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Review

A laboratory guide to *in vitro* fertilization of oceanic squids

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ABSTRACT

In vitro fertilization of oceanic squid is a necessary step to develop their larval culture and creates new opportunities to study and understand cephalopod development, taxonomy and ecology. The techniques described here in the form of a laboratory guide represent an attempt to refine and standardize the general methodology by indicating suitable laboratory materials, sources and preservation of gametes, and methods for fertilization and egg incubation. Twelve oceanic squid species have been fertilized *in vitro* to date; we outline a generalized experimental protocol and suggest that the reader consider particular species-specific modifications. Inadequate egg chorion expansion and premature hatching are identified as major challenges for *in vitro* fertilization. Recommendations for future research include studies on optimal gamete concentration, gamete preservation and determination of the functions of female oviducal and nidamental glands. The greatest obstacles to improving fertilization success in squids are the lack of standard methodologies and the paucity of information on both endogenous and exogenous factors controlling the fertilization process. This review is a first step toward overcoming these challenges.

Contents

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1. Introduction

Oceanic squids (Teuthida: Oegopsida) are one of the most diverse groups of cephalopods, with more than 240 species described, occupying key trophic roles as predators in the open ocean ecosystem (Clarke, 1996; Jereb and Roper, 2010). Some of these species undergo high fishing pressure and their catches represent half of the total cephalopod world captures (Boyle and Rodhouse, 2005; FAO, 2010). But despite accumulated knowledge on the biology and ecology of juvenile, subadult and adult forms, information on eggs and paralarvae is still very limited and, for most species, does not exist at all.

This is probably due to their oceanic life and spawning mode. Neritic loliginid squid species attach dense egg masses to hard surfaces in shallow waters which are easily accessible to humans; by contrast, most oceanic squids produce (or are suspected to produce) ellipsoid translucent balloons from one to four meters in diameter containing from tens to hundreds of thousands of eggs. These egg masses are released in the open ocean, making them difficult for researchers to detect and access (see among others Bower and Sakurai, 1996; O'Shea et al., 2004; Roberts et al., 2011; Staaf et al., 2008; Young et al., 1985a and Table 1). Unusual cases of species spawning free eggs (Young et al., 1985b) or cylindrical egg masses (Guerra et al., 2002; Miyahara et al., 2006b) or even brooding egg masses in the female's arms (Bjorke et al., 1997; Seibel et al., 2000, 2005) have also been documented.

In view of the very low probability of collecting wild egg masses and the difficulties involved in obtaining them from spawning of captive broodstock maintained in aquaria (Bower and Sakurai, 1996; O'Dor and Balch, 1985; Staaf et al., 2008), *in vitro* fertilization techniques have provided an alternative method for obtaining valuable information on the early development of oceanic squids and are a necessary step to develop their larval culture. Eggs of twelve oceanic squid species (Table 1) have been fertilized artificially in the laboratory, enabling the description of embryo morphology, determination of the duration of embryonic life under different conditions and use of hatchling specimens for paralarval identification and taxonomy (Sakai et al., 1998; Sakurai et al., 1995; Watanabe et al., 1996; Yatsu et al., 1999). These techniques have also facilitated the investigation of the development of internal organs of embryos and hatchlings (Shigeno et al., 2001a,b), somatic growth using statoliths (Balch et al., 1988; Sakai et al., 1998, 2004; Yatsu et al., 1999), and swimming behaviour of paralarvae (Staaf et al., 2008). These laboratory studies reveal the temperature ranges in which normal embryogenesis is possible and help to delimit potential spawning areas in the wild

Table 1
Studies on the eggs, egg masses and embryonic development of oegopsid oceanic squids. Wild and aquaria spawned egg masses as well as laboratory *in vitro* fertilization experiments are indicated.

