HOKKAIDO UNIVERSITY

Title	SPAWNING ECOLOGY AND EARLY LIFE HISTORY OF THE NEON FLYING SQUID
Author(s)	Dharmamony, Vijai
Degree Grantor	北海道大学
Degree Name	博士(水産科学)
Dissertation Number	甲第11777号
Issue Date	2015-03-25
DOI	https://doi.org/10.14943/doctoral.k11777
Doc URL	https://hdl.handle.net/2115/58617
Type	doctoral thesis
File Information	Vijai_Dharmamony.pdf

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SPAWNING ECOLOGY AND EARLY LIFE HISTORY OF THE NEON FLYING SQUID

A DISSERTATION SUBMITTED TO THE

GRADUATE SCHOOL OF FISHERIES SCIENCES
HOKKAIDO UNIVERSITY, JAPAN

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY
(FISHERIES SCIENCES)

BY
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JANUARY, 2015

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Abstract

The neon flying squid *Ommastrephes bartramii*, is an oceanic species that is widely distributed in the North Pacific, with the winter-spring cohort spawning around the Hawaiian Islands. Very little is known about its reproduction, development, and early life stages, as it is a true oceanic dweller for which experiments in land-based laboratories are challenging. In the present study I explored the spawning pattern and early life stages of the winter-spring cohort.

I aimed to examine the reproductive characteristics of the winter-spring cohort of *O. bartramii* near the Hawaiian Islands, using individuals collected during 1990 - 2000 and 2013. The findings of this study, combined with the results of previous studies, are expected to clarify whether this species is a single or multiple spawner. Squid were collected aboard the T/S Hokusei Maru and the R/V Kaiyo Maru. Sampling aboard the T/S Hokusei Maru was performed by manual jigging, gillnetting, and trawling on 11 cruises from February to March every year from 1990 to 2000 between 20° and 35°N and 150°W and 180° longitude. Collection aboard the R/V Kaiyo Maru was conducted during December 2013 north of Hawaii, between 24° and 33°N and between 173° and 155°W. A total of 719 (622 males, 97 females) individuals were collected by the T/S Hokusei Maru, in addition to 11 individuals by the R/V Kaiyo Maru. Female spawning status was determined from somatic indices and histological characteristics of the ovaries.

At all developmental stages, the ovaries of spawned females contained oocytes, while oviduct fullness was not correlated with body size. Thus, because the eggs mature asynchronously, with multiple filling and evacuation events, this species is considered an intermittent spawner. Mature males with developed accessory glands were also present within the distribution range of healthy, spawned females, suggesting that mating occurs between spawning events. The data indicate that, the first spawning event occurs at a mantle length of ~520–540 mm. Subsequently, the squid forage and grow, and refill the oviducts, before spawning again.

Studies on the relationships between the early life stages of the *O.bartramii* and oceanographic conditions are essential for understanding the spatial and temporal distribution patterns of this ecologically and economically important species. Eggs of the neon flying

squid were artificially fertilized and incubated at temperatures found in the known distribution range of this species (16–26°C). Artificial fertilization of was performed on board the R/V Kaiyo Maru using standard techniques. Fresh seawater collected from 500 and 1000 m depth was fortified with antibiotics (25 mg L^{-1} each of ampicillin and streptomycin) and used as the experimental medium.

The present study establishes an atlas for the normal development of *O. bartramii* from fertilized eggs to paralarvae. The stages are mainly based on morphological features and can be distinguished clearly under a stereomicroscope. These observations of embryonic development correspond closely with those of two other ommastrephids, *Todarodes pacificus* and *Illex argentinus*. To observe the species-specific effects of the oviducal gland powder on chorion expansion, four batches of *O. bartramii* fertilized eggs (n = 400) were prepared. Two batches were provided with oviducal gland jelly water (OGW) prepared from *T. pacificus*, and the other two batches were provided with OGW prepared from *O. bartramii*. One day after incubation, during the water exchange process, one batch started with *T. pacificus* OGW was changed to *O. bartramii* OGW, and the reverse interchanging procedure was conducted for one batch started with *O. bartramii* OGW. The remaining two batches were provided with the same OGW throughout the experiment. Embryos treated with OGW from a single species throughout the experiment successfully reached the hatching stage. However, the chorion of the embryos for which the OGW provision was switched began to contract and did not survive longer than a day. These results indicate that the overall chemical nature of the oviducal gland was species-specific, although the chorion expansion inducer was the same across species. The addition of oviducal gland jelly also induced partial parthenogenesis in the early stages of embryonic development through the expansion of the perivitelline space and blastodisc formation.

Complete organogenesis with normal successive cleavage and the distinct continuity of morphological features occurred only between 18° and 25°C. Experimental rearing, cruise-collected specimens, and oceanographic data (sea surface temperature) confirmed that the optimal temperature range for *O. bartramii* paralarval survival around Hawaii is 18–25°C. Embryos reared at 16°C showed abnormal organogenesis; however, normal development resumed when embryos were transferred to 22 or 24°C after blastoderm formation.

Hatchlings showed a variety of swimming behaviors, which may allow squid to regulate their thermal distribution in the wild. Furthermore, ball formation and associated chromatophore

expansion might represent an aposematic adaptation to imitate unpalatable prey. Although prey items initially consumed by hatchlings were not identified, I discuss the possible role of the proboscis (fused tentacles) in feeding. This study highlights the flexible strategy of ommastrephid embryo development and illustrates how information on paralarval behavior and oceanographic data (sea surface temperature) may be combined to improve our understanding of the factors that influence survival.

Preface

The results of this study have been documented in three manuscripts written for peer-reviewed scientific journals. Chapter 2 is from a paper published in the journal *Scientia Marina* (permission not obtained from publisher, as it is an open access article). Chapter 3 is from a manuscript submitted to *Zoomorphology*. Chapters 4 and 5 are from a manuscript submitted to *Marine Ecology Progress Series*. In some cases, texts and figures of the submitted manuscripts were slightly modified and adapted to format this thesis. All cited literature are listed in the References section.

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Acknowledgements

I would like to express my special appreciation and thanks to my advisor Professor Dr. Yasunori Sakurai, you have been a tremendous mentor for me. I would like to thank you for encouraging my research and for allowing me to grow as a researcher. Your advice on both research as well as on my career have been priceless. Without your guidance and persistent help this dissertation would not have been possible.

I am grateful to Dr. John Bower -my first contact in Japan- for being a mentor and a good friend. Discussions with you were always fruitful. Eiley's Irish Pub is still waiting for our many visits!

I am thankful to Dr. Mitsuo Sakai for allowing me to participate in cruises on board RV Kaiyo Maru and for the insight and thoughtful discussions without which these studies would have been less worthwhile.

Dr. Sei-ichi Saitoh, Dr. Yutaka Watanuki, Dr. Jun Yamamoto, Hae-Kyun Yoo, Christopher Aura, Irene Alabia, Tetsuro Kubo, Hajime Matsui and as well as all members in my lab, provided invaluable help in setting a research life in Japan and provided support to carry out the topics covered in this thesis. I also remember with gratitude, the people from the Fishery Agency of Japan who accompanied me on board cruises in Pacific, especially Toshie Wakabayashi and Yoshiki Kato for their invaluable help. I received financial support from Japanese Ministry of Education, Culture, Sports, Science and Technology (MEXT) through Japanese Government (Monbukagaku-sho) scholarship for which I am grateful.

This study was supported by the Research Program on Climate Change Adaptation (RECCA) under the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan, and by the “Project on the Evaluation of the Status of International Fishery Resources” by the Fisheries Agency of Japan.

Finally, I thank my wife Puneeta Pandey for being a constant support for me. All those embryo and paralarval drawings in this thesis came from her. I am not mentioning my daughter Anusha’s contribution, as she always steals away my time, with her cute little smile.

Dedicated to my father and inspiration

Muthayya Nadar Dharmamony

“If it's not documented... It never happened!”

Chapter 1. Introduction

Squids are broadly divided into the primarily nearshore Myopsida and the mostly oceanic Oegopsida. Myopsids provide most of our knowledge of squid biology, including reproduction and development (Gilbert et al. 1990). Much less is known about the oegopsids. Among the oegopsids, the family Ommastrephidae is of particular interest. Ommastrephid squid consume a substantial amount of epipelagic fish (Markaida & Sosa-nishizaki 2003, Watanabe et al. 2004, Field et al. 2007) and constitute the dominant diets of many marine megafauna (Klimley et al. 1993, Rosas-Alayola et al. 2002, Ruiz-Cooley et al. 2004, Barlow & Forney 2007). This group also supports the world's largest cephalopod fisheries, which have been expanding as fish stocks decline (Caddy & Rodhouse 1998). Management of these fisheries is challenged by limited ecological knowledge, particularly regarding questions of reproduction and early development (Staaf 2010).

Ommastrephids exhibit the following ecological traits of r-strategists (Nigmatullin & Markaida 2008): monocyclia (a complete life cycle within one year), small eggs (~1 mm), very high fecundity, intermittent multi-batch individual spawning (except for *Todarodes pacificus*) and prolonged population spawning, planktonic rhynchoteuthion paralarvae and juveniles, very high growth rates, complex intraspecific spawning structure with presence of different seasonal spawning groups, significant long-term fluctuations in abundance (by an order of magnitude or more), and complete annual renewal of the population composition. The most important factor that determines the pattern of abundance, dynamics and distribution of exploitable ommastrephid populations is the availability of planktonic paralarvae and juveniles that inhabit subsurface depths for about 2 to 2.5 months (Nigmatullin et al. 2003). These stages are characterized by a high mortality rate. Therefore, spawning

success and paralarval survival rates are the principal factors that determine recruitment level (Sakurai et al. 2000, Goto 2008).

Another important ecological-population feature of these squids is their one-year life cycle. These two key factors govern the problems and specifics of ommastrephid fishery biology and the annual abundance dynamics. All ommastrephids form schools from the juvenile stage. Schools range in size from two to several thousand, generally of equal-sized individuals (Wormuth 1976, Shevtsova et al. 1979, Dunning 1998, Yatsu & Mori 2000, Brunetti et al. 2006, Roper et al. 2010).

Most ommastrephid species are characterized by long daily vertical migrations (hundreds of metres) (Murata & Nakamua 1998) and ontogenetic, seasonal, horizontal migrations, both parallel to the coastline, up to 1000 to1500 miles long, and perpendicular (bathymetric) migrations from 10 to 50 miles and even to 150 miles offshore (Nigmatullin et al. 2003). During these migrations squids traverse the boundaries of a diversity of climatic zones and ecosystems. Throughout the life cycle, with continuous body size increases, most ommastrephids "permeate" the trophic pyramid, consecutively transferring from consumers of II to III orders to consumers of IV to VI orders and respectively changing the taxonomic and ecological spectrum of their food organisms (prey), enemies (predators) and parasites (Shevtsova et al. 1979, Nigmatullin et al. 2003). In some oceanic communities, large adult ommastrephids actually are top-level predators.

Ommastrephids are strongly active predators (Shevtsova et al. 1979, Roper et al. 2010). Their food composition depends on their mantle length (ML): in young, small squid, prey consist predominantly of meso- and macroplanktonic crustaceans and juvenile fishes; in middle-sized squid prey consists of micronektonic fishes (mainly myctophids) and marcroplanktonic euphausiids and shrimps; and large-sized individuals prey mostly on fishes

and, squids, and to a lesser degree, shrimps. Cannibalism is common (Roper et al. 2010). Ommastrephids have a high general and active metabolism, and adult squid have a daily food consumption rate of 6 to 12% of the body weight (Wormuth 1976, Yatsu & Mori 2000). Protein is the main substrate for energy metabolism. The unique combination of ecological and physiological traits confirms that ommastrephid squids are one of the most important elements in the "rigid framework" of highly mobile predators that unites local ecosystems into ecosystems of the next higher rank, and they function as "ecosystem enzymes" (Shevtsova et al. 1979): they significantly accelerate all ecosystem-related processes. The total instantaneous ommastrephid biomass is around 55 million tonnes on average; the total yearly production is about 400 million tonnes (production/biomass coefficient is P/B = 5 in inshore species and P/B = 8 in oceanic species), and the total annual food consumption is around 1,000 million tonnes (Dunning 1998, Nigmatullin et al. 2003, Roper et al. 2010). Ommastrephids are food for numerous animals – sea birds, marine mammals and especially large predacious teleosts (alepisaurids, gempilids, coryphaenids, tunas, billfishes, swordfishes, etc.) and sharks (Brunetti et al. 2006, Dunning 1998, Nigmatullin et al. 2003, Roper et al. 2010, Shevtsova et al. 1979, Wormuth 1976, Yatsu & Mori 2000).

Ommastrephes bartramii is an extremely widely distributed ommastrephid species with an oceanic bi-subtropical (anti-tropical) worldwide distribution (Murata 1990). It inhabits the great subtropical ocean gyres in subtropical and partly temperate oceanic waters, but it is excluded from the equatorial waters of all 3 oceans (Yatsu et al. 1998). Its principal range is in subtropical water masses. In the temperate and partly tropical zones, *O. bartramii* penetrates into warm and cold boundary currents, respectively. It occurs at sea surface temperatures from 10° to 25°C, generally over bottom depths greater than 200 m. Three major

populations, or subspecies, not yet formerly described, inhabit three massive, but isolated, regions (Sinclair 1991, Guerra et al. 2010, Kurosaka et al. 2012, Wakabayashi et al. 2012):

1. The North Atlantic subspecies
2. The Southern Hemisphere conspecific group
3. The North Pacific subspecies

These three geographically isolated populations are differentiated by spermatophore morphology, by their size structure (Zalygalin et al., 1983; Nigmatullin et al., 2003) and by substrate-inhibitory traits of optic ganglia cholinesterases (Shevtsova et al., 1979).

Spawning occurs almost year round in the North Pacific. Two principal intraspecific groups (cohorts) are distinguished by the time of reproduction: 1) a fall or autumn cohort that spawns in autumn with the hatching period from September to February, and 2) a winter-spring cohort that spawns in winter with the hatching period principally from January to May and sometimes to August. Both groups have a one-year life cycle. These two cohorts each are subdivided into two subgroups (stocks) by size composition, paralarval distribution and parasitic helminth infection rates (Bower & Ichii 2005) **(**Figure 1-1**).**

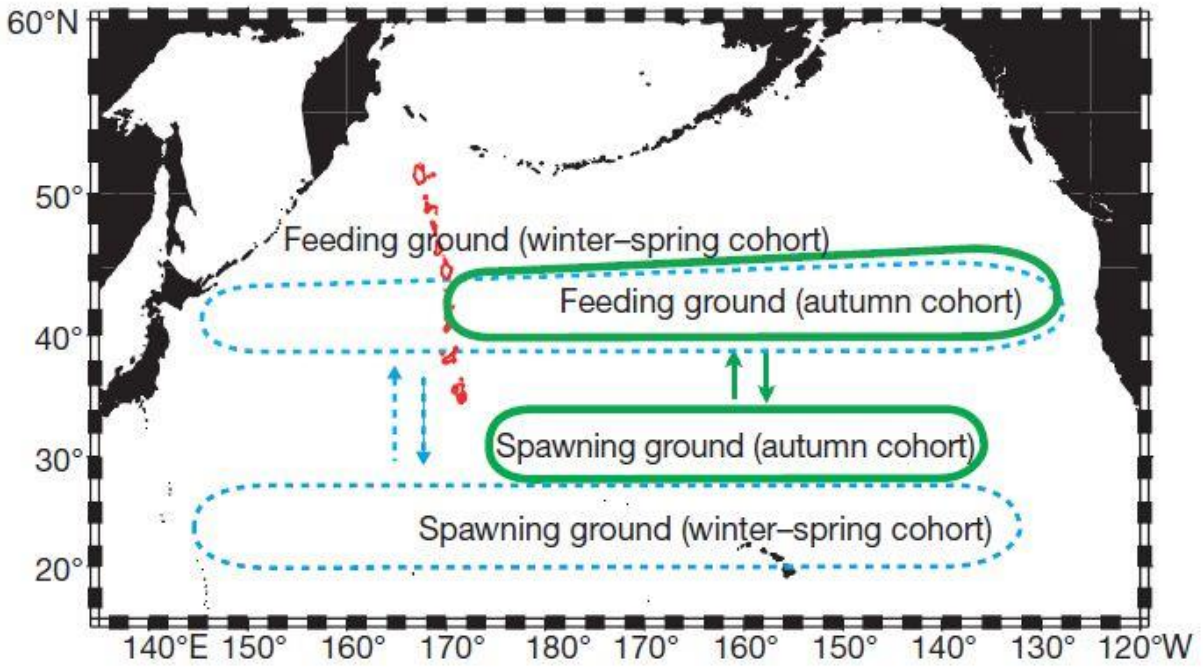

Figure 1-1. Migration patterns of the autumn and winter–spring cohorts of neon flying squid (*Ommastrephes bartramii*) in the North Pacific (from Ichii et al. 2011)

The size of mature females of the autumn spawning group is greater than 460 mm ML, while the size at maturity of the winter-spring spawning group of mature females is less than 490 mm ML. The size of squid of the autumn group during summer is much larger than that of the winter–spring group (380 - 460 mm ML and 160 - 280 mm ML, respectively), and they also differ in maximum size (600 and 490 mm ML, respectively). Males are non-migratory; they remain in subtropical waters and do not migrate to the northern grounds. Migrating squid are capable of covering 5 to 10 km per day. It has been observed that the parasite load of *O. bartramii* is sufficiently different geographically to differentiate between populations associated with eastern and western Pacific feeding grounds (Bower & Ichii 2005).

In spite of its worldwide distribution, *Ommastrephes bartramii* is fished commercially only in the North Pacific Ocean where its populational structure and abundance seem to be maximized for exploitation (Yatsu & Mori 2000, Roper et al. 2010).

Ommastrephid squids spawn large number of eggs enclosed in gelatinous masses, which are thought to later settle in the pycnocline until hatching. In spawning millions of eggs, of which only a few reach maturity, a single female injects enormous numbers of planktonic predators into the ecosystem. Egg masses for *O. bartramii* have never been observed. Eggs measure about 0.9 x 1.1 mm in size (Sakurai et al., 1995). Spawning in the North Pacific occurs virtually all year long (Yatsu et al. 1998). Hatchlings measure about 1.1 mm ML (Yatsu & Mori 2000). A 1-mm hatchling can grow to a full size adult in less than one year, a transition that must be fuelled by a tremendous quantity of prey. Little is known, however, about the diet of early stages of *O. bartramii*, and indeed of ommastrephids in general. This is partially due to morphological differences between hatchlings and adults (Staaf 2010). Hatchling squid possess the standard adult anatomy of mantle, fins, head and arms, but the relative dimensions of these structures are strikingly different from those of adults. In addition, squids often do not hatch with functional feeding tentacles; they grow as the hatchling gradually develops into a juvenile. These anatomical differences have led to use of the term paralarvae to refer to hatchling cephalopods, since this taxonomic group has no true larval stages (Young & Harman 1988). During the unique paralarval stage of ommastrephids, called the rhynchoteuthion, the two tentacles grow first as a single fused proboscis, which splits later in development. Use and development of this structure are not well understood (Shea 2005, Staaf 2010).

Paralarvae of *O. bartramii* have been captured over a broad stretch of the North Pacific from 140°E and 130°W between 25°N and 35°N (Yatsu et al. 1998) and as far south as 20°N in Hawaiian waters (Young et al. 2000). Paralarvae appear to occur mostly in the upper 25 m during the day and night (Young & Hirota 1990, Saito & Kubodera 1993). In the North Pacific paralarvae have been captured over a broad stretch of from 140°E and 130°W

between 25° and 35°N (Yatsu et al. 1998). In the Hawaiian Archipelago, paralarvae are caught where sea surface temperatures were 21°-24°C (Bower 1994).

In this thesis, I studied the spawning pattern and development of *O. bartramii* paralarvae. A hypothesis for the spawning pattern of the mature female *O. bartramii* is presented in chapter 2. In chapter 3, embryonic and paralarval development are addressed. Chapter 4 deals with the effects of temperature on embryonic development. Finally the behavior of hatched paralarvae is discussed in chapter 5. Embryos observed for chapters 3, 4 and 5 were obtained through artificial fertilization.

Chapter 2. Spawning pattern

2.1. Abstract

The neon flying squid, *Ommastrephes bartramii*, is an oceanic squid species that is widely distributed in the North Pacific, with the winter-spring cohort spawning around the Hawaiian Islands. Here, I investigated the spawning characteristics of *O. bartramii* by analyzing various reproductive parameters of 730 individuals (622 males, 108 females) collected in this region. Female spawning status was determined from the somatic indices and histological characteristics of the ovaries. At all developmental stages, the ovaries of spawned females contained oocytes, while oviduct fullness was not correlated with body size. Thus, because the eggs mature asynchronously, with multiple filling and evacuation events, this species is considered an intermittent spawner. Mature males with developed accessory glands were also present within the distribution range of healthy spawned females, indicating that mating occurs between spawning events. My data indicate that, the first spawning event for *O. bartramii* near Hawaii occurs at a ML of ~520–540 mm. Subsequently, the squid forage and grow, and refill the oviducts before spawning again.

2.2. Introduction

All living cephalopods are considered semelparous (i.e. characterized by a single reproductive event; Boyle 1983, Calow 1987, Rodhouse 1998), with the only exception being *Nautilus*, which has a life span of more than 20 years (Rocha et al. 2001). However, recently, several authors (Nigmatullin et al. 1996, Nigmatullin 2002, Nigmatullin 2011) have reported complex reproductive patterns that cannot be categorized as semelparous, particularly in tropical and subtropical oegopsid squids belonging to the family Ommastrephidae. For example, intermittent spawning without somatic growth between spawning events has been

reported for *Illex coindetii* (González & Guerra 1996), *Todaropsis eblanae* (Rasero et al. 1995), and *Illex illecebrosus* (O'Dor & Dawe 1998). Furthermore, intermittent spawning with somatic growth between spawning events has been reported for Ommastrephidae (Reznik & Bessmertnaya 1993, Nigmatullin & Laptikhovsky 1994, Nesis 1996, Laptikhovsky & Nigmatullin 2005, Nigmatullin & Markaida 2009). Recently, iteroparity has also been reported in the squid *Kondakovia longimana* (Laptikhovsky et al. 2013).

The neon flying squid, *Ommastrephes bartramii*, is an oceanic squid that occurs in subtropical and temperate waters worldwide (Roper et al. 2010). The population in the North Pacific includes an autumn spawning cohort and a winter-spring spawning cohort (Yatsu et al. 1998). Individuals live for one year, migrating between the spawning grounds in tropical waters and foraging grounds in sub-Arctic waters during this period (Murata & Nakamura 1998, Yatsu et al. 1998, Chen & Chiu 2003, Bower & Ichii 2005). The main spawning and nursery ground of the autumn cohort occurs in the subtropical frontal zone (STFZ) of the North Pacific Ocean. This location is characterized by enhanced productivity in winter because of its proximity to the transition zone chlorophyll front (TZCF). In comparison, the spawning and nursery ground of the winter-spring cohort occurs within the subtropical domain (STD), which is less productive (Ichii et al. 2009). The STFZ is defined by a salinity range of 34.6 to 35.2 at the sea surface, generally occurring at ~29 to 34°N latitude, with the STD occurring south of the STFZ (Roden 1991, Ichii et al. 2009). *O. bartramii* hatchlings are considered to be paralarvae (i.e. young cephalopods in the planktonic stages) from hatching to one month of age (Bigelow and Landgraph 1993), juveniles and subadults from one month of age until they reach a ML of ~25 cm (Ichii et al. 2009), and adults above 25 cm ML.

The oocytes develop in the ovary, which extends from the posterior end of the digestive gland to approximately the posterior end of the mantle. Ova are released from the

ovary and accumulate in the oviducts. The nidamental glands produce the outer layer of the egg mass (Bower & Sakurai 1996). Females store viable sperm in the buccal area; thus, mating and spawning do not need to coincide (Harman et al. 1989). Closely related *Dosidicus gigas* and *Sthenoteuthis pteropus* ommastrephid females spawn in pelagic waters, producing spherical egg masses (Laptikhovsky & Murzov 1990, Staaf et al. 2008). For two *O. bartramii* collected from the Atlantic Ocean, Laptikhovsky (2011) calculated a potential fecundity of 4.9 and 3.7 million eggs, respectively. The sperm duct of males transfers sperm from the testis located anterior of the spermatophoric organ, where spermatophores are formed. Spermatophores are tubular structures stored in the spermatophoric sac (Needham's sac), which opens into the mantle cavity through the penis (Jereb & Roper 2010). In cephalopods, allometric growth relationships between somatic, reproductive, and digestive components change during ontogeny, depending on the state of maturity and nutritional status (Boyle & Rodhouse 2005). These allometric growth relationships may provide a way of directly determining the spawning status of *O. bartramii* collected from the sea.

In this study, I aimed to examine the reproductive characteristics of the winter-spring cohort of *O. bartramii* near the Hawaiian Islands, using individuals collected during 1990 - 2000 and 2013. The findings of this study, combined with the results of previous studies, are expected to clarify whether this species is a single or multiple spawner.

2.3. Materials and Methods

Sampling

Squid were collected near Hawaii aboard the TS Hokusei Maru (Hokkaido University, Japan) and R/V Kaiyo Maru (Fisheries Agency, Japan). Sampling aboard the TS Hokusei Maru was performed by manual jigging, gillnetting, and trawling on 11 cruises from February to March every year from 1990 to 2000 between 20° and 35°N and 150°W and 180° longitude

(Fig. 2-1). Collection aboard the R/V Kaiyo Maru was conducted during December 2013 north of Hawaii, between 24° and 33°N and between 173° and 155°W (Fig. 4-1b). A total of 719 (622 males, 97 females) individuals were collected by the TS Hokusei Maru (from 78 stations), in addition to 11 individuals (all females) by the R/V Kaiyo Maru (from 8 stations). All squid were used in the analysis. Monthly data from the 1990–2000 survey years (excluding 2013) were combined because interannual variation in ML is thought to be minimal (Murata & Hayase 1993, Yatsu et al. 1998).

Analysis of the reproductive system

For the analysis, I selected females larger than 400 mm and males larger than 300 mm ML, to ensure that only mature squid were included in the analysis. Dorsal ML (to the nearest 1 mm) was measured, and the following parameters were estimated: body weight (to the nearest 1 g), stomach weight, and digestive gland weight (Laptikhovsky & Nigmatullin 1992, Laptikhovsky & Nigmatullin 1993). I obtained the testis weight and accessory gland weight of males (Nigmatullin et al. 2003). I also obtained the ovary weight, oviduct weight, nidamental gland length, nidamental gland weight, and mating status (copulated or not) of females. Just 11 of the female squid collected by the R/V Kaiyo Maru were used for the analysis of oocyte size and potential fecundity (PF). To determine the number of oocytes in each ovary, I collected three subsamples (100 mg) from the ovary surface, ovary core, and intermediate layer. Each subsample was weighed and observed in a Bogorov chamber under a binocular microscope (Nigmatullin & Markaida 2009). Potential fecundity was calculated as the sum of total oocyte (>0.05 mm in diameter) number in the ovary and total egg number in the oviducts (Nigmatullin et al. 1995, Nigmatullin 1997, Laptikhovsky & Nigmatullin 1999, Nigmatullin & Laptikhovsky 1999). Oviducal load (OL) is the number of eggs in the oviducts (Nigmatullin & Markaida 2009).

