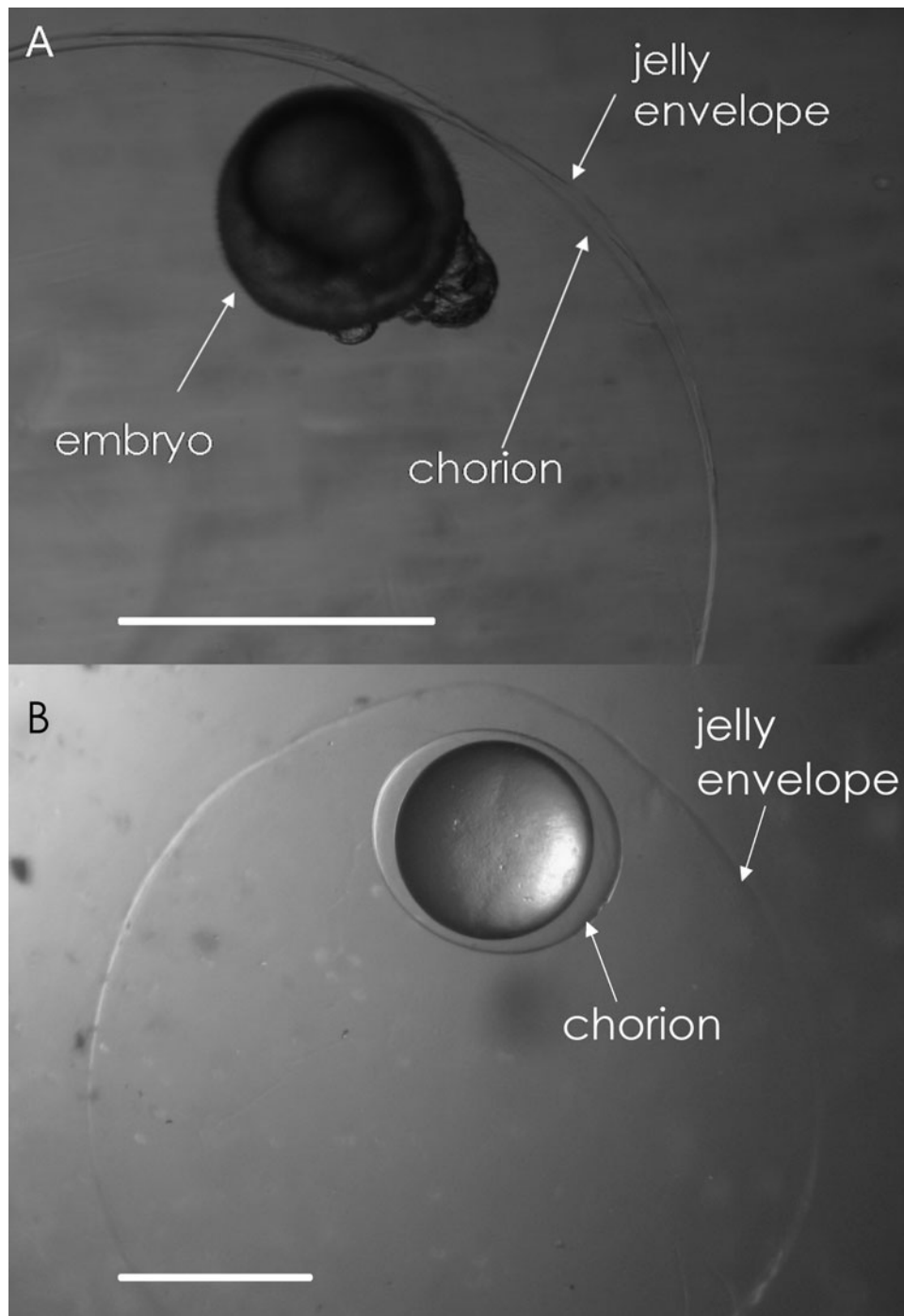


Fig. 4. (A) Developing embryo (Stage 25) from the wild egg mass and (B) an unfertilized egg deposited in captivity. The unusual envelope surrounding the chorion is similarly sized, although the chorion of the wild egg is much more expanded. Scale bars are approximately 1 mm.

infection, and many showed developmental abnormalities. Viable hatchlings began to emerge from portions of the successfully fertilized egg masses 3 days after spawning while being maintained at room temperature. None of the loose eggs hatched successfully.