Species	Fertilization type	Geographic area	Reference
Abralia trigonura	Wild eggs	North Pacific	Bigelow, 1992
Abralia sp.	*In vitro*	North Pacific	Arnold and O' Dor, 1990
Abraliopsis sp.	*In vitro*	North Pacific	Arnold and O' Dor, 1990
Brachioteuthis sp.	Wild eggs	North Pacific	Young et al., 1985b
Dosidicus gigas	*In vitro*	SE Pacific	Yatsu et al., 1999
	Wild egg mass, spawning in aquaria and *in vitro*	NE Pacific	Staaf et al., 2008, 2011
Enoploteuthinae	Wild eggs	North Pacific	Okiyama and Kasahara, 1975; Young and Harman, 1985
Gonatus fabricii	Wild egg mass	NE Atlantic	Bjorke et al., 1997
Gonatus onyx	Wild egg mass	NE Pacific	Seibel et al., 2000, 2005
Illex argentinus	*In vitro*	SW Atlantic	Sakai and Brunetti, 1997; Sakai et al., 1998, 2004, 2011
Illex coindetii	Wild egg mass	Mediterranean	Naef, 1928
	Spawning in aquaria	Mediterranean	Boletzky et al., 1973
	In vitro	Mediterranean	Villanueva et al., 2011
Illex illecebrosus	Spawning in aquaria	NW Atlantic	Balch et al., 1985; Durward et al., 1980; O'Dor and Balch, 1985
	Spawning in aquaria and *in vitro*	NW Atlantic	O'Dor et al., 1982
Lycoteuthidae	Wild egg mass	SW Indian	Roberts et al., 2011
Nototodarus gouldi	Wild egg mass	SW Pacific	O'Shea et al., 2004
Ommastrephes bartramii	*In vitro*	North Pacific	Sakurai et al., 1995
Sthenoteuthis oualaniensis	Spawning in aquaria	Arabian Sea	Chesalin and Giragosov, 1993
	In vitro	North Pacific	Sakurai et al., 1995
Sthenoteuthis pteropus	Wild egg mass	Eastern Atlantic	Laptikhovsky and Murzov, 1990
Sthenoteuthis sp.	*In vitro*	North Pacific	Arnold and O' Dor, 1990
Thysanoteuthis rhombus	Wild egg mass	East Atlantic and Mediterranean	Guerra et al., 2002; Sanzo, 1929
	Wild egg mass	East Pacific	Sabirov et al., 1987
	Wild egg mass	West Pacific	Billings et al., 2000
	Wild egg mass	NW Pacific	Misaki and Okutani, 1976; Miyahara et al., 2006a,b; Suzuki et al., 1979; Watanabe et al., 1998
	In vitro	North Pacific	Arnold and O' Dor, 1990
Todarodes pacificus	*In vitro*	NW Pacific	Hayashi, 1960; Ikeda and Sakurai, 2004; Ikeda et al., 1993; Ikeda and Shimazaki, 1995; Sakurai et al., 1995, 1996; Shigeno et al., 2001a,b; Soeda, 1952, 1954, 1956; Watanabe et al., 1996;
	Spawning in aquaria	NW Pacific	Bower and Sakurai, 1996; Hamabe, 1962, 1963
Todaropsis eblanae	*In vitro*	Mediterranean	R. Villanueva, unpublished
Watasenia scintillans	Spawning in aquaria	NW Pacific	Hayashi, 1995

(O'Dor et al., 1982; Sakurai et al., 1996; Staaf et al., 2011). Ultimately, artificial fertilization may provide material for larval rearing experiments to study the poorly understood physiology and ecology of young oceanic squid. *In vitro* fertilization of two shallow water loliginid squids, *Doryteuthis pealeii*, (Crawford, 1985; 2000, 2001, 2002, 2003; Kao, 1985; Klein and Jaffe, 1984; Wadeson and Crawford, 2003) and *Heterololigo bleekeri* (Iwata et al., 2011) has facilitated research like that described above. However, due to the aforementioned ease of obtaining naturally spawned loliginid eggs, *in vitro* work on loliginids has not been widespread.

The 5th International Symposium on Pacific squid, held during October 2010 in La Paz, Mexico, included a workshop entitled "Considerations for *in vitro* embryonic development in *Dosidicus gigas*: theory and practice." As a conclusion of the workshop, participants compiled their published and unpublished methodologies into a user-friendly "cooking recipe" to be used in future research. The following sections describe these methods and protocols and highlight areas that need further investigation. Our objective is to establish a laboratory protocol that ensures maximal success of *in vitro* fertilization of oceanic squid, while identifying the main gaps in knowledge that are inhibiting further success. Most knowledge of *in vitro* fertilization of squid comes from a few species of the family Ommastrephidae; the reader should consider that these techniques could be subjected to particular modifications when other families are studied.

2. Laboratory guide to *in vitro* fertilization of oceanic squids

2.1. Species-specific considerations

Before planning any *in vitro* fertilization experiments, a minimal knowledge of the biology of the selected species and the oceanographic conditions within its geographic range is necessary. Knowledge of the species' maturity season(s) and spawning grounds helps to identify the best time to collect mature individuals and carry out experiments. Furthermore, it is helpful to know a well-defined sexual maturity scale and maturity characteristics of the species, in order to identify and select the best functionally mature individuals as sources of gametes. The location of sperm storage in copulated females also varies between species (see below). Reproductive anatomy in Ommastrephid squids are showed in Fig. 1.

Oocyte size and egg incubation temperature influence the relative duration of embryonic development in different species of cephalopods (Boletzky, 1994; Laptikhovsky, 1999), and may determine the duration of designed experiments. For example, oceanic squid species with relatively small oocytes, such as *Todarodes pacificus*, whose oocyte length is 0.83 mm (Watanabe et al., 1996), need 2 days to hatch when incubated at 26 °C and 8 days to hatch at 14 °C (Sakurai et al., 1996). In contrast, a deep-sea species such as *Gonatus onyx*, with relatively large eggs of 2–3 mm in length, may need up to 9 months to develop at 3 °C, the temperature at which spawning females were collected (Seibel et al., 2000). Information about the oceanographic characteristics of the species range and the depth ranges of known (or suspected) spawning are important parameters to be taken into account when setting up laboratory temperature ranges. Suitable salinity should also be chosen based on the species range.

