frontiers | Frontiers in Marine Science

TYPE Original Research
PUBLISHED 02 December 2022
DOI 10.3389/fmars.2022.1039775

OPEN ACCESS

EDITED BY
Roger Villanueva,
Institute of Marine Sciences, (CSIC), Spain

REVIEWED BY
Jaruwat Nabhitabhata,
Prince of Songkla University, Thailand
Catalina Perales-Raya,
Centro Oceanográfico de Canarias (IEO-CSIC), Spain

*CORRESPONDENCE
Jeffrey Jolly
Jeffrey.A.Jolly@gmail.com
Daniel Rokhsar
dsrokhsar@gmail.com

†PRESENT ADDRESS
Jeffrey Jolly,
Marine Climate Change Unit, Okinawa Institute of Science and Technology Graduate University, Okinawa, Japan
Yuko Hasegawa,
Brain Functional Dynamics Collaboration Laboratory, Rikagaku Kenkyūjyo (RIKEN) Center for Brain Science, Saitama, Japan
Chikatoshi Sugimoto,
Department of Biology, Keio University, Yokohama, Kanagawa, Japan

SPECIALTY SECTION
This article was submitted to Marine Biology, a section of the journal Frontiers in Marine Science

RECEIVED 08 September 2022
ACCEPTED 11 November 2022
PUBLISHED 02 December 2022

CITATION
Jolly J, Hasegawa Y, Sugimoto C, Zhang L, Kawaura R, Sanchez G, Gavriouchkina D, Marlétaz F and Rokhsar D (2022) Lifecycle, culture, and maintenance of the emerging cephalopod models *Euprymna berryi* and *Euprymna morsei*. *Front. Mar. Sci.* 9:1039775. doi: 10.3389/fmars.2022.1039775

Lifecycle, culture, and maintenance of the emerging cephalopod models *Euprymna berryi* and *Euprymna morsei*

Jeffrey Jolly[1*†], Yuko Hasegawa[1†], Chikatoshi Sugimoto[1†], Lin Zhang[1], Risa Kawaura[1], Gustavo Sanchez[1,2], Daria Gavriouchkina[1], Ferdinand Marlétaz[1,3] and Daniel Rokhsar[1,4,5*]

[1]Molecular Genetics Unit, Okinawa Institute of Science and Technology Graduate University, Okinawa, Japan, [2]Graduate School of Integrated Science for Life, Hiroshima University, Hiroshima, Japan, [3]Centre for Life's Origins and Evolution, Department of Genetics, Evolution and Environment, University College London, London, United Kingdom, [4]Department of Molecular and Cell Biology, University of California, Berkeley, Berkeley, CA, United States, [5]Chan-Zuckerberg BioHub, San Francisco, CA, United States

Cephalopod research remains limited by the inability to culture species under laboratory conditions for multiple generations to provide continuous access to animals at all stages of the life cycle. Here, we describe a multi-generational laboratory culture system for two emerging cephalopod models: the hummingbird or Berry's bobtail squid, *Euprymna berryi* Sasaki, 1929, and Morse's bobtail squid, *Euprymna morsei* Verrill, 1881, which are primarily found off mainland Japan. *E. berryi* wild adults were spawned and raised to the third filial generation, and *E. morsei* wild adults were spawned and raised to the second filial generation in a closed system at 20°C. We report growth and survivorship data for a cohort of 30 individuals across the first generation raised in captivity. *E. berryi* and *E. morsei* grew exponentially during the first 90 and 60 days post-hatching, respectively. Survivorship at the first spawning event for *E. berryi* and *E. morsei* was 90% and 77%. *E. berryi* and *E. morsei* females spawned after days 112 and 71 days post-hatching, respectively. We describe the life history of each species and how to distinguish sexes. We discuss the challenges of cephalopod culture and how culturing these species address those problems.

KEYWORDS

***Euprymna*, *Euprymna berryi*, *Euprymna morsei*, cephalopod, bobtail squid, model organism, aquaculture, developmental biology**

Introduction

Cephalopods are widely recognized as the most behaviorally complex invertebrates, attracting attention in the fields of neuroscience, development, and evolution (O'Brien et al., 2018). They have unique characteristics, including adaptive camouflage (Chiao et al., 2015), efficient motor control relative to other mollusks (Levy et al., 2017), and high levels of RNA editing (Liscovitch-Brauer et al., 2017) and transposon activity (Albertin et al., 2015). Furthermore, their capacity to perform complex tasks resembling that of some vertebrate species promoted their inclusion in European Union legislation for animal experimentation and welfare under Directive 2010/63/EU at the same level as vertebrate organisms (European Parliament and Council of the European Union, 2010; Sykes et al., 2012; Smith et al., 2013; Fiorito et al., 2015).

The study of cephalopod development and evolution is a growing area of research that has led to increasing demand for embryos and animals at all stages of their life cycle (Lee et al., 2003; Peyer et al., 2014; Koenig et al., 2016; Navet et al., 2017; Tarazona et al., 2019). While for many purposes wild-caught animals can be studied, and hatchings raised to juvenile or later stages in the laboratory, multigenerational cultures have only been initiated for some cephalopods including octopus (Iglesias et al., 2004; Rosas et al., 2014; Vidal et al., 2014; Maldonado et al., 2019; Grearson et al., 2021), sepioids (Minton et al., 2001; Walsh et al., 2002; Nabhitabhata, 2014), sepiolids (Boletzky et al., 1971; Nabhitabhata et al., 2005; Jones and Richardson, 2010; Nabhitabhata and Nishiguchi, 2014; Sanchez et al., 2019), and the myopsid squid *Sepioteuthis lessoniana* (Forsythe et al., 1994). Large-scale multigenerational cephalopod culture systems are not only a necessity for forward genetics but is also desirable for targeted approaches like CRISPR-Cas genome editing (Jinek et al., 2012; Doudna and Charpentier, 2014). In cephalopods, gene knockouts by genome editing have been accomplished in the progeny of wild-caught *Doryteuthis pealeii* (Crawford et al., 2020). Multigenerational cultures will thus help to move forward the field of development and evolution on cephalopods.

In general, each cephalopod species has unique biological characteristics, morphology, and lifestyle that determine which phenomena can be readily studied, as well as disadvantages in terms of difficulty of culture conditions and difficulty of maintenance (Figure 1). For example, cephalopods generally have a high metabolism and food conversion rate but limited fat

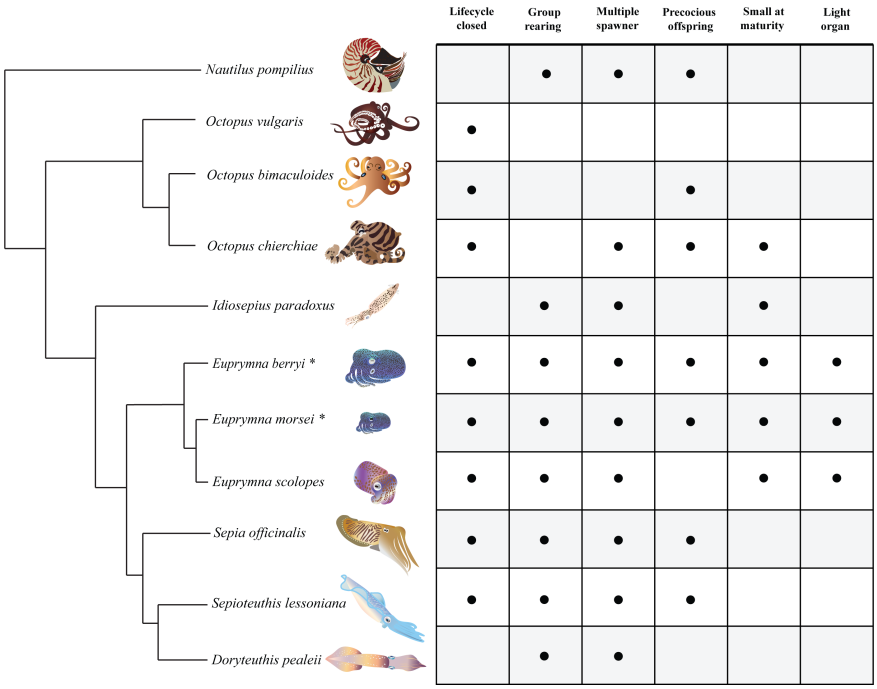


FIGURE 1
Advantageous Culture Traits of Several Cephalopod Models. Comparison of cephalopod species previously used in laboratory experiments. "Lifecycle closed" refers to a species being cultured across at least one generation. An animal is considered capable of group rearing if minimal aggression and cannibalism is observed, and the stress of group rearing prevents successful culturing efforts. "Multiple spawner" indicates normal multiple spawning events completed by one female. "Precocious offspring" refers to hatchling behaviors similar to adults (including predation). "Small at maturity" refers to an animal with a dorsal mantle length less than 6 cm. Some cephalopod species have evolved a light organ that is bioluminescent. The tree is based on results published by Anderson and Lindgren (2020).

reserves, requiring frequent feeding (Iglesias et al., 2014; Vidal et al., 2014). Physically larger species such as *Sepia officinalis*, *Sepioteuthis lessoniana*, and *Octopus vulgaris* therefore require correspondingly large aquaria and amounts of food which rapidly become impractical for many laboratory budgets without dedicated marine facilities. Furthermore, most cephalopods are active visual hunters that prefer live prey, which can be costly and labor intensive to provide (Villanueva et al., 2017). Moreover, many cephalopods have evolved different ranges of sociality, with most of the octopus species being solitary and many squids performing group-like behaviors (Sugimoto et al., 2013; Iglesias et al., 2014). Some species may even practice cannibalism (Ibáñez and Keyl, 2010), preventing the culture of more than single animals per tank. Cephalopods that are relatively small at maturity, avoid cannibalism, and are not entirely solitary therefore have advantages for small-scale laboratory culture.