I used the allometric growth relationships of somatic and reproductive components to determine the spawning status of *O. bartramii* directly. Specifically, I calculated and analyzed the somatic indices of female squid and the volume of oocytes in the ovaries to determine spawning status. The somatic indices used in this study were calculated using the definitions shown in Table 2-1. Reproductive maturity was assessed based on the maturity stages I through VII proposed by Nigmatullin (1989). These stages were adjusted for *O. bartramii*, whereby stages I and II were defined as immature, stage III as early maturing, stage IV as late-maturing, stage V as mature, stage VI as spawning state (after the first spawn), and stage VII as spent. Stage VII squid was not collected during any of the cruises. Statistical analyses were performed using R software (R Core Development Team 2013).

2.4. Results

Somatic indices

The somatic indices of 622 males and 97 females (1990–2000 data) were analyzed. Females (ML: 526 ± 4.5 mm, mean ± SE) were larger than males (ML: 333 ± 0.7 mm). The size distribution of males and females is shown in Figure 2-2. All females with >400 mm ML had eggs in the oviducts, indicating that all individuals had attained stage IV or above. Another indicator of sexual maturity in females is the relative size of the nidamental glands (Durward et al. 1979, Okutani & Tung 1978). The nidamental glands of mature females were relatively large, opaque, and white. The glands ranged in size from 68 to 190 mm, and represented 0.9% to 4% of the total body weight in individuals of >400 mm ML. The nidamental gland index differed significantly among maturity stages IV, V, and VI ($P < 0.01$, analysis of variance [ANOVA]; Fig. 2-3). Nidamental gland weight increased from the late-maturing stage to the mature stage and decreased after spawning (spawning state). These data indicate the readiness of the reproductive system to produce eggs continuously (Fig. 2-4).

Nidamental gland weight was positively correlated ($r = 0.56$, $P < 0.01$) with ML. Within each of the three stages (IV, V, and VI), neither ML ($F_{2,83} = 1.8$, $P > 0.05$, ANOVA) nor body weight ($F_{2,83} = 2.9$, $P > 0.05$, ANOVA) differed significantly. Stage VI females ranged in size from 516 to 572 mm ML.

The oviduct somatic index (ODSI) distribution of females was scattered (Fig. 2-5). This index showed no correlation with ML (Spearman rank coefficient $r = 0.148$, $P > 0.05$) in females with >400 mm ML. Although there was considerable scatter in the ODI among mature individuals, there was no evidence that any female was dying. All mature females had food in their stomach. In addition, the weight of the stomach content in stage VI females (spawning state) was similar to that of stage IV and V females ($F_{2,74} = 1.85$, $P > 0.05$, ANOVA; Figs. 2-6 and 2-7). The ovary index was also not correlated with ML (Spearman rank coefficient $r = 0.031$, $P > 0.05$). Females of advanced maturity stages had large ovaries (OSI: 4.34 ± 0.83%, Figs. 2-7 and 2-8, Table 2-2).

There was a negative correlation between the testis index and ML (Spearman rank coefficient $r = -0.48$, $P < 0.001$) of males. However, there was no significant correlation between the accessory gland index and ML (Spearman rank coefficient $r = -0.04$, $P > 0.05$, Fig. 2-9) of males.

Oocyte size and fecundity

The oocytes in the ovaries of mature females were at various stages of development, indicating asynchronous development. It was possible to distinguish six size groups of oocytes that corresponded to the stages of oocyte development (after Nigmatullin et al. 1995). Size group I corresponded to the second phase of provitellogenesis, in which oocytes were polygonal and the centrally situated nucleus was oval and large. Group II corresponded to the

third phase of provitellogenesis. Oocytes were either oval or goblet-shaped, and the cytoplasm volume was greater compared to the preceding size group. Group III corresponded to the intercalary period of oocyte development. Oocytes were leaf-like, of dark color, with numerous shallow longitudinal grooves together with follicle cells that protruded into the grooves. The nucleus was not visible in this group. Group IV corresponded to the first and second phases of trophoplasmatic growth. Oocytes were covered with reticulate grooves, and were dark in color. Group V corresponded to the third phase of trophoplasmatic growth. Oocytes were rounded and yellow and the reticulate grooves had almost disappeared. Group VI corresponded to the fourth phase of the trophoplasmatic growth. Oocytes had a smooth surface and were oval. Data on the sizes of oocytes are given in Table 2-3. The percentage of oocytes belonging to different size groups changed as the reproductive system developed (Fig. 2-10). In immature females, oocytes of size group I were prevalent (76%), with the remainder belonging to size groups II and III. Ripe oocytes were absent in maturing females. Mature and spawning state females always had group I oocytes, but with different percentages (Fig. 2-10). The relative number of ripe oocytes (group VI) was always low (<1%) in mature and spawning state females, because they are immediately transferred into the oviducts after extrusion into the coelom. Individual potential fecundity varied considerably, ranging from 7 million oocytes in immature females to 2.4–7.0 million oocytes in maturing and mature females (Table 2-4).

2.5. Discussion

Structure of the reproductive organs

The results of this study indicate that *O. bartramii* is an intermittent spawner that exhibits somatic growth between spawning events, as previously reported for *S. oualaniensis* (Harman et al. 1989), *T. rhombus* (Nigmatullin et al. 1995), *Photololigo* sp. (Moltschaniwskyj

1995), and other higher ommastrephids (Reznik & Bessmertnaya 1993, Nigmatullin & Laptikhovsky 1994, Zuyev et al. 2002, Laptikhovsky & Nigmatullin 2005, Nigmatullin 2011). The primary evidence supporting this suggestion is the lack of a strong correlation between body size and the number of mature oocytes in mature females, coupled with proof of the occurrence of multiple filling and evacuation events in the oviduct. Ova are stored in the oviducts; hence, the fullness of the oviducts may be used as an indicator of repeated spawning. However, the fullness of the oviducts was not statistically correlated with body size in my study. Single spawners should show a gradual increase in oviduct fullness as size increases (Young et al. 1997), but this trend was not detected in the present data. Variability in oviduct fullness presents convincing evidence of batch spawning in females that grow substantially after reaching sexual maturity and continue to produce mature oocytes throughout this growth period.

O. bartramii females reach sexual maturity at ~300 mm ML (Yatsu et al. 1998, Brunetti et al. 2006, Li et al. 2011), after which spawning occurs under favorable conditions. Because I did not find any sexually immature females of >400 mm ML, substantial growth must occur in females after attaining maturity (Yatsu et al. 1998, Li et al. 2011). A positive correlation is expected between ML and oviduct fullness (ODSI) for single spawning squids (e.g., *T. pacificus*, Ikeda et al. 1993), because the oocytes are produced and then develop in the ovaries before being transferred to the oviducts at stage VI for storage until spawning (Moltschaniwskyj 1995). However, the lack of correlation between ML and ODSI indicates that oocytes are present in the oviduct throughout stages IV, V, and VI. The OSI value showed the presence of oocytes in the ovaries of females with mature ML ranges (400–600 mm). This finding indicates that all stages of oocytes may be simultaneously present in the ovary. The somatic indices of 11 females at three maturity stages (late maturing, mature, and

spawning state) collected from a single station in 1992 are presented in Fig. 2-7. For these females, the OSI values were within a narrow range (2.5–4.0). Mature squid had high oviduct content but minimal stomach content (SCI: 0 to 0.2). In contrast, spawning state squid had smaller oviduct content but substantial stomach content (SCI: 1 to 4). Spawning state squid also had high HSI values (6.58 ± 2.05) showing the active digestive system. Thus, female *O. bartramii* seem to grow after reaching maturity, and continue to produce eggs throughout this period. Therefore, oocytes are expected to accumulate in the oviducts, unless spawning occurs (Moltschaniwskyj 1995). However, oviduct fullness and ovary fullness (OSI) were generally unrelated to body size. This result is indicative of batch spawning. The presence of oocytes of all maturity stages indicates that oocytes are produced continuously and not in a single batch. After stomach content analysis, females appeared to forage irrespective of ML and maturity stage (Figs. 2-6 and 2-7). These results indicate that the *O. bartramii* develops ova continuously, and not in a single batch (Nigmatullin & Laptikhovsky 1994, Nigmatullin et al. 1995). This finding contrasts with the single spawning mode where all oocytes mature simultaneously (O'Dor 1983, Moltschaniwskyj 1995, Nixon 1983, Sakurai et al. 2000).

The nidamental glands of females produce the outside layer of egg masses during spawning (Bower & Sakurai 1996). Nidamental glands become enlarged and functional at sexual maturity. A single spawning female should show a gradual increase in nidamental gland size with increasing ML. In the current study, variation in nidamental gland weight and the fullness of oviducts indicated that the nidamental gland cycle is repeated through stages IV to V. This phenomenon occurs when the oviducts are filled and emptied in a squid exhibiting repeated spawning.

The skewed distribution of females might be an artifact of the capture techniques used. Mature females occupy deeper waters, making them less vulnerable to jigging (Young et al.

1997) or trawling. Also, females are larger so they are presumably more likely to fall off fishing hooks and better able to avoid trawls. Therefore, large-size classes (>550 mm ML) may be under-represented in the present data. The size difference between the sexes in my dataset is consistent with that reported in previous studies (Ichii et al. 2009, Yatsu et al. 1998).

Spawning pattern

The mean ML size of *O.bartramii* at maturity is ~300–330 mm for males and 370–570 mm for females (Yatsu et al. 1998). Furthermore, females tend to be larger than males of the same age (Ichii et al. 2009). Yatsu et al. (1998) reported a peak in oviduct fullness for *O. bartramii* at ~540 mm ML. This finding may indicate the size at which *O.bartramii* first spawn. Young et al. (1997) also reported asynchronous ovulation based on the size-frequency distribution of oocytes in the ovaries of 12 squid. From the results of these previous studies and my own findings, I hypothesize that the first spawning event in the *O.bartramii* occurs at ~520–540 mm ML (Fig. 2-11). Subsequently, the female feeds, undergoes somatic growth, and refills the oviduct. The second spawning event commences after the female has again passed through maturity stages IV and V (Fig. 2-11). Since my sample size was small, for calculating exact somatic growth between spawning events future studies are required. Multiple spawning has also been reported for related ommastrephids, including *S. oualaniensis* (Harman et al. 1989), *S. pteropus* (Laptikhovsky & Nigmatullin 2005), and *Ornithoteuthis antillarum* (Arkhipkin et al. 1998). Ichii et al. (2009) suggested that the extended spawning season of the *O.bartramii* might represent a risk-spreading strategy to reduce the chance of population collapse. The extended spawning season is achieved by dispersing the population through numerous seasonal cohorts and, possibly, through the occurrence of multiple spawning events. The lack of correlation between the body size and the accessory gland somatic index in males indicates that males continuously produce

spermatophores. In the current study, these mature males were scattered throughout the distribution range of healthy spawning-state females. Thus, it is possible that mating occurs between spawning events.

Based on my findings, I suggest that the spawning pattern of *O. bartramii* is similar to the pattern proposed for *T. rhombus* (Nigmatullin et al. 1995), *D. gigas* (Nigmatullin & Markaida, 2009), and both *Sthenoteuthis* species (Nigmatullin & Laptikhovsky 1994, Zuyev et al. 2002, Laptikhovsky & Nigmatullin 2005). I suggest the following life-history model for female *O. bartramii*. Specifically, the maximum number of oocytes forms by the end of stage II of maturity in immature individuals. At subsequent developmental stages, the total number of oocytes remains constant, but they start to develop asynchronously. As soon as the oocytes ripen, they are released from the follicles into the sexual coelom and are transferred to the oviducts, where they accumulate. At the same time, the nidamental and oviduct glands develop. When the oviducts are full of ripe eggs, spawning occurs, as long as the SST range is favorable (21 to 25°C).

Spawning strategies

Because of the high levels of potential fecundity, the presence of primary and secondary oocytes in the ovary of mature females indicates that *O. bartramii* invests in intermittent spawning, as described for *T. rhombus* (Nigmatullin et al. 1995), *D. gigas* (Nigmatullin & Markaida, 2009) and both *Sthenoteuthis* (Nigmatullin & Laptikhovsky, 1994, Zuyev et al. 2002, Laptikhovsky & Nigmatullin 2005). In other words, feeding occurs between spawning events (i.e. during the interval between each successive filling and evacuation event in the oviducts) at the spawning ground, fueling both somatic growth and new oocyte development. In a lifetime they might release just two or three large egg masses, and spawning rates might vary individually. Future studies should focus on determining the

duration of maturity, the rate at which the oviducts fill between spawning events, and the length of intervals between spawning events. The spawning/nursery ground for the winter-spring cohort (subtropical domain) is an oligotrophic region where the flux of nutrients into the euphotic zone is probably the lowest of any oceanic environment (Cullen 1982, Eppley & Peterson 1979). Batch spawning may represent an adaptation to highly unstable environments, where a high rate of paralarval survival depends on favorable, but fortuitous and temporary, oceanographic conditions (Rocha et al. 2001). I subscribe to the concept proposed by Harman et al. (1989), whereby batch spawning might be a common strategy among tropical and subtropical squids.

Table 2-1. Definitions of somatic indices in *Ommastrephes bartramii* (BW = body weight in g) (Kidokoro & Sakurai 2008)

	Index	Definition
SCI	Stomach Content Index	$Stomach\ content\ weight \times 100/BW$
TSI	Testis Somatic Index	$Testis\ weight \times 100/BW$
OSI	Ovary Somatic index	$Ovary\ weight \times 100/BW$
ODSI	Oviduct Somatic Index	$Oviduct\ weight \times 100/BW$
AGSI	Accessory Gland Somatic Index	$Accessory\ gland\ weight \times 100/BW$
NSI	Nidamental Gland Somatic Index	$Nidamental\ gland\ weight \times 100/BW$
HSI	Hepato Somatic Index	$Digestive\ gland\ weight \times 100/BW$

Table 2-2. Mean ± SD and range values for the size (mantle length, ML) and weight (body weight, BW) of female *Ommastrephes bartramii*. Mean values are shown for: the stomach content index (SCI), ovary somatic index (OSI), nidamental gland somatic index (NSI), and Hepato somatic index (HSI)

Maturity stage	ML (mm)			BW (g)			Mean indices (%)			
	Min.	Mean ± SD	Max.	Min.	Mean ± SD	Max.	SCI	OSI	NSI	HSI
VI	416	493.6 ± 53.9	572	2165	3645.6 ± 1057.3	5650	2.6 ± 1.49	3.8 ± 0.76	2.3 ± 0.78	6.58 ± 2.05
V	415	507 ± 61.2	595	2260	4154.6 ± 1034.7	4865	1.8 ± 2.2	4.2 ± 0.93	3.8 ± 0.75	6.08 ± 1.45
IV	420	522.1 ± 54.25	600	2100	4371.6 ± 1141.7	6700	1.7 ± 1.67	4.6 ± 0.64	2.5 ± 0.50	7.58 ± 2.07

Table 2-3. Diameter of size groups of oocytes from the ovary

Size group	Diameter (mm)		
	Range	Mean	SD
I	0.05-0.09	0.07	0.045
II	0.09-0.28	0.18	0.072
III	0.27-0.45	0.36	0.051
IV	0.45-0.9	0.62	0.081
V	0.8-0.98	0.92	0.084
VI	0.9-1.1	0.99	0.028

Table 2-4. Means of potential fecundity (PF, in millions), oviductal load (OL, in thousands), Ovary index (OSI, in %), and oviduct somatic index (ODSI, in %) of *O. bartramii* collected in 2013 around Hawaii region

Stage of maturity	ML (mm)	Weight (g)	Ovary (g)	Oviducts (g)	PF	OL	OSI	ODSI
VI	520	4144	176.5	69.5	3.44	114	4.26	1.68
VI	552	4620	145	29.5	3.21	60	3.14	0.64
VI	566	5290	204	233	4.52	224	3.86	4.40
V	552	5034	234	411	7.05		4.65	8.16
V	559	5480	201	405.5	6.56		3.67	7.40
V	562	5196	260	470	7.22		5.00	9.05
IV	474	3564	154	113	3.56		4.32	3.17
IV	492	3908	141	40	2.39		3.61	1.02
IV	550	5162	272	156.5	5.53		5.27	3.03
IV	510	4640	200	140	4.65		4.31	3.02
III	424	2112	23		7.1		1.09	

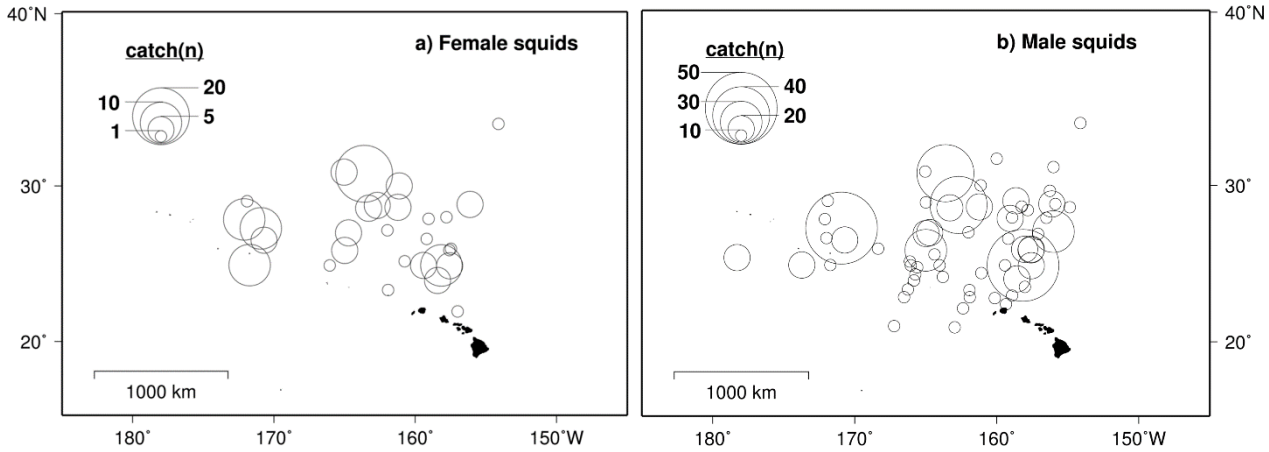

Figure 2-1. Spatial distribution of the cumulative catch of *Ommastrephes bartramii* females (a) and males (b) by the TS Hokusei Maru near Hawaii

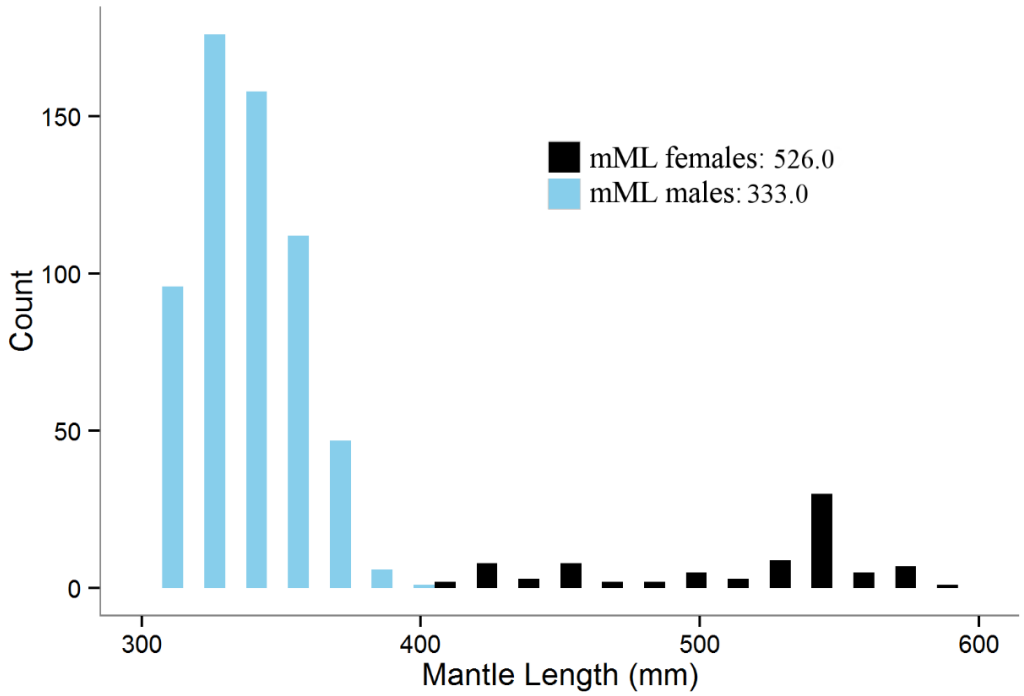

Figure 2-2. Mantle length distribution of female and male *Ommastrephes bartramii*; mML: mean mantle length

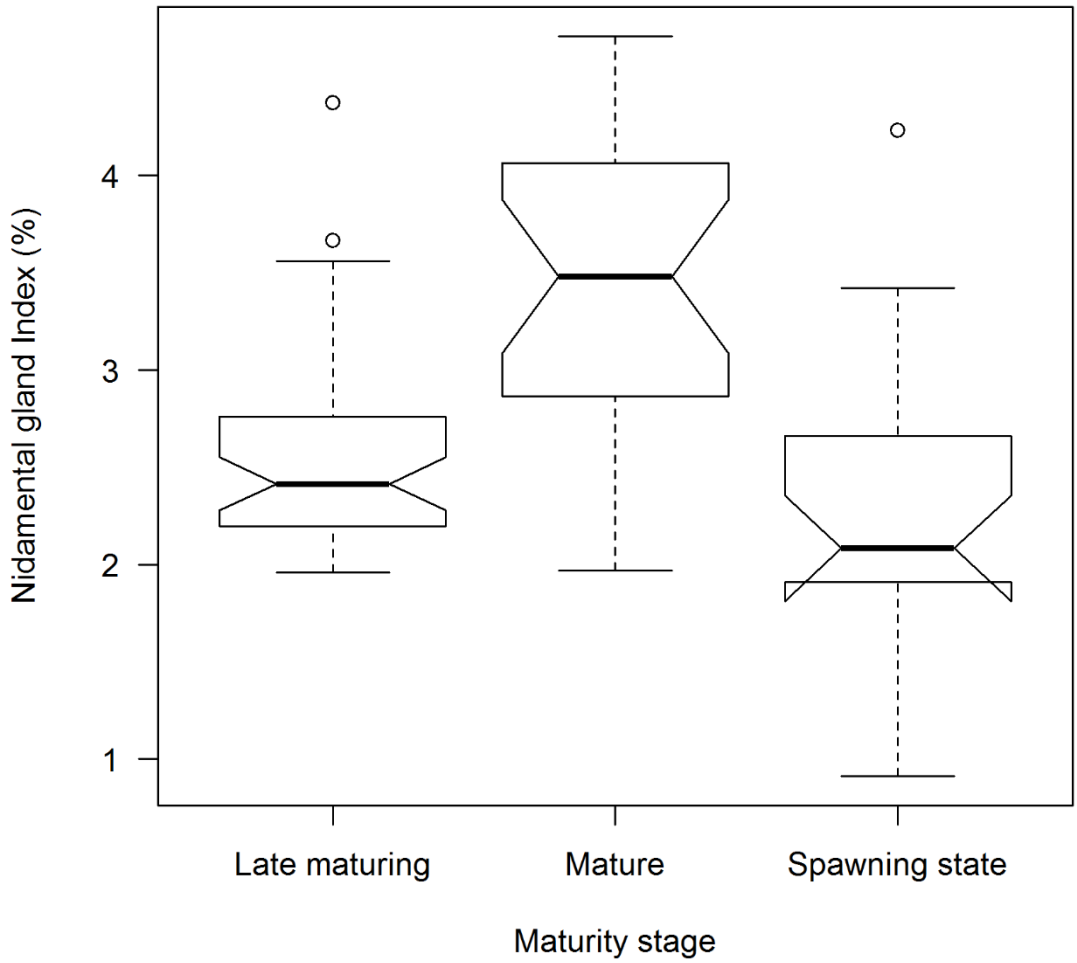

Figure 2-3. Box plots showing variation in nidamental gland somatic index among the three stages of maturity for *Ommastrephes bartramii*. The median (horizontal black lines within box plots) and its confidence interval (notches) also indicated. There were significant differences among the three stages ($P < 0.01$, analysis of variance [ANOVA])

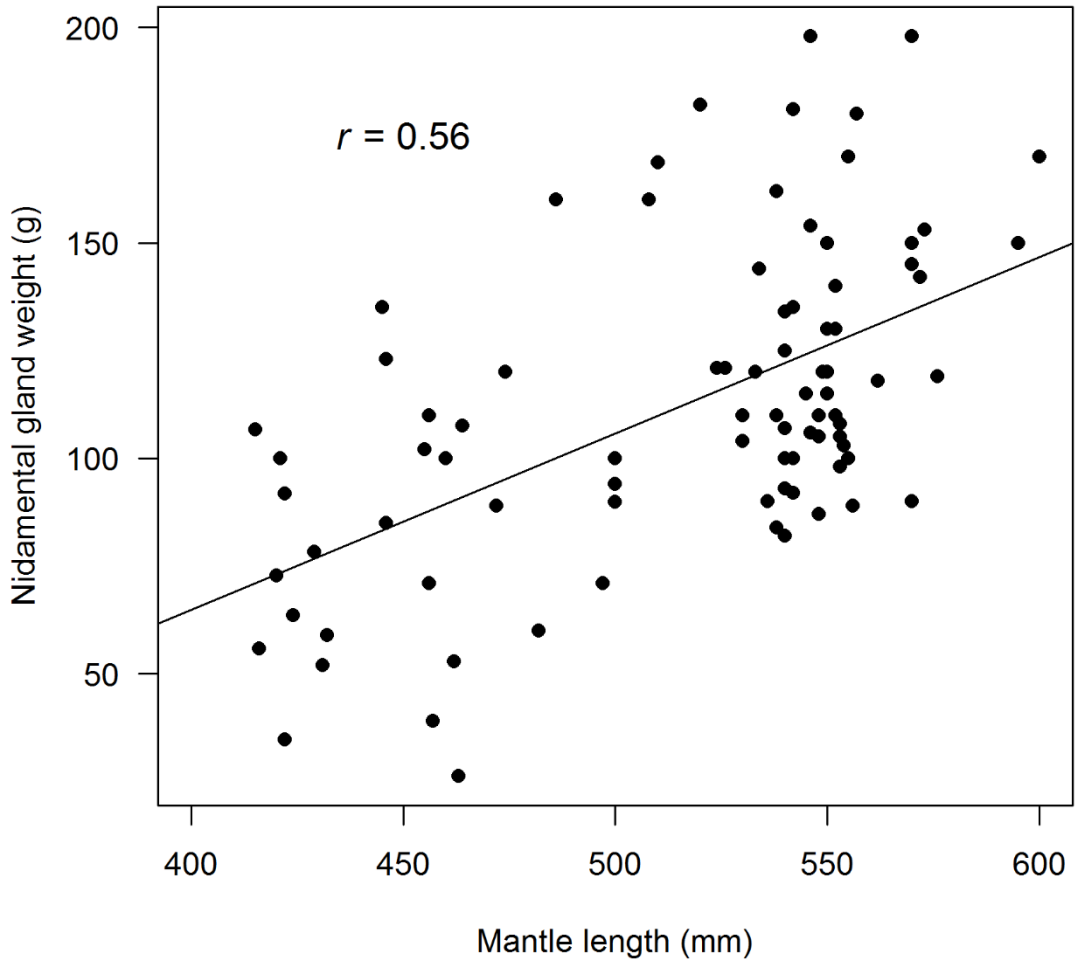

Figure 2-4. The relationship between mantle length and nidamental-gland weight of the *Ommastrephes bartramii* female. Mantle length was significantly correlated with nidamental-gland weight ($r = 0.56$, $P < 0.01$)