2.2. Sources and preservation of gametes

Using as many adult females and males as possible at the same time is recommended in order to select the most suitable gametes, as there can be significant individual variability in gamete viability. The female and male sources of gametes used in each fertilization should always be recorded. A few hours after fertilization (see below), the fertilization rates of the different progenitors can be checked in order to select the most suitable group of embryos to continue incubating. The sequence of laboratory steps for the gamete collection, *in vitro* fertilization and egg incubation of oceanic squid are showed in Fig. 2.

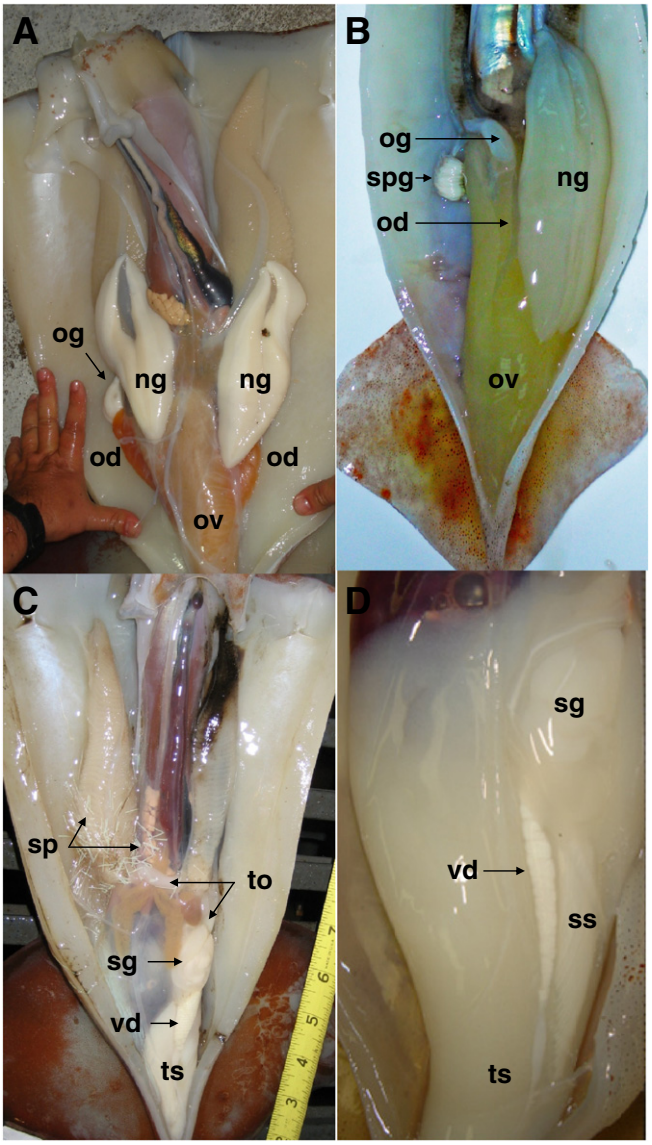

Fig. 1. Reproductive anatomy of Ommastrephid squids. A, mature female *Dosidicus gigas*; B, mature female *Illex coindetii* (note that nidamental glands have been displaced laterally to shown the spermatangia); C, mature male *Dosidicus gigas*; D, mature male *Illex argentinus*. Abbreviations: ng, nidamental gland; od, oviduct; og, oviducal gland; ov, ovary; sg, spermatophoric gland; sp, spermatophores (released); spg, spermatangia; ss, spermatophoric sac; to, terminal organ (passes under the gill); ts, testis; vd, vas deferens.

2.2.1. Oocytes

In the present paper we use the term *oocyte* to refer the unfertilized female gametocyte. Oocytes are stored inside the oviducts of a mature female, ready to be spawned. The term *egg* is used here to denote the zygote and resulting embryo obtained after fertilization. Oocytes can be obtained from both oviducts of dissected mature females; no difference in oocyte maturity between right and left oviducts has been reported to date for oceanic squid.