Reproductive (Rocha et al., 2001) and life history traits, including early mode of life (Boyle, 2005; Villanueva et al., 2016), vary among cephalopod species. For most cephalopods, the diet during their early life in their natural habitats is still unknown, limiting the selection of suitable prey to raise them. Some cephalopods lay only very few eggs, e.g., *Eumandya parva* (Sanchez et al., 2019), and others can produce hundreds or thousands of immature planktonic paralarvae with high mortality, e.g., *Octopus vulgaris* (Villanueva, 1995), and whose size is too tiny to feed with standard prey in laboratory settings.

Bobtail squid from the subfamily Sepiolinae, i.e., sepiolida clade (Anderson and Lindgren, 2020), are a group of nocturnal cephalopods with relatively small size, correspondingly limited nutritional requirements, short life span, benthopelagic early mode of life, and ability to live at high densities without cannibalism. Female bobtail squid can also mate several times with different males and store spermatangia for around two months for future spawning (Squires et al., 2013; Drerup et al., 2020). These characteristics make them suitable for laboratory culture and a potential model organism for developmental, physiological, behavioral, and genetic assays. Their small size is also ideal for advanced imaging (Kerbl et al., 2013).

Thanks to the pioneering efforts of McFall-Ngai and Ruby (Boettcher and Ruby, 1990; Montgomery and McFall-Ngai, 1994; Boettcher et al., 1996; Ruby, 1996; Ruby and Lee, 1998; McFall-Ngai, 1999), the Hawaiian bobtail squid *Euprymna scolopes* Berry 1913 has been widely adopted as a model for bacterial-metazoan symbiosis in which luminescent *Allivibrio fischeri* colonize the light organ of these and related species (Boettcher et al., 1996; McFall-Ngai, 1999; McFall-Ngai, 2014). These efforts fostered studies of bobtail squid diversity (Jones et al., 2006) and development (Lee et al., 2003) both morphologically (Lee et al., 2009b) and at the molecular level (Callaerts et al., 2002; Hartmann et al., 2003; Lee et al., 2003; Sanchez et al., 2021). The genome of *E. scolopes* (Belcaid et al., 2019) has become a reference to study other species in the *Euprymna* clade (Heath-Heckman and Nishiguchi, 2021; Schmidbaur et al., 2022). Many bobtail squid have also been investigated in studies of associative learning, behavior, and the heritability of personality and fitness traits (Steer et al., 2004; Sinn and Moltschaniwskyj, 2005; Sinn et al., 2006; Sinn et al., 2008; Zepeda et al., 2017). Despite these advantages, a disadvantage of *E. scolopes* for multigenerational culture is the high mortality of its larval stage (Lee et al., 2009b).

Two species of bobtail squid that are abundant in mainland Japanese waters have the potential to become laboratory models: the hummingbird or Berry's bobtail *Euprymna berryi* Sasaki, 1929, and Morse's bobtail *Euprymna morsei* Verrill, 1881 (Figures 2A–G). The distribution of these sympatric species extends from Japanese waters southward along the coast of China and westward into the Indian Ocean (Raj and Kalyani, 1971; Okutani and Horita, 1987; Reid and Jereb, 2005; Sundaram and Sreeram, 2008). The spawning season for *E. berryi* is late April to July in Aichi, Japan (Choe, 1966a), and March and December in Taiwan (Huang, 2006). Adults of *E. berryi* have been found from April to June in the southern and the Pacific Ocean side of mainland Japan swimming near the water surface at night, while adults have been found as deep as 60 m on the Pacific side of mainland Japan. In a trawl survey off Nobeoka Bay in Miyazaki prefecture, Toriyama et al. found a relatively similar amount of *E. morsei* across the year with the highest catch from April to June and the lowest from January to March (Toriyama et al., 1970). However, some trawl surveys could have confused both species due to their very similar morphology. Although most fishermen do not discriminate between the two species, they are distinguished by several morphological differences as well as molecular markers (Sanchez et al., 2019). *E. morsei* (mantle length ≤ 4 cm) is considerably smaller than *E. berryi* (mantle length ≤ 5cm) (Reid and Jereb, 2005). *E. morsei* males have enlarged suckers on the ventral sucker rows of arms II, III, and IV, whereas *E. berryi* have enlarged suckers on both dorsal and ventral sucker rows of arms II and IV (Okutani and Horita, 1987; Norman and Lu, 1997). *E. morsei* have chromatophores on the dorsal surface of the fins, while *E. berryi* have chromatophores on both dorsal and ventral surfaces (Okutani and Horita, 1987). Finally, the tentacular suckers in *E. morsei* have a cylindrical shape but in *E. berryi* resemble a smoking pipe (Okutani and Horita, 1987; Reid and Jereb, 2005; Huang, 2006).

Several previous studies described culturing attempts of *E. berryi*, *E. morsei*, and other members of the genus *Euprymna* (Supplementary Table 1). *E. berryi* was reared for two months (Choe, 1966a), and *E. morsei* was raised to reproductive maturity (Ikeda et al., 2003). *Euprymna scolopes* was successfully raised to the second generation (Hanlon et al., 1997). Several species of *Sepiola* and *Euprymna* have been cultured to the second generation (Boletzky et al., 1971; Jones and Richardson, 2010; Sanchez et al., 2019). Further, *Euprymna tasmanica* and