Artificial fertilization

Artificially fertilized eggs suffered a great deal of microbial contamination which was only somewhat ameliorated by treatment with antibiotics. Chorion expansion occurred to

a lesser extent in these eggs than in either the captive-spawned or the wild eggs, and changed relatively little over the course of development (Table 1). No outer envelope like that in the egg masses was ever seen. Some successful hatchlings were obtained from these eggs.

Growth and development

Hatchlings from artificially fertilized eggs, captive-spawned masses, and the wild egg mass were all morphologically similar to *D. gigas* hatchlings previously described by Yatsu

Table 1. Ratio of chorion diameter (CD) to embryo total length (TL) for eggs from artificial fertilization, captive-spawned egg masses and naturally deposited wild egg mass at different developmental stages (stage after Watanabe *et al.*, 1996). Values of mean \pm standard deviation are indicated. N indicates number of embryos measured.

Embryo source	Stage	N	CD:TL
Artificial fertilization 2006	21–23	4	1.06 \pm 0.03
Artificial fertilization 2007	1	5	1.05 \pm 0.02
	5	5	1.15 \pm 0.05
	10	5	1.08 \pm 0.06
	15	5	1.16 \pm 0.04
	20	5	1.17 \pm 0.11
	25	5	1.08 \pm 0.09
Captive-spawned 2006	21–23	4	1.20 \pm 0.18
Captive-spawned 2007	19–20	4	1.25 \pm 0.12
Wild egg mass	25	10	2.08 \pm 0.13

et al. (1999), which were in turn quite similar to other ommastrephid hatchlings (e.g. Sakurai *et al.*, 1995).

During the first 3 days post-hatching, the wild paralarvae increased markedly in mantle length, mantle width, and head width, and the proboscis appeared and grew considerably in length (Table 2). However, after 3 days, the paralarvae remained constant in most dimensions, shrinking somewhat in mantle length. The initial growth was fuelled by yolk consumption, and the subsequent lack of growth was likely due to starvation. While maintained in the laboratory, the paralarvae exhausted their yolk reserves and, despite offerings of zooplankton and phytoplankton, at no time did they exhibit obvious feeding behaviours. Ingested material was not found in their guts upon post-mortem examination.

Swimming kinematics (Jar 1)

After hatching from their eggs, the wild paralarvae swam easily within the large egg mass, maintaining their position by jetting up, then sinking down. Eventually they escaped from the mass and began to move actively within the water column, where they sank quickly (-0.46 ± 0.02 cm/s, $N = 3$) upon cessation of swimming activity. Some jetted powerfully towards the surface and maintained height, but most sank to the bottom, where they remained oriented with the posterior mantle pointing up, jetting frequently but gaining no altitude.

Swimming direction was always vertical or nearly so. Swimming speed in 3–6 day old paralarvae varied from simply maintaining position in the water column (a net velocity of 0 cm/s, generated by the animal's swimming velocity counteracting its sinking velocity) to 0.51 cm/s, the fastest net upward swimming speed maintained over several seconds. Rates of mantle contraction ranged from 2.2–4.2 Hz, and the magnitude of mantle contraction varied from 28.17%–39.54%.

Table 2. Wild egg mass paralarval dimensions (in mm) through development. Mean \pm standard deviation. Age is given in days post-hatching. N indicates number of paralarvae measured.