2.2.2. Spermatozoa

Sperm can be collected from two sources: mature males and copulated females. Males of oceanic squid produce spermatophores in the spermatophoric glands; the spermatophores are then

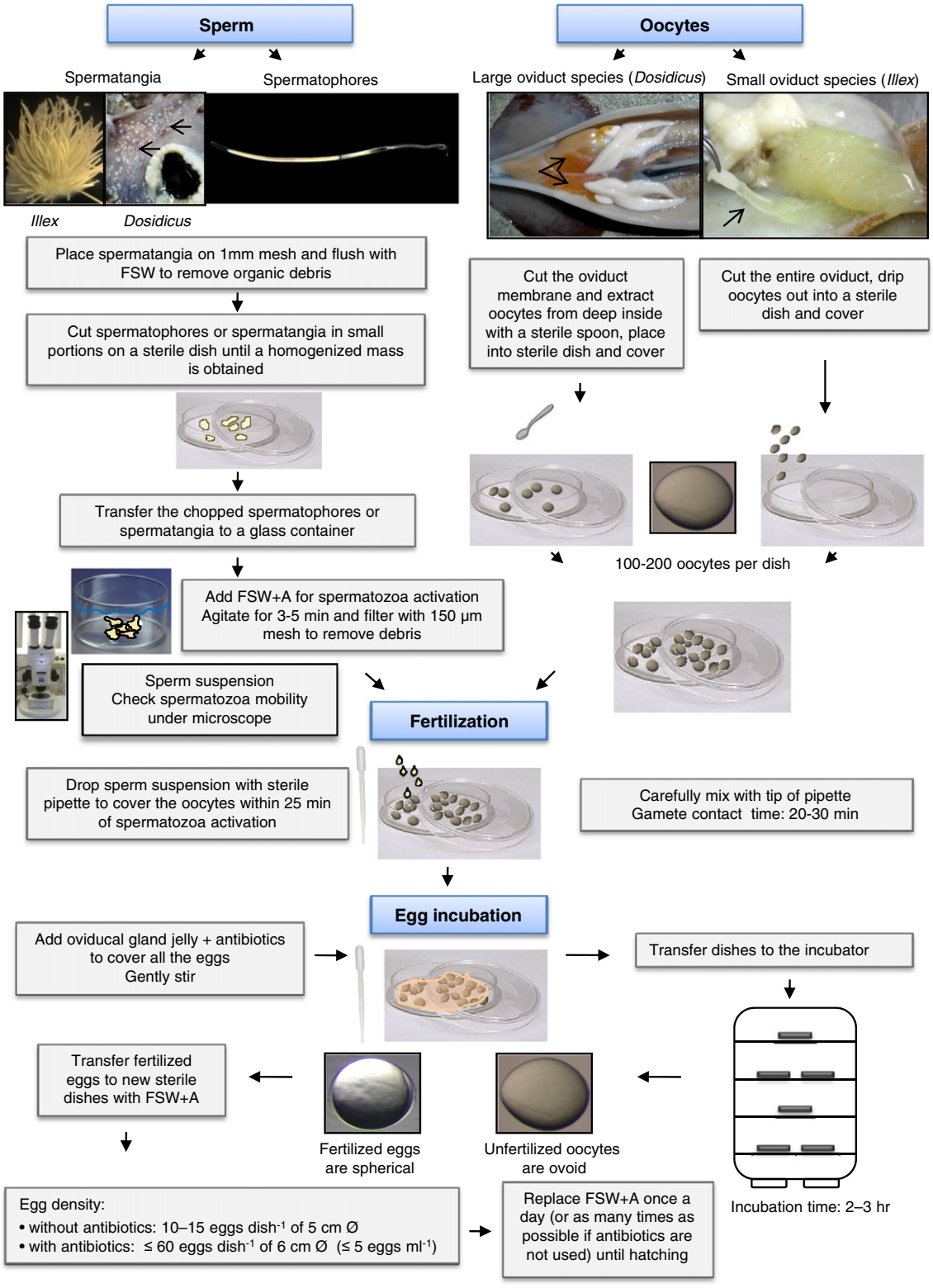

Fig. 2. Schematic sequence of laboratory steps for the gamete collection, *in vitro* fertilization and egg incubation of oceanic squid. FSW, filtered seawater; FSW + A, filtered seawater with antibiotics.

accumulated in the spermatophoric sac (Needhman's sac) (Nigmatullin et al., 2003). During mating, the spermatophores undergo the so-called spermatophoric reaction, a complex process of evagination of the ejaculatory apparatus of the spermatophore that leads to the extrusion and attachment of the spermatangium (i.e., everted spermatophore containing the sperm mass) on different areas of the female body (Marian, 2011, 2012; Nesis, 1995). Depending on the species, the spermatangia (everted spermatophores) of copulated females can be found on: 1) the external body surfaces of the females as modified seminal receptacles on the buccal membrane and outer lip (Ikeda and Sakurai, 2004; Ikeda et al., 1993); 2) implanted in the anterior dorsal and ventral rugose, semi-gelatinous mantle tissue

(Hoving et al., 2008); 3) inserted in unmodified tissues on the skin of the arms, head and mantle (Guerra et al., 2004; Hoving and Laptikhovsky, 2007; Hoving et al., 2004, 2012; Norman and Lu, 1997); 4) attached internally on seminal receptacles situated on the nuchal cartilage (Hoving et al., 2007); 5) implanted into the mantle muscle layers (Hoving et al., 2010; Nesis et al., 1998), or 6) attached to the inner surface of the mantle near the bases of the gills (Brunetti, 1990; Sakai et al., 1998).