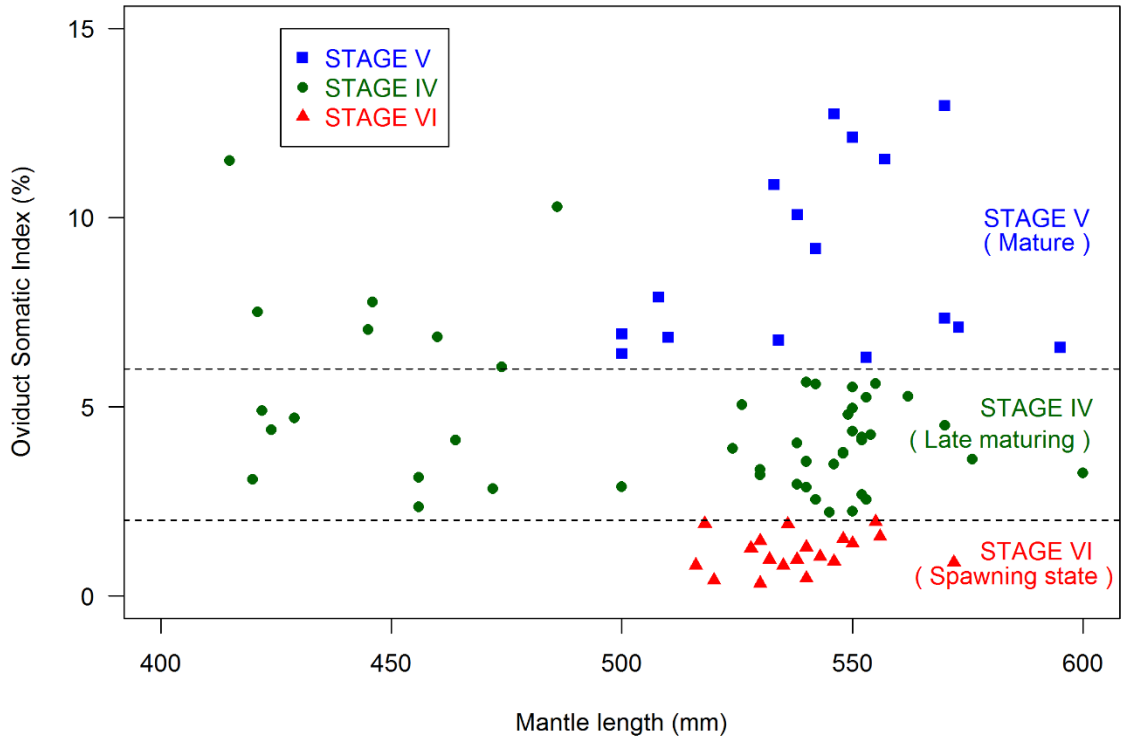

Figure 2-5. Mantle length distribution in relation to the oviduct somatic index and stages of maturity for *Ommastrephes bartramii* female

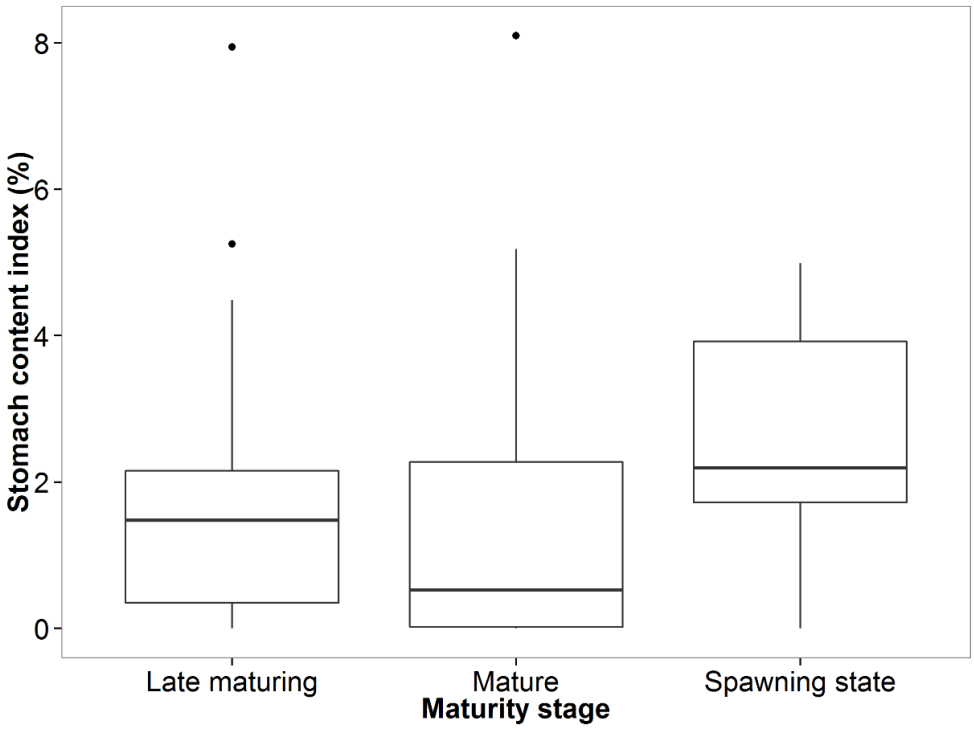

Figure 2-6. Boxplot showing variation in stomach content (SCI) among the three stages of maturity of *Ommastrephes bartramii*. There were no significant differences among the three stages ($F_{2,74}$ = 1.85, P > 0.05, ANOVA)

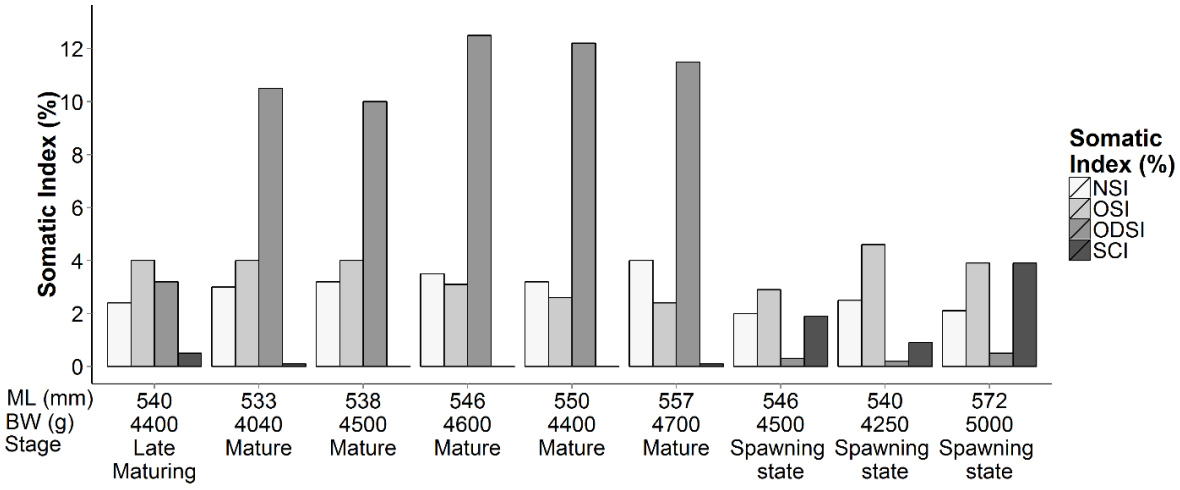

Figure 2-7. Somatic indices of the squid *Ommastrephes bartramii* at the three stages of maturity (late maturing, mature, and spawning state) collected during February 1992. BW: body weight; ML: mantle length; NSI: nidamental gland somatic index; ODSI: oviduct somatic index; OSI: ovary somatic index; SCI: stomach content index

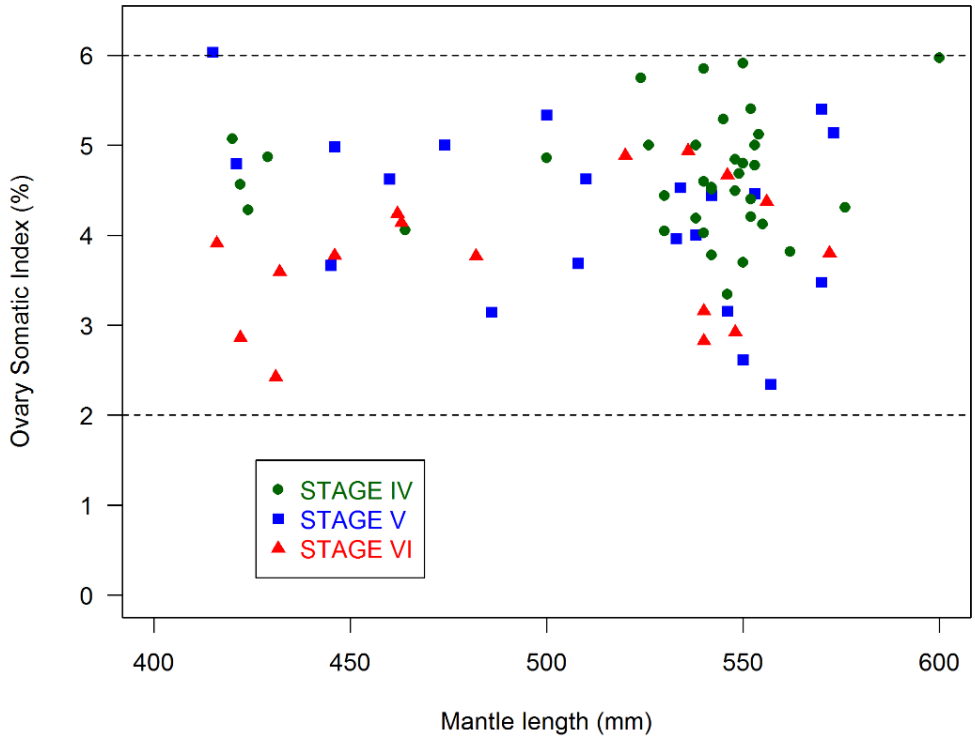

Figure 2-8. Mantle length distribution in relation to the ovary somatic index and maturity stages of *Ommastrephes bartramii*

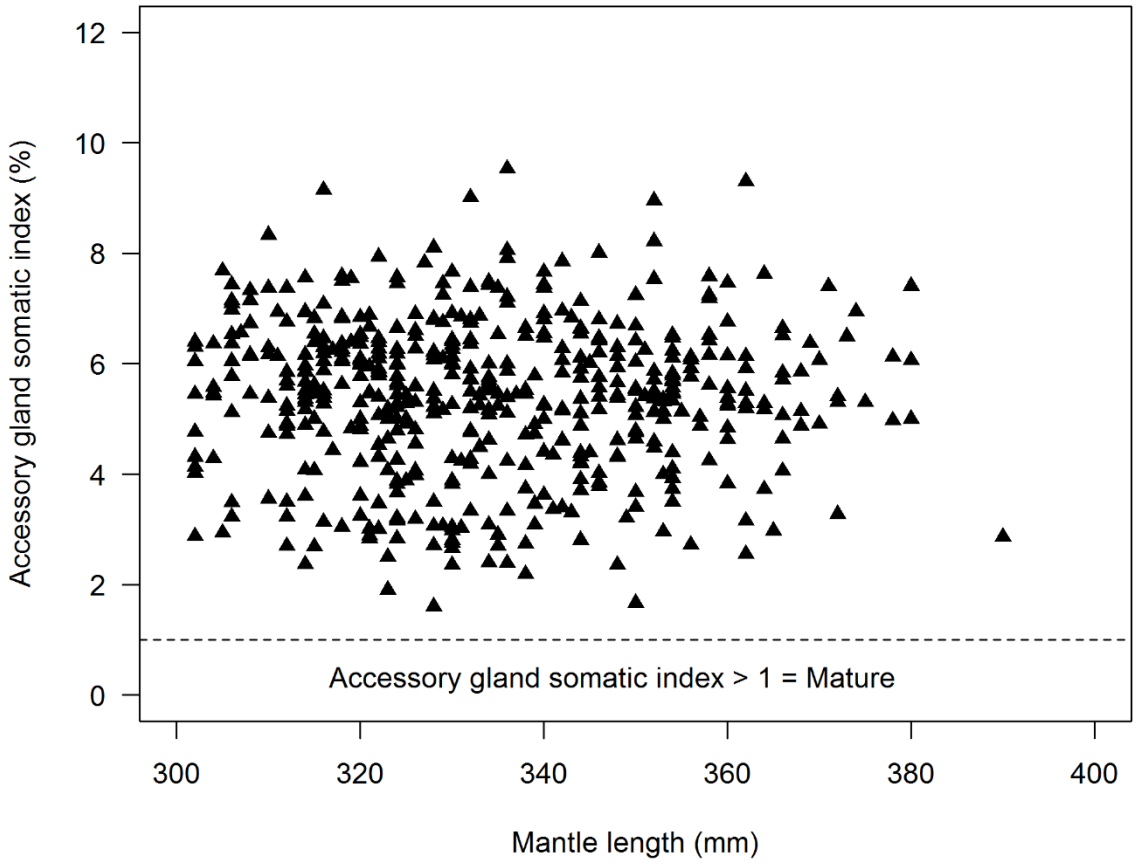

Figure 2-9. Mantle length distribution with the accessory gland somatic index of the male squid *Ommastrephes bartramii*. There was no significant correlation

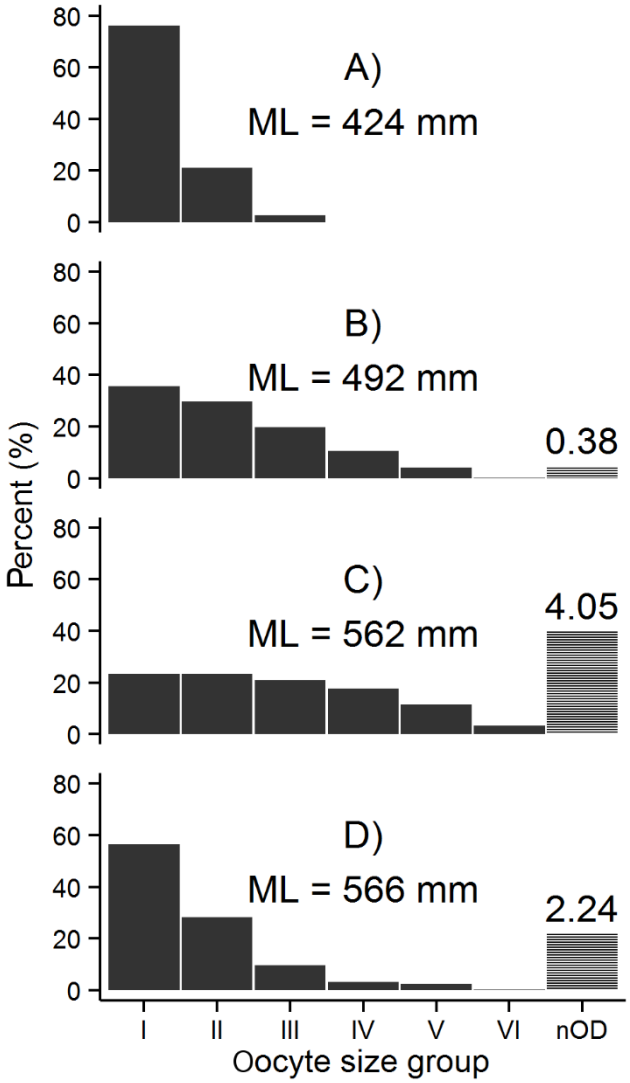

Figure 2-10. Frequency distribution of the different size groups of oocytes within the ovary of *Ommastrephes bartramii* in (A) immature, (B) maturing, (C) mature, and (D) spawning-state (after the first spawning event) females. ML: mantle length, nOD: number of oocytes in oviducts (million)

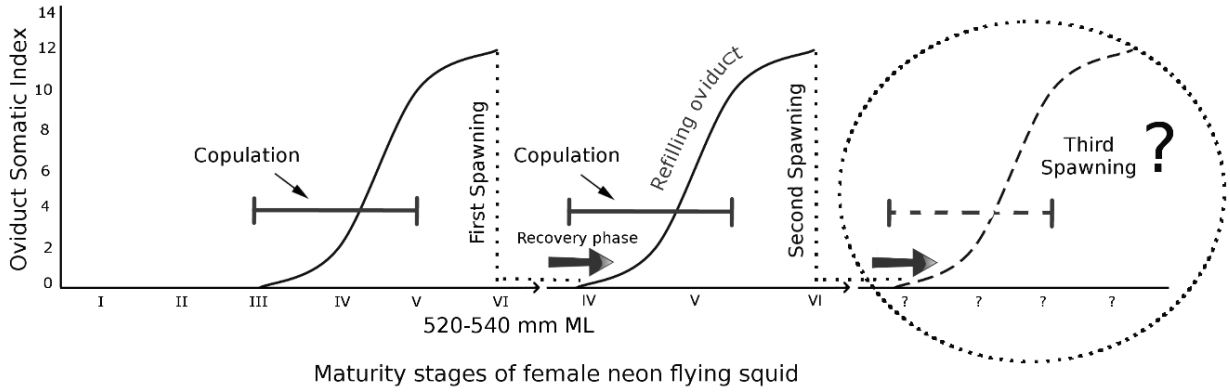

Figure 2-11. Schematic of the spawning events of *Ommastrephes bartramii*. I assumed that the first spawning event occurs at ~520–540 mm ML (mantle length). Subsequently, the female feeds, undergoes somatic growth, and refills the oviduct, before the second spawning event commences

Chapter 3. Embryonic and paralarval development

3.1. Abstract

Embryonic development was observed in three ommastrephids: *Ommastrephes bartramii*, *Sthenoteuthis oualaniensis*, and *Eucleoteuthis luminosa*. All studied embryos were obtained through artificial fertilization conducted on board the research vessel Kaiyo Maru during a cruise around the Hawaiian Islands in December 2013. The embryonic and paralarval stages of *O. bartramii* are explained in detail. The hatchling stages were compared between all three species, as well as with information for *Illex argentinus* hatchlings from the literature. The species-specific effects of the oviducal gland powder on chorion expansion were analyzed. The results indicate that the overall chemical nature of the oviducal gland was species-specific, although the chorion expansion inducer was the same across species. The addition of oviducal gland jelly also induced partial parthenogenesis in the early stages of embryonic development through the expansion of the perivitelline space and blastodisc formation.

3.2. Introduction

Developmental studies of ommastrephid squids are very limited, and few have been conducted after the preliminary work by Naef (1928) on *Illex coindetii* and O'Dor et al. (1982) on *I. illecebrosus*. Watanabe et al. (1996) provided a detailed description of the embryonic development of the ommastrephid squid *Todarodes pacificus* from the fertilized egg to paralarval stage. These authors used the criteria established by Naef (1928) and the features proposed for *Loligo* by Arnold (1965, 1990). Sakai et al. (1998) later extended Watanabe et al.'s (1996) descriptions to *Illex argentinus*. Information on the early life of *Ommastrephes bartramii*, including its spawning, embryonic, paralarval, and juvenile stages,

is relatively scarce, although it is the most commercially important oceanic squid. The present study thus aims to explore the embryonic and paralarval development of *O. bartramii* under laboratory conditions.

Artificial fertilization is especially important for the developmental analysis of pelagic squids that are not easily maintained in aquaria until spawning (Sakurai et al. 1995). Eggs collected from the ovaries of mature female and fertilized artificially permit the study of embryonic development from the earliest stages onward (Boletzky 2003). I obtained all embryos for this experiment through artificial fertilization. This study represents the first reported use of artificial fertilization in *Eucleoteuthis luminosa*. Additionally, I employed a staging series to more accurately assess embryonic development. This technique is appropriate because different embryos, even those within a single clutch, develop at slightly different rates (Kimmel et al. 1995).

The immediate microenvironment of the developing embryo is defined by the chorion microstructure, which also determines the functioning of the chorion as a filter (Boletzky 2003). In the natural egg mass, the embryo starts developing inside the chorion, which is enwrapped in gelatinous material secreted by the oviducal and nidamental glands (Boletzky 1998). Oviducal gland jelly is necessary for the formation of the first perivitelline space at fertilization (Ikeda & Shimazaki 2009). Oviducal gland jelly from one species has been shown to successfully induce chorion swelling in another species (Yatsu et al. 1999). However, the species-specific effects of oviducal gland jelly on embryonic development have not been studied.

The embryonic development of cephalopods is characterized by the yolkiness of their eggs, which measure from nearly 1 mm to approximately 30 mm in diameter (Boletzky 2003). Cleavage in cephalopods is known to be partial and discoidal; the process involves only the cytoplasmic cap at the animal pole of the zygote (Kao 1985). From the outset of

embryogenesis, the ambient temperature determines the speed of development and thus influences the duration of the process (Laptikhovsky 1991). Development is faster at higher temperatures (O'Dor et al. 1982, Sakurai et al. 1996, Boletzky 2003). The posthatching stages are designated paralarvae; their distribution is pelagic in near-surface waters during the day, and their mode of life differs distinctively from that of older conspecifics (Young & Harman 1988).

Theis chapter reports observations on the embryonic growth and effects of oviducal gland jelly in *O. bartramii*. Hatchlings of *O. bartramii* were compared with those of other ommastrephid species (*Sthenoteuthis oualaniensis* and *E. luminosa*), as well as with hatchling information from the literature (*I. argentinus*).

3.3. Materials and Methods

Mature, copulated female specimens of *O. bartramii*, *S. oualaniensis*, and *E. luminosa* were captured with jigs on hand lines on board the research vessel (RV) Kaiyo Maru (Fisheries Agency, Japan) in December 2013. Collections were made in the northern Hawaiian region between 20° and 35°N and between 150°W and 180°, near the spawning grounds of the squid.

Artificial fertilization

Copulated females were decapitated and their mantles were opened with a ventral cut to collect ripe eggs from the oviduct; viable sperm (deposited during copulation) were collected from the buccal area of the same females. Artificial fertilization of *O. bartramii*, *S. oualaniensis*, and *E. luminosa* was performed on board the RV Kaiyo Maru using standard techniques (Sakurai et al. 1995, Sakai et al. 2011, Villanueva et al. 2012). Fresh seawater collected from 500 and 1000 m depth and fortified with antibiotics (25 mg L^{-1} each of ampicillin and streptomycin) was used as a medium (Staaf et al. 2011). Petri dishes containing 150–300 fertilized *O. bartramii* eggs were maintained in incubators at 16°, 18°, 19°, 20°, 21°,

22°, 23°, 24°, 25°, and 26°C (±0.2°C). *S. oualaniensis* eggs were maintained at 20°, 21°, 22°, 23°, 24°, 25°, and 26°C (±0.2°C), and *E. luminosa* eggs were maintained at 18°, 19°, 20°, and 22°C. Treatment temperatures were chosen based on the ecologically relevant range for each species.

To induce chorion swelling during development, I added externally prepared oviducal gland jelly water (OGW). Oviducal gland powder was prepared from two sources: oviducal glands extracted from mature *T. pacificus* females before the cruise and frozen at -20°C, and fresh oviducal glands extracted from mature *O. bartramii* females collected on board. Lyophilized oviducal gland powder was used to make OGW (Sakurai et al. 1995). To study the effects of different oviducal gland powders, four batches of fertilized *O. bartramii* eggs (n = 400) were prepared. Two batches were provided with OGW prepared from *T. pacificus*, and the other two were provided with OGW prepared from *O. bartramii*. One day after incubation, during the water exchange process, one batch that started with *T. pacificus* OGW was changed to *O. bartramii* OGW, and the reverse interchanging procedure was conducted for one batch that started with *O. bartramii* OGW. The remaining two batches were provided with the same OGW throughout the experiment. This experiment was conducted in three cycles at eight incubation temperatures from 18° to 25°C.

For accurate descriptions of embryonic development, and especially for comparisons between the embryogenesis of different species, a staging system is indispensable (Boletzky 2003). I adapted the staging scheme and definitions of Watanabe et al. (1996), which were originally proposed for *T. pacificus*. Several of the descriptions for early cleavage stages (stages 6–9) are based on Boletzky's (1989) descriptions for *Loligo pealei*.

3.4. Results

Differential effects of oviducal gland jelly

*O. bartramii e*mbryonic survival was observed under treatment with OGW from two species. Embryos treated with OGW from a single species throughout the experiment successfully reached the hatching stage. However, chorions of the embryos treated with OGW from two species began to contract, and the embryos did not survive longer than a day (Fig. 3-1).

Developmental stages

For cephalopods, the speed of cleavage and subsequent embryonic development are strictly temperature dependent (Boletzky 1989). Here, I present the development of *O. bartramii* at 22°C.

Meiosis and blastodisc formation (Stages 1–3) (Fig. 3-2)

Perivitelline space expansion and blastodisc formation were observed in unfertilized eggs after the addition of OGW (partial parthenogenesis; Staaf et al. 2011). Watanabe et al. (1996) reported these stages in *T. pacificus* only after fertilization.

Unfertilized ova. Oval shape. The long axes ranged between 0.86 and 0.95 mm (mean 0.91 ± 0.028 SD), and the short axes ranged between 0.73 and 0.77 mm (mean 0.75 ± 0.022 SD).

Stages 1–3 (fertilized and unfertilized eggs). The chorion swells with the expansion of the previtelline space, and the micropyle is visible at the animal pole. The cytoplasm begins to stream toward the animal pole, segregating the blastodisc from the yolk-rich vegetal cytoplasm. This segregation continues with the first and second meiotic division.