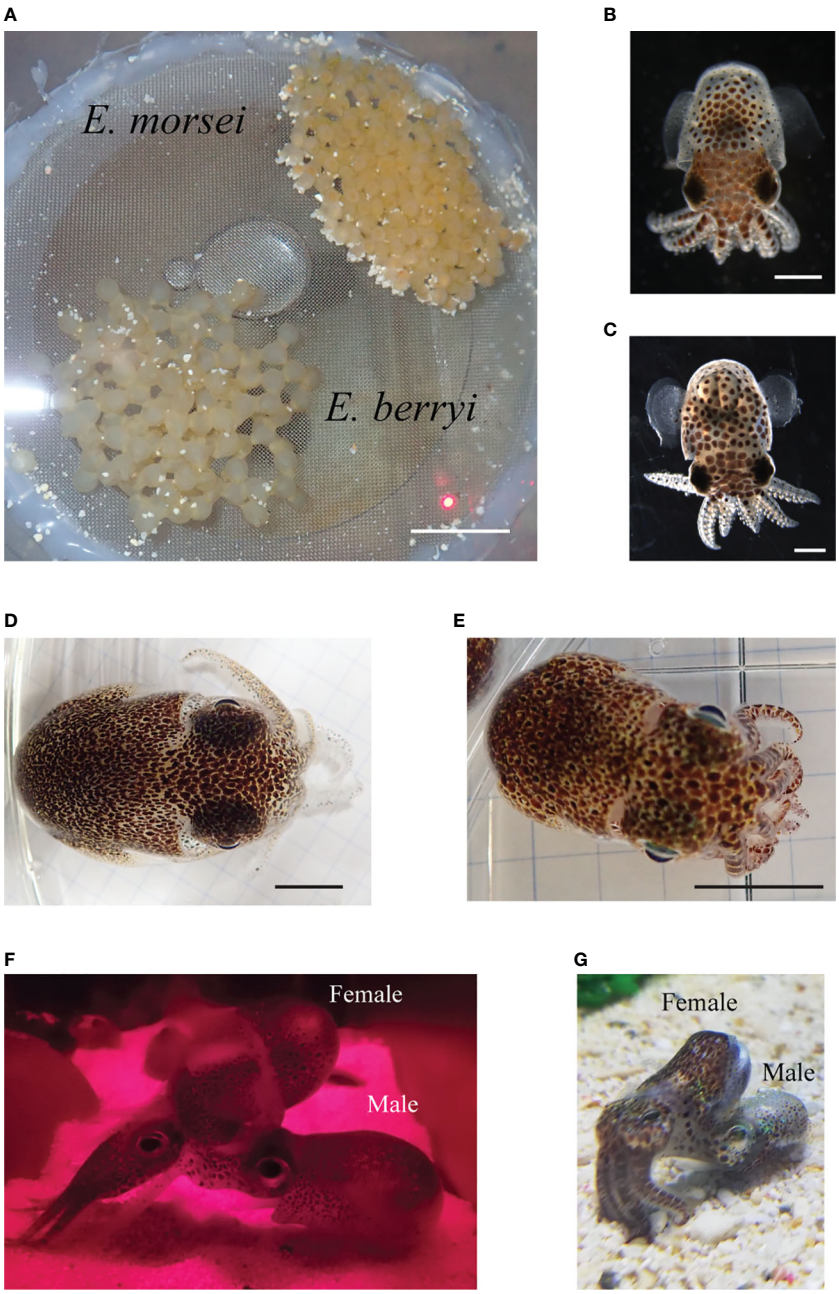


FIGURE 2
E berryi and *E morsei* at different life stages. **(A)** Egg clutches of *E berryi* (left) and *E morsei* (right). Scale bar is 5 cm. **(B)** *E berryi* at one day post-hatching (dph). Scale bar is 1 mm. **(C)** *E morsei* at one dph. Scale bar is 1mm. **(D)** Mature *E berryi* at 130 dph. Scale bar is 1 cm. **(E)** Mature *E morsei* at 70 dph. Scale bar is 1 cm. **(F)** *E berryi* mating. The male (on the right) grasps the female from the ventral side to engage mating. **(G)** *E morsei* mating. The smaller male on the right grasps the female from the ventral side to engage mating.

Euprymna hyllebergi have been cultured to the third generation (Nabhitabhata et al., 2005; Nabhitabhata and Nishiguchi, 2014).

Here we report our efforts to develop multigenerational cultures of *E. berryi* and *E. morsei*. We closed the lifecycle of both species in a recirculating aquaculture system and measured growth and survivorship for thirty individuals of each species under the same aquarium conditions. This work, along with the development of genomic resources for *E. berryi* (Gavriouchkina et al., 2022), provides a foundation for the future development of *E. berryi* and *E. morsei* as laboratory model organisms.

Materials and methods

Broodstock collection

Both *E. berryi* and *E. morsei* were obtained from vendors or from wild collections in southern mainland Japan. Adults are available from vendors seasonally. Adults survived long-distance shipping with commercial couriers using oxygen-saturated seawater and styrofoam-insulated packaging. Adult females of *E. berryi* and *E. morsei* were collected from Mie prefecture, Japan from February to June with a set net, and transported using overnight shipping services. Animals were individually packed in 15 L round-bottom transparent 3 mm plastic bags containing 5-7 L of oxygen saturated filtered seawater with excess volume filled with pure oxygen and shipped in expanded polystyrene foam boxes similar to *E. scolopes* (Hanlon et al., 1997; Cecere and Miyashiro, 2022). Transit time until arrival in the lab was less than 48 h. Upon arriving in Okinawa, animals were acclimated to the temperature (20°C or 23°C) and salinity (~35 gL^{-1} i.e., parts per thousand) of our culture system. Adults from each species were housed separately. Upon spawning, eggs were removed to two-liter tanks like previous methods (Sanchez et al., 2019). Recently spawned eggs – within the first ten days post spawning - were shipped internationally within 72 h using similar methods to shipping adults apart from using 5 L bags containing 1.5-2 L oxygen saturated filtered seawater. To prevent widespread fouling, eggs were monitored on a daily basis and nonviable eggs removed. Hatchlings were housed in two liter tanks for approximately the first month after hatching.

Culture system

The tank system assembled for culturing bobtail squids is a closed tank system that consists of a 200 L filter tank, five 70 L tanks (60 cm x 35 cm x 35 cm), five 2 L tanks (20 cm x 13 cm x 13 cm), two protein skimmers, an ultraviolet sterilizer, and contains filtered natural seawater from the OIST Seragaki Marine Science Station (Supplementary Figure 1). Flow rate for larger tanks is 4.5 to 5 L min^{-1}, and 160 to 180 ml min^{-1} for smaller tanks. Cleaning and partial exchange of 10% seawater are performed daily, and larger 50% water changes are performed biweekly. Water temperature from 2017 to February 2018 was maintained at 23°C with a chiller and heater and thereafter maintained at 20°C. All measurements were taken from animals kept at 20°C. The following water parameters were maintained between the ranges and monitored daily: salinity - 33 to 37 gL^{-1}, pH - 8.2 to 8.4. The following parameters were measured at least weekly nitrate - 0 to 20 mg L^{-1}, i.e., parts per million (ppm), nitrite - 0 to 0.5 mg L^{-1} (ppm), ammonia (NH_3/NH_4) - 0 to 0.25 mg L^{-1} (ppm).

Artificial plants, coral rubble, and PVC pieces were added to tanks to provide egg laying substrate and refuge. Beach sand collected from nearby beaches was added to aquarias. Sand was autoclaved and rinsed in reverse osmosis-treated water before introducing into aquaria. Enough substrate was given to allow the animals to bury completely. Without any burying substrate, skin lesions can form on the ventral side of the animal from friction with the aquarium floor. Aquaria were spot cleaned daily with siphons. The use of coarse sand collected from beaches or from vendors is adequate to allow the animal refuge while remaining easy to siphon.

Blue light-emitting diodes (450 nm wavelength) were used to create twelve hour light-twelve hour dark diurnal cycles in the laboratory. The photoperiod was shifted similarly to previous methods (Franklin et al., 2014) so "night" begins at noon local time to facilitate feeding and experimentation. We used red light-emitting diodes (665 nm) to observe and feed animals during "night" when they were most active.

Feeding and maintenance conditions

Animals were fed *ad libitum*, with new shrimp added once daily. From hatching to 40 dph (days post-hatching), both species were fed mysids (*Neomysis* spp.). Mysids were maintained in a separate tank and fed *Artemia* sp. nauplii once a day prior to being fed to hatchlings. After 40 dph, both species were fed glass shrimp (*Palamonetes* spp.), and the freshwater marsh shrimp-*Caridina* spp.

Both species were also trained to consume cut frozen shrimp (e.g., black tiger prawn) upon reaching maturity.

Frozen shrimp were thawed and cut before presenting to a squid with forceps and moved to simulate living prey. Afterward, contact was made between frozen food and the inner portion of the squid's arms until the squid either showed signs of stress or voluntarily grasped the frozen food (Supplementary Video 1). After approximately one week of continuous training, squids could attack falling frozen food spontaneously.

In ongoing culture we provide adequate space, refugia, and burying substrate to minimize stress. Adults are kept in a ratio at or greater than 1:1 males to females. Our heuristic for determining appropriate space is to ensure each animal has at least two mantle lengths distance between animals. For our tank dimensions (approximately 70 L, 60 cm x 35 cm x 35 cm and water height of 31 cm), we do not exceed eight fully mature *E. berryi* individuals per tank (eight squids per 2,100 cm^2 floor area, 65,100 cm^3 water volume). Assuming a maximum potential mantle length of 6 cm at maturity (Okutani and Horita, 1987), each animal is therefore given a cube of water that is 12 cm on each side, equivalent to 1700 cm^3. *E. morsei* reaches a smaller size at maturity, with a dorsal mantle length (DML) less than 4 cm (Okutani and Horita, 1987; Reid and Jereb, 2005), and therefore can be kept at higher densities than *E. berryi*. To avoid reproductive attempts and aggression from males, females can be separated from males after mating. We have not observed

any overt changes in behavior when adults of either species are isolated.

Survivorship and growth rate

Thirty F1 hatchlings of *E. berryi* and *E. morsei* were isolated on the first day after hatching and reared to monitor their survivorship and growth rate. To measure growth rate, wet weight (WW, g) and DML (mm) were measured in five randomly selected individuals approximately every 10 d to maturity to prevent additional stress due to handling. We measured only five individuals each to minimize handling stress on the cohorts. Squid were placed in a transparent reservoir containing aquarium water atop graph paper and imaged using an Olympus TG-5 camera. The FIJI variant of ImageJ (Schindelin et al., 2012) was used for image calibration and DML measurement. All data is expressed as Mean ± SD. Survivorship was calculated (Leverich and Levin, 1979) as the percentage of surviving individuals, I(t) by:

$$I(t) = 100\left(\frac{Ns(t)}{No}\right)$$

Where $N_s(t)$ is the number of survivors at time t and N_o is the initial cohort size.

At 115 dph, eleven *E. berryi* were removed from the study due to lack of space and prey items, and $N_o$was then adjusted from 30 to 19 for *E. berryi*. All 27 surviving *E. berryi* were first removed from the aquaria, then eight of each males and females were selected and placed back in the aquaria. The animals selected were the first animals to be collected. The other 11 *E. berryi* were removed and euthanized *via* overdose to the anesthetic ethanol (Abbo et al., 2021). Animals were immersed in a bath of 1% ethanol in filtered seawater. Over a period of thirty minutes, ethanol was gradually introduced until reaching a final concentration of 5% followed by mechanical destruction of the brain (Fiorito et al., 2015; Abbo et al., 2021).