Age	N	DML	Mantle width	Proboscis length	Head width
0	10	1.02 \pm 0.08	0.80 \pm 0.04	0.0	0.60 \pm 0.05
3	4	1.55 \pm 0.17	1.29 \pm 0.13	0.55 \pm 0.06	0.79 \pm 0.06
6	4	1.36 \pm 0.08	1.27 \pm 0.05	0.55 \pm 0.08	0.75 \pm 0.09

Other behavioural observations

A commonly observed behaviour in 3–6 day old paralarvae was extension of the proboscis (Figure 5). The proboscis stretched to $252 \pm 38\%$ of its original length in 375 ± 77 ms ($N = 12$). Retraction to the original length was considerably slower and more variable. Although it is tempting to interpret proboscis extensions in the context of feeding attempts, their occurrence appeared to be spontaneous and independent of the presence of potential food items in the water.

Chromatophore activity, on the other hand, was often stimulated by external disturbance. Of the 27 instances of chromatophore activity observed during a 10 minute video sample, 5 occurred when a paralarva bumped into the surface of the water, 8 occurred when it touched the bottom of the aquarium, and the remainder appeared to occur spontaneously during normal swimming. Chromatophore activity generally involved all of the chromatophores more or less at once. They expanded, remained that way for a variable length of time, usually less than one second, and then contracted again (Figure 6). Occasionally, the chromatophores expanded and contracted sequentially at this rate for two or three cycles.

A less common response to disturbance was a dramatic cessation of normal vertical swimming and commencement of jetting in a tight circle, one to several times, before resuming the usual jetting up and sinking down. Even more rarely, paralarvae were observed retracting their heads into the mantle cavity. This only occurred when paralarvae were resting on the bottom, never while swimming in the water column.

DISCUSSION

Considerations of spawning and reproductive behaviour

Previously reported diameters of ommastrephid egg masses ranged from 0.4–1.0 m in *Illex illecebrosus* (Durward *et al.*, 1980; Balch *et al.*, 1985) and *Todarodes pacificus* (Bower & Sakurai, 1996) to 1.0–2.0 m for *Nototodarous gouldi* (O'Shea *et al.*, 2004). The major diameter of the ellipsoid *D. gigas* egg mass was estimated to be 3–4 m, making it by far the largest of any squid yet reported.

Our estimate of the number of eggs in the natural egg mass ranges from 0.6 million to 2 million, which is consistent with estimates of up to 1.2 million eggs from full oviducts of large-sized females (Nigmatullin *et al.*, 1999; Nigmatullin & Markaida, 2002). This is nearly an order of magnitude more eggs than have been found in any ommastrephid (or any squid) egg mass to date (*Illex illecebrosus*: 100,000 eggs (Durward *et al.*, 1980) and *Todarodes pacificus*: 21,000–200,000 eggs (Bower & Sakurai, 1996)). As a multiple spawner (Rocha *et al.*, 2001), with a potential fecundity ranging from 5 to 32 million oocytes (Nigmatullin & Markaida, 2002), the reproductive output of a single *D. gigas* female could be between 3 and 20 egg masses, each of a size comparable to the one discussed here.

The ecological consequences of this enormous quantity of offspring are noteworthy. Presumably the tiny hatchlings suffer high mortality early in life, and they are undoubtedly an important food source for a variety of small predators,