Cleavage (Stages 4–10) (Fig. 3-3)

Cleavage is meroblastic. The first cleavage furrow marks the future symmetry plane of the embryo (Boletzky 1989), appearing near the polar bodies extruded after fertilization (Crawford 1985). During cleavage, the cells divide but remain interconnected by cytoplasmic

bridges (Crawford 2000). The first cleavage (stage 4) furrow is vertically oriented and appears normal until the 16-cell stage. The following several cleavages are oriented to the first one (Boletzky 1989). The second cleavage (stage 5) furrow is roughly perpendicular to the first. The third cleavage (stage 6) furrows are unequal in the posterior half and symmetrical in the right and left halves. The fourth cleavage (stage 7) is roughly parallel to the second furrow; the inner four cells are established as blastomeres, while the surrounding 12 cells remain contiguous at their outer margins as blastocones. At the end of the fifth cleavage (stage 8), 14 blastomeres and 18 blastocones are formed. The fifth cleavage planes are radial, except in the blastocones adjacent to the second furrow and in the posterior-medial blastocones. At stage 9, all blastocones and the blastomeres abutting the second furrow in the fifth cleavage are cleaved transradially. The orientation in the central blastomeres varies. At stage 10, the cleavage continuous asynchronously, and only the central cells remain recognizable (Boletzky 1989, Watanabe et al. 1996, Sakai et al. 1998).

Germ layers and blastoderm (Stages 11–15) (Fig. 3-4)

Stages 11–15 mark the segregation of germ layers and growth of the blastoderm. These stages are similar to those observed by Watanabe et al. (1996) in *T. pacificus* and Sakai et al. (1998) in *I. argentinus*.

At stage 11, a ring-shaped group of cells appears around the yolk mass below the margin of the blastoderm, which is caused by the marginal superposition of the single early layer of the germ layer. Cell size becomes smaller and almost uniform. The growth of the inner layer continues in stage 12, spreading toward the animal pole below the outer layer. The papilla-like yolk mass apex resembles a truncated cone in the animal pole, and the blastoderm covers half of the egg. The epibolic process continues, and the outer monolayer grows over and beyond the peripheral ring, which forms an inner layer. The inner layer spreads towards the animal pole below the outer layer. At stage 13, the blastoderm covers approximately two-

thirds of the egg. The papilla-like yolk apex gradually becomes smaller when the inner germ layer reaches a position near the animal pole. Stage 14 marks the spreading of the blastoderm edge, which forms a ring around the vegetal pole and covers over three-quarters of the egg. At stage 15, the edge of the spreading blastoderm nearly reaches the vegetal pole, and the papilla-like yolk apex disappears.

Organogenesis (Figs. 3-5 & 3-6)

At the onset of organogenesis, the thin extraembryonic blastopore lip grows over the yolk mass and becomes a densely ciliated coat. During and after the formation of the outer yolk sac envelope, the organ rudiments of the embryo proper appear as increasingly distinct tissue concentrations. At first, the areas that represent future organ complexes can be recognized, and later the individual rudiments composing them become distinct. The bilateral symmetry, which is somewhat obscured during the later cleavage stages and in the early gastrula, again becomes very clear as soon as the organ areas can be distinguished. The actual shaping of the embryonic body starts with a series of folds leading to the formation of the shell sac in the mantle, the eye and statocyst vesicles, and the stomodaeum. These processes are enhanced by the progressive radial contraction of the entire embryo cap (Boletzky 1987). The growth process shaping the mantle, fins, and funnel complex are characterized by essentially laminar spreading, which differs from folding/invaginations (Boletzky 2003).

Stage 16 The yolk sac envelope and embryo proper increase in size. The primordium of the shell gland is evident as a projection. Chorion expansion begins.

Stage 17 Eye and mouth primordia are evident with the vertical expansion of the embryo. Mantle and funnel development begins, as do invaginations of the shell gland.

Stage 18 Elevation of the mantle, funnel, eyes, tentacle clubs, and arms II begins. The shell gland is closed. The embryo revolves slowly in the perivitelline space. Chorion expansion continues.

Stage 19 Formation of the mantle margin and mantle cavity begins. The funnel folds are clearly visible, as are the primordia of the tentacle clubs and arms II. Yellow pigmentation of eyes begins.

Stage 20 Primordia of the statocysts appear, as do faint primordia of arms I. Retina pigmentation begins. The mantle cavity spreads into the dorsum. The anterior parts of the funnel folds unite by the margin. Faint chromatophores become visible on the ventral and dorsal mantle margins.

Stage 21 Funnel folds are completely fused, and statocyst invagination begins. The optic stalks increase in height.

Stage 22 Three rows of chromatophores are visible on the mantle. The funnel folds fuse and form a triangular shape with a slit at the midline. The first sucker primordia appear on the tentacle clubs.

Stage 23 Four rows of chromatophores are visible on the mantle. The mantle reaches the posterior margin of the funnel. The funnel folds form a complete tube.

Stage 24 The mantle occasionally contracts. Optic ganglia appear on the posterior sides of the eye vesicles. Four sucker primordia on each tentacle and one sucker primordium on each arm are clearly visible. The chromatophores on the mantle become larger and more distinct.

Stage 25 Yolk is transferred from the yolk sac of the cephalic region to the mantle cavity. The primordia of ventral organs, such as the gills, branchial hearts, systemic heart, stomach, caecum, and anal knoll, become prominent as three swellings. Bases of the two tentacle clubs come together in the midline. The embryo slowly glides backwards by ciliary action along the expanded chorion. Four rows of red and brown chromatophores are clearly visible, with one very large chromatophore at the anteroventral side of the mantle.

Stage 26 Embryo movement inside the chorion becomes more active and faster. The yolk sac contracts at the cephalic region. A split becomes evident in the posterior apex of the internal yolk sac. The tentacles are nearly fused.

Posthatching (paralarval stages) (Figs. 3-6 & 3-7)

Stage 27 Hatching. Arms I and II each possess a single large sucker. Primary lids cover the eye vesicles. The tentacles are completely fused. The yolk sac at the cephalic region has nearly disappeared, and the gutter of the posterior apex of the inner yolk sac deepens. Statoliths are evident in both statocysts. The funnel locking apparatus begins to form on the posterior edges of the funnel. The ink sac is first visible as a small vesicle on the primordium of the rectum, but no ink is present.

Stage 28 Two lateral proboscis suckers begin to grow more quickly than the arm suckers. Fin primordia appear on the apex of the mantle. The primordia of arms IV are first visible as two faint swellings on the bases of the fused tentacles between both eyes in the ventral view. Head chromatophores are first visible. The heart-gill complex is clearly visible, and the branchial hearts occasionally pulsate. Ink begins to be concentrated in the ink sac.

Stage 29 Very large lateral proboscis suckers become very clear. The fused tentacles begin to elongate as a proboscis. The digestive and salivary glands are visible. Faint swellings of anal papillae appear on the anterior margin of the rectal primordium. The primordia of the gills and branchial hearts are evident. The digestive gland is visible as a faint swelling under the rectal primordium. The stomach is visible. The ink sac fills with ink, which the hatchling ejects when stimulated.

Stage 30 The inner yolk sac has contracted and separated from the posterior end of the mantle. The eyes are covered by primary lids. The intestine continues to grow, and anal papillae are evident. The proboscis is extensible.

Stage 31 Embryos continuously move very actively in the petri dish and frequently squirt ink when disturbed. The inner yolk sac becomes smaller, and its posterior part lies behind the stomach. The caecum is visible, and the digestive gland grows larger. The stomach shows peristaltic movement. The suckers of the proboscis exhibit primordia of chitinous rings.

Stage 32 The inner yolk sac becomes smaller. The proboscis stretches and contracts with sucker movement. Branched gills are visible.

Stage 33 All internal organs increase in size. Proboscis suckers can adhere to the walls of the petri dish, and the embryos can hold their positions during mild mechanical stimulation.

Stage 34 Seven days after hatching. The yolk is almost entirely consumed. The embryos are very weak and slow swimming, but their fins are well developed, with active flapping. All paralarvae died once the yolk was completely exhausted.

Comparison with *S. oualaniensis* and *E. luminosa*

Comparisons of *O. bartramii* hatchlings with those of *S. oualaniensis* and *E. luminosa* are shown in Table 3-1. The early stages of development (until stage 15) were similar between the three species. Details for the paralarval stages of *S. oualaniensis* and *E. luminosa* were not observed, as experiments on these species, always a secondary priority on board, were limited by time and available resources.

3.5. Discussion

Effects of oviducal gland jelly

Yatsu et al. (1999) successfully used oviducal gland powder prepared from *O. bartramii* to induce chorion expansion in *Dosidicus gigas* eggs. Staaf et al. (2008) used oviducal gland powder from *D. gigas* but found that it was no more effective than that from *O. bartramii*. In the present study, oviducal gland powder prepared from *T. pacificus* caused

chorion expansion in *O. bartramii, S. oualaniensis,* and *E. luminosa.* These results suggest that the oviducal gland may contain no species-specific component that induces chorion expansion. These observations may be broadly indicative for ommastrephids and perhaps for other oegopsid squids (Staaf et al. 2011). However, when the source of oviducal gland jelly was changed after the embryos started to grow, chorion expansion was arrested, and the embryo showed deformities (Fig. 3-1). These changes resulted in embryo mortality and indicated that the chemical nature of the oviducal gland may be species-specific, although the chorion expansion inducer itself may not differ. For comparison, the egg capsules of the cuttlefish *Sepia officinalis* are known to release a species-specific pheromonal peptide that acts as a specific spawning stimulator in other sexually mature females (Zatylny et al. 2000). Future studies are necessary to identify the structure and function of the inducer and species-specific compounds.

Partial parthenogenesis

Perivitelline space expansion and blastodisc formation were observed in unfertilized eggs after the addition of OGW. Staaf et al. (2011) observed this phenomenon in *D. gigas* and proposed the renaming of stages 1–3 in Watanabe et al. (1996) from "Fertilization and Meiosis" to "Meiosis-Blastodisc" (Fig. 3-2). In nature, spawned eggs (both fertilized and unfertilized) are always in contact with the oviducal gland, as the gelatinous substance composing the egg mass consists of secretions from the oviducal and nidamental glands (Boletzky 1998). Unfertilized eggs thus have the chance to develop partial parthenogenesis. The implications of such development in the wild have yet to be understood. This phenomenon may be a common trait among members of the family Ommastrephidae. It is important to determining whether parthenogenesis inhibits artificial fertilization (Staaf et al. 2011).

Embryonic growth

The present study establishes an atlas for the normal development of *O. bartramii* from fertilized eggs to paralarvae. The stages are mainly based on morphological features and can be distinguished clearly under a stereomicroscope. These observations of embryonic development correspond closely with those of other ommastrephids, including *T. pacificus* (Watanabe et al. 1996) and *I. argentinus* (Sakai et al. 1998).

Although the cleavage phase of *Loligo* is similar to that of the ommastrephids, a significant difference occurs in blastoderm formation. In ommastrephids, the cleavage furrows reach the equator of the eggs, whereas the furrow length of *Loligo* represents only a fraction of the egg perimeter (Watanabe et al. 1996). Until stage 15, embryonic development was similar across all studied ommastrephids and exhibited a meroblastic cleavage pattern. Stage 16 marks the onset of embryogenesis, and species-specific characters were expected to develop from this stage onward. Some of the species-specific characters used for the identification of paralarvae collected from the wild had already started appearing in the hatching stage, such as, the large lateral proboscis suckers and chromatophore patterns of *O. bartramii* (Young & Hirota 1990) and *E. luminosa* (Wakabayashi et al. 2002). Conversely, several characters were common across the species, such as the retarded appearance of arms IV in embryogenesis and complete absence of arms III.

The natural hatching stage is unknown for any ommastrephid. At the hatching stage in the laboratory, ommastrephids have many characters in common, with several species-specific characters (Table 3-1). In my experiments, hatching occurred primarily in stages 26-28, with a few individuals also hatching at stage 25. Similar results were obtained for *S. oualaniensis* and *E. luminosa* (Fig. 3-8). The body size of *O. bartramii* at hatching was very small (Table 3-1), as was expected due to the smaller egg size of this species in comparison with that of other ommastrephids. All hatchlings possessed a large amount of unconsumed inner yolk while hatching. This yolk content allowed the hatchlings to remain alive for at least

seven days after hatching without feeding. In nature, hatching may occur at a later stage after more inner yolk has been consumed, because on board conditions involve continuous mechanical stimulation due to the movement of the ship, which has been known to induce early hatching (Watanabe et al. 1996, Sakai et al. 1998). This flexibility of hatching conditions suggests the existence of a hatching competence phase rather than a morphological and/or physiologically well-defined hatching stage (Boletzky 1994). This flexibility may offer a means of optimizing post-hatching survival (Boletzky 2003).

For the Oegopsida, the differentiation of the respiratory and digestive organs is relatively delayed due to their comparatively small yolk sac and egg sizes. These characters could be related to a reproductive strategy of paralarval dispersion in the open ocean (Watanabe et al. 1996). Due to the recent standardization of artificial techniques (Villanueva et al. 2012), ommastrephid embryos can be easily obtained in large numbers for early-stage studies. These embryos are also translucent, allowing for the observation of internal structures in vivo, and may be a useful model system for developmental biology studies.

Table 3-1. Comparison of stage 27 (average hatching stage) for *Ommastrephes bartramii* with the hatching stages of other ommastrephids

Character	*Ommastrephes bartramii*	*Sthenoteuthis oualaniensis*	*Eucleoteuthis luminosa*	*Illex argentinus* (Sakai et al. 1998)
Hatching	Stage 27	Stage 26	Stage 26	Stage 30
Mean size at hatching (mm)	1.15	1.31	1.07	1.59
Arm sucker	Small	Large	Small	Small
Ratio of proboscis suckers (medial:lateral）	1:2	1:1	1:1.5	1:1
Ink sac	Very large	Large	Large	Small
Fin primordia	Stage 28	Stage 28	Stage 28	Stage 23
Arm III	No	No	No	No
Arm IV	Retarded	Retarded	Retarded	Retarded

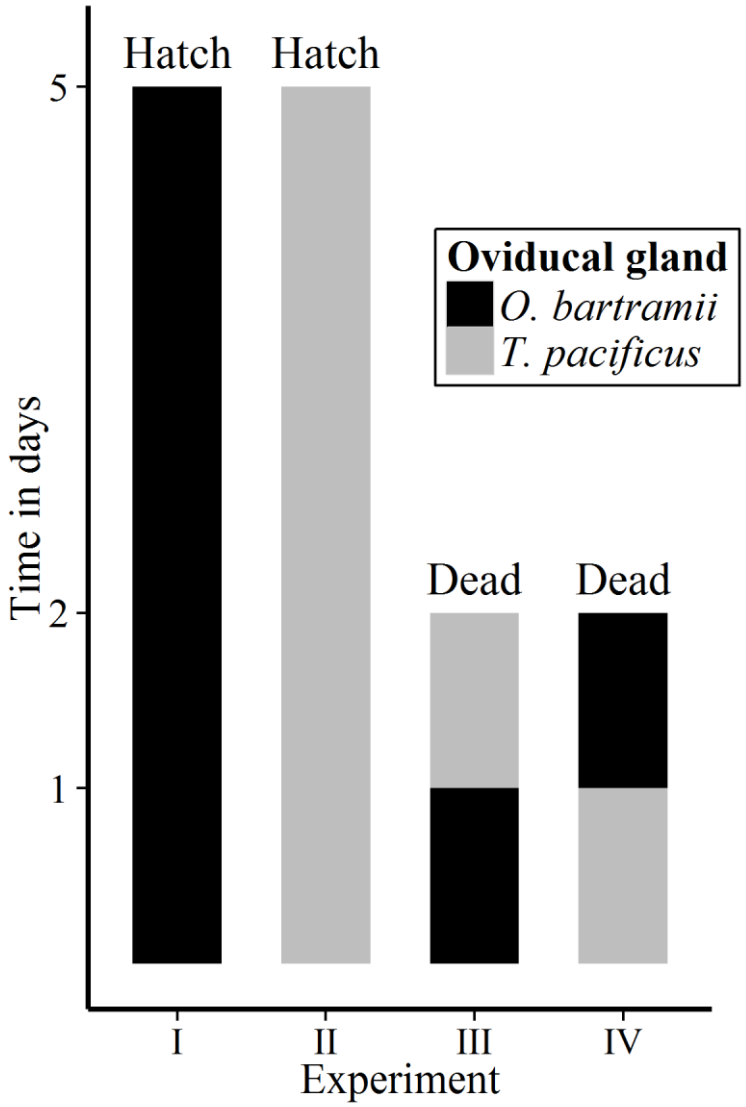

Figure 3-1. *Ommastrephes bartramii* embryonic survival under treatment with oviducal gland jelly water (OGW) from *O. bartramii* and *Todarodes pacificus* at 22°C: Embryos treated with OGW from a single species throughout the experiment successfully reached the hatching stage. Embryos for which the OGW origin was switched did not survive for over a day. Exp I: OGW prepared from *O. bartramii*, exp II: OGW prepared from *T. pacificus*, exp III: started with OGW from *O. bartramii*, and then switched to *T. pacificus*, exp IV: started with OGW from *T. pacificus*, and then switched to *O. bartramii*

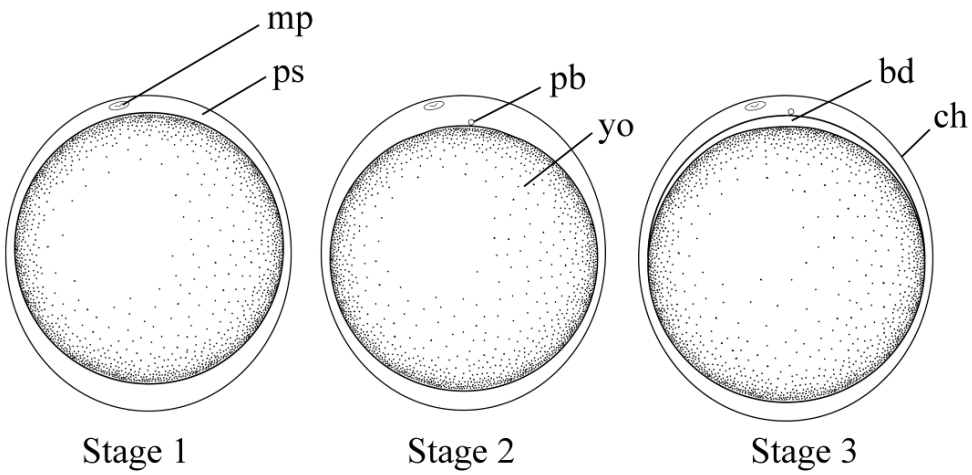

Figure 3-2. Meiosis and blastodisc formation of an *Ommastrephes bartramii* embryo in the lateral view. mp: micropyle; ps: perivitelline space; pb: polar body; yo: yolk; bd: blastodisc; ch: chorion

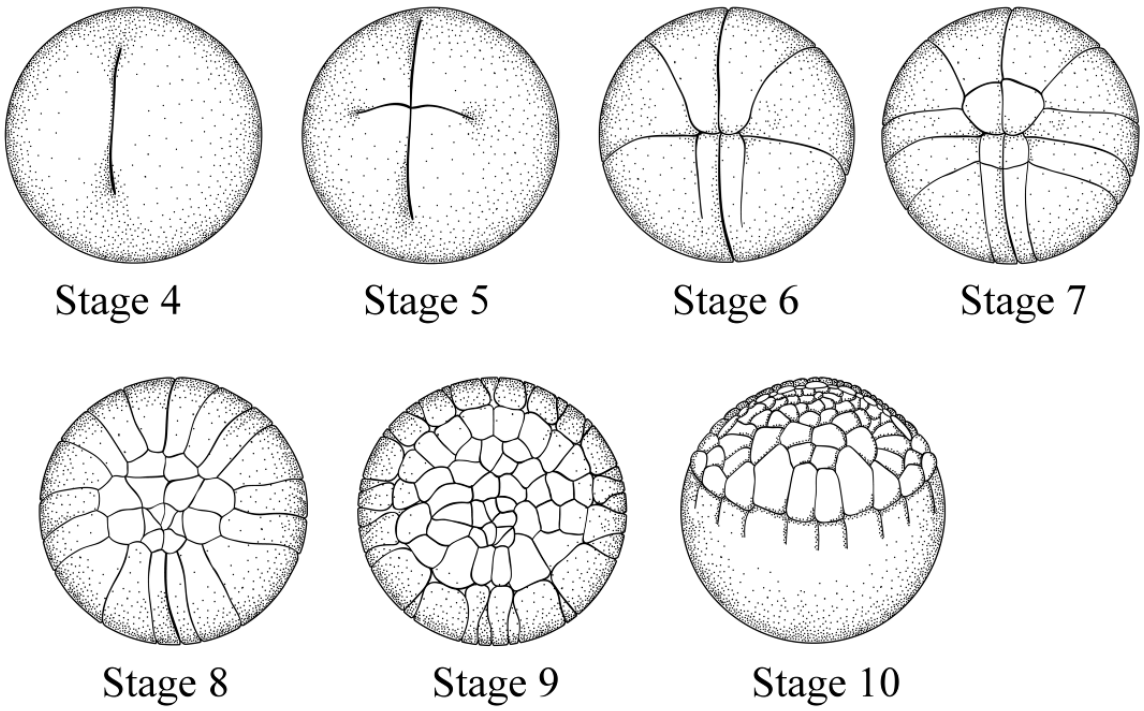

Figure 3-3. Embryonic development of *Ommastrephes bartramii* through the cleavage stages. The chorion is omitted

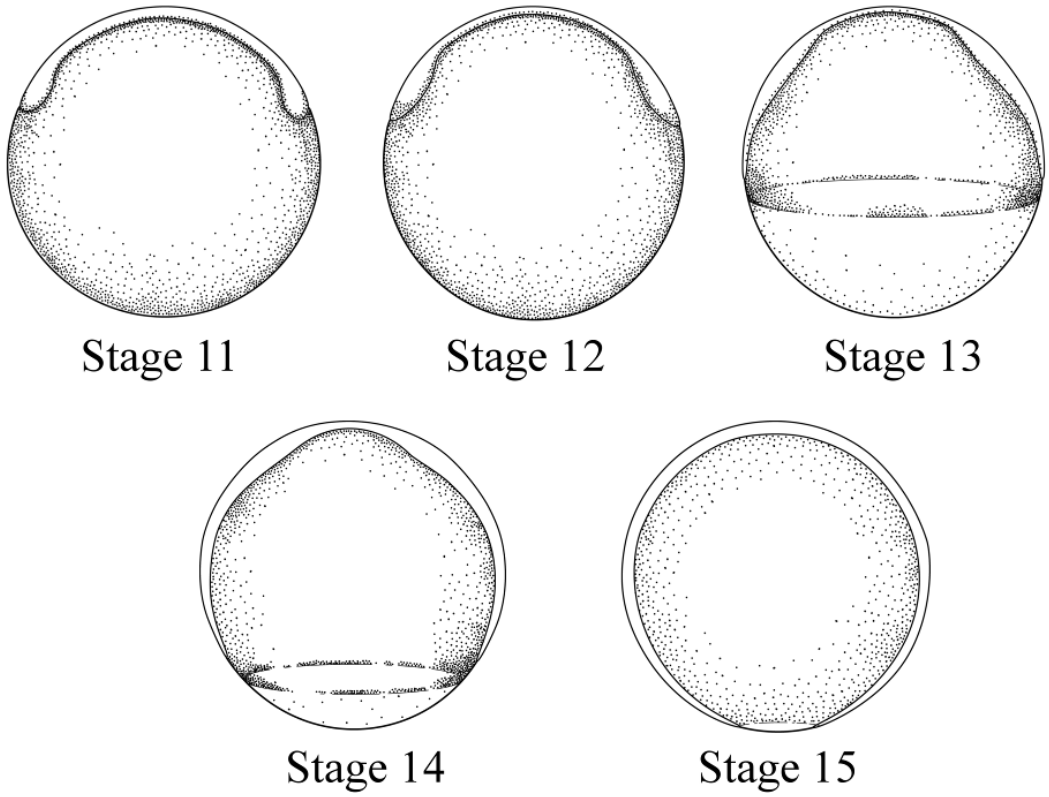

Figure 3-4. Embryonic development of *Ommastrephes bartramii* through the segregation of germ layers and growth of the blastoderm. The chorion is omitted

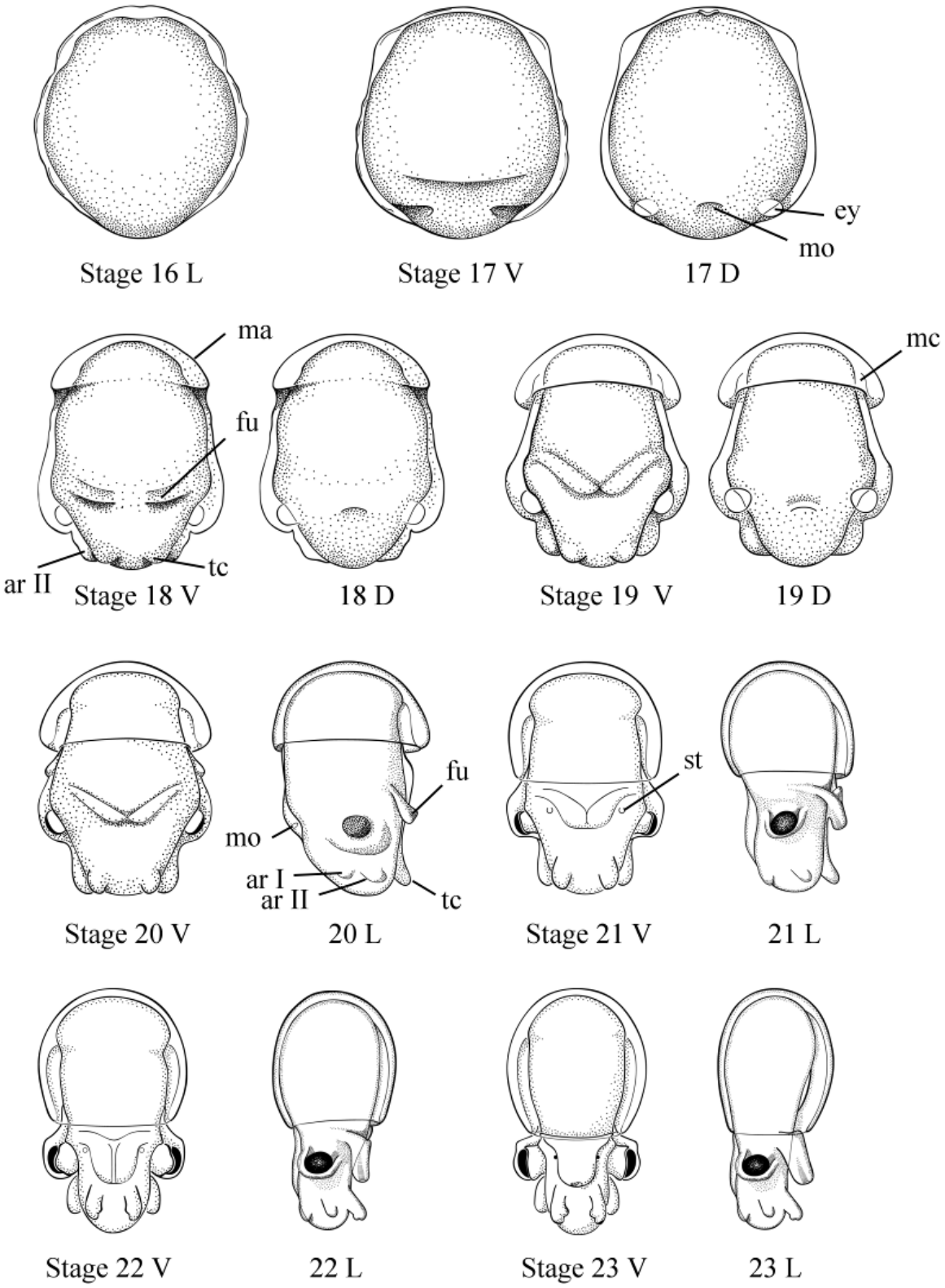

Figure 3-5. Embryonic development of *Ommastrephes bartramii* from stage 16 to stage 23. D, L, or V with a stage number indicates a dorsal, lateral, or ventral view, respectively. The chorion is omitted. ar: arm; ey: eye; fu: funnel; ma: mantle; mc: mantle cavity; mo: mouth; st: statocyst; tc: tentacle club