Observations

Behavioral observations of *E. berryi* were made visually from March 2017 to May 2020. *E. morsei* observations were from March 2018 to May 2020. Both species were cultured during that time for other experiments.

Results

Culture

We cultured *E. berryi* and *E. morsei* under conditions similar to those used for other bobtail squid (Hanlon et al., 1997; Nabhitabhata et al., 2005; Jones and Richardson, 2010; Sanchez et al., 2019) (**Methods**). We spawned wild-caught adults of both species and cultured *E. berryi* to the third filial generation and *E. morsei* to the second filial generation. Growth rate and survivorship were tracked for the first filial generation (Figure 3).

Egg masses

Both *E. berryi* and *E. morsei* were collected during the Japanese spring season and we observed mating and spawning of wild-caught adults in the laboratory. We shipped recently spawned eggs internationally and they hatched and were raised without overt abnormalities. Both species laid a large number of eggs per clutch, usually exceeding 200 eggs for *E. berryi* and 100 eggs for *E. morsei* (Figure 2A). Wild-caught *E. berryi* laid an average of 235 ± 75.8 eggs per clutch (n=7), while wild-caught *E. morsei* laid 153 ± 26.5 eggs per clutch (n=4). Eggs are encapsulated within a jelly coat and laid individually in a clutch. The jelly coat of eggs of both species have an orange tint due to a dye secreted by the maternal accessory nidamental gland. Non-viable eggs have an opaque white appearance.

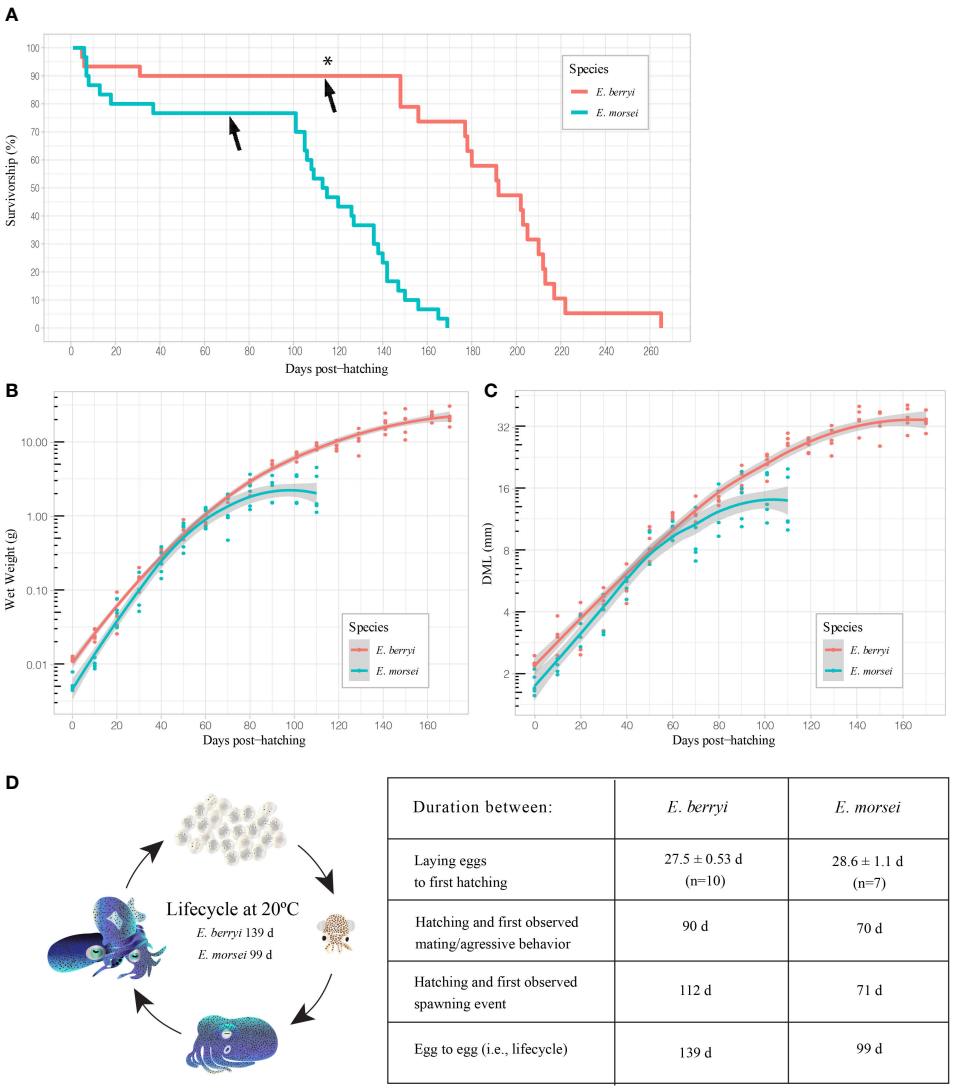
The period between spawning events for wild *E. berryi* at 20°C was 6.7 ± 2.7 d (n=18) and was not recorded for *E. morsei*. Both species demonstrated intermittent terminal spawning and spawned separate clutches continually once reaching sexual maturity (Figure 3A). On one occasion one isolated wild-caught female *E berryi* laid 9 fertilized clutches over a period of 59 d without any additional mating in the laboratory (presumably using stored spermatangia). *E. berryi* was observed to live longer in captivity after capture than *E. morsei* and benefited from more spawning events. *E. berryi* has higher survivorship and fecundity than *E. morsei* (Figure 3).

We observed no physical or behavioral abnormalities in sequential generations; however, survivorship of later generations of *E. berryi* and *E. morsei* immediately after hatching was noticeably reduced for some clutches. On some occasions, we observed eggs laid outside of the jelly coat and some clutches with many unfertilized eggs.

Growth

Growth of *E. berryi* and *E. morsei*, measured by WW or DML, was approximately exponential for the first 90 and 60 dph (Table 1) before reaching species-specific plateaus by 80 and 140 dph, respectively (Figures 3B, C).

We observed a large range of both DML and WW at later stages in both *E. berryi* and *E. morsei* (Figures 3B, C). Average *E. berryi* WW and DML were 17.32 ± 3.82 g and 32.14 ± 4.90 mm for males (n=16), and 23.06 ± 4.90 g and 36.26 ± 3.67 mm for females (n=14). Average *E. morsei* WW and DML were 1.41 ± 0.15 g and 11.45 ± 1.24 mm for



Duration between:	*E. berryi*	*E. morsei*
Laying eggs to first hatching	27.5 ± 0.53 d (n=10)	28.6 ± 1.1 d (n=7)
Hatching and first observed mating/agressive behavior	90 d	70 d
Hatching and first observed spawning event	112 d	71 d
Egg to egg (i.e., lifecycle)	139 d	99 d

FIGURE 3
Survivorship, growth rate, and developmental timelines for *E berryi* and *E morsei*. **(A)** Survivorship for each species. For each species, initial population size was 30 individuals. The asterisk (*) represents an artificial reduction in total population size for *E berryi* from 30 to 19 individuals due to limited tank space. Arrows indicate the first spawning event for each species. **(B)** Growth rate comparing wet weight (g) to dph on semi-log scale. **(C)** Growth rate comparing dorsal mantle length (mm) to age on semi-log scale. **(D)** Comparison of the lifecycle and time between developmental landmarks of both *E berryi* and *E morsei*.

males (n=6), and 3.70 ± 0.42 g and 18.95 ± 2.20 mm for females (n=6). The average female *E. berryi* weighed 1.33 times larger than males and were 1.13 times longer. The average female *E. morsei* weighed 2.63 times larger than males and were 1.65 times longer.

Survivorship

Survivorship was 93% for *E. berryi* and 80% for *E. morsei* for the first 30 days after hatching (Figure 3A). Survivorship was stable until shortly after spawning began. Thereafter, survivorship declined steadily from ~101 dph in *E. morsei* and ~148 dph in *E. berryi*. The oldest *E. berryi* and *E. morsei* in our laboratory culture were males and lived 265 dph and 169 dph, respectively. *E. berryi* outlived *E. morsei* and took longer to reach spawning age by 42 dph.

Mating and spawning

Sexual maturity was noted when males became aggressive towards conspecifics. No courtship behavior was observed in

TABLE 1 Exponential growth curve equations for *E. berryi* 0-90 days post hatching (dph) and *E. morsei* 0-60 dph.

Exponential growth curve from 0 to 90 dph	**a**	**T**	**R^2**
E. berryi WW (g) = a $e^{d/T}$	0.015 g	14.8 d	0.984
E. berryi DML (mm) = a $e^{d/T}$	2.31 mm	42.6 d	0.977
Exponential growth curve from 0 to 60 dph			
E. morsei WW (g) = a $e^{d/T}$	0.0054 g	10.85 d	0.985
E. morsei DML (mm) = a $e^{d/T}$	1.70 mm	33.2 d	0.991

a is the growth parameter either wet weight (WW, g) or dorsal mantle length (DML, mm), T is time (d), R^2 is the coeffient of determination.

either species. Aggression appeared similar to mating, i.e., a male would assault, grapple, and possibly bite a conspecific. At 20°C this was first observed at 90 dph in *E. berryi* and 70 dph in *E. morsei*. During mating the female is first attacked by the male and the male attempts to grab the ventral head of the female, i.e., the male-to-female neck position. The male maintains control of the female and inserts the hectocotylus holding spermatophores into the mantle of the female (Figures 2F, G).