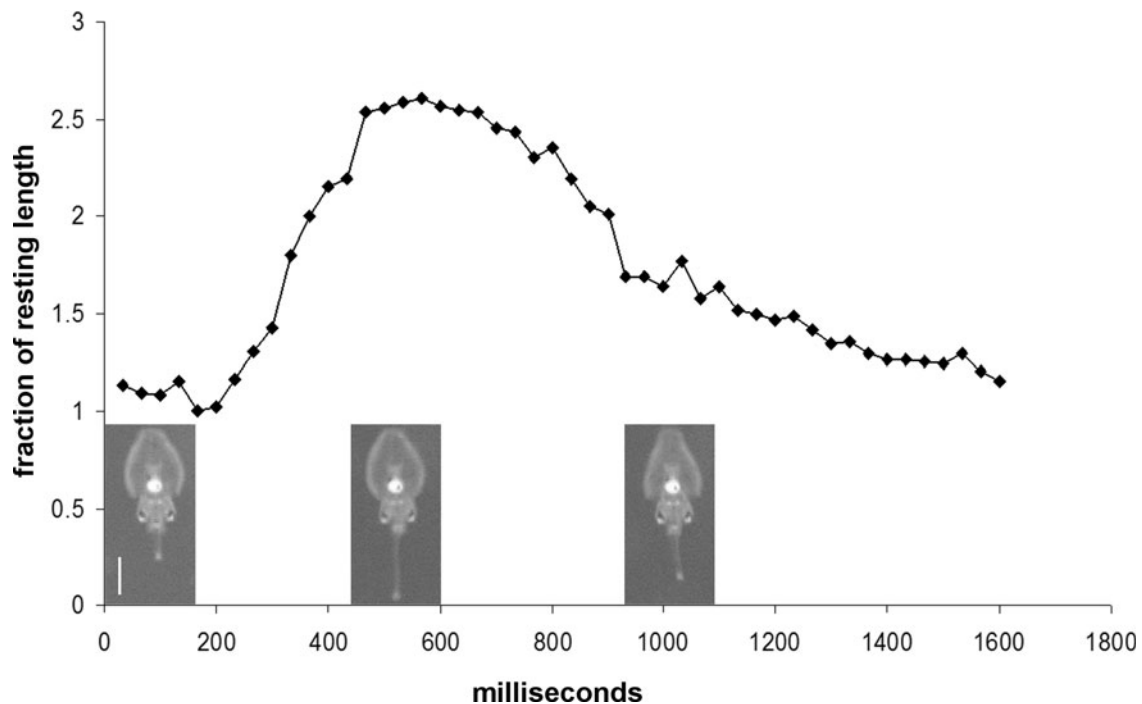


Fig. 5. Representative proboscis extension derived from a video clip (29.97 Hz frame rate). Inset frames are before extension, at the peak of extension, and during retraction. Scale bar in first frame is approximately 1 mm.

just as adults provide crucial sustenance for oceanic megafauna. Furthermore, as a short-lived, highly fecund species, *D. gigas* can be extremely responsive to environmental variation (Gilly & Markaida, 2007). When growth conditions are good and predation is low, *D. gigas* has the potential for explosive population growth. But when conditions are poor, affected populations may crash. This susceptibility is attested to by the highly variable fishery in the Gulf of California in relation to El Niño events (Bazzino *et al.*, 2007).

Given the high population density and abundance of adult *D. gigas* (Nigmatullin *et al.*, 2001), it is surprising that only one egg mass of this species has ever been reported. As a comparison, the most frequently encountered oceanic squid egg masses belong to a non-ommatrephid oegopsid, *Thysanoteuthis rhombus* (Young *et al.*, 1985a). Over 30 of this species' egg masses have been reported over the last 20 years (Sabirov *et al.*, 1987; Guerra & Rocha, 1997; Watanabe *et al.*, 1998; Guerra *et al.*, 2002; Miyahara *et al.*, 2006), although the adults are only sparsely distributed throughout their range (Nigmatullin & Arkhipkin, 1998). What could account for the difference? The cylindrical egg masses of *T. rhombus* can reach 1.8 m in length, but diameters do not exceed 0.3 m and they

contain only 32,000–76,000 eggs (Nigmatullin *et al.*, 1995), making them considerably smaller than the egg mass of *D. gigas*. Development to hatching occurs in 5–7 days (Sabirov *et al.*, 1987), only two days longer than *D. gigas*. As the egg masses of *T. rhombus* are neither larger nor longer-lived, what could make them more easily discovered?

Thysanoteuthis rhombus eggs are found in superficial surface waters (Nigmatullin & Arkhipkin, 1998) and apparently float at the surface until hatching (Miyahara *et al.*, 2006). Such floating egg masses should be visible from a ship, which is in fact how the discoveries have generally been made (Guerra *et al.*, 2002). The few reported ommastrephid egg masses have all occurred at depths too great to permit discovery through surface observations (Laptikhovskiy & Murzov, 1990; O'Shea *et al.*, 2004; this study). Given the difficulty of systematic exploration of a three-dimensional volume rather than a two-dimensional sea surface, and the limited visibility in water compared with air, it is likely that ommastrephid egg masses, though large, will remain rare finds.