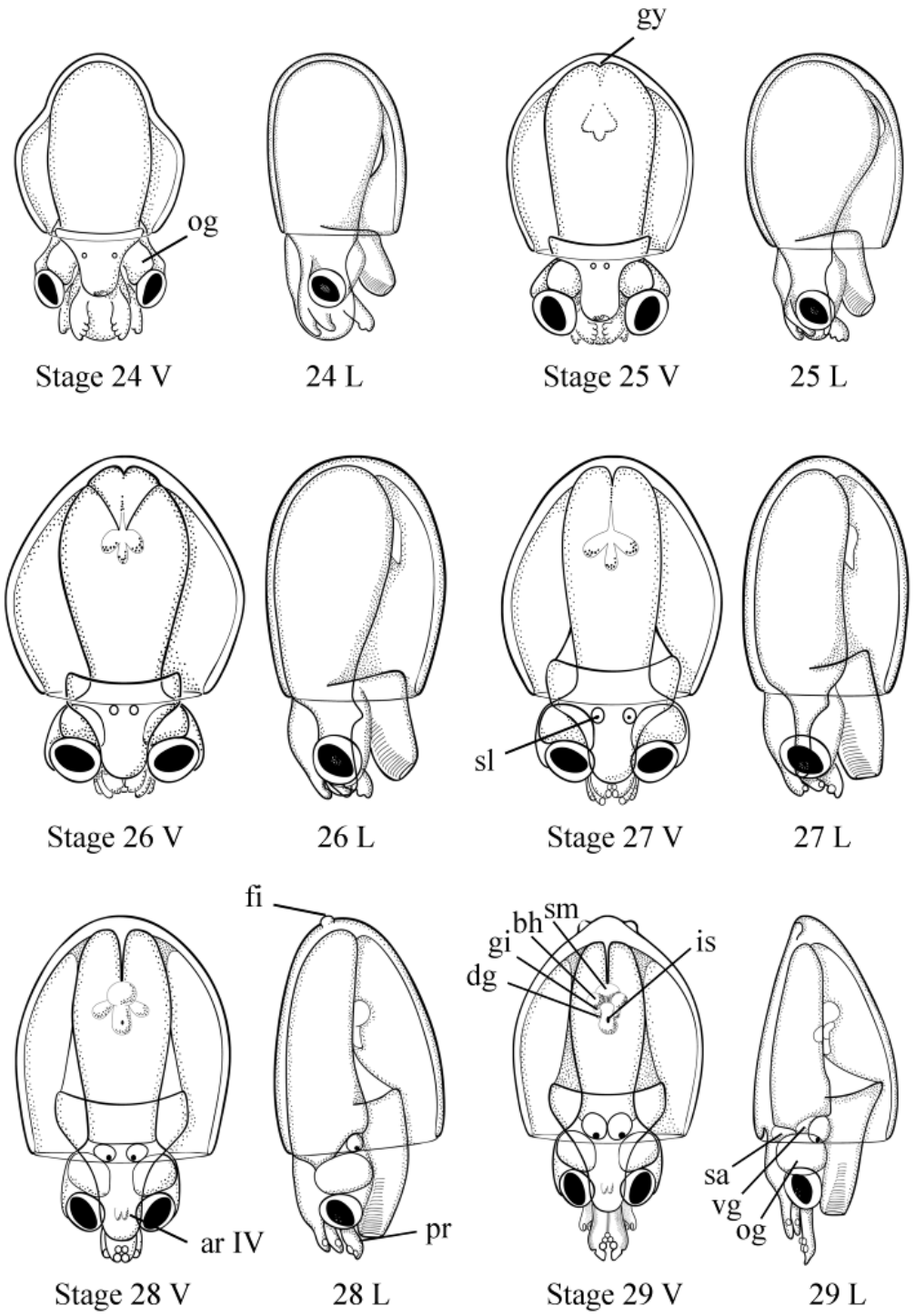

Figure 3-6. Embryonic development of *Ommastrephes bartramii* from stage 24 to stage 29. D, L, or V with a stage number indicates a dorsal, lateral, or ventral view, respectively. The chorion is omitted. ar: arm; bh: branchial heart; dg: digestive gland; fi: fin; gi: gill; gy: gutter of the inner yolk sac; og: optic ganglion; pr: proboscis; sa: salivary gland; sm: stomach; vg: visceral ganglion

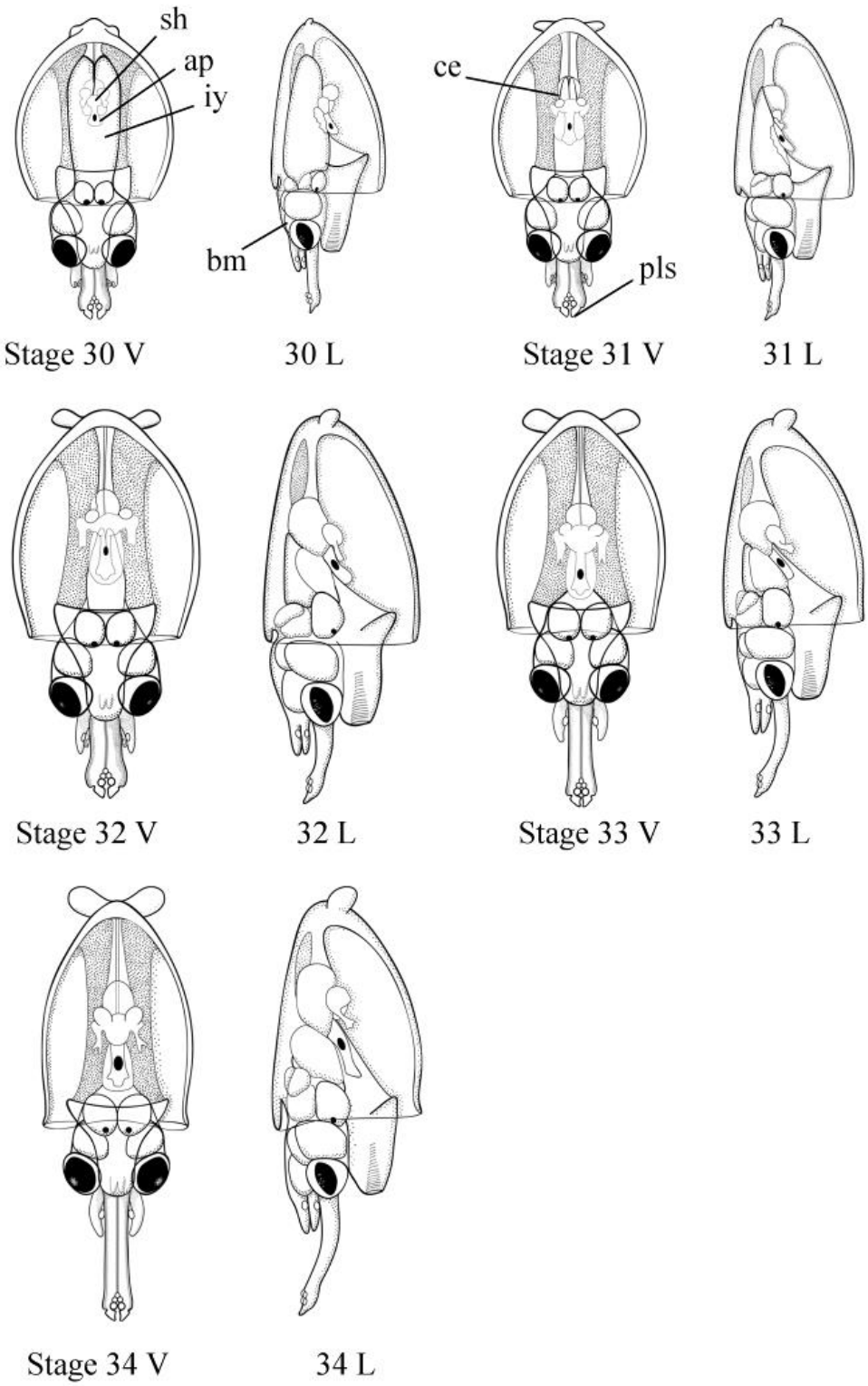

Figure 3-7. Embryonic development of *Ommastrephes bartramii* from stage 30 to stage 34. D, L, or V with a stage number indicates a dorsal, lateral, or ventral view, respectively. ap: anal papilla; bm: buccal mass; ce: caecum; iy: inner yolk; pls: proboscis lateral sucker; sh: systematic heart

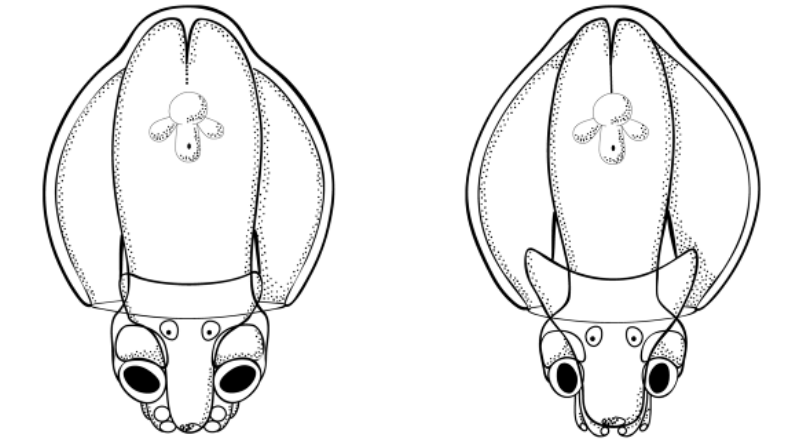

Figure 3-8. Hatchling stages of *Sthenoteuthis oualaniensis* and *Eucleoteuthis luminosa*

Chapter 4. Effects of temperature on embryonic development

4.1. Abstract

Studies on the relationships between the early life stages of the *O. bartramii* and oceanographic conditions are essential for understanding the spatial and temporal distribution patterns of this ecologically and economically important species. In this study, *O. bartramii* eggs were artificially fertilized and incubated at temperatures found in its known distribution range (16–26°C). Complete organogenesis with normal successive cleavage and the distinct continuity of morphological features was limited to between 18° and 25°C. Experimental rearing, cruise-collected specimens, and oceanographic data confirmed that the optimal temperature range for paralarval survival around Hawaii is 18–25°C. Embryos reared at 16°C showed abnormal organogenesis; however, normal development resumed when the embryos were transferred to 22° or 24°C after blastoderm formation. This study highlights the flexible strategy of ommastrephid embryo development and illustrates how information on paralarval behavior and oceanographic data (sea surface temperature) may be combined to improve our understanding of the factors that influence the survival of this species.

4.2. Introduction

Ommastrephid squids are the most abundant, widely distributed, and ecologically active family of cephalopods (Roper et al. 2010). This group support the largest fisheries for invertebrates in the world; however, little is known about their embryonic development and paralarval behavior—characteristics that are critical for better understanding their life history (Staaf et al. 2011).

Cephalopods are ecological opportunists adapted to exploit favorable environmental conditions (Rodhouse 2013). The embryonic development of cephalopods is highly temperature dependent, and the existence of a temperature minimum for development is common in ommastrephid (e.g., 12.5°C for *Illex illecebrosus* [O'Dor et al. 1982], 14°C for *Todarodes pacificus* [Sakurai et al. 1996], 13°C for *Illex argentinus* [Sakai et al. 1999] and 15°C for *Dosidicus gigas* [Staaf et al. 2011]). Nevertheless, the thermal limits of oceanic species, such as *O. bartramii*, are largely unknown. Thermal limits determine the spawning grounds, recruitment levels, and spatiotemporal ranges of paralarvae (O'Dor et al. 1982, Sakurai et al. 2000, Staaf et al. 2011); thus, it is important to understand these ranges and factors that might influence them. In particular, embryonic development and its dependence on temperature are of particular interest in the context of global warming and the resultant warming of oceans (Rodhouse 2013, Rosa et al. 2012, Xavier et al. 2014).

The North Pacific population of *O. bartramii* comprises two seasonal cohorts (autumn and winter–spring). The population makes an annual round-trip migration between its subtropical spawning grounds and its northern feeding grounds near the Subarctic Boundary (Yatsu et al. 1998, Bower & Ichii 2005). The main spawning and nursery ground of the autumn cohort occurs in the subtropical frontal zone (STFZ) of the North Pacific Ocean. This location is characterized by enhanced productivity in winter because of its proximity to the transition zone chlorophyll front (TZCF). In comparison, the spawning and nursery ground of the winter-spring cohort occurs within the subtropical domain (STD), which is less productive (Ichii et al. 2009). The STFZ is defined by a sea-surface salinity range of 34.6 to 35.2, which generally occurs at ~29 to 34°N, with the STD occurring south of the STFZ (Ichii et al. 2009, Roden 1991). Prevailing regional oceanographic features (particularly fronts) in combination

with temperature impose limits on the geographical dispersal of cephalopod paralarvae (O'Dor et al. 1982, Villanueva et al. 2012, Moreno et al. 2008).

The probability for the successful collection of wild egg masses and live paralarvae of ommastrephids is very low; moreover, it is also difficult to obtain these resources by spawning captive brood stock maintained in aquaria. As an alternative, artificial fertilization techniques have provided valuable information about the early development of oceanic squids, and may provide material for larval rearing experiments that would help improve our understanding of the physiology and ecology of paralarvae (Sakurai et al. 1995, Villanueva et al. 2012). In the first rearing experiments of *O. bartramii* paralarvae under different temperatures, Sakurai et al. (1995) reported that the duration of embryonic development to hatching decreased with increasing temperature. However, because this study focused on developing an artificial fertilization technique, the optimal thermal limits for embryonic development were not assessed (Sakurai et al. 1995).

Ommastrephids are pelagic species, spawn fragile, neutrally buoyant egg masses, have planktonic paralarvae, and use large-scale current patterns for larval transport and migration (Bower & Sakurai 1996, Sakurai et al. 2000, Boyle & Rodhouse 2005, Staaf et al. 2008). The planktonic rhynchoteuthion paralarval stage experience high mortality rates; therefore, spawning success and paralarval survival rates (which are influenced by large interannual habitat variability) are the principal factors that determine recruitment levels (Roper et al. 2010, Ichii et al. 2011). Growth and ontogenetic changes in squid paralarvae are largely linked to water temperature (Sakurai et al. 2000, Staaf et al. 2011). Rosa et al. (2012) observed the embryonic development of loliginid squid under projected near-future warm conditions and found that abiotic conditions inside the eggs promote metabolic suppression followed by premature hatching. Moreover, less-developed hatchlings showed a greater

incidence of malformations. However, only limited data on environment-related changes in survival are available for ommastrephid paralarvae. Because temperature influences the embryonic growth (Sakurai et al. 1995) and the distribution of the paralarvae of this species in the surface layers (Harman & Young 1985, Saito & Kubodera 1993, Sakurai et al. 1995), sea surface temperature (SST) is an important factor determining the distribution range and survival of paralarvae.

The thermal limits for successful embryonic development in *O. bartramii* are unknown. The present study aims to identify the thermal range for successful development until hatching in vitro and verify the results through the field collection of specimens in relation to relevant oceanographic data (SST).

4.3. Materials and Methods

Sampling

Mature, copulated females (n = 11; dorsal mantle length (DML), 533 ± 32.4 mm, mean ± SD) were captured with jigs on hand lines on board the RV Kaiyo Maru (Fisheries Agency, Japan) in December 2013. Sampling was conducted in the northern Hawaiian region between 20 and 35^{o}N and between 150 ^{o}W and 180 around its spawning ground (STFZ) (Figs. 4-1a & 4-1b). Paralarvae were collected during the same cruise by horizontal towing using a multiple opening and closing net with an environmental sensing system (MOCNESS, 1 m2, 0.335-mm mesh size), a Nackthai net (0.50-mm mesh size), and a ring net (2-m diameter, 0.526-mm mesh size). For details on net operations, see Sakai et al. (2014). The paralarvae were immediately removed from the catch and identified on board based on morphological (Sweeney et al. 1992) and DNA analyses (Wakabayashi et al. 2006).

In addition to the 2013 sampling, long-term data on mature and post-spawning females (n = 85; DML, 526.7 ± 33.6mm, mean ± SD) collected on board theTS Hokusei Maru

(Hokkaido University) were used to determine the favorable SST range. Reproductive maturity was assessed based on the maturity stages I through VII proposed by Nigmatullin (1989). These stages were adjusted for *O. bartramii* (Vijai et al. 2014), whereby stages I and II were defined as immature, stage III as early-maturing, stage IV as late-maturing, stage V as mature, stage VI as spawning state (after the first spawn), and stage VII as true spent. Stage VII squid were not collected during any of the cruises. Sampling on board the TS Hokusei Maru was performed by manual jigging, gill-netting, and trawling during 11 cruises from February to March each year from 1990 to 2000, between 20 and 35°N, and between 150 °W and 180°. The spatial and temporal sampling ranges of the RV Kaiyo Maru and TS Hokusei Maru coincided with the spawning grounds and season of the winter–spring cohort of *O. bartramii* (Bower & Ichii 2005).

Artificial fertilization

Artificial fertilization of *O. bartramii* was performed on board the RV Kaiyo Maru using standard techniques (Sakurai et al. 1995, Sakai et al. 2011, Villanueva et al. 2012). Fresh seawater collected from 500 and 1000 m depth was fortified with antibiotics (25 mg L^{-1} each of ampicillin and streptomycin) and used as the experimental medium (Staaf et al. 2011).

Embryonic development

Petri dishes containing 150–300 fertilized eggs were maintained in incubators at 16°, 18°, 19°, 20°, 21°, 22°, 23°, 24°, 25°, and 26°C (±0.2°C). Treatment temperatures were chosen based on the ecologically relevant range for *O. bartramii*. For all experiments, the on-off cycle of white timer-controlled halogen lamps coincided with the diurnal photoperiod (14 h light, 10 h dark). Water was changed twice daily by pipetting out fluid until the embryos were minimally covered and then adding clean seawater containing antibiotics. Culture

experiments were conducted in four cycles for all 10 incubation temperatures. Embryos were characterized according to the developmental stages described by Watanabe et al. (1996) for *T. pacificus*. Approximately 800 developing *O. bartramii* embryos incubated at 16° and 18°C were transferred to higher temperatures (22° and 24°C) after stage 15 to observe any variation in development from the initial treatment temperature. Development of *O. bartramii* paralarvae after stage 29 (n = 300) was also observed and recorded in aerated circular kreisel tanks (38 × 24.5 cm; 28 L). The tanks were kept in temperature-controlled (22° to 25°C) rooms to maintain water temperature. Post-hatching paralarvae were maintained under these conditions without feeding for approximately 4–7 days until the internal yolk was completely absorbed.

Embryonic development rates were compared for the different incubation temperatures. Embryonic development and mortality rates at each temperature were determined under a dissecting microscope and were based on the percentage of normally developing embryos in each dish (Sakurai et al. 1996). During every water change, the developmental stages were observed and dead eggs and paralarvae were counted and removed. The period of embryonic development was defined as the time from fertilization until 50% hatching (Sakurai et al. 1996). One-way ANOVA, followed by Tukey's HSD test with $\alpha = 0.05$ were used to analyze the number of 8-day-old paralarvae that survived under the various temperature treatments. All data were verified for normality and homogeneity of variances using Bartlett's test and Fligner–Killeen test with $\alpha < 0.01$, respectively. A generalized additive model (GAM) was applied on paralarval survival data. The predictor variables (incubation temperatures) were modelled as cubic splines and the degree of smoothing was estimated using the mgcv package in R (Wood 2006). All statistical analyses

were performed using the R language and environment for statistical computing (R Core Team 2013).

Sea surface temperature

Satellite-derived data were used to study the interannual variability of SST in winter from 1990 to 2000 and in 2013 in the North Pacific Ocean. Monthly average SST data were compiled from the advanced very high-resolution radiometer (AVHRR) Pathfinder v. 5.0 datasets produced by the Physical Oceanography Distributed Active Archive Center (PO.DAAC) at NASA's Jet Propulsion Laboratory (ftp://podaac-ftp.jpl.nasa.gov/OceanTemperature/avhrr/L3/pathfinder_v5/monthly/), with a spatial resolution of ~0.25° for both latitude and longitude. SST was also recorded in situ using conductivity–temperature–depth (CTD) casts at all stations. The GMT software package (http://gmt.soest.hawaii.edu/) was used to produce contour lines depicting SST distribution.

4.4. Results

Temperature effects

Embryonic development

The fertilization percentage in the artificial fertilization experiments ranged from 16% (at 16°C) to 98% (at 22°C), and development was highly temperature dependent. At 16°C, normal embryonic development was observed only until stage 15; beyond that development became abnormal. Normal development with high survival rates (>50%) until hatching was observed between 18° and 25°C (Fig. 4-2a). Mean survival rates on day 8 of larvae reared at different temperatures were significantly different (1-way ANOVA: $F_{9,21} = 8.245$, $p < 0.001$). Pairwise comparisons revealed that survival rates at 16° and 26°C were significantly lower than those at all other temperatures (Tukey's HSD: $p < 0.05$). The GAM indicated that temperature was significant ($p < 0.001$), and explained 88.5% of variation in paralarval

survival (Fig. 4-2b). Embryonic development between 16° and 18°C was slower (Fig. 4-3a) than that at higher temperatures, with embryos incubated at 26°C suffering from high mortality (84%) after 24 h (Fig. 4-3b). Mortality was low between 18° and 25°C (Fig. 4-3b).

Hatching occurred at stages 26 to 28 at all temperatures except 16°C; no embryos survived beyond stage 21 at 16°C. Abnormal hatching occurred in approximately 40% of all embryos at 18°C. At 26°C, the mantles of all developing larvae were completely inverted after stage 15. These paralarvae had exposed viscera, were unable to swim, and survived for less than 1 day after hatching (Figs 4-4a & 4-4b).

Differential effects of temperature

Cleavage and blastoderm formation occur until stage 15, and were observed at all treatment temperatures. Stage 16 represents the onset of organogenesis, with the transition from stage 15 to stage 16 being highly varied, ranging from a maximum of 20 h at 26°C to a maximum of 75 h at 16°C. At 16°C, there was no chorion expansion after stage 15, and development beyond this stage was mostly (90%) abnormal, with retarded arm and mantle development. All eggs transferred from 16°C to higher temperatures (22 and 24°C) after stage 15 developed normally and hatched successfully (Fig. 4-5a). Development rates of embryos transferred from 18°C to higher temperatures (22 and 24°C) increased, and these embryos hatched 4 days earlier than embryos maintained at 18°C (Fig. 4-5b).

Sea surface temperature

Figure 4-1b shows the distribution of female squid specimens collected by the RV Kaiyo Maru in relation to SST. *O. bartramii* females and paralarvae were captured at temperatures between 18 and 25°C. Figure 4-6a shows paralarval distribution in relation to in

situ SST. Mature and spent *O. bartramii* females collected by the TS Hokusei Maru were distributed at SST between 16° and 24°C (Fig. 4-6b).

4.5. Discussion

Temperature effects

Embryonic development

This study identified the potential thermal limits for *O. bartramii* to complete successful in vitro development (18–25°C), in addition to confirming the significance of temperature for embryonic development. Within the optimal range, the duration of embryonic development to hatching decreased with increasing temperature and vice versa. The large number of embryos that developed abnormalities outside of these thermal limits may have been due to hypo- or hyper-embryogenesis (Sakurai et al. 1996) coupled with hypoxic stress (Rosa et al. 2012). Since cephalopods are poikilotherms, warmer water temperatures under a global warming scenario would accelerate growth rates (subject to the availability of sufficient oxygen), shorten life spans, and increase turnover (Rodhouse 2013, Pecl & Jackson 2008), which might, in turn, drive changes in the life-history parameters of this species.

The identification of the thermal limits for this species during the early stages of development explained the distribution of ommastrephid species around Hawaii. Egg masses of *O. bartramii* have never been observed in nature; however, based on paralarval distribution data, SSTs ranging from 21 to 25°C are considered favorable temperatures for egg hatching in the North Pacific (Hayase 1995, Bower 1996, Mori et al. 1999). My rearing experiment results indicated that the lower limit of favorable temperature is 18°C. Although I was unable to observe embryonic growth at 17°C, Sakurai et al. (1995) obtained hatchlings at this temperature, but observed frequent developmental abnormalities and cessation of development during the early stages of embryogenesis. Furthermore, the cruises (TS Hokusei

Maru) collected mature (about to spawn) and spawned state (post-spawning) *O. bartramii* females at SSTs ranging from 16° to 25°C (Fig. 4-7), in addition to paralarvae at SSTs ranging from 18° to 25°C (RV Kaiyo Maru, Fig. 4-8). These observations provide evidence that spawning and hatching might occur at this temperature range.

Temperature requirements restrict the spatial and temporal distribution of spawning in ommastrephid squid. Temperature requirements regulate the distribution and migration ranges of ommastrephids, including *I. illecebrosus* (O'Dor et al. 1982), *I. argentinus* (Sakai et al. 1999), *T. pacificus* (Sakurai et al. 2000), and *D. gigas* (Staaf et al. 2011). As an r-strategist species, this requirement may be a limiting factor in the range of *O. bartramii*, and may explain why it migrates to subtropical regions for spawning. SST varies interannually in the North Pacific (Tanimoto et al. 1993, Sheng 1999, Deser et al. 2010, Kosaka & Xie 2013), with major anomalies occurring in the STFZ (Qiu et al. 2014). Paralarval mortality is known to depend on SST (Sakai et al. 2004). Because the lifespan of ommastrephid squids is just one year, this variation affects the distribution and spawning area of these species. Year-to-year fluctuations in oceanographic factors and production cycles also account for large variations in recruitment success (Jennings et al. 2001). Sinclair's (1988) hypothesis regarding the variation in recruitment emphasizes the importance of the relationship between the time of spawning and stable oceanographic features that retain larvae in favorable environments.