Ikeda et al. (1993) studied the fertilizing capacity of spermatozoa from different parts of the mature male reproductive system and from copulated females of *T. pacificus*, and concluded that spermatophores from the male's spermatophoric sac and spermatangia from copulated females have similar fertilization rates. These authors found that sperm from the male's vas deferens had less fertilizing capacity; however, Sakai et al. (2011) found high fertilization rates from sperm collected from the male vas deferens in *Illex argentinus*.

2.2.3. Age and preservation of gametes

Most *in vitro* experiments to date have been carried out on board oceanographic vessels immediately after capture of adult squid (Arnold and O' Dor, 1990; Sakai and Brunetti, 1997; Sakai et al., 1998; Sakurai et al., 1995; Staaf et al., 2008; Yatsu et al., 1999) or from squid maintained in aquarium (Ikeda et al., 1993; Sakurai et al., 1996; Watanabe et al., 1996) and in both cases showed high fertilization rates. Fertilizations conducted 4–5 h after squid capture and death have also demonstrated good fertilization rates, when whole individuals are maintained on ice (mean: 67%, range: 28–94%) (Villanueva et al., 2011). Whole individuals stored at 0 °C also maintain a high fertilization capacity (>72% within 24 h) (Sakai et al., 2011). Use of gametes from recently dead squids is recommended; however, gametes from oviducts and spermatophoric sacs placed in sealed plastic bags and preserved at 0–4 °C can serve as relatively good experimental material for up to two days (Staaf et al., 2011). This time period may allow transport of gametes from the sea to a land laboratory with better working facilities, or exchange of material between laboratories.

Even greater longevity has been observed in sperm, but not in oocytes. Spermatangia of the oceanic squid *Dosidicus gigas* stored in filtered seawater at 12 °C showed spermatozoa motility for up to 5 days (Huffard et al., 2007). Intact spermatophores of the cuttlefish *Sepia apama* placed in plastic tubes with filtered seawater and refrigerated at 4 °C showed spermatozoa motility after two months (Naud and Havenhand, 2006). Long term sperm refrigeration has been not been studied in squids, but similar characteristics may be suspected. Preliminary results on the cryopreservation of *Illex coindetii* sperm consisted of freezing the whole spermatophore in liquid nitrogen, using dimethyl sulfoxide as cryoprotectant agent (Robles et al., submitted for publication). These spermatophores stored at 4 °C in filtered seawater during two days after squid capture and then freezing in liquid nitrogen allows the recovery of only a low percentage (10%) of viable and motile spermatozoa.

2.3. Laboratory materials

2.3.1. Laboratory tools

Microbial infection is a major problem to avoid during fertilization and egg incubation experiments, so the use of sterile material is essential. Laboratory working surfaces, needles, forceps and scissors should be cleaned with ethanol prior to use. Gloves and masks are recommended. To date, all successful egg incubations have been performed at a small scale using Petri dishes. Small or medium-sized sterile dishes are recommended (50 to 90 mm diameter). If a dish is infected, all the eggs inside will die within a few hours; multiple small dishes therefore result in higher survival rates than one large dish. Incubators for dishes are useful to maintain a stable temperature as well as a clean incubation environment.

2.3.2. Seawater

Use of 0.2 μm filtered seawater (FSW) is recommended. FSW can be stored before use in autoclaved glass bottles in darkness at 4 °C. Recently, the addition of antibiotics (25 mg l^{-1} each of ampicillin and streptomycin) to FSW has provided high embryonic survival rates (Staaf et al., 2008; Villanueva et al., 2011). However, previous studies had also obtained high survival without antibiotics by changing FSW as frequently as possible to reduce microbial growth. When using antibiotics, the mix of FSW + antibiotic should be made new every day before use to avoid antibiotic degradation. All FSW + antibiotic used during the experiment should be collected for suitable waste treatment.

2.3.3. Oviducal gland jelly and other substances to obtain chorion expansion

Obtaining expansion of the primary egg envelope, or chorion, is a major challenge of *in vitro* fertilization. This requires the presence of oviducal gland jelly in the incubation medium, as demonstrated by Ikeda et al. (1993). Fresh, frozen or freeze-dried oviducal gland can be used. To prepare fresh and frozen gland, pieces of tissue are chopped with seawater and stirred just before fertilization. Freeze-dried material should be shaken or blended to obtain fine powder, which can be stored frozen until use. Freeze-dried oviducal gland is highly recommended because the freeze-drying process reduces pathogens, the powder is easy to transport and store, and dry weight of the powder can be used to determine the exact quantities to add to seawater. Good embryo survival and chorion expansion was observed with concentrations of 1 g l^{-1} of oviducal gland powder in seawater (Sakurai et al., 1995; Villanueva et al., 2011). Oviducal gland powder produces a mucus-like jelly solution after being stirred with FSW. This solution often contains some organic debris, which should be removed by filtering with a 100 μm mesh before the solution is added to the eggs. The active component or characteristic of the oviducal gland that helps chorion expansion remains unknown, but appears to function across species. Within ommastrephids, oviducal gland powder from one species can be used successfully with the eggs of another species (Sakurai et al., 1995; Yatsu et al., 1999).