Spawning events began shortly after the night cycle began. Females spawned on the substrate provided including the tank walls, PVC pipes, rocks, and on imitation plants. As described above, for both species, the female laid each egg individually as part of a large clutch (Supplementary Video 2). Spawning began at night and continued into the day. Females have been observed laying eggs cooperatively on the same substrate simultaneously. Females typically consumed less prey one day before spawning. Females repeatedly laid egg clutches every few days until reaching a late senescent life stage (Figure 3A). Some individuals spawned within three days of shipment. Mature *E. berryi* females were observed spawning fertilized clutches repeatedly over a period of 100 days at 20°C. No parental care was observed in either species. Egg clutch morphology was different for both species. *E. morsei* eggs were more densely packed in a clutch, whereas *E. berryi* eggs were more spaced out (Figure 2A).

Sexual dimorphism

Sexual dimorphism was visually evident at 100 dph for *E. berryi* and 70 dph for *E. morsei*. The sex of the animal can be determined by its side profile, size, suckers, and the morphology of the first left-arm (Supplementary Figure 2). Males are smaller than females for both species. The size difference is more pronounced in *E. morsei* than *E. berryi*. The side mantle profile in males is sharper in males than in females (Supplementary Figures 2A, B). Fully mature females generally have a bulbous mantle, because of the presence of oocytes in their ovaries, and can be distinguished from males visually in a minimally invasive manner. Males of both species can further be distinguished from females by observing the first arm pair (Supplementary Figure 2C). Males have a modified first left arm known as the hectocotylus which is shorter than the opposing arm and curls slightly outward. Males of both species have large suckers on some rows of certain arms and modified suckers on the hectocotylus (Supplementary Figure 2D), whose patterns can be used to discriminate species of *Euprymna* (Norman and Lu, 1997). Females have uniform sucker sizes. Female first arms and suckers (Supplementary Figure 2E) are indistinguishable from one another (Norman and Lu, 1997).

Senescence

Males of both species generally outlived females and displayed similar signs of senescence. Characteristics of early senescence include nonfunctional and faded chromatophores, greater susceptibility to infections, and loss of appetite. Signs of later stages of senescence include complete cessation of eating and burying, loss of equilibrium, and continuous labored ventilation.

Discussion

Two promising cephalopod model organisms

The utility and prominence of *E. scolopes* as a model cephalopod species was discussed by Lee et al. (2009c) who also suggested that, as genomic information becomes available for different cephalopod species, the availability of broodstock and embryos becomes a primary factor in choosing a model system. Here we have explored the culturing of two related Japanese bobtail squid species, *E. berryi* Sasaki, 1929, and *E. morsei* Verrill, 1881. We find that *E. berryi* and *E. morsei* have comparable life cycles in captivity to *E. scolopes* (Hanlon et al., 1997), *E. hyllebergi* (Nabhitabhata et al., 2005), *E. tasmanica* (Nabhitabhata and Nishiguchi, 2014), *E. parva*, and *E. brenneri* (Sanchez et al., 2019) (Table 2). Both *E. berryi* and *E. morsei* can be raised in laboratory settings and are intermittent terminal spawners, spawning repeatedly once reaching sexual maturity (Figure 3A). They are therefore well-suited for evo-devo studies,

physiological assays, behavioral assays, laboratory culture, and have the potential to be used for gene editing (Crawford et al., 2020). *E. berryi* has higher survivorship and fecundity than what is reported for other sepiolids including *E. morsei*, *E. scolopes* (Hanlon et al., 1997), and *S. atlantica* (Jones and Richardson, 2010). These characteristics are crucial for establishing genetic lines with mutations that potentially decrease fitness.

Broodstock

Adult wild *E. berryi* and *E. morsei* can both be shipped using commercial couriers from their native range in southern mainland, with oxygen-saturated seawater and styrofoam-insulated packaging as described for *E. scolopes* (Cecere and Miyashiro, 2022), and acclimate well to aquarium conditions. *E. berryi* and *E. morsei* are usually caught using a set net round 30m deep, although both species have been collected with dip nets at night near the surface. *E. berryi* is also caught from the shore by recreational fishermen, and by commercial fishermen by trawling for sale to fish markets. An existing commercial fishery is potentially useful to obtain large numbers of specimens either living, for seeding propagation in captivity or studying behavior, or dead animals for morphological comparisons, isotope analysis, and population genetics. Because *E. morsei* is a relatively smaller cephalopod, this species is less familiar to the fishing community, which hinders the collection of wild specimens. *E. morsei* is also similar in size to the adult forms of the sympatric species *Lusepiola birostrata* and *Eumandya parva* making identification challenging for nonexperts (Takayama and Okutani, 1992; Bello, 2020). Differences in egg clutch morphology have been used to distinguish sympatric species (Sanchez et al., 2019), and can aid in identifying and collecting eggs in the field.

Proper care of sepiolid eggs is necessary to prevent fouling and maintain high hatching rates (Lee et al., 2009a). Females of *E. scolopes* host bacterial consortium in their accessory nidamental gland that is secreted to eggs during spawning to protect them from predation (Kerwin et al., 2019). We indirectly observed the same feature of *E. berryi* and *E. morsei*; specifically, we noted orange-dyed accessory nidamental glands, whose pigments are generated from carotenoids produced by symbiotic bacterial communities (Pichon et al., 2005). Eggs can be kept in incubating tanks with constant water flow in dark conditions to further inhibit microbial growth (Choe, 1966a). Our eggs were maintained at constant conditions with minimal disturbance as failure to do so can cause premature hatching and decreased survivorship (Hanlon et al., 1997).

The stocking density and male:female ratio are important to consider in cephalopod culture. Crowding has been shown to induce stress and decrease fecundity in *Sepia officinalis* (Forsythe et al., 2002), *Sepioteuthis lessoniana* (LaRoe, 1971; Boal and Gonzalez, 1998) and *Euprymna scolopes* (Hanlon et al., 1997). As most bobtail squid adopt benthic lifestyles quickly (Table 2), the ratio of animals to floor area is also relevant. A low male:female ratio can reduce stress from mating events and prevent forced copulation. We achieved mating and spawning with a low (1:1-1:2) male to female ratio though it is preferable to separate males from females as males become aggressive, similar to *E. tasmanica* (Nabhitabhata and Nishiguchi, 2014).

Few studies exist on the effects of inbreeding depression on cephalopod culture. *Sepia officinalis* grown for seven consecutive generations developed decreased fertility in later generations, and the seventh generation failed to produce viable offspring (Forsythe et al., 1994). There are also accounts of a decreased size

TABLE 2 Comparison of life cycle and culture traits of cultured *Euprymna* spp.

Species	*E. berryi*[a]	*E. morsei*[a]	*E. scolopes*[b]	*E. tasmanica*[c]	*E. hyllebergii*[d]	*E. brenneri*[e]	*E. parva*[e]
Known distribution	West Pacific, East Indian Oceans	West Pacific, East Indian Oceans	Central Pacific/Hawaii	South Indopacific/ Australia	East Indian Ocean/Thailand	West Pacific Ocean/Okinawa	West Pacific Ocean/East Asia
Temperature (°C)	20	20	23	20	28	24	24
Clutch size (eggs)	137-362	121-175	50 - 250	25-500	108-464	–	47
Embryonic Phase (d)	28	29	20	29	14	–	22
Survivorship - First 30 days	93%	80%	73%	–	–	–	–
Hatchling behavior	Benthic	Benthic	Planktonic	Benthic	Planktonic	Planktonic	Benthic
Exponential Growth Phase (d)	90	60	83	44	30	–	–
First Mating Behavior (d)	90	70	61	60	66	83	–
Lifecycle (d)	139	99	80	–	80	–	90
Max Lifespan (d)	265	169	139	–	125	99	–

(a - this study; b - Hanlon et al., 1997; c - Nabhitabhata and Nishiguchi, 2014; d - Nabhitabhata et al., 2005; e - Sanchez et al., 2019).

at maturity for cephalopods cultured to multiple generations (Iglesias et al., 2014). Thus, maintaining the genetic diversity of a colony, by careful interbreeding of separate subpopulations, or the introduction of new alleles by the steady addition of wild animals to the culture, may be necessary to support healthy laboratory colonies. *Euprymna hyllebergi* and *Euprymna tasmanica* were cultured for three generations without the introduction of wild-caught specimens. Growth rates were similar across generations and no obvious abnormalities relating to inbreeding were observed (Nabhitabhata and Nishiguchi, 2014). No physical or behavioral abnormalities were observed in both species in sequentially cultured generations; however, sometimes survivorship immediately after hatching was noticeably reduced for some clutches due to some unknown phenomenon similarly reported in *E. scolopes* (Hanlon et al., 1997). Additionally, rare clutches contained many unfertilized eggs and aberrant jelly coats, and more work should be done to understand and improve these traits. Based on our findings, it should be feasible to maintain a culture of both *E. berryi* and *E. morsei* for several generations. Genetic diversity can be maintained by introducing wild caught individuals seasonally (February to June) when vendors in Japan are able to supply additional animals.