Differential effects of temperature

Several phases of squid development have been demonstrated to be differentially affected by temperature (O'Dor et al. 1982). I recorded evidence of the differential effects of exposure to low temperature. Although cleavage and blastoderm formation were normal at all temperatures, organogenesis appeared to be sensitive to temperatures outside the optimal thermal limits. Experiments investigating embryonic growth at 16°C with subsequent transfer

to higher temperatures (22° and 24°C) indicated that underdeveloped eggs may remain in stasis until the temperature increases (O'Dor et al. 1982). Further research is required to determine the duration and temperature range under which such stasis may be maintained. The normal development of embryos transferred from 16°C to 22° or 24°C also suggests that this squid may be able to withstand larger temperature fluctuations. The adaptation of embryos to these varying conditions may allow squids to survive for extended periods, even when their egg masses are released at temperatures below 16°C, and to continue normal development when favorable temperature conditions return, which is a likely scenario in nature.

Depending on their density, egg masses accumulate in the mid-water pycnocline (Bower & Sakurai 1996, Sakurai et al. 2000, Staaf et al. 2008), with squid paralarvae that hatch from the egg mass in the mid-water the isopycnic surface layer being recruited to surface waters (Bakum & Csirke 1998, Sakurai et al. 2000). At the spawning grounds near Hawaii, paralarvae of *O. bartramii* are distributed in the surface layers, where the pycnocline temperature is approximately 15–17°C (Bingham & Lukas 1996, Seki et al. 2002) during the putative spawning season of the winter-spring cohort (Jan–May) (Yatsu et al. 1997), which is lower than the ideal range of SST identified here. This observation indicates that paralarvae may hatch at lower temperatures in the pycnocline, but survive in habitats at higher SSTs. My findings indicate that putative spawning and hatching temperatures in the waters around Hawaii range from 16° to 25°C, and that paralarval distribution in the surface layers occurs from 18° to 25°C. Spawning may also occur at lower temperatures (<16°C).

The ability of *O. bartramii* paralarvae to adapt to higher temperatures has not been previously studied; however, such information is critical for understanding how early developmental stages respond to global warming. This study identified the upper temperature

limit beyond which deformity of body structures occurs, and would have deleterious effects on embryonic survival and growth by creating challenges to feeding (Rosa et al. 2012). The inability of *O. bartramii* paralarvae to survive at high temperatures (26°C) may explain its absence from tropical waters. A temperature increase in the present subtropical spawning area as a result of global warming may result in the shifting of distribution range of this squid species to higher latitudes (Cheung et al. 2009, Chen et al. 2011).

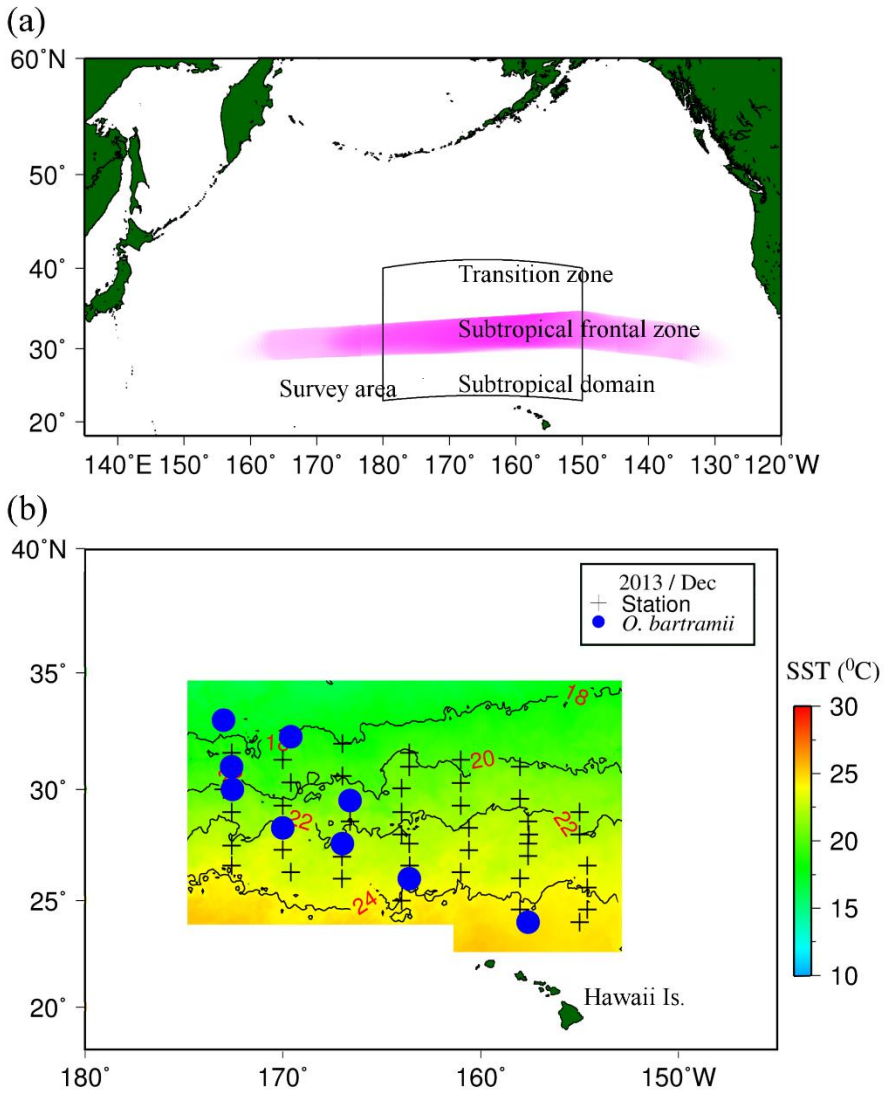

Figure 4-1. (a) The box represents the surveyed area of the RV Kaiyo Maru for *Ommastrephes bartramii* adults and paralarvae in December 2013, and the major surrounding oceanographic regions (Ichii et al. 2011) in the North Pacific Ocean. (b) Sampling locations and corresponding sea surface temperature (SST) contours where copulated female squid specimens were jigged

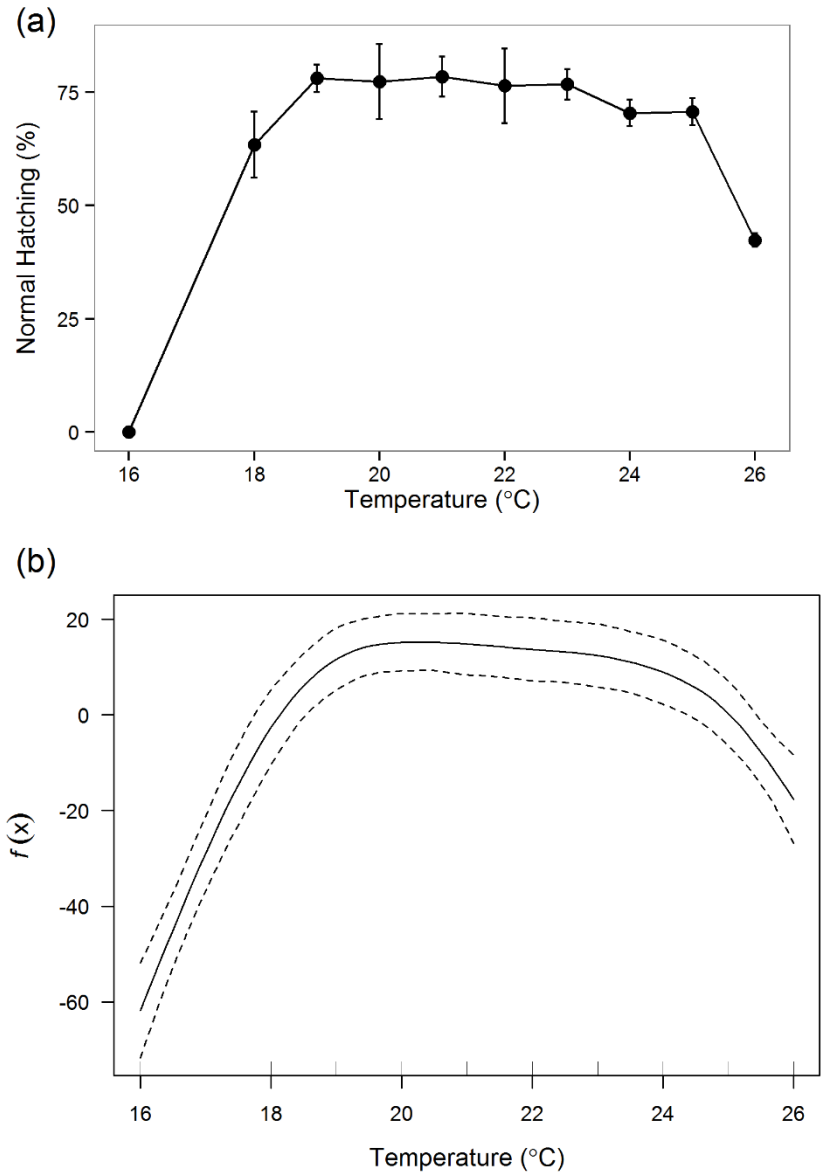

Figure 4-2. (a) Percentage of normal hatching (bars indicate 95% confidence interval) in *Ommastrephes bartramii* plotted against incubation temperature at stage 26. (b) Generalized additive model plot for *O. bartramii* showing the effect of temperature on survival. Fitted line (solid line) and 95% confidence interval (area between dashed lines) are shown

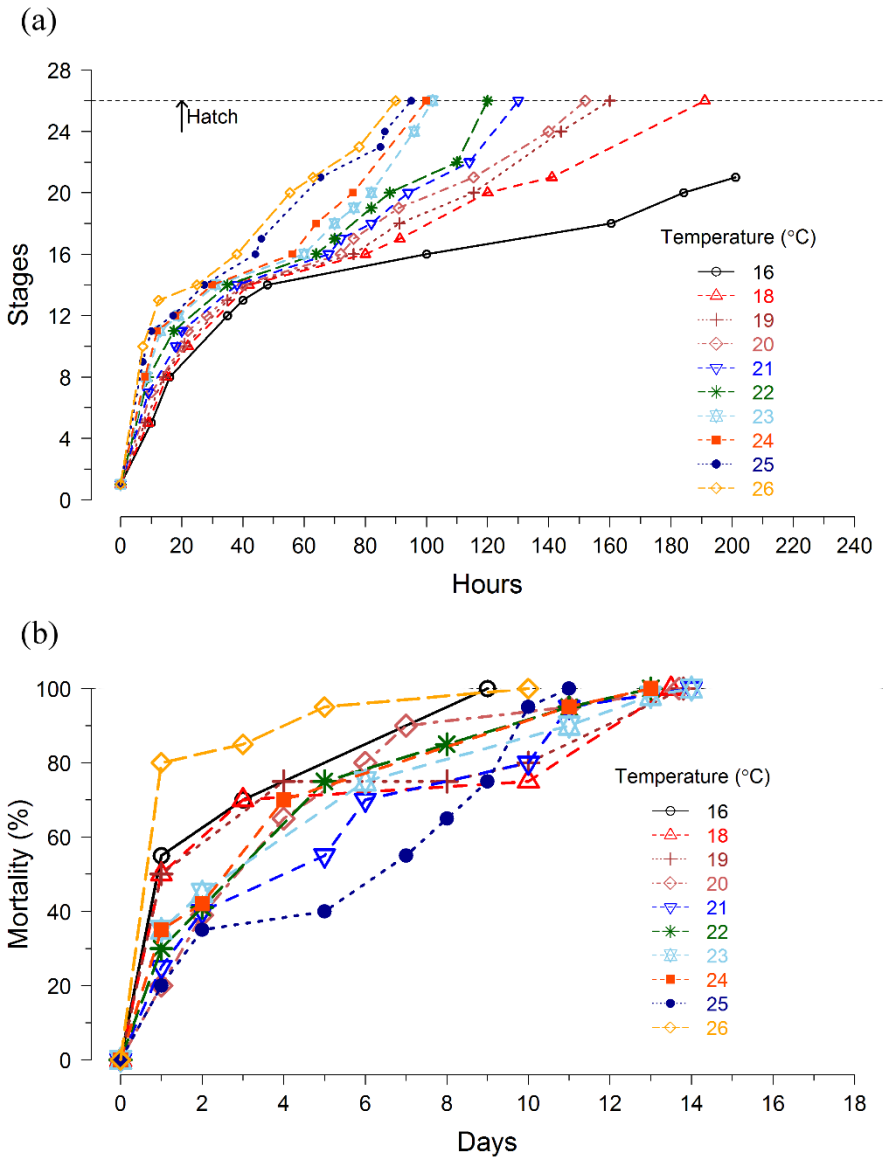

Figure 4-3. (a) Relationship between egg mortality rates and time (days) after fertilization for 10 incubation temperatures. (b) Relationship between development of *Ommastrephes bartramii* eggs and time for 10 incubation temperatures. Horizontal dashed line represents the hatching stage

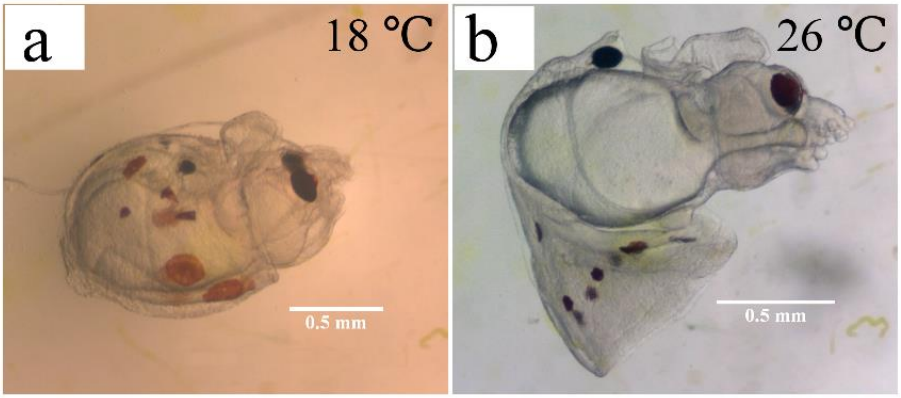

Figure 4-4. Types of abnormalities found in *Ommastrephes bartramii* embryos incubated at two different temperatures. (a) Retarded growth; (b) complete body deformity

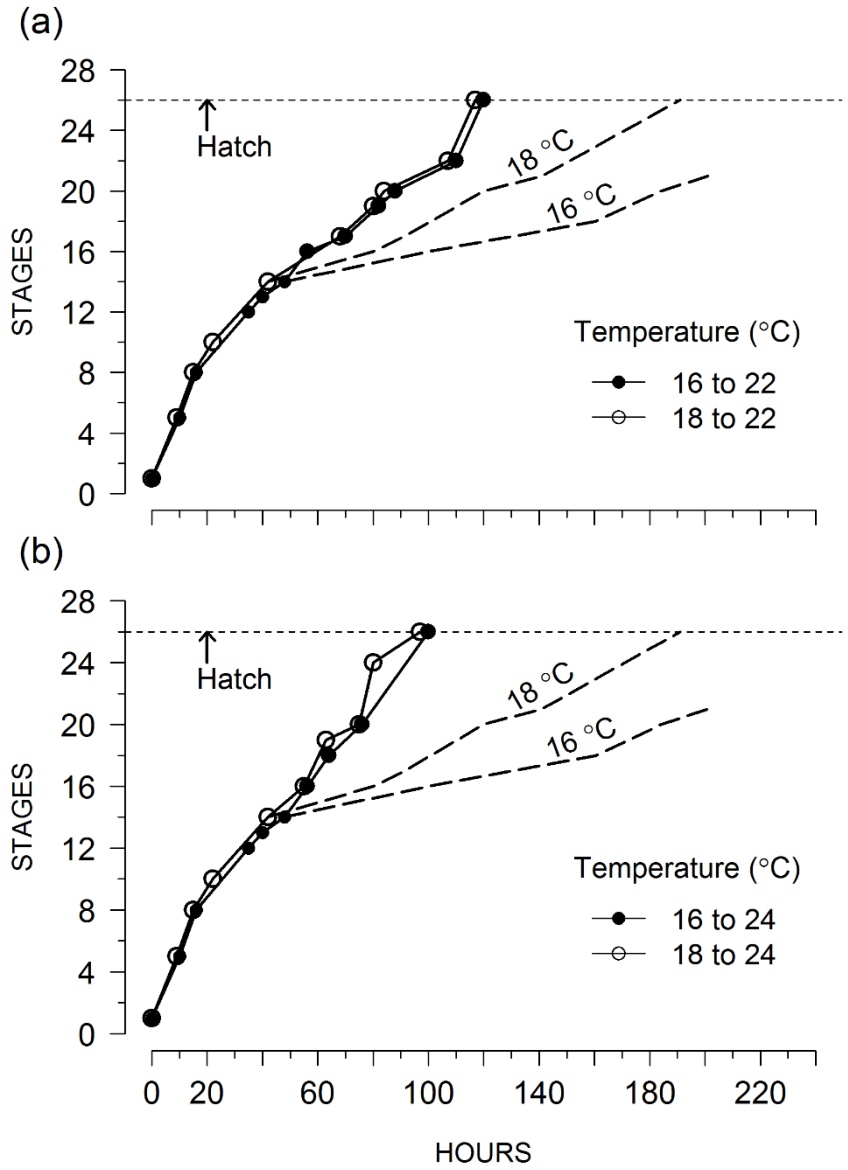

Figure 4-5. Relationship between development of *Ommastrephes bartramii* eggs and time for different incubation temperatures. (a) Eggs transferred from incubation temperature of 16 or 18°C to 22°C; (b) eggs transferred from incubation temperature of 16 or 18°C to 24°C. Dashed lines (derived from Figure 4-3b) represent the developmental trajectory at constant (unchanged) temperature

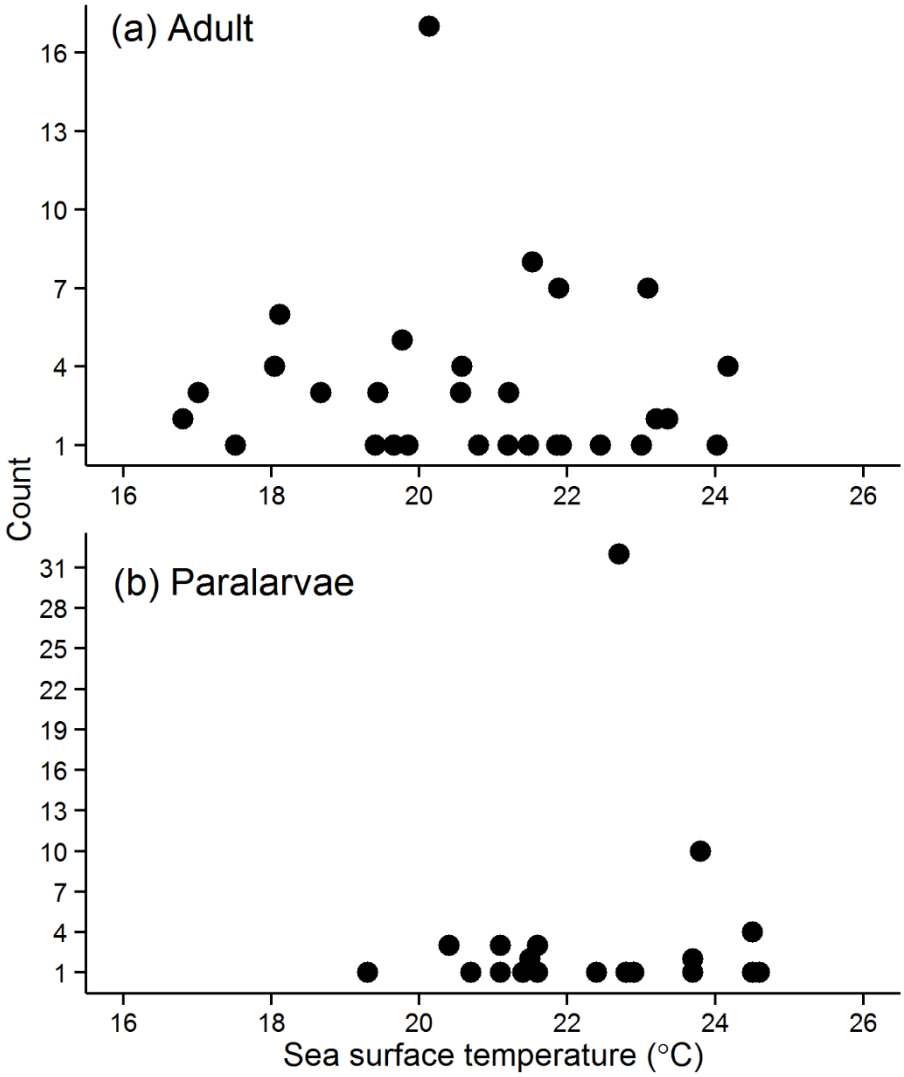

Figure 4-6. (a) Numbers of *Ommastrephes bartramii* adults (mature and spent) collected on board the TS Hokusei Maru, and (b) paralarvae collected on board the RV Kaiyo Maru plotted against *in situ* sea surface temperature (SST)

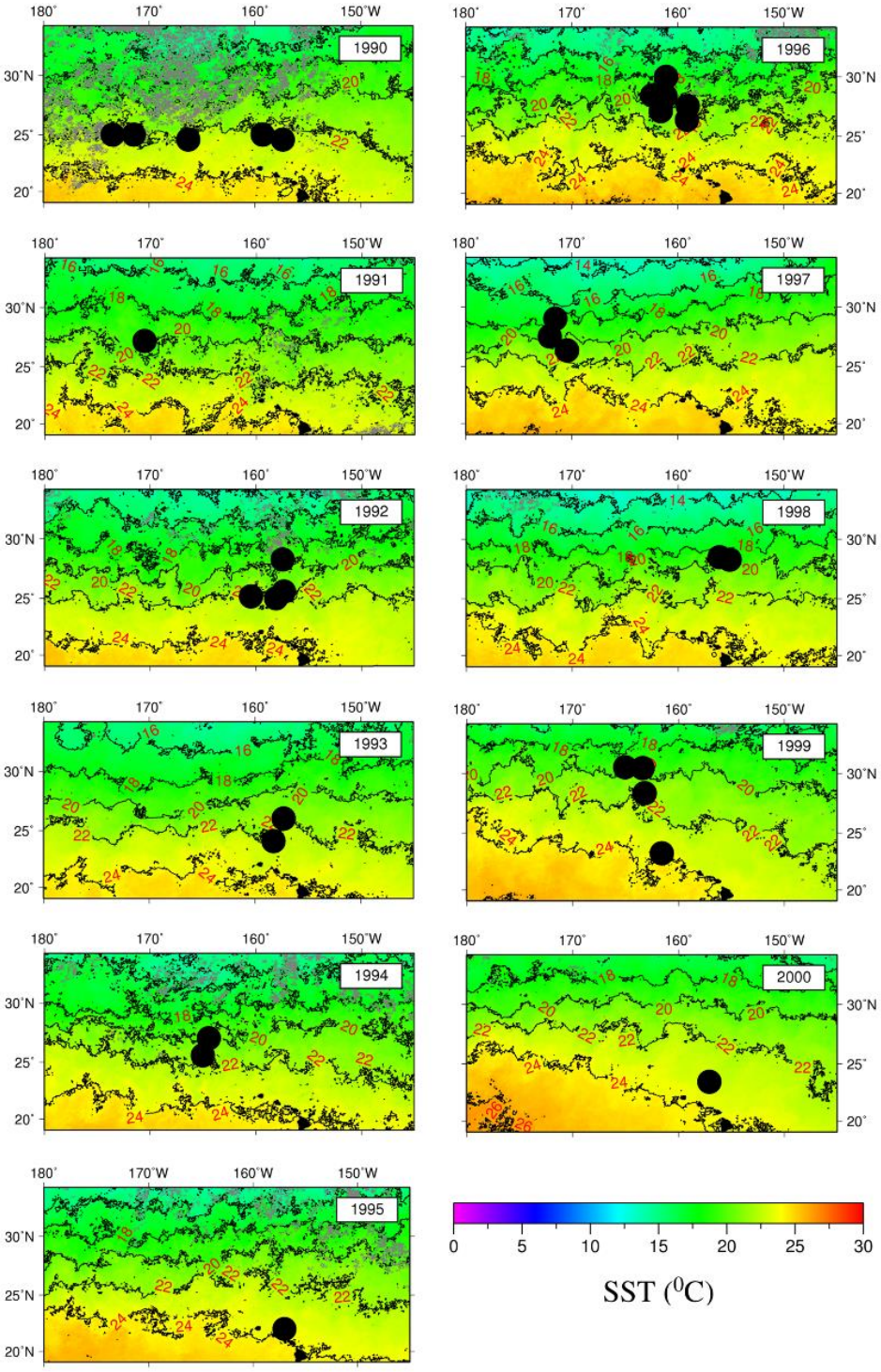

Figure 4-7. TS Hokusei Maru sampling locations (black dots) where mature and spent females were captured. Sea surface temperature (SST) data are composited monthly data for February for all years except for 1991, which shows March data

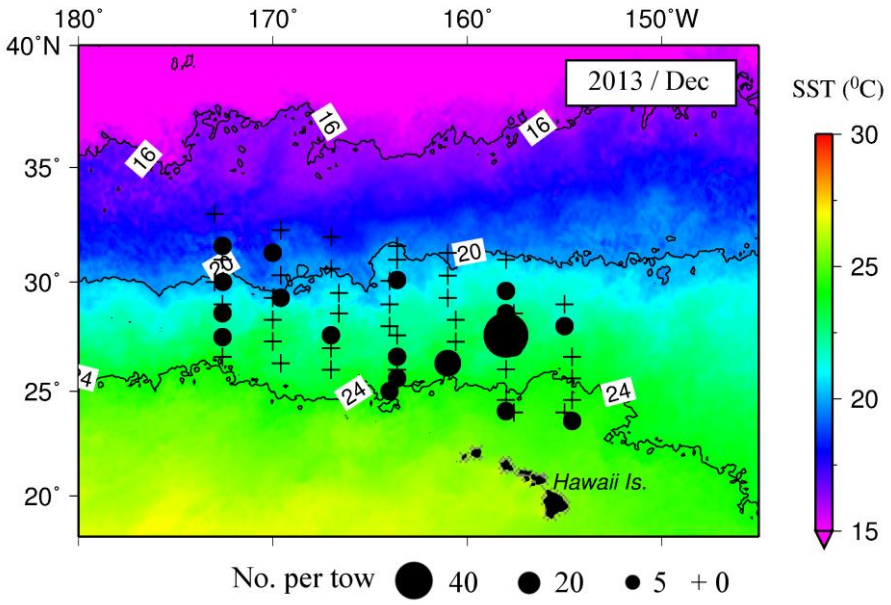

Figure 4-8. Locations and sea surface temperature (SST) contours where *Ommastrephes bartramii* paralarvae were collected

Chapter 5. Some aspects of the behavior of paralarvae

5.1. Abstract

Squid are the largest jet propellers in nature as adults, but as paralarvae they are some of the smallest, faced with the inherent inefficiency of jet propulsion at a low Reynolds number. In this study I describe the paralarval behavior of *Ommastrephes bartramii*, observed under laboratory conditions. Paralarvae demonstrated a variety of swimming behaviors, including active jetting, passive sinking, and pulsing. These behaviors may allow the squid to control their distribution in the wild. Ball forming behavior and associated chromatophore expansion might represent an aposematic adaptation to imitate unpalatable prey. Although I could not identify the initial prey items consumed by hatchlings, I discuss the possible role of the proboscis in feeding. Proboscises had well-developed suckers that could be used to direct the mucus mass or other food particles into the mouth until the onset of raptorial feeding.