As an alternative to the use of oviducal gland jelly, *in vitro* fertilization of *Doryteuthis pealeii* was obtained with suitable chorion expansion and hatching by using plastic Petri dishes lined with a cushion of 0.2% agarose to prevent adhesion of the eggs (Klein and Jaffe, 1984) and agarose plus 0.5% bovine serum albumin in FSW (Crawford, 2002). However, agarose and BSA were not successful in obtaining chorion expansion for the oceanic squid *Dosidicus gigas* (Staaf et al., 2011).

2.3.4. Anaesthetic

Ethics and welfare laboratory protocols should be followed during experimentation with live cephalopod embryos and paralarvae resulting from fertilizing experiments (Mather and Anderson, 2007; Moltschaniwskyj et al., 2007). Anaesthetic should be used to reduce pain when necessary, particularly before sacrificing individuals. Magnesium chloride ($MgCl_2$) is an effective anaesthetic and narcotizing agent for cephalopods, and over-anaesthesia can be used to euthanize individuals (Messenger et al., 1985; Mooney et al., 2010). Alternatives to $MgCl_2$ include chilling and increasing concentrations of ethanol from 1 to 5%.

2.4. Step-by-step recipe for in vitro fertilization and egg incubation

2.4.1. Activation of spermatozoa

As noted before, spermatophores or spermatangia can be used as sperm sources. Spermatophores can be collected by dissecting the spermatophore sac of mature males. Spermatangia are located on external surfaces of the female body (see above) and sometimes may have mud or organic debris. In this case, place whole spermatangia on a 1-mm mesh and vigorously flush with seawater to remove organic debris from their surface before collecting the sperm. Cut

sperm sources in small portions (in ex., ≤2 mm) on a Petri dish using scissors or razor blades until a homogenized mass is obtained. For spermatozoa activation, sperm should be in contact with FSW. Transfer the chopped spermatophores or spermatangia to a glass container and add FSW (optional step: FSW with ampicillin and streptomycin, 25 mg l^{-1}) to obtain a milky sperm suspension that should be gently agitated for 3–5 min. Filter this solution with 150 μm mesh to remove debris of spermatophores or spermatangia. Add the sperm suspension to the oocytes within 25 min of spermatozoa activation (Villanueva et al., 2011). Spermatozoa mobility can be checked under a microscope at 200–400× magnification.

2.4.2. Collection of oocytes

For large oviducts, cut the membrane of the oviduct and extract the oocytes from deep inside, using a sterile plastic spoon to avoid microbial infection (Sakurai et al., 1995). For small oviducts, cut the entire oviduct, then pick up the pieces with tweezers and let the oocytes drip out into sterile Petri dishes. In each dish, a small drop containing about one to two hundred oocytes will be sufficient. Cover the dish with a lid as soon as oocytes are deposited. Before the addition of sperm, oocytes can remain some minutes virtually dry on the dish (range 8 to 12 min), which does not affect their capacity to be fertilized (Villanueva et al., 2011).

Optional step: Some species, such as *T. pacificus*, may need oocyte hydration before fertilization. For hydration, oocytes need to be placed in a container with seawater for a few minutes (Sakurai et al., 1995).

2.4.3. Fertilization

Add drops of sperm suspension using a sterile pipette, enough to cover the pile of oocytes. Carefully mix the oocytes and sperm suspension with the tip of the pipette to homogenize the medium, avoiding damage to the oocytes. A gamete contact time of 20–30 min seems to be sufficient for fertilization. Temperature during the fertilization process should be controlled. High room temperatures may affect gamete quality and/or early embryo development, depending on the species' natural temperature range.

Optional step: Oocytes collected from the oviduct, washed several times in FSW and added directly to the sperm suspension resulted in high fertilization rates in *D. pealeii* (Crawford, 2002).

Optional step: If the quantity of available sperm is limited, fertilization success may be increased by using a longer contact time between gametes, as observed in fishes (Butts et al., 2009).