Egg masses for experimental work may be more reliably obtained in captivity. In two consecutive years' field work during May/June, we found only one natural egg mass from

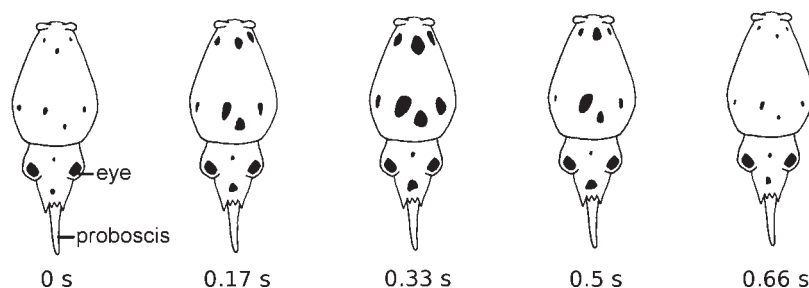


Fig. 6. Drawings from video frames (29.97 Hz frame rate) illustrating roughly synchronous activity of chromatophores on the dorsal mantle surface which occurred when the paralarva touched the water surface.

30 blue-water dives, but five females spawned in captivity (of about twice that many maintained overnight). The small size of these captive-spawned masses (1–2 l) relative to the wild egg mass (3130 l) suggests they may have been ‘incomplete’ egg masses (Bower & Sakurai, 1996).

Although these captive spawning events were unnatural and probably stress-induced, they impart valuable information. Our morphological study of the post-spawning females raises an important question concerning identification of post-spawning animals taken from the wild. As no ripe eggs were visible in the ovary or oviducts, these post-spawning females could easily have been classified as ‘maturing’ stage IV, even though they had already spawned at least once (in captivity). Accordingly, it may be necessary to reconsider the meaning of the established maturity stages (Lipiński & Underhill, 1995) within the context of intermittent spawning. It has been previously suggested that 20% of eggs remain in the oviducts after spawning (Nigmatullin & Markaida, 2002), but our observations of empty oviducts indicate that all eggs may be evacuated in a single spawning event, at least in captivity. If a similar phenomenon were to occur in the wild, an adult female might repeatedly pass through the final maturity stages (IV and V) during the reproductively active part of her life (IV–V-spawning–IV–V-spawning, etc.). The present scheme of maturity stages does not recognize this possibility, since functional maturity and active spawning are assigned to stage V alone. This could lead to underestimating the fraction of actively spawning females in a sampled population.

Nature of the egg mass

Eggs within the wild egg mass as well as those expelled in captivity were surrounded by a diffuse, gelatinous matrix that was not present when eggs were artificially fertilized. There was no indication of microbial infection of the wild egg mass, or of the eggs sampled from it, but infection was clearly a problem with captive spawning and artificial fertilization. Although the holding tanks onboard the ship undoubtedly supported a different microbial community than that in the open ocean, it is likely that susceptibility to infection increases greatly if the egg mass is incomplete or damaged (captive-spawned eggs) or absent (artificially fertilized eggs). An intact matrix around the eggs probably serves to prevent microbial infection. This idea is supported by previous studies of captive-spawned ommastrephid eggs that were more susceptible to microbial infection if the surface of an egg mass was physically damaged (Durward *et al.*, 1980; Bower & Sakurai, 1996). Egg capsules of other squid species are also known to be resistant to infection and predation, but the mechanisms involved are not well known (Atkinson, 1973; Biggs & Epel, 1991).

Each individual egg in a spawned egg mass of *D. gigas* was surrounded by a distinct envelope that was external to the chorion, but no such structure was evident in artificially fertilized eggs. Although the envelope was of a similar overall diameter in both wild- and captive-spawned eggs, chorion expansion was greater in wild eggs, and the thickness of the surrounding envelope layer was correspondingly less. At present the structural nature of the envelope is unknown, and we are not aware of any explicit description of a similar structure for other species of squid.