5.2. Introduction

Marine larvae tend to propel themselves either with ciliary action or muscular flexion of the body or fins. Jet-propelled squid paralarvae are a striking exception. Jet propulsion in paralarvae, as in adult squid, is accomplished with contractions of circular muscle fibers of the mantle. Water is expelled from the mantle through a muscular funnel that can be aimed to direct the jet and therefore the direction of swimming. Owing to the energetic loss of accelerating a relatively small jet of water to high speed, as well as the costly refilling period, during which there is no active thrust, squid jet propulsion is inherently inefficient in comparison to undulatory locomotion in fish (Staaf et al. 2014).

Little is known about Ommastrephid embryonic development and paralarval behavior characteristics that are critical to a meaningful understanding of their life history (Staaf et al. 2011). Among oegopsid families, ommastrephids are unique in passing through a post-hatching paralarval stage called a rhynchoteuthion, in which the tentacles are fused into a proboscis. The most important factor determining patterns of abundance and the dynamics and distribution of ommastrephid populations is the availability of planktonic rhynchoteuthion paralarvae and juveniles that inhabit subsurface depths for approximately 2 to 2.5 months. These stages experience high mortality rates; therefore, spawning success and paralarval survival rates (which are influenced by large interannual variability in habitat) are the principal factors that determine recruitment (Roper et al. 2010, Ichii et al. 2011). Growth and ontogenetic changes of squid paralarvae are largely linked to water temperature (Sakurai et al. 2000, Villanueva 2000, Staaf et al. 2011). Rosa et al. (2012) observed embryonic development of loliginid squid under projected near-future warm conditions and found that abiotic conditions inside the eggs promoted metabolic suppression followed by premature hatching; in addition, the less-developed hatchlings showed a greater incidence of malformations. However, limited data on environment-related changes in survival are available for ommastrephid paralarvae. Prevailing regional oceanographic features (especially fronts) in combination with temperature impose limits on the geographical dispersal of cephalopod paralarvae (O'Dor et al. 1982, Moreno et al. 2008, Villanueva et al. 2012). All ommastrephids have a pelagic lifestyle characterized by extrusion of fragile, neutrally buoyant egg masses, release of larvae into the surface plankton, and use of large-scale current patterns for larval transport and assisted migration of populations (Bower & Sakurai 1996, Sakurai et al. 2000, Boyle & Rodhouse 2005, Staaf et al. 2008, Kato et al. 2014, Nishikawa et al. 2014). Neutrally dense gelatinous egg masses are thought to retain their location in the water column by floating at the interface between water layers of slightly different densities,

the isopycnic surface or pycnocline (Sakurai et al. 2000, Boyle & Rodhouse 2005). The planktonic paralarvae, post-paralarvae, and juveniles of *O. bartramii* inhabit surface waters from 0 to 100 m during both day and night (Roper et al. 2010). Because temperature influences embryonic growth (Sakurai et al. 1995) and because paralarvae are distributed in the surface layers (Harman & Young 1985, Saito & Kubodera 1993, Sakurai et al. 1995), sea surface temperature (SST) is an important factor determining the distribution range and survival of paralarvae. Therefore, I examined SST in relation to the distribution of adults (mature and spent) and paralarvae.

To understand post-hatching behaviors of paralarvae, I attempted preliminary observations of swimming activities. Adult squids generally employ a unique, dual-mode locomotory system that involves a pulsed jet and fin movements (Bartol et al. 2008). The pulsed jet is formed by first expanding the mantle radially, causing water to fill the mantle cavity through intake regions at the anterior margin. Then, muscles around the circumference of the mantle contract. This contraction increases the pressure in the mantle cavity, driving water out of the cavity via the funnel. The bending of the funnel below the body allows squids to swim either arms- or tail-first. Repetition of these muscle patterns results in a pulsed jet (Bartol et al. 2008, Staaf 2010). Squid paralarvae have saccular, rounded bodies, relatively large funnel apertures, and rudimentary fins, which forces hatchlings to rely more heavily on the jet than on the fins for propulsion (Hoar et al. 1994). Paralarvae operate at low and intermediate Reynolds numbers (*Re*) (Vidal & Haimovici 1998), whereas juveniles and adults operate at higher *Re* and generally have more streamlined bodies, larger fins, and relatively small funnel apertures (Bartol et al. 2008). Ommastrephid paralarvae are known to begin active swimming right after hatching (Sakai 2007, Staaf et al. 2014).

Information about the diet of ommastrephid paralarvae is limited. These young squid are coated with a mucus layer that is thought to harbor prey items (O'Dor et al. 1985, Vidal & Haimovici 1998). Dietary analysis by Vidal & Haimovici (1998) revealed the presence of the same microorganisms in the paralarval digestive tract that were present in the mucus layer and on the proboscis suckers. Feeding attempts were made by Sakurai et al. (1995) on 2 and 3-day-old *O. bartramii* and *S. oualaniensis* hatchlings by adding copepods, fish eggs, and squid eggs to the culture bottles. None of these prey items was consumed, probably because they were not appropriate choices, or because paralarvae were maintained under inappropriate conditions or hatchlings were insufficiently developed. Villanueva et al. (2014) suggested that high-priority research targets in cephalopod culture include the development of sustainable artificial foods and control of production. I also conducted feeding experiments in an attempt to identify initial prey items of ommastrephid paralarvae. In addition, observations on the peculiar paralarval behavior of withdrawing the head into mantle were carried out.

5.3. Materials and Methods

Sampling

Mature, copulated *O. bartramii* females (n = 11) of the winter-spring cohort were captured with jigs on hand lines on board the RV Kaiyo Maru in December 2013. Collections were made north of the Hawaiian Islands between 20 and 35°N and between 150°W and 180°, around its spawning ground (Figure 4-1, previous chapter).

Artificial fertilization

Copulated females were decapitated, and their mantles were opened with a ventral cut to collect ripe eggs from the oviduct; viable sperm (deposited during copulation) were collected from the buccal area of the same females. Artificial fertilization was performed on board using standard techniques (Sakurai et al. 1995, Sakai et al. 2011, Villanueva et al.

2012). Fresh seawater collected from 500 and 1000 m depth and fortified with antibiotics (25 mg L^{-1} each of ampicillin and streptomycin) was used as a medium (Staaf et al. 2011).

Swimming behavior

The swimming behavior of *O. bartramii* paralarvae was observed and recorded in kreisel tanks (38 × 24.5 cm; 28 L) (Fig. 5-1), petri dishes (radius = 4.3 cm; height = 2 cm), and long cylindrical tubes (radius = 1 cm; height = 1.5 m). After hatching, approximately 20 paralarvae were maintained in each petri dish. Post-hatching paralarvae that attained stage 30 or 31 were transferred by pipette to a 2-L bottle and those that demonstrated active hop-and-sink behavior (n = 300) were further transferred to circular kreisel tanks and cylindrical tubes for observation of swimming behavior. A black background was placed behind the kreisel tanks and cylindrical tubes to enhance the visibility of the nearly transparent paralarvae (Staaf et al. 2014). Paralarval behavior of all species was observed in petri dishes. Mechanical stimulation (flashing light and agitation of water) was applied to elicit startle responses in the paralarvae.

Swimming behavior of paralarvae in the petri dishes was observed under a stereomicroscope (Nikon SMZ 1500). Live videos from the microscope were recorded with a Canon PowerShot A400 IS digital camera. Videos of paralarvae in the kreisel tanks were taken with a Sony HDR-CX590V handycam. All video footage was annotated, reviewed, and analyzed. Selected sequences from the 30 frames s^{-1} videos were captured with VLC 2.1 and exported as frames into ImageJ, the National Institute of Health's public domain program (http://rsbweb.nih.gov/ij/) to observe swimming behavior and ball formation. Swimming speed was measured as the number of MLs travelled per second, based on frame rate (Staaf et al. 2014). Because the exact distance from the camera to the squid could not always be determined, dorsal ML was used to calibrate all measurements (Staaf 2010).

Feeding experiments

Attempts were made to feed the *O. bartramii* paralarvae maintained in kreisel tanks (radius = 19 cm; width = 24.5 cm), with dissolved organic matter (DOM), marine snow, and the water used to wash the ring net after normal plankton sampling. These foods were chosen because the mucus covering rhynchoteuthion paralarvae commonly contains phytoplankton and zooplankton detritus, and the paralarvae are believed to feed directly on the mucus or on the microorganisms that colonize it (O'Dor et al. 1985, Vidal & Haimovici 1998).

5.4. Results

Swimming behavior

Muscular contractions in the developing embryos began at stage 23. Before hatching, the developing embryo always maintained a ventral-side-up posture in the dish. At hatching (stage 26), the egg membrane ruptured and the larvae left, posterior end of the mantle first, by a pulsating movement. Soon after that (within 8–10 s), the paralarvae started swimming by horizontal jetting, always with the ventral side up. Swimming was achieved by jet propulsion with associated mantle contractions as shown in Figure 5-2. From stage 30, paralarvae of all species demonstrated a variety of swimming behaviors, categorized as maintenance jets, vertical jets, horizontal jets, escape jets, passive sinking, and pulsing. The categorization was modified from the report of Staaf et al. (2014) for the ommastrephid *D. gigas*. Maintenance jetting (observed in the kreisel tanks, Petri dishes, and cylindrical tubes) consisted of individual jets counteracting sinking to maintain their vertical position in the water column. Paralarvae were always observed to sink if not swimming actively. Prolonged periods of repeated jetting that exceeded the velocity of passive sinking were observed in vertical jets, with maximum measured speeds of 0.48 cm s^{-1}. The paralarvae moved upward during vertical jets, frequently to the water surface, which resembled the hop-and-sink behavior described in *D. gigas* paralarvae (Staaf 2010, Staaf et al. 2014). Horizontal jets observed in petri dishes

consisted of repeated jets during which the ventral side was usually facing up. Wall effects of the petri dishes limited the horizontal jets in a diagonal path. Circular jetting—the occurrence of several rapid jets (approximately 1.2 cm s^{-1}) in a circular path (0.5-cm radius)—was observed. Less frequently (six times), stage 33–34 paralarvae swam with their lateral side up during horizontal jets; this was observed on the surface and never in the water column. Escape jets, the most rapid form of propulsion, were individual jets and appeared to be similar to the escape jets of adult squid. The maximum speed measured in an escape jet was 3.12 cm s^{-1}. Resting paralarvae responded to physical contact from other paralarvae by escape jets, prompting the assumption that this was a "play" behavior. Passive sinking was observed when paralarvae that were swimming actively by vertical jets stopped jetting; these larvae were carried passively by small currents generated by aeration bubbles, which had little effect during active swimming. Passive sinking usually lasted for 3–4 s after which the paralarvae resumed hop-and-sink behavior. Pulsing behavior observed in the petri dishes consisted of continuous contractions and expansions of the mantle with fin flaps, but zero velocity gain in any direction.

Jetting was achieved by mantle contraction. The opening and closing of a mantle aperture during a jet is shown in Figure 5-2. The width of the mantle and mantle aperture, normalized by ML, are shown in Figure 5-2. The frequency of mantle aperture closure was longer for maintenance jets than for vertical jets. Inside the kreisel tank, cylindrical tubes, and cylindrical tanks, paralarvae relied predominantly on high-frequency low-velocity vertical jets as they moved up and down in the water column, as reported for other squid (Bartol et al. 2008, Staaf et al. 2014). Paralarvae moved up during mantle contractions and sank during mantle refilling, with net vertical displacement determined by the duration of the refilling phase. This behavior was usually followed by periods of sinking that lasted much longer (3–4

s) than a single refilling period, and during which no active swimming was observed. Although the swimming position was vertical, lateral movements were achieved by diagonal propulsion in the water column, probably by maneuvering the funnel nozzle. In the kreisel tanks, paralarvae displayed lateral swimming while approaching the bottom of the tanks. The proboscis was always in the stretched position during vertical and horizontal jets, presumably for balance. Spontaneous extension of the proboscis was also frequent. During "rest periods" (10 min to 2 h) of healthy paralarvae, the proboscis suckers were attached to the wall of the kreisel tank, maintaining the body in an inactive state at an inclined angle in the water column. The same behavior was observed in the petri dishes, and the sucker grip was strong enough to withstand mild agitations. Paralarvae released into the kreisel tanks survived 3 days longer on average (range, 1–7 days) compared to those in petri dishes.

Ball formation

Any sudden provocation (flash of light, agitation of water, or drop of ethanol) resulted in rapid retraction of the head into the mantle, with paralarvae remaining in a ball shape (Fig. 5-3). Ball formation was always accompanied by chromatophore expansion. With chromatophores expanded, surface area increased more than six-fold (probably to its maximum extent), and head contracted into the mantle (Fig. 5-3), the animal looked like a "large orange" under the microscope (Young 1972). Distortions in the shape and relative position of parts while the animals were in the ball formation are illustrated in Figure 5-3. If the duration of the provocation was longer, the "ball" posture was maintained for a longer period and sank, remaining in the bottom of the dish; maintenance of the ball posture ceased when the provocation was withdrawn. The recovery phase involved eversion of the proboscis, head, funnel, and tail back to their usual positions. Recovery of normal shape from the ball shape required seconds to several minutes. While in mild provocation (gentle agitation of water), ball formation became part of normal swimming (4 to 5 times min^{-1}). These

considerable distortions in shape and in the relative position of body parts occurred without any apparent damage. Inking was another startle response. Three to four hours after hatching, the ink sac became more prominent and was clearly visible through the mantle wall. Paralarvae (from stage 29) occasionally spouted ink into the water and darted away from the ink cloud when provoked.

Feeding experiment

Attempts at feeding the paralarvae were not successful. The control (without food) and experimental animals showed no significant difference in survival rate ($P > 0.05$). Food particles were found attached to their mucus covering (Fig. 5-4), but there was no evidence of the animals consuming these particles.

5.5. Discussion

Swimming behavior

Behavioral traits (e.g., vertical migration) could allow larvae to enter water masses that are moving in different directions at different rates, and could control the rate and direction of larval transport (Weinstein 1988). Squid paralarvae can occur in great abundance in oceanic surface waters (Boyle & Rodhouse 2005). The vertical jets demonstrated by the paralarvae may explain their ascent mechanism from the mid-water hatching area to the surface water where they feed. These jets could also be used to effect diel vertical migrations, allowing the young cephalopods to sink to deeper depths during the day to avoid visual predators and rise to the surface at night (Staaf et al. 2008). Staff et al (2010) calculated that a paralarval migration of 15 m would take less than 1 h for *D. gigas* at the observed swimming speed of 0.5 cm s^{-1}. Vertical swimming experiments conducted by Yoo et al. (2014) with *T. pacificus* paralarvae at various temperatures revealed that the squid always had a preference for surface layers with higher temperatures. Hop-and-sink behavior may be an adaptation for conserving energy (Staaf 2010). Rapid jetting in paralarvae resembles burst-and-coast

swimming in jellyfish (Alben et al. 2013) since there is a burst of thrust as the mantle contracts and water is expelled, followed by a coasting phase as the mantle refills.

Squid paralarvae are transported by surface currents (Boyle & Rodhouse 2005, Roberts & van den Berg 2005). Movement of paralarvae with respect to the small circular currents generated by aeration bubbles inside the kreisel tanks could mirror the phenomenon that occurs in natural environments with currents. The young squid can actively resist the current by vertical jet propulsion, or can passively drift with the current. Directional swimming in the water column was achieved via two mechanisms: by bending the funnel to the desired direction before jetting and by starting to swim vertically at an inclined angle pointing in the desired direction. The effects of temperature on *O. bartramii* were observed by maintaining one kreisel tank constant at 23°C and another at room temperature, which fluctuated between 22° and 27°C. Neither survival nor swimming activity of paralarvae differed between the constant and fluctuating temperature regimes. *T. pacificus* paralarvae are known to be active swimmers within a temperature range between 19.5° and 23.5°C (Yamamoto et al. 2012). The wide range of temperature tolerance in paralarvae may increase their chances of survival in the wild.

Maintenance jetting can be achieved by leaking water through the mantle aperture rather than expelling it solely through the funnel (Staaf et al. 2014). Escape jet locomotion was the fastest swimming behavior (3.12 cm s^{-1}) and probably enables squid to avoid predation. Staaf et al. (2008) reported that swimming speed in 3- to 6-d-old *D. gigas* paralarvae varied from simply maintaining their position in the water column (net velocity of 0 cm s^{-1}, generated by the animal's swimming velocity counteracting its sinking velocity) to 0.51 cm s^{-1}, the fastest net upward swimming speed maintained over several seconds. These researchers also observed a disturbance-provoked 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. Although fin flapping was observed, its role in movement was limited, and directional swimming was achieved by controlling the funnel nozzle (Staaf 2010). Pulsing may be a predatory behavior (Staaf 2010), as in *Doryteuthis opalescens* hatchlings in which predation on *Artemia* spp. was sometimes prefaced by frequent mantle contractions (Preuss et al. 1997). Before hatching, the embryo's anatomy and center of gravity cause it to be oriented with the dorsal side towards the inner wall of the chorion (Marthy 1994). After hatching, the swimming paralarvae maintained the same position (ventral side up) in the petri dishes, probably for the same reason.

Some paralarvae reared in water from 1000 m were devoid of one or both statoliths, presumably because of the lower concentration of calcium (or increased acidity) at that depth (statoliths are mainly composed of calcium carbonate in the crystal form of aragonite) (Hulet 1984, Bidwell et al. 1986, Hanlon et al. 1989). These paralarvae exhibited abnormal swimming behaviors because of the critical role statoliths play in maintaining balance, and never survived for more than one day after hatching.

Ball formation

When disturbed, the strong head-retractor muscles enable paralarvae to rapidly withdraw their head and proboscis ventrally into the mantle cavity (Shigeno et al. 2001). Ball formation in gonatid paralarvae is considered a defensive body posture that mimics the appearance (size and color) of the small hydromedusan jellyfish *Aglantha digitalis* (Arkhipkin & Bizikov 1996). Hydromedusan jellyfish are capable of stinging, so this resemblance discourages visual predators. Alteration of body posture by rolling into a ball was considered to be a defensive mechanism in cranchiids (Nixon 1983). The defensive body posture achieved by forming a ball helps to increase the chance of survival during the vulnerable

paralarval phase (Arkhipkin & Bizikov 1996). Ball formation has been observed in chemically preserved samples (e.g., *Cranchia scabra*, Young 1972; *T. pacificus*, Shigeno et al. 2001). Withdrawal of the head into the mantle cavity during fixation illustrates the highly contractive capacity of head-retractor muscles: before death, the paralarvae responded to irritants (alcohol and formalin) by adopting the ball posture. Ball formation observed in a single drop of water may have been due to hypoxia. O'Dor et al. (1985) observed a complete withdrawal of the head into the mantle with simultaneous contraction of the mantle lip in rhynchoteuthion paralarvae of *Illex illecebrosus*, and interpreted this to represent a feeding behavior that enabled the squid to feed upon detritus adhered to the mantle's mucous covering. I observed the suckers of the proboscis close to the mouth in the ball state, so this may also be a feeding behavior. The sinking nature of the ball posture may help to move the squid away from danger zones. Since head-withdrawal behavior is provoked by mechanical (flash of light, agitation of water) or chemical (alcohol and formalin) stimulation, this behavior is presumably a defensive reflex in ommastrephids and other oceanic squid paralarvae.

Chromatophore expansion associated with the ball posture can generate aposematic coloration. Warning colors and patterns are an adaptation in prey species that provide a signal to predators of the potential cost of making an attack. Orange coloration is associated with chemical defenses in at least three phyla (Young & Bingham 1987), and 60% of unpalatable marine invertebrate larvae are red or orange (Lindquist & Hay 1996). Aguado & Marin (2007) reported orange coloration as aposematic, and Valdes et al. (2013) reported bright color as aposematic in marine molluscs. Because ommastrephid paralarvae lack morphological defenses, the orange color and ball shape adopted by paralarvae when threatened by predators may imitate unpalatable organisms that share the same habitat.

My feeding experiments failed to identify the initial prey of the paralarvae. Paralarvae might have been unable to feed due to the absence of a functional buccal mass or digestive system as a result of premature hatching (stage 26) and smaller size. Hatchlings also had an available energy source in the remaining yolk content. In natural environments, normal hatching from undisturbed egg masses occurs when all of the yolk has been used (after stage 34) and when the digestive system is developed (O'Dor et al. 1982, Bower 1997). Future research could be directed towards finding appropriate prey items for ommastrephid paralarvae so that they can be reared under laboratory conditions to the adult stage like other myopsid paralarvae. Each transition period will be critical for paralarval survival, but the transition from suspension feeding to raptorial feeding will likely have the highest mortality because it requires major changes in food type, morphology, and feeding behavior (Uchikawa et al. 2009). The ability of the proboscis to attach to the walls of the dishes and tank indicates that well-developed suckers are formed after stage 30 and supports the hypothesis of proboscis-based suspension feeding proposed by O'Dor et al. (1985). In *O. bartramii*, raptorial feeding by arm-strikes starts at MLs of 13 mm (Uchikawa et al. 2009); until then, the proboscis could be used to direct the mucus mass or other food particles into the mouth.

Figure 5-1. Kreisel tanks used for the swimming and feeding experiments of *Ommastrephes bartramii* paralarvae. Total tank volume is 28 L with key dimensions as follows: radius = 38 cm; width = 24.5 cm. Tank is constructed of clear acrylic and black backgrounds behind the tanks enhanced the visibility of the nearly transparent paralarvae

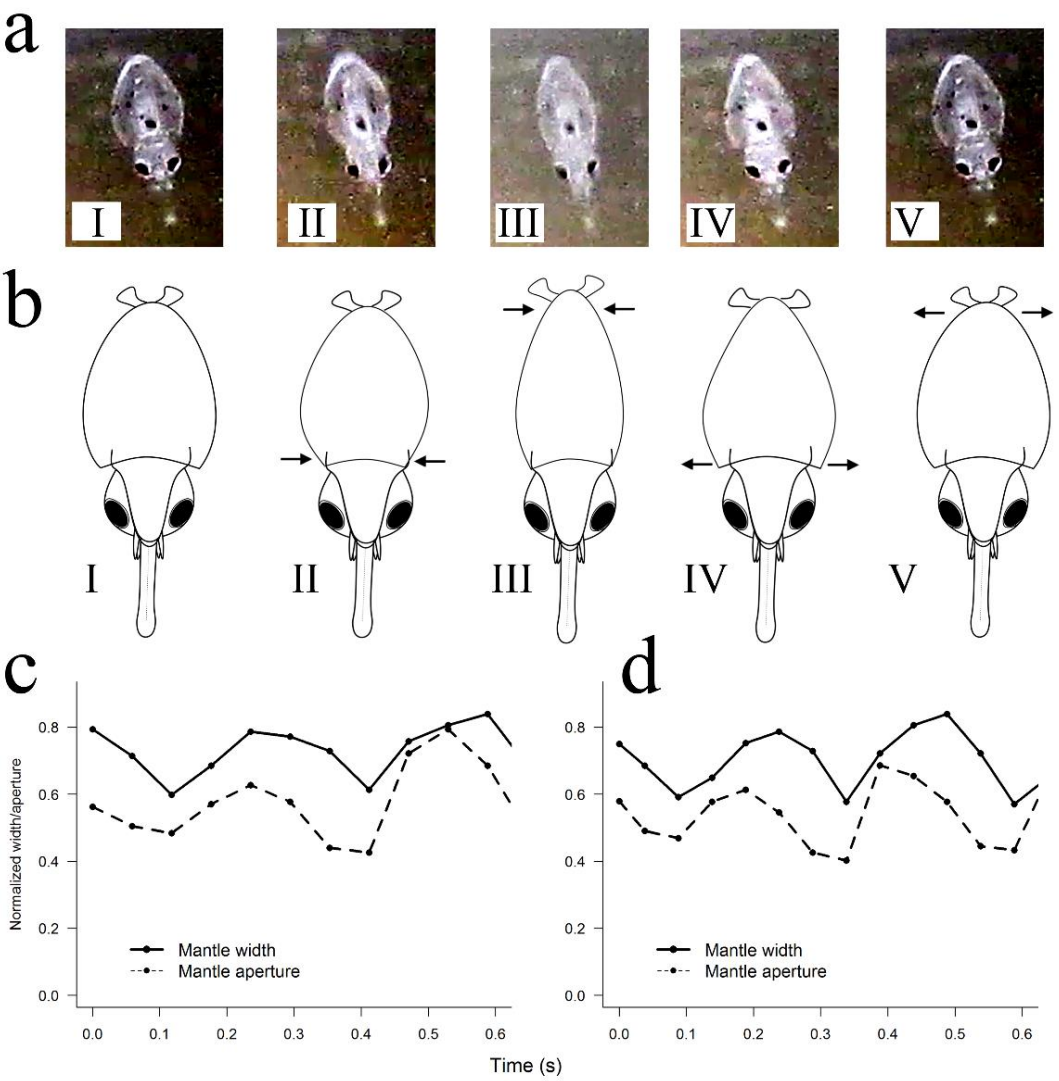

Figure 5-2. (a) Video frames and (b) diagrams of opening and closure of the mantle aperture around the head during a single jet. Arrows show the direction of movement of the mantle. (c) and (d) representative traces from standard videos (30 frames s^{-1}) of maintenance jetting and vertical jetting, respectively. Normalized mantle width and aperture are shown

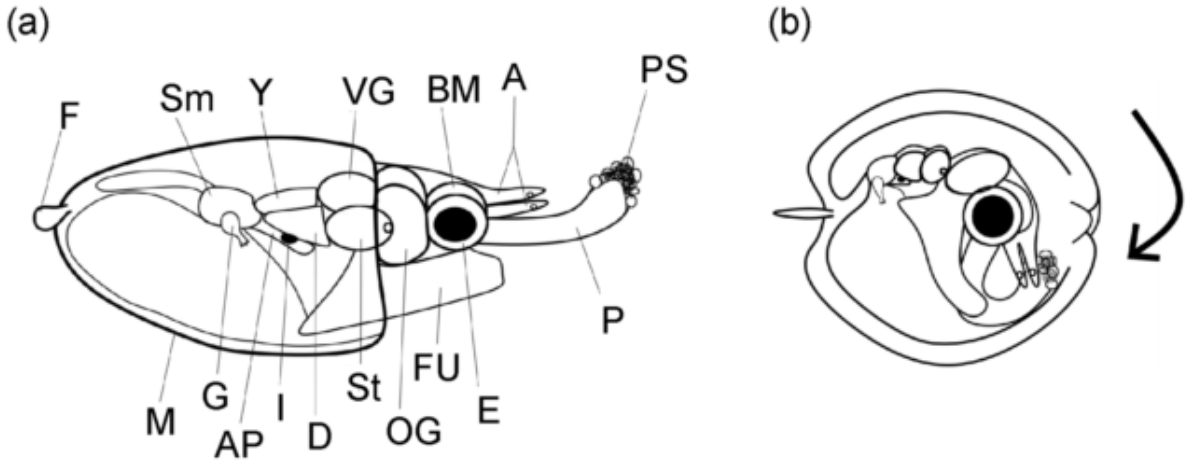

Figure 5-3. Ball formation. (a) Diagrammatic representation of a stage-33 *Ommastrephes bartramii* paralarvae (a, arms I and II; ap, anal papilla; bm, buccal mass; d, digestive gland; e, eye; f, fin; fu, funnel; g, gill; I, ink sac; m, mantle; og, optic ganglion; p, proboscis; ps, proboscis sucker; sm, stomach; st, statocyst; vg, visceral ganglion; y, inner yolk). (b) Diagram showing the retracted proboscis and head forming the ball posture. Internal organs and other parts are similar to those labeled in Fig 7a. Arrow shows the direction of retraction (towards the ventral side)

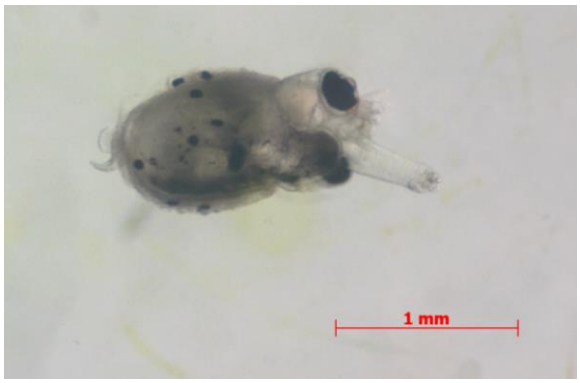

Figure 5-4. Paralarvae covered with dissolved organic matter (DOM) during feeding experiment

Conclusions

Among oegopsids, the family Ommastrephidae is of particular interest. Ommastrephid squid dominate the diets of many marine megafauna and support the world's largest cephalopod fisheries, which have been expanding as fish stocks decline (Caddy & Rodhouse 1998). Unfortunately, management of these fisheries is challenged by limited ecological knowledge, particularly regarding questions of reproduction and early development (Staaf 2010).