2.4.4. Egg incubation

After fertilization, add oviducal gland jelly to cover all the eggs, filling approximately half of the Petri dish. Gently stir again. Transfer dishes to incubators. Two to three hours after fertilization (depending on temperature), successfully developing eggs should be identifiable by one of several methods. Observation of cleavage is unambiguous. In some cases, developing eggs can also be distinguished by chorion expansion and a relatively spherical shape in comparison with the ovoid shape of the unfertilized oocytes. At this point, successfully fertilized eggs can be transferred with pipettes to new dishes at the desired egg density. For incubations using FSW without antibiotics, densities of 10–15 eggs per dish of 50 mm diameter can be incubated at 22 °C (Sakurai et al., 1995). Sakai et al. (2004) reported egg incubations at temperatures ranging from 11.4 to 25.4 °C in polystyrene multi-well plates with 6 wells of 35 mm diameter and 15 ml volume, at a density of 20 eggs in 10 ml seawater (2 eggs ml^{-1}). Seawater in the wells was changed at least four times a day. When using antibiotics, a conservative egg density of ≤60 eggs per dish of 60 mm diameter (≤5 eggs ml^{-1}) and a daily change of seawater is recommended at an incubation temperature of 17 °C (Villanueva et al., 2011).

Depending on the species, care should be taken when transferring eggs. Mechanical stress imposed by aspiration of recently fertilized eggs of *Doryteuthis pealeii* using microhematocrit pipettes does not affect initial stages of development (Kao, 1985). However, Sakai et al. (2011) showed that embryos of *I. argentinus* at developing stages 5 to 12 (from second cleavage until blastoderm stage; embryos aged 4.5 to 11.5 h after fertilization at 20 °C) were the most sensitive to mechanical stress produced by aspiration, and caution should be taken when handling eggs during this critical period. FSW (optional: with antibiotics) can be replaced every day using sterile pipettes until hatching. Dead embryos should be removed, and counted, if necessary, to estimate mortality rates. During daily water changes, take care to avoid temperature shocks, and never leave the eggs without water. High room temperatures during water changes may affect embryo development and survival. The experimental design should include an adequate number of replicates for each treatment in order to permit statistical analysis of the results.

Optional step: If necessary, after the addition of oviducal jelly, eggs can remained more than 2–3 h in this medium until the first FSW change, without apparent damage. For example, recently fertilized eggs of *I. coindetii* maintained for 19 h in oviducal jelly + antibiotics until the first FSW change and incubated at 17 °C resulted in a 44 ± 22 survival rate to hatchling (R. Villanueva, unpublished).

Optional step: Depending on the experimental objective, more additions of oviducal jelly during embryonic development may be applied to increase chorion expansion. A second oviducal jelly addition before organogenesis in *I. coindetii* embryos resulted in a larger egg diameter, partially delayed hatching and heavier hatchling squids, in comparison with treatments receiving only one jelly addition (Villanueva et al., 2011).

3. Future research

The *in vitro* techniques described here are relatively simple and can be used on oceanographic vessels and in land laboratories, but they may require some improvement and adaptation to each different squid species. In other groups of molluscs and fishes, experimental techniques greatly influence fertilization success (Butts et al., 2009; Song et al., 2009). Fertilization techniques in squids need to overcome the previous lack of a standardized methodology and procedures will benefit from further clarification of such important fertilization parameters as optimal oocyte concentration, seawater fertilization volume and spermatozoa: oocyte ratio. Little progress has been made in elucidating the motility of squid spermatozoa (Iwata et al., 2011; Laptikhovsky, 1990; Laptikhovsky and Nigmatullin, 1996; Wang et al., 2011) and the spermatozoa density necessary for suitable squid oocyte fertilization has not been determined. Up to now an excess of sperm has probably been used in order to guarantee fertilization success.

Methods to determine gamete quality and density, critical gamete contact time and spermatozoa motility are well established in marine fishes (see among others: Chereguini et al., 1999; Cosson et al., 2008; Rurangwa et al., 2004; Suquet et al., 1995; Tvedt et al., 2001) and we expect that many of these techniques can be adapted to squids. However, certain reproductive characteristics of cephalopods may make *in vitro* fertilization easier than in fish. For example, the existence of a spermatozoa attractant as observed in the cuttlefish *Sepia officinalis* may facilitate fertilization by increasing chances of gamete contact, and opens new experimental approaches in this field (Zatylny et al., 2002). In addition, the biflagellate spermatozoa of *Illex* squid enable them to swim at high velocity of 130–140 μm s^{-1} (Laptikhovsky and Nigmatullin, 1996), which probably enhances gamete contact and increases fertilization rates. This spermatozoa velocity is close to the 167 μm s^{-1} recorded for *Heterololigo bleekeri* (Iwata et al., 2011) and higher than that of other cephalopod species, most of which fall between 25 and 65 μm s^{-1} (Laptikhovsky, 1990).