Chorion expansion was much greater in the eggs from the naturally deposited mass than in the artificially fertilized eggs

(Table 1). Chorion expansion in artificially fertilized eggs requires the addition of powdered oviducal gland (Ikeda *et al.*, 1993b) but clearly this is less effective than the oviducal gland jelly produced naturally by a spawning female. Even natural jelly, however, may not be sufficient for full expansion if produced under stressed conditions, or with an incomplete egg mass, as can be seen from the lesser chorion expansion of the captive-spawned masses when compared to the wild egg mass (Table 1).

The exact mechanism of egg mass formation and the origins of the gelatinous matrix and individual envelopes remain unclear. Bower & Sakurai (1996) speculated on the origin of the jelly in captive-spawned egg masses of *Todarodes pacificus*: ‘Externally, the masses were covered with a jellylike secretion, presumably from the nidamental gland, and the interior of the masses, which contained the eggs, consisted of a jelly presumably secreted by the oviducal gland.’ Unfortunately, it is not clear how these components correspond to the matrix and envelopes described in the present study. Although the natural egg mass from *D. gigas* was viewed only underwater by divers, no external skin or barrier of any type was noted. Moreover, following withdrawal of the sample jar, the matrix material appeared to immediately coalesce around the exit point and show no sign of having been penetrated by a hand and collecting vessel.

Following the suggestion by Bower & Sakurai (1996), we propose that each egg within the *D. gigas* egg mass is indeed coated with material from the oviducal gland as it passes through, and that this material forms the individual envelopes and acts to support fertilization and chorion expansion. Following passage through the oviducal gland, the coated eggs are then mixed with concentrated jelly from the nidamental glands, which eventually forms the gelatinous matrix in which the eggs are suspended. This entire mass is then extruded out the siphon, and presumably transferred by the arms to the buccal area, where it is mixed with sperm from spermatangia. Reaction with seawater then leads to an overall expansion of the egg mass that probably continues as the embryos develop, eventually leading to the observed watery consistency of the wild egg mass.

Location of the natural egg mass in the water column

The egg mass may have been extruded by the female at the depth of discovery, or it may have risen or sunk to that depth. The rapidly decreasing salinity at this depth supports the assertion of O’Dor *et al.* (1982a) that oegopsid egg masses normally accumulate at mid-water pycnoclines. The resting depth of the egg mass is determined by its density, which is influenced by the physical and chemical properties of the jelly and the amount of seawater that it absorbs. Over time, evolutionary pressures have probably influenced the density of pelagic egg masses so that they float in an environment that is favourable for embryonic development and hatching. Although the peak in oxygen occurs below the depth of the egg mass, near-surface waters still provide a reasonably well-oxygenated environment, which may be important for proper embryonic development (Strathmann & Strathmann, 1995).

The temperature at this depth was 25–27°C, just above the range of temperatures reported to result in successful development of artificially fertilized eggs of *Ommastrephes bartramii* and *Sthenoteuthis oualaniensis* (Sakurai *et al.*, 1995). Because CTD data in the present study were not obtained from

precisely the location of the egg mass, the exact temperature of natural incubation cannot be given, but it is unlikely that it was substantially higher than the range stated above.

Several analyses have addressed the rate of egg development in cephalopods as a function of egg size and temperature (Laptikhovskiy, 1991, 1999; Boletzky, 1994). Small eggs at warm temperatures tend to develop to hatching in only a few days, a trend which is consistent with our observations here, and with other studies of ommastrephid development. *Todarodes pacificus* eggs (0.83 mm) hatched after almost 4 days at 23°C, (Watanabe, 1996), and *D. gigas* eggs (1.3 mm) kept at 18°C began hatching after 6 days (Yatsu *et al.*, 1999). Rapid development at warm temperatures may be advantageous due to a variety of factors, including reduced maternal investment in yolk.