Little information is available on the early life of *Ommastrephes bartramii*, including spawning, embryos, paralarvae and juvenile stages, despite its commercial importance. The objectives of this dissertation were to observe the spawning pattern, and the embryonic and paralarval development of this species. My data for analysis came from two sources, first, secondary data records collected on board Hokkaido University's TS Hokusei Maru during 1990 to 2000. Second, from experiments done on board the RV Kaiyo Maru of the Fishery Research Agency of Japan, during 2012-2014, which I did myself.

Chapter 2 explored the spawning pattern of *O. bartramii* around the Hawaiian Islands. All living cephalopods are considered semelparous (i.e. characterized by a single reproductive event; Boyle 1983, Calow 1987, Rodhouse 1998), with the only exception being *Nautilus*, which lives more than 20 years (Rocha et al. 2001). However, recently, several authors (Nigmatullin et al. 1996, Nigmatullin 2002, Nigmatullin 2011) have reported complex reproductive patterns that cannot be categorized as semelparous, particularly in tropical and subtropical oegopsid squids belonging to the family Ommastrephidae. Before this study, there was no clear idea of the spawning events of *O. bartramii*. For this study I recorded the somatic indices and histological characteristics of the ovaries of 730 squids (622 males, 108

females), from which their spawning status was determined. My results indicate that *O. bartramii* is an intermittent spawner, investing in multiple filling and evacuation events of the oviducts. The presence of mature males with developed accessory glands in the same areas as healthy spent females (after the first spawning event) suggests that mating may occur between spawning events.

On the basis of the findings, I proposed an initial life-history model of female development and spawning for *O. bartramii*. The hypothesis says that first spawning event occurs at a ML of ~520–540 mm for Hawaiian *O. bartramii*. Subsequently, the squid forage and grow, and refill the oviducts, before the second spawning event occurs. In a lifetime they might release just two or three large egg masses, and spawning rates might vary individually. Future studies should focus on determining the duration of maturity, the rate at which the oviducts fill between spawning events, and the length of intervals between spawning events.

For chapters 3, 4 and 5, artificial fertilization experiments were conducted. The probability of successful collection of wild egg masses and live paralarvae of ommastrephids is very low and it is difficult to obtain these resources by spawning captive brood stock maintained in aquaria. As an alternative, artificial fertilization techniques have provided valuable information about the early development of oceanic squids and may provide material for larval rearing experiments that will enable study of the poorly understood physiology and ecology of paralarvae (Villanueva et al. 2012).

Developmental studies of ommastrephid squids are very limited. Detailed descriptions of embryonic development are available for only two ommastrephid squid (*Todarodes pacificus* and *Illex argentinus*) (Watanabe et al. 1996, Sakai et al. 1998). In chapter 3, I established an atlas for the normal development of *O. bartramii* from fertilized eggs to paralarvae. The stages are mainly based on morphological features and can be distinguished

clearly under a stereomicroscope. These observations of embryonic development correspond closely with those of other ommastrephids, including *T. pacificus* (Watanabe et al. 1996) and *I. argentinus* (Sakai et al. 1998). Until stage 15, embryonic development was similar across all studied ommastrephids and exhibited a meroblastic cleavage pattern. Stage 16 marks the onset of embryogenesis, and species-specific characters were expected to develop from this stage onward. Some of the species-specific characters used for the identification of paralarvae collected from the wild had already started appearing in the hatching stage, such as, the large lateral proboscis suckers and chromatophore patterns of *O. bartramii* (Young & Hirota 1990) and *E. luminosa* (Wakabayashi et al. 2002). Conversely, several characters were common across the species, such as the retarded appearance of arms IV in embryogenesis and complete absence of arms III.

The natural hatching stage is unknown for any ommastrephid. At the hatching stage in the laboratory, ommastrephids have many characters in common, with several species-specific characters. In my experiments, hatching occurred for *O. bartramii* primarily in stages 26-28, with a few individuals also hatching at stage 25. Similar results were obtained for *S. oualaniensis* and *E. luminosa.* The body size of *O. bartramii* at hatching was very small, as was expected due to the smaller egg size of this species in comparison with that of other ommastrephids. All hatchlings possessed a large amount of unconsumed inner yolk at hatching. This yolk content allowed them to remain alive for at least 7 days after hatching without feeding. In nature, hatching may occur at a later stage after more inner yolk has been consumed, because on board conditions involve continuous mechanical stimulation due to the movement of the ship, which has been known to induce early hatching (Watanabe et al. 1996, Sakai et al. 1998). This flexibility of hatching conditions suggests the existence of a hatching competence phase rather than a morphological and/or physiologically well-defined hatching

stage (Boletzky 1994). This flexibility may offer a means of optimizing posthatching survival (Boletzky 2003).

The species-specific effects of the oviducal gland powder on chorion expansion were also analyzed in chapter 3. *O. bartramii e*mbryonic survival was observed under treatment with oviducal gland jelly water (OGW) from two different species. Embryos treated with OGW from a single species throughout the experiment successfully reached the hatching stage. However, the chorion of the embryos for which the OGW provision was switched began to contract and did not survive longer than a day. My results suggest that the oviducal gland may contain no species-specific component that induces chorion expansion. These observations may be broadly indicative for ommastrephids and perhaps for other oegopsid squids (Staaf et al. 2011). However, when the source of oviducal gland jelly was changed after the embryo started to grow, chorion expansion was arrested, and the embryo showed deformities. These changes resulted in embryo mortality and indicated that the chemical nature of the oviducal gland may be species-specific, although the chorion expansion inducer itself may not differ.

Perivitelline space expansion and blastodisc formation were observed in unfertilized eggs after the addition of OGW. Staaf et al. (2011) observed this phenomenon in *D. gigas* and proposed the renaming of stages 1–3 in Watanabe et al. (1996) from "Fertilization and Meiosis" to "Meiosis-Blastodisc". In nature, spawned eggs (both fertilized and unfertilized) are always in contact with the oviducal gland, as the gelatinous substance composing the egg mass consists of secretions from the oviducal and nidamental glands (Boletzky 1998). Unfertilized eggs thus have the chance to develop partial parthenogenesis. The implications of such development in the wild have yet to be understood. This phenomenon may be a common trait among members of the family Ommastrephidae. It is important to determine whether parthenogenesis inhibits artificial fertilization (Staaf et al. 2011).

In chapter 4, I tried to explore the effects of temperature on the embryonic development of *O. bartramii*. From the outset of embryogenesis, ambient temperature sets the pace of developmental progress and thus influences the duration of embryogenesis (Laptikhovsky 1991). Within the species-specific range of temperature adaptation, development is faster at higher than at lower temperatures. At the lower and higher limits of this temperature niche, the incidence of embryo deformities increases substantially (Gowland et al. 2002).

The existence of a temperature minimum for development is a general phenomenon of the ommastrephid group (e.g. 12.5°C for *Illex illecebrosus* [O'Dor et al. 1982], 14°C for *T. pacificus* [Sakurai et al. 1996], and 15°C for *Dosidicus gigas* [Staaf et al. 2011]). Thermal limits for successful embryonic development in *O. bartramii* was unknown because they are true ocean dwellers for which experiments in land-based laboratories are challenging. Thermal limits determine the spawning grounds, recruitment levels, and spatiotemporal ranges of paralarvae (O'Dor et al. 1982, Sakurai et al. 2000, Staaf et al. 2011), and thus are important to understand. The embryonic development of these species, and its dependence on temperature, are of particular interest in the context of global warming and the resulting warming of the oceans (Rosa et al. 2012, Xavier et al. 2014).

I identified the possible thermal range of 18–25°C for complete *in vitro* development of *O. bartramii*. The duration of embryonic development through hatching decreased with increasing temperature and increased with decreasing temperature. These findings agreed with the results for other ommastrephid species, *I. illecebrosus*, *T. pacificus*, and *D. gigas*, which also have thermal limits. The higher rate of abnormal embryos that occurred outside of these thermal limits could have been due to hypo- or hyper embryogenesis (Sakurai et al. 1996) coupled with hypoxic stress (Rosa et al. 2012). The better survival rates that occurred in the

kreisel tanks compared to the petri dishes were presumably due to the opportunity for free swimming in the tanks. In addition, lower water volumes can cause stress and abrasion of skin and fins against the walls of petri dishes.

Furthermore, two lines of evidence support these thermal limits. First, the cruises collected mature (about to spawn and post-spawning) *O. bartramii* females from SSTs of 16° to 25°C. Second, paralarvae were also collected from the range of 18° to 25°C. This indicates that spawning and hatching might have occurred around this temperature range.

Ommastrephids produce neutrally buoyant but denser than seawater egg masses by embedding small eggs in large volumes of gel produced by the nidamental glands (O'Dor & Balch 1985). Depending on its density, these egg masses accumulate at the mid-water pycnocline (Bower & Sakurai 1996, Sakurai et al. 2000, Staaf et al. 2008), and squid paralarvae that hatch from the egg mass floating mid-water at the isopycnic surface are recruited into surface waters (Bakum & Csirke 1998, Sakurai et al. 2000). In the spawning grounds near Hawaii, paralarvae of *O. bartramii* are distributed in the surface layers where the pycnocline temperature is approximately 15–17°C (Bingham & Lukas 1996, Seki et al. 2002) during the putative spawning season of the winter-spring cohort (December–March) (Bower & Ichii 2005), which is lower than the ideal range of SST identified here. This indicates that paralarvae may hatch at lower temperatures in the pycnocline but survive in habitats at higher SSTs. My findings indicate that putative spawning and hatching temperature in waters around Hawaii can range from 16° to 25°C, and that paralarval distribution in the surface layers can occur from 18° to 25°C. The chance of spawning at a lower temperature (<16°C) is also a possibility.

Behavioural aspects of ommastrephid paralarvae beyond temperature effects were least studied. In chapter 5, I did a preliminary investigation on the paralarval activities soon

after hatching. This information on paralarval behavior characteristics is critical for a meaningful understanding of their life history. My observations on paralarvae were focussed on three aspects: a) swimming activities, b) peculiar ball formation, and c) feeding.

Muscular contractions in the developing embryos began at stage 23. Before hatching, the developing embryo always maintained a ventral-side-up posture in the dish. At hatching (stage 26), the egg membrane ruptured and the larvae left, posterior end of the mantle first, by a pulsating movement. Soon after that (within 8–10 s), the paralarvae started swimming by horizontal jetting, always with ventral side up. Swimming was achieved by jet propulsion with associated mantle contractions. From stage 30, paralarvae demonstrated a variety of swimming behaviors, categorized as maintenance jets, vertical jets, horizontal jets, escape jets, passive sinking, and pulsing. The categorization was modified from the report of Staaf et al. (2014) for the ommastrephid *D. gigas*. Maintenance jetting (observed in the kreisel tanks, Petri dishes, and cylindrical tubes) consisted of individual jets counteracting sinking to maintain their vertical position in the water column. Paralarvae were always observed to sink if not swimming actively. Prolonged periods of repeated jetting that exceeded the velocity of passive sinking were observed in vertical jets, with maximum measured speeds of 0.48 cm s^{-1}. The paralarvae moved upward during vertical jets, frequently to the water surface, which resembled the hop-and-sink behavior described in *D. gigas* paralarvae (Staaf 2010, Staaf et al. 2014). Horizontal jets observed in petri dishes consisted of repeated jets during which the ventral side was usually facing up. Wall effects of the petri dishes limited the horizontal jets in a diagonal path. Circular jetting—the occurrence of several rapid jets (approximately 1.2 cm s^{-1}) in a circular path (0.5-cm radius)—was observed. Less frequently (six times), stage 33–34 paralarvae swam with their lateral side up during horizontal jets; this was observed on the surface and never in the water column. Escape jets, the most rapid form of propulsion,

were individual jets and appeared to be similar to the escape jets of adult squid. The maximum speed measured in an escape jet was 3.12 cm s^{-1}. Resting paralarvae responded to physical contact from other paralarvae by escape jets, prompting the assumption that this was a "play" behavior. Passive sinking was observed when paralarvae that were swimming actively by vertical jets stopped jetting; these larvae were carried passively by small currents generated by aeration bubbles, which have little effect during active swimming. Passive sinking usually lasted for 3–4 s after which the paralarvae resumed hop-and-sink behavior. Pulsing behavior observed in the petri dishes consisted of continuous contractions and expansions of the mantle with fin flaps, but zero velocity gain in any direction.

Behavioral traits (e.g., vertical migration) could allow larvae to enter water masses that are moving in different directions at different rates, and could control the rate and direction of larval transport (Weinstein 1988). Squid paralarvae collect in great abundance in oceanic surface waters (Boyle & Rodhouse 2005). The vertical jets demonstrated by the paralarvae may explain their ascent mechanism from the mid-water hatching area to the surface water where they feed. These jets could also be used to effect diel vertical migrations, allowing the young cephalopods to sink to deeper depths during the day to avoid visual predators and rise to the surface at night (Staaf et al. 2008). Staff et al (2010) calculated that a paralarval migration of 15 m would take less than 1 h for *D. gigas* at the observed swimming speed of 0.5 cm s^{-1}. Vertical swimming experiments conducted by Yoo et al. (2014) with *T. pacificus* paralarvae at various temperatures revealed that the squid always had a preference for surface layers with higher temperatures. Hop-and-sink behavior may be an adaptation for conserving energy (Staaf 2010). Rapid jetting in paralarvae resembles burst-and-coast swimming in jellyfish (Alben et al. 2013) since there is a burst of thrust as the mantle contracts and water is expelled, followed by a coasting phase as the mantle refills.

When disturbed, the strong head-retractor muscles enable paralarvae to rapidly withdraw their head and proboscis ventrally into the mantle cavity (Shigeno et al. 2001). Ball formation in gonatid paralarvae is considered a defensive body posture that mimics the appearance (size and color) of the small hydromedusan jellyfish *Aglantha digitalis* (Arkhipkin & Bizikov 1996). Hydromedusan jellyfish are capable of stinging, so this resemblance discourages visual predators. Alteration of body posture by rolling into a ball is considered to be a defensive mechanism in cranchiids (Nixon 1983). The defensive body posture achieved by forming a ball helps to increase the chance of survival during the vulnerable paralarval phase (Arkhipkin & Bizikov 1996). Ball formation has been observed in chemically preserved samples (e.g., *Cranchia scabra*, Young 1972; *T. pacificus*, Shigeno et al. 2001). Withdrawal of the head into the mantle cavity during fixation illustrates the highly contractive capacity of head-retractor muscles: before death, the paralarvae responded to irritants (alcohol and formalin) by adopting the ball posture. Ball formation observed in a single drop of water may have been due to hypoxia. O'Dor et al. (1985) observed a complete withdrawal of the head into the mantle with simultaneous contraction of the mantle lip in rhynchoteuthion paralarvae of *Illex illecebrosus*, and interpreted this to represent a feeding behavior that enabled the squid to feed upon detritus adhered to the mantle's mucous covering. I observed the suckers of the proboscis close to the mouth in the ball state, so this may also be a feeding behavior. The sinking nature of the ball posture may help to move the squid away from danger zones. Since head-withdrawal behavior is provoked by mechanical (flash of light, agitation of water) or chemical (alcohol and formalin) stimulation, this behavior is presumably a defensive reflex in ommastrephids and other oceanic squid paralarvae.

Chromatophore expansion associated with the ball posture can generate aposematic coloration. Warning colors and patterns are an adaptation in prey species that provide a signal

to predators of the potential cost of making an attack. Orange coloration is associated with chemical defenses in at least three phyla (Young & Bingham 1987), and 60% of unpalatable marine invertebrate larvae are red or orange (Lindquist & Hay 1996). Aguado & Marin (2007) reported orange coloration as aposematic, and Valdes et al. (2013) reported bright color as aposematic in marine molluscs. Because ommastrephid paralarvae lack morphological defenses, the orange color and ball shape adopted by paralarvae when threatened by predators may imitate unpalatable organisms that share the same habitat.

My feeding experiments failed to identify the initial prey of the paralarvae. Other probable reasons for this result were inability to feed because of the absence of a functional buccal mass or digestive system as a result of premature hatching (stage 26) and smaller size. Hatchlings also had an available energy source in the remaining yolk content. In natural environments, normal hatching of undisturbed egg masses occurs when all of the yolk has been used (after stage 34) and when the digestive system is developed (O'Dor et al. 1982, Bower 1997). Future research could be directed towards finding appropriate prey items for ommastrephid paralarvae so that they can be reared under laboratory conditions to the adult stage like other myopsid paralarvae. Each transition period will be critical for paralarval survival, but the transition from suspension feeding to raptorial feeding will likely have the highest mortality because it requires major changes in food type, morphology, and feeding behavior (Uchikawa et al. 2009). The ability of the proboscis to attach to the walls of the dishes and tank indicates that well-developed suckers are formed after stage 30 and supports the hypothesis of proboscis-based suspension feeding proposed by O'Dor et al. (1985). In *O. bartramii*, raptorial feeding by arm-strikes starts at MLs of 13 mm (Uchikawa et al. 2009); until then, the proboscis could be used to direct the mucus mass or other food particles into the mouth.

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Appendix A. Fecundity estimation

Gravimetric method

$F = nG/g$

Where, F=fecundity, n= number of eggs in the subsample, G= total weight of the ovary and oviduct, g= weight of the subsample in the same units.

Volumetric method

$F = nV/v$

Where, F=fecundity, n= number of eggs in the subsample, V= total volume of the ovary and oviduct, v= volume of the subsample in the same units.

Appendix B. *Ommastrephes bartramii* paralarval stages

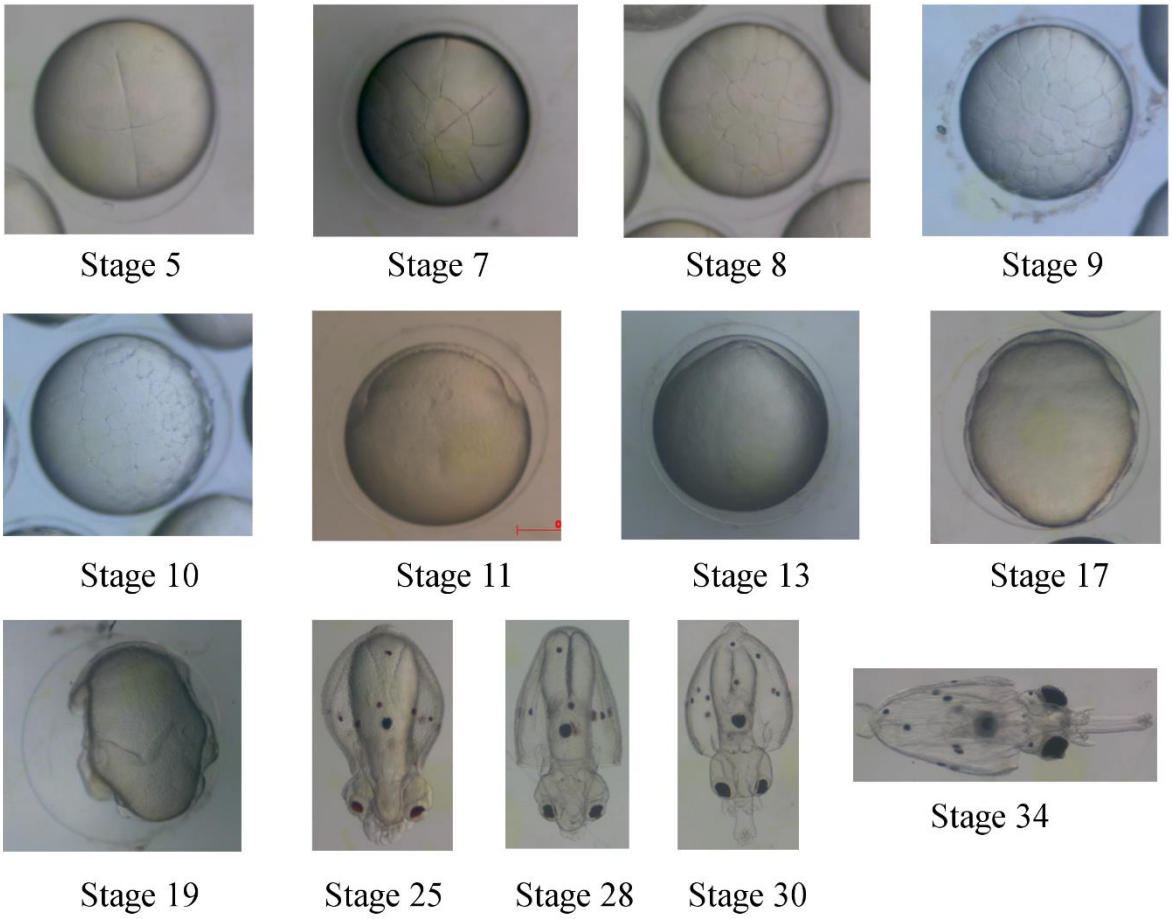

Figure A-1. Images showing some of the developmental stages of *Ommastrephes bartramii* photographed on board the RV Kaiyo Maru

Appendix C. Gear used for paralarval collection

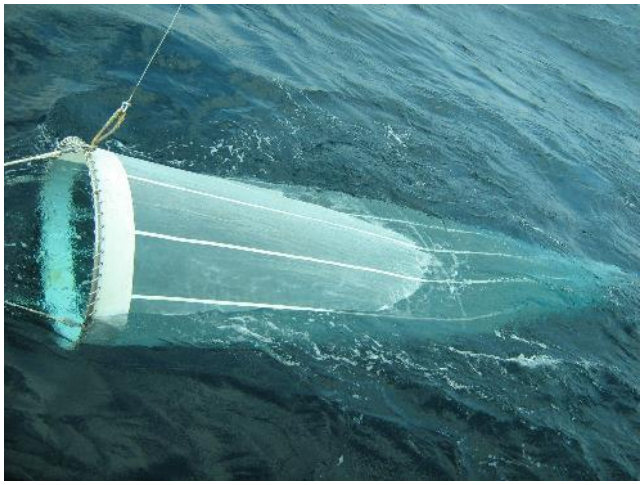

Figure A-2. Ring net (2-m diameter, 0.526-mm mesh size)

Figure A-3. Nackthai net (0.50-mm mesh size)

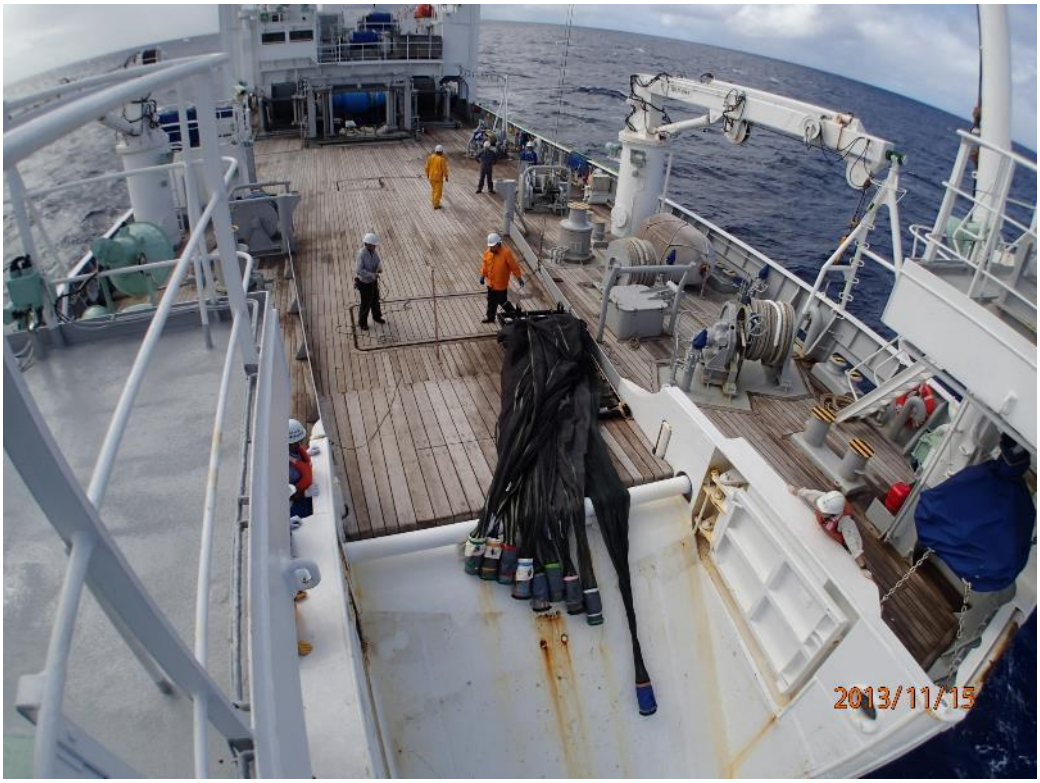

Figure A-4. Multiple opening and closing net with an environmental sensing system (MOCNESS, 1 m^2, 0.335-mm mesh size)