Prey and hunting

Activity patterns were similar to what is described for other *Euprymna* species (Hanlon et al., 1997; Nabhitabhata et al., 2005; Nabhitabhata and Nishiguchi, 2014; Sanchez et al., 2019; Drerup et al., 2020) and animals became active at "night" - emerging from the substrate and discarding the sand coat for hunting and mating. During the "day", animals bury themselves under the sandy substrate for shelter and to avoid predators (Hanlon et al., 1997; Rodrigues et al., 2010; Drerup et al., 2020). An alternating 12 hour light-dark cycle is sufficient to mimic natural diurnal cycles (Franklin et al., 2014). As for other *Euprymna*, *E. berryi* and *E. morsei* cover their body, head, and arms with sand but leave their eyes exposed (Nabhitabhata et al., 2005; Hanlon and Messenger, 2018; Sanchez et al., 2019; Drerup et al., 2020).

While adults can be fed frozen shrimp, hatchlings and juveniles require live food, similar to other *Euprymna* spp. (Choe, 1966b; Hanlon et al., 1997; Ikeda et al., 2003; Nabhitabhata and Nishiguchi, 2014; Sanchez et al., 2019). To feed hatchlings of either species, mysids can be collected from inshore locations and reared on a diet of *Artemia* spp. (Lussier et al., 1988). Hatchlings of both species attacked adult mysids often larger than themselves similar to other species of *Euprymna* (Okutani and Horita, 1987; Hanlon et al., 1997; Nabhitabhata et al., 2005; Sanchez et al., 2019). While adults were taught to spontaneously grasp frozen prey, they would sometimes ignore non-moving prey. Fully mature adult females consumed more food relative to adult males possibly due to ongoing egg production.

Hatchling behavior

For both *E. berryi* and *E.morsei*, hatching from an egg clutch occurs over a period of several days. *E. berryi* settled and established a benthic lifestyle shortly after hatching in agreement with (Choe, 1966a) and similar to *Euprymna tasmanica* (Nabhitabhata and Nishiguchi, 2014) and *Eumandya parva* (Sanchez et al., 2019). *E. berryi* and *E. morsei* exhibited a brief nektobenthic paralarval stage similar to what is described for *Euprymna hyllebergi* (Nabhitabhata et al., 2005) and unlike hatchling behavior of *Euprymna scolopes*, *Eumandya pardalota*, and *Euprymna brenneri* (Hanlon et al., 1997; Sanchez et al., 2019) which displayed surface swimming phototaxic paralarval stages the first month after hatching (Table 2). In our culture, *E. berryi* and *E. morsei* could consume prey within 24 hours after hatching, two days earlier than previously reported for *E. berryi* (Choe, 1966a).

Growth and sexual dimorphism

E. berryi and *E. morsei* followed similar growth patterns to other *Euprymna* spp., including early growth stages of *E. scolopes* (Hanlon et al., 1997) and *E. hyllebergi* (Nabhitabhata et al., 2005) (Table 2). For comparison, *E. scolopes* raised at 23°C experienced exponential growth from hatchling to 83 dph (Hanlon et al., 1997). *E. hyllebergi* demonstrated an exponential growth phase the first 30 dph when raised at 28°C (Nabhitabhata et al., 2005). *E. tasmanica* raised at 20°C experienced exponential growth from 7 to 44 dph was followed by approximately linear growth from 58 to 140 dph (Moltschaniwskyj and Carter, 2010). As with other bobtail squid, adult males of *E. berryi* and *E. morsei* can be definitively distinguished from females by their characteristically modified first left arm, the hectocotylus, which is shorter than the opposing arm and curls outward; female left and right first arms are indistinguishable (Okutani and Horita, 1987; Norman and Lu, 1997). Sexual dimorphism becomes visually evident ~90-100 dph for *E. berryi* and ~70 days for *E. morsei*, concurrent with aggressive behavior in males.

Survivorship

Both *E. berryi* and *E. morsei* recorded higher survivorship in the first 30 dph (93% and 80%) compared to the 73% survivorship reported for *E. scolopes* (Hanlon et al., 1997). Neither species exhibited long-lived pelagic paralarval stages after hatching, which could contribute to higher survivorship in captivity relative to *E. scolopes*. *E. morsei* was previously reared at

22.5°C and survived for 97 to 128 dph (Ikeda et al., 2003). Our *E. morsei* grew more slowly and lived longer, possibly due to being cultured at a lower temperature (20°C) and thus having a lower metabolic rate (Iglesias et al., 2014). *E. berryi* recorded the longest lifespan of any cultured *Euprymna* spp. (Table 2).

Reproductive maturity and mating behavior

Mating behavior is similar to what was observed in other *Euprymna* spp. without any obvious courtship behavior (Moynihan, 1983; Hanlon et al., 1997; Nabhitabhata et al., 2005; Squires et al., 2013; Sanchez et al., 2019; Drerup et al., 2020). Females stored spermatangia deposited during matings similarly to other *Euprymna* spp. (Hanlon et al., 1997; Squires et al., 2013). As females were observed laying eggs cooperatively on the same substrate simultaneously; it is necessary to separate females to track parental lineage without genotyping. Male squids were sometimes aggressive towards conspecifics; therefore, crowding should be avoided especially as squids reach sexual maturity.

Spawning

Both *E. berryi* and *E. morsei* are multiple spawners similar to other members of the genera *Euprymna* and *Sepiola* (Huang, 2006; Rodrigues et al., 2011; Squires et al., 2013). Adult females of both species laid egg clutches every few days until reaching a late senescent life stage similar to other *Euprymna* species (Hanlon et al., 1997; Nabhitabhata et al., 2005; Squires et al., 2013). Spawning events were observed within three days of shipment of wild animals, possibly stimulated by the stress of transport (Cecere and Miyashiro, 2022). Mature *E. berryi* were observed spawning fertilized clutches repeatedly over a period of 100 days at 20°C. Egg clutch morphology differs across sepiolids, and may be used to differentiate sympatric species (Sanchez et al., 2019). Similarly, we found eggs more densely packed in *E. morsei* clutches than in *E. berryi* (Figure 2A).

Concluding remarks

Protocols established for *E. scolopes* are readily adapted for *E. berry* and *E. morsei*. Some existing protocols, including *in situ* hybridization (Lee et al., 2009e), micro-CT (Kerbl et al., 2013), immunohistochemistry (Lee et al., 2009d), and hemocyte collection (Collins and Nyholm, 2010) have already been described in *E. berryi* (Gavriouchkina et al., 2022), and protocols for infection with symbiotic bacteria (Naughton and Mandel, 2012), behavioral assays, injury treatment, and electrophysiology (Howard et al., 2019) are expected to be transferable from *E. scolopes* other bobtail species.

Established cultures of *E. berryi* and *E. morsei* will allow for comparative studies among bobtail squids in the genus *Euprymna*. Genomic and transcriptomic data are publicly available for both *E. berryi* and *E. morsei* (Sanchez et al., 2019) and other related species (Sanchez et al., 2021), and a genome sequence of *E. berryi* has recently been reported (Gavriouchkina et al., 2022). The widespread distribution of *E. morsei* and *E. berryi* in conjunction with the ability to ship adults and recently spawned eggs should allow more researchers access to these model bobtail squids, and also offers opportunities to find adaptations acquired by different populations.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://figshare.com/search?q=10.6084%2Fm9.figshare.21063211.

Ethics statement

Japan has no specific regulations regarding cephalopods used for research purposes and cephalopods do not fall under the Japanese legislation 'Act on Humane Treament and Management of Animals (Ogden et al., 2016). All procedures were approved by the OIST Animal Care and Use Committee (approval ID: 2018-204). Procedures and animal cultural protocols followed the guidelines set by Directive 2010/63/EU for cephalopods (Fiorito et al., 2015) and animal welfare guidelines set by OIST Animal Care and Use Committee. Efforts were made to provide the highest quality care and reduce the suffering of animals.

Author contributions

JJ led the project and all aspects of husbandry. JJ, YH, LZ, RK, GS, and CS were involved in husbandry efforts. YH and CS were involved in obtaining wild specimens. JJ, GS, and YH identified species. JJ and CS took growth measurements. DG, FM, GS, and DR provided guidance. JJ, GS, and DR wrote the manuscript. All authors contributed to the article and approved the submitted version.

Funding

This project was funded by the Okinawa Institute of Science and Technology (OIST) to the Molecular Genetics Unit. DR is also grateful for the generous support of the Marthella Foskett-Brown Chair in Biology at the University of California, Berkeley,

and from the Chan-Zuckerberg BioHub. GS is grateful for the support of the 22K15085 Grant-in-Aid for Early-Career Scientists (KAKENHI). FM was supported by a JSPS Kakenhi grant (#19K06620). CS was supported by JSPS KAKENHI Grant-in-Aid for Early-Career Scientists, Grant Number 19K15901.

Acknowledgments

We thank Oleg Simakov and Eric Edsinger for helping to initiate bobtail squid studies at OIST, Bret Grasse, Taylor Sakmar, Spencer Nyholm, Bethany Rader, and Robyn Crook for guidance regarding bobtail squid culturing, Keishu Asada for assisting with culturing efforts, Michael Kuba and Tamar Gutnik for guidance on general cephalopod culturing, and Chika Azama for providing logistical support. We appreciate Aki Masunaga's beautiful illustrations created for this manuscript. We thank the local fishermen in Hiroshima and Mie prefectures for providing information on the seasonal abundance of bobtail squid and for providing animals for this study. Finally we thank the reviewers whose suggestions improved this manuscript.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2022.1039775/full#supplementary-material

References

Abbo, L. A., Himebaugh, N. E., DeMelo, L. M., Hanlon, R. T., and Crook, R. J. (2021). Anesthetic efficacy of magnesium chloride and ethyl alcohol in temperate octopus and cuttlefish species. *J. Am. Assoc. Lab. Anim. Sci.* 60, 556–567. doi: 10.30802/AALAS-JAALAS-20-000076

Albertin, C. B., Simakov, O., Mitros, T., Wang, Z. Y., Pungor, J. R., Edsinger-Gonzales, E., et al. (2015). The octopus genome and the evolution of cephalopod neural and morphological novelties. *Nature* 524, 220–224. doi: 10.1038/nature14668

Anderson, F. E., and Lindgren, A. R. (2020). Phylogenomic analyses recover a clade of large-bodied decapodiform cephalopods. *Mol. Phylogenet. Evol.* 156, 107038. doi: 10.1016/j.ympev.2020.107038

Belcaid, M., Casaburi, G., McAnulty, S. J., Schmidbaur, H., Suria, A. M., Moriano-Gutierrez, S., et al. (2019). Symbiotic organs shaped by distinct modes of genome evolution in cephalopods. *Proc. Natl. Acad. Sci. U. S. A.* 116, 3030–3035. doi: 10.1073/pnas.1817322116

Bello, G. (2020). Evolution of the hectocotylus in sepiolinae (Cephalopoda: Sepiolidae) and description of four new genera. *Eur. J. Taxon.* 1–53. doi: 10.5852/ejt.2020.655

Boal, J. G., and Gonzalez, S. A. (1998). Social behavior of individual oval squids (Cephalopoda, teuthoidea, loliginidae, *Sepioteuthis lessoniana*) within a captive school. *Ethology* 104, 161–78. doi: 10.1111/j.1439-0310.1998.tb00059.x

Boettcher, K. J., and Ruby, E. G. (1990). Depressed light emission by symbiotic *Vibrio fischeri* of the sepiolid squid *Euprymna scolopes*. *J. Bacteriol.* 172, 3701–3706. doi: 10.1128/jb.172.7.3701-3706.1990

Boettcher, K. J., Ruby, E. G., and McFall-Ngai, M. J. (1996). Bioluminescence in the symbiotic squid *Euprymna scolopes* is controlled by a daily biological rhythm. *J. Comp. Physiol. A* 179, 65–73. doi: 10.1007/BF00193435

Boletzky, S. V. (1975) *The reproductive cycle of sepiolidae (Mollusca, cephalopoda). pubblicazioni della stazione zoologica, napoli (Italy) 39.* Available at: http://agris.fao.org/agris-search/search.do?recordID=XE7600708.

Boletzky, S. V., Boletzky, M. V., Frösch, D., and Gätzi, V. (1971). Laboratory rearing of sepiolinae (Mollusca: Cephalopoda). *Mar. Biol.* 8, 82–87. doi: 10.1007/BF00349349

Boyle, P. R. (2005). *Cephalopods: ecology and fisheries* (Oxford, UK: Ames, Iowa: Blackwell Pub). Available at: https://openlibrary.org/books/OL17173099M/Cephalopods.

Callaerts, P., Lee, P. N., Hartmann, B., Farfan, C., Choy, D. W. Y., Ikeo, K., et al. (2002). HOX genes in the sepiolid squid *Euprymna scolopes*: implications for the evolution of complex body plans. *Proc. Natl. Acad. Sci. U. S. A.* 99, 2088–2093. doi: 10.1073/pnas.042683899

Cecere, A. G., and Miyashiro, T. I. (2022). Impact of transit time on the reproductive capacity of *Euprymna scolopes* as a laboratory animal. *Lab. Anim. Res.* 38, 25. doi: 10.1186/s42826-022-00135-2

Chiao, C.-C., Chubb, C., and Hanlon, R. T. (2015). A review of visual perception mechanisms that regulate rapid adaptive camouflage in cuttlefish. *J. Comp. Physiol. A Neuroethol. Sens. Neural Behav. Physiol.* 201, 933–945. doi: 10.1007/s00359-015-0988-5

Choe, S. (1966a). On the eggs, rearing, habits of the fry, and growth of some Cephalopoda. *Bull. Mar. Sci.* 16, 330–348.

Choe, S. (1966b). On the growth, feeding rates and the efficiency of food conversion for cuttlefishes and squids. *Korean J. Syst. Zool.* 9, 12–20.

Collins, A. J., and Nyholm, S. V. (2010). Obtaining hemocytes from the Hawaiian bobtail squid *Euprymna scolopes* and observing their adherence to symbiotic and non-symbiotic bacteria. *J. Vis. Exp.* 36, 1–3. doi: 10.3791/1714

Crawford, K., Diaz Quiroz, J. F., Koenig, K. M., Ahuja, N., Albertin, C. B., and Rosenthal, J. J. C. (2020). Highly efficient knockout of a squid pigmentation gene. *Curr. Biol* 30 (17), 3484–3490. doi: 10.1016/j.cub.2020.06.099

Doudna, J. A., and Charpentier, E. (2014). Genome editing. the new frontier of genome engineering with CRISPR-Cas9. *Science* 346, 1258096. doi: 10.1126/science.1258096

Drerup, C., Sykes, A. V., and Cooke, G. M. (2020). Behavioural aspects of the spotty bobtail squid *Euprymna parva* (Cephalopoda: Sepiolidae). *J. Exp. Mar. Bio. Ecol.* 530-531, 151442. doi: 10.1016/j.jembe.2020.151442

European Parliament and Council of the European Union. (2010). Directive 2010/63/EU of the European parliament and of the council of 22 September 2010 on the protection of animals used for scientific purposes. *Off. J. Eur. Union* 53, 33–79.

Fiorito, G., Affuso, A., Basil, J., Cole, A., de Girolamo, P., D'Angelo, L., et al. (2015). Guidelines for the care and welfare of cephalopods in research -a consensus based on an initiative by CephRes, FELASA and the Boyd group. *Lab. Anim.* 49, 1–90. doi: 10.1177/0023677215580006

Forsythe, J. W., DeRusha, R. H., and Hanlon, R. T. (1994). Growth, reproduction and life span of *Sepia officinalis* (Cephalopoda: Mollusca) cultured through seven consecutive generations. *J. Zool.* 233, 175–192. doi: 10.1111/j.1469-7998.1994.tb08582.x

Forsythe, J., Lee, P., Walsh, L., and Clark, T. (2002). The effects of crowding on growth of the European cuttlefish, *Sepia officinalis* linnaeuReared at two temperatures. *J. Exp. Mar. Bio. Ecol.* 269, 173–185. doi: 10.1016/S0022-0981(02)00006-0

Franklin, A. M., Squires, Z. E., and Stuart-Fox, D. (2014). Does predation risk affect mating behavior? an experimental test in dumpling squid (*Euprymna tasmanica*). *PloS One* 9, e115027. doi: 10.1371/journal.pone.0115027

Gavriouchkina, D., Tan, Y., Ziadi-Künzli, F., Hasegawa, Y., Piovani, L., Zhang, L., et al. (2022). A single-cell atlas of bobtail squid visual and nervous system highlights molecular principles of convergent evolution. *bioRxiv* 2022, 5.26.490366. doi: 10.1101/2022.05.26.490366

Grearson, A. G., Dugan, A., Sakmar, T., Sivitilli, D. M., Gire, D. H., Caldwell, R. L., et al. (2021). The lesser pacific striped octopus, *Octopus chierchiae*: An emerging laboratory model. *Front. Mar. Sci.* 8. doi: 10.3389/fmars.2021.753483

Hanlon, R. T., Claes, M. F., Ashcraft, S. E., and Dunlap, P. V. (1997). Laboratory culture of the sepiolid squid *Euprymna scolopes*: A model system for bacteria-animal symbiosis. *Biol. Bull.* 192, 364–374. doi: 10.2307/1542746

Hanlon, R. T., and Messenger, J. B. (2018). *Cephalopod behaviour 2nd edn.* (Cambridge: Cambridge University Press). doi: 10.1017/9780511843600

Hartmann, B., Lee, P. N., Kang, Y. Y., Tomarev, S., de Couet, H. G., and Callaerts, P. (2003). Pax6 in the sepiolid squid *Euprymna scolopes*: evidence for a role in eye, sensory organ and brain development. *Mech. Dev.* 120, 177–183. doi: 10.1016/s0925-4773(02)00456-2

Heath-Heckman, E., and Nishiguchi, M. K. (2021). Leveraging short-read sequencing to explore the genomics of sepiolid squid. *Integr. Comp. Biol.* 61, 1753–1761. doi: 10.1093/icb/icab152

Howard, R. B., Lopes, L. N., Lardie, C. R., Perez, P. P., and Crook, R. J. (2019). Early-life injury produces lifelong neural hyperexcitability, cognitive deficit and altered defensive behaviour in the squid *Euprymna scolopes*. *Philos. Trans. R. Soc Lond. B Biol. Sci.* 374, 20190281. doi: 10.1098/rstb.2019.0281

Huang, C. P. (2006). Species diversity of the genus Euprymna Steenstrup, 1887 in Taiwan and reproductive cycle of *Euprymna berryi* Sasaki, 1929. *Master's thesis* (Taichung, Taiwan: National Chung Hsing University), 1929.

Ibáñez, C. M., and Keyl, F. (2010). Cannibalism in cephalopods. *Rev. Fish Biol. Fish.* 20, 123–136. doi: 10.1007/s11160-009-9129-y

Iglesias, J., Fuentes, L., and Villanueva, R. (2014). *Cephalopod culture* (Dordrecht: Springer Netherlands). 1a. doi: 10.1007/978-94-017-8648-5_23

Iglesias, J., Otero, J. J., Moxica, C., Fuentes, L., and Sánchez, F. J. (2004). The completed life cycle of the octopus (*Octopus vulgaris*, cuvier) under culture conditions: Paralarval rearing using *Artemia* and zoeae, and first data on juvenile growth up to 8 months of age. *Aquac. Int.* 12, 481–487. doi: 10.1023/B:AQUI.0000042142.88449.bc

Ikeda, Y., Sakurazawa, I., Sakurai, Y., and Matsumoto, G. (2003). Initial trials of squid rearing, maintenance and culture at the brain science institute of RIKEN. *Aquac. Sci.* 51, 391–400. doi: 10.11233/aquaculturesci1953.51.391

Jinek, M., Chylinski, K., Fonfara, I., Hauer, M., Doudna, J. A., and Charpentier, E. (2012). A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science* 337, 816–821. doi: 10.1126/science.1225829

Jones, B. W., Lopez, J. E., Huttenburg, J., and Nishiguchi, M. K. (2006). Population structure between environmentally transmitted vibrios and bobtail squids using nested clade analysis. *Mol. Ecol.* 15, 4317–4329. doi: 10.1111/j.1365-294X.2006.03073.x

Jones, N. J. E., and Richardson, C. A. (2010). Laboratory culture, growth, and the life cycle of the little cuttlefish *Sepiola atlantica* (Cephalopoda: Sepiolidae). *J. Shellfish Res.* 29, 241–246. doi: 10.2983/035.029.0121

Kerbl, A., Handschuh, S., Nödl, M.-T., Metscher, B., Walzl, M., and Wanninger, A. (2013). Micro-CT in cephalopod research: Investigating the internal anatomy of a sepiolid squid using a non-destructive technique with special focus on the ganglionic system. *J. Exp. Mar. Bio. Ecol.* 447, 140–148. doi: 10.1016/j.jembe.2013.02.022

Kerwin, A. H., Gromek, S. M., Suria, A. M., Samples, R. M., Deoss, D. J., O'Donnell, K., et al. (2019). Shielding the next generation: Symbiotic bacteria from a reproductive organ protect bobtail squid eggs from fungal fouling. *MBio* 10, 1–18. doi: 10.1128/mBio.02376-19

Koenig, K. M., Sun, P., Meyer, E., and Gross, J. M. (2016). Eye development and photoreceptor differentiation in the cephalopod *Doryteuthis pealeii*. *Development* 143, 3168–3181. doi: 10.1242/dev.134254

LaRoe, E. T. (1971). The culture and maintenance of the loliginid squids *Sepioteuthis sepioidea* and *Doryteuthis plei*. *Mar. Biol.* 9, 9–25. doi: 10.1007/BF00348813

Lee, P. N., Callaerts, P., and de Couet, H. G. (2009a). Culture of Hawaiian bobtail squid (*Euprymna scolopes*) embryos and observation of normal development. *Cold Spring Harb. Protoc.* 2009, db.prot5323. doi: 10.1101/pdb.prot5323

Lee, P. N., Callaerts, P., and de Couet, H. G. (2009b). The embryonic development of the Hawaiian bobtail squid (*Euprymna scolopes*). *Cold Spring Harb. Protoc.* 2009, db.ip77. doi: 10.1101/pdb.ip77

Lee, P. N., Callaerts, P., De Couet, H. G., and Martindale, M. Q. (2003). Cephalopod hox genes and the origin of morphological novelties. *Nature* 424, 1061–1065. doi: 10.1038/nature01872

Lee, P. N., McFall-Ngai, M. J., and Callaerts, P. (2009c). The Hawaiian bobtail squid (*Euprymna scolopes*): a model to study the molecular basis of eukaryote-prokaryote mutualism and the development and evolution of morphological novelties in cephalopods. *Cold Spring Harbor* 4 (11), 1–9. doi: 10.1101/pdb.emo135

Lee, P. N., McFall-Ngai, M. J., Callaerts, P., and de Couet, H. G. (2009d). Confocal immunocytochemistry of embryonic and juvenile Hawaiian bobtail squid (*Euprymna scolopes*) tissues. *Cold Spring Harb. Protoc.* 2009, db.prot5320. doi: 10.1101/pdb.prot5320

Lee, P. N., McFall-Ngai, M. J., Callaerts, P., and de Couet, H. G. (2009e). Whole-mount in situ hybridization of Hawaiian bobtail squid (*Euprymna scolopes*) embryos with DIG-labeled riboprobes: II. embryo preparation, hybridization, washes, and immunohistochemistry. *Cold Spring Harb. Protoc.* 2009, db.prot5322. doi: 10.1101/pdb.prot5322

Leverich, W. J., and Levin, D. A. (1979). Age-specific survivorship and reproduction in *Phlox drummondii*. *Am. Nat.* 113, 881–903. doi: 10.1086/283443

Levy, G., Nesher, N., Zullo, L., and Hochner, B. (2017). "Motor Control in Soft-Bodied Animals: The Octopus", in Ed. *The Oxford handbook of invertebrate neurobiology*. Ed J. H. Byrne (Oxford: Oxford University Press). doi: 10.1093/oxfordhb/9780190456757.013.36

Liscovitch-Brauer, N., Alon, S., Porath, H. T., Elstein, B., Unger, R., Ziv, T., et al. (2017). Trade-off between transcriptome plasticity and genome evolution in cephalopods. *Cell* 169, 191–202.e11. doi: 10.1016/j.cell.2017.03.025

Lussier, S. M., Kuhn, A., Chammas, M. J., and Sewall, J. (1988). Techniques for the laboratory culture of *mysidopsis* species (Crustacea: Mysidacea). *Environ. Toxicol. Chem.* 7 (12), 969–977. doi: 10.1002/etc.5620071203

Maldonado, E., Rangel-Huerta, E., González-Gómez, R., Fajardo-Alvarado, G., and Morillo-Velarde, P. S. (2019). *Octopus insularis* as a new marine model for evolutionary developmental biology. *Biol. Open* 8, 1–11. doi: 10.1242/bio.046086

McFall-Ngai, M. J. (1999). Consequences of evolving with bacterial symbionts: Insights from the squid-vibrio associations. *Annu. Rev. Ecol. Syst.* 30, 235–256. doi: 10.1146/annurev.ecolsys.30.1.235

McFall-Ngai, M. (2014). Divining the essence of symbiosis: insights from the squid-vibrio model. *PloS Biol.* 12, e1001783. doi: 10.1371/journal.pbio.1001783

Minton, J. W., Walsh, L. S., Lee, P. G., and Forsythe, J. W. (2001). First multi-generation culture of the tropical cuttlefish *Sepia pharaonis* Ehrenberg, 1831. *Aquac. Int.* 9, 379–392. doi: 10.1023/A:1020535609516

Moltschaniwskyj, N. A., and Carter, C. G. (2010). Protein synthesis, degradation, and retention: mechanisms of indeterminate growth in cephalopods. *Physiol. Biochem. Zool.* 83, 997–1008. doi: 