Colder temperatures, on the other hand, may not be favourable for development. For example, some ommastrephid paralarvae are unable to tolerate temperatures below 18°C (O'Dor *et al.*, 1982b; Miyanaga & Sakurai, 2006). This characteristic could be shared by *D. gigas* paralarvae, as the egg mass was found in water well above this lower limit. Although *D. gigas* adults regularly undergo rapid vertical migrations, moving quickly from extremely warm temperatures (>26°C) to much colder temperatures (5–6°C) (Gilly *et al.*, 2006b), the ability to withstand such large changes in temperature may be absent in hatchlings and arise later in development.

Paralarval development and behaviour

Growth rates observed in hatchlings from the wild egg mass were comparable to those described for the hatchlings of artificially fertilized eggs (Yatsu *et al.*, 1999), which showed 'considerable variation within and among ages.' Positive growth in wild hatchlings ceased between 3 and 6 days (Table 2), but Yatsu *et al.* (1999) found that growth of artificially fertilized hatchlings appeared to continue through Day 7, and the yolk of these hatchlings was not exhausted until 8 days after hatching. The disparity could potentially be a result of the fact that artificially fertilized eggs tend to hatch two stages earlier than eggs in an egg mass (Watanabe *et al.*, 1996), and so growth characterized as 'hatchling growth' in artificially fertilized hatchlings may occur while wild embryos are still embryos. In addition, wild hatchlings in this study were maintained at slightly elevated temperatures (up to 20°C) when compared to the hatchlings of Yatsu *et al.* (18°C). This may have accelerated metabolism and yolk utilization.

Like other ommastrephids, *D. gigas* development time is short (3 days) and hatchlings are small (1 mm) when compared to myopsids (25–75 days and 1.6–7 mm; summary in Watanabe *et al.*, 1996) and even other oegopsids such as *Gonatus onyx* (up to 9 months and 3.2–3.5 mm; Seibel *et al.*, 2000). As one might expect based on these features, ommastrephid hatchlings are less developmentally advanced, with development of digestive and respiratory organs delayed until after hatching (Watanabe *et al.*, 1996; Yatsu *et al.*, 1999; Shigeno *et al.*, 2001).

In particular, the eyes of ommastrephid hatchlings are not as morphologically developed as those of squid that hatch at larger sizes (Shigeno *et al.*, 2001; authors' personal observations). Laboratory studies of the myopsid *Doryteuthis opalescens* have revealed that hatchlings are active visual predators (Hurley, 1976; Chen *et al.*, 1996). It is possible that *D. gigas* hatchlings are not capable of similar visually

guided prey capture simply because their eyes are insufficiently developed. This feature may be relevant to the fact that feeding of ommastrephid paralarvae has never been successful. Due to the unusual rhynchoteuthion morphology, it has been suggested that ommastrephid hatchlings are not immediately raptorial feeders, as are many other cephalopod paralarvae, and instead go through an initial dependence on particulates or dissolved organic material (O'Dor *et al.*, 1985; Vidal & Haimovici, 1998).

However, *D. gigas* paralarvae displayed swimming (jetting) abilities comparable to those reported for *Doryteuthis opalescens* (Preuss *et al.*, 1997), consistent with the recent finding that loliginid and ommastrephid squids have similar metabolic rates throughout their common size-range (Seibel, 2007). If the swimming abilities of newly hatched *D. gigas* paralarvae are not used initially for prey capture, they may instead serve to avoid predation. The warm, well-lit and well-oxygenated surface waters where the egg mass was discovered may be good for development, but leave hatchlings vulnerable to visual predators, particularly during daylight.

One solution would be to sink to slightly greater depths during the day and return to the near-surface environment during the night. Evidence exists for a diel migration in the paralarvae of at least one ommastrephid, *Sthenoteuthis oualaniensis*. Piatkowski *et al.* (1993) found that paralarvae of this species were concentrated between 30–75 m depth during the day, whereas at night they were distributed fairly evenly between these depths and the surface. A migration of 15 m, from the egg mass depth of 16 m into a slightly deeper daytime zone, would take less than 1 hour at 0.5 cm/s. As the hatchling squid grew, the extent of this diel migration could gradually increase to the large distance seen in adults.

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