Fertilization in rare oceanic squid species may be facilitated in the future through the conservation and storage of sperm by refrigeration and cryopreservation. Naud and Havenhand (2006) showed that

cuttlefish spermatophores refrigerated for two months at 4 °C still contain mobile spermatozoa. Similar capabilities may be suspected of squid sperm. Techniques for the cryopreservation of squid sperm are available (Robles et al., submitted for publication) and promise to be a useful tool for *in vitro* fertilization programs as in other aquatic invertebrates and marine fishes (see among others: Bart et al., 2006; Cabrita et al., 2010; Gwo, 2000; Vuthiphandchai et al., 2007), allowing squid sperm availability throughout the year. Techniques for cephalopod gamete preservation, particularly female gametes, should be explored. For example, chilled storage (2 °C) of unfertilized fish oocytes packed into sealed polyethylene bags without a gas-filled space resulted in 50% of fertilization rate after 20 days (Komrakova and Holtz, 2011), and squid oocytes may have similar potential.

Premature hatching seems to be a common event in oceanic squid fertilized *in vitro* (Sakai et al., 1998; Villanueva et al., 2011; Watanabe et al., 1996; Yatsu et al., 1999). Watanabe et al. (1996) noted that eggs from wild egg masses of *T. pacificus* hatched two stages later than artificially fertilized eggs and suggested that wild eggs were enwrapped by nidamental jelly in addition to oviducal jelly, and this natural gelatinous matrix may delay hatching. Research on the function(s) of the oviducal and nidamental glands in the formation of natural egg masses and natural egg development will probably suggest improvements to *in vitro* fertilization techniques in the near future. It is assumed that the oviducal mucosubstance, which is essential for chorion expansion in fertilized eggs, surrounds each egg when spawned (Ikeda et al., 1993). Kimura et al. (2004) showed that the water-soluble fraction of the mucosubstance of the nidamental gland forms the egg mass surface layer and the insoluble fraction forms the fibrils within the egg mass of *T. pacificus* egg masses. In the same species, Ikeda and Shimazaki (1995) showed that nidamental gland jelly does not induce the formation of perivitelline space at fertilization. However, chorionic expansion was obtained in *Abralia*, *Abraliopsis, Sthenoteuthis* and *Thysanoteuthis* by using freeze-dried nidamental gland of *Loligo* sp. (Arnold and O' Dor, 1990) and in *Doryteuthis pealeii* by adding a cushion of 0.2% agarose (Klein and Jaffe, 1984) or agarose plus 0.5% bovine serum albumin (Crawford, 2002). These different results suggest that probably more than one factor contribute to chorionic expansion, and further research is needed.

The use of antibiotics (ampicillin and streptomycin) to avoid microbial infections is recommended because their experimental use seems to produce higher embryo survival rates. However, the existence of secondary effects of these antibiotics on the embryo is unknown and different antibiotics and concentrations should be explored. Antimicrobial activity of the accessory nidamental gland and egg cases has been detected in loliginid squid and may inhibit microbial growth on the egg capsule through several mechanisms, such as reducing ciliary activity (Atkinson, 1973; Barbieri et al., 1997; Biggs and Epel, 1991). This antimicrobial activity has been attributed to the higher levels of unsaturated fatty acids present on accessory nidamental glands of ripe individuals (Gomathi et al., 2010), suggesting that the accessory nidamental gland may help loliginid embryos to resist infection. Although oegopsid squid lack these accessory glands (Young et al., 1998) the oegopsids nidamental glands should be studied for antimicrobial properties.

To date, all incubations of embryonic squid have been done at a small scale, using Petri dishes. Investigations into larger egg incubation systems will help to reduce working time and facilitate the collection of large numbers of hatchlings. Furthermore, larger scale systems may permit the rearing of oceanic squid paralarvae, which has not yet been successful for any species.

4. Conclusions

Egg masses of oegopsid squid are spawned in the open ocean, making them difficult to detect. In view of the challenges involved in acquiring naturally spawned eggs, *in vitro* fertilization techniques have provided an alternative method for obtaining valuable information on the eggs and paralarvae of oceanic squids. A laboratory guide for the *in vitro* fertilization of oceanic squid is provided here with the aim of recommending laboratory materials, sources and preservation of gametes, and improving existing methods for fertilization and egg incubation. Adequate egg chorion expansion and premature hatching are identified as major challenges for *in vitro* fertilization. Future research on the functions and properties of the oviducal and nidamental glands of the mature females will probably suggest improvements to obtain suitable egg chorion expansion, likely reducing premature hatchling. Methods to determine gamete quality should be established as well as procedures to determine important fertilization parameters such as optimal gamete density, critical gamete contact time and spermatozoa: oocyte ratio. Development of new tools, including the preservation of gametes by refrigeration and cryopreservation, will enhance future studies. *In vitro* fertilization techniques will continue to be a necessary step to develop the larval culture of this group of cephalopods and essential in obtaining comparative material for paralarval taxonomy, as well as opening possibilities for the culture of oceanic squid — so ecologically critical, and yet still so poorly understood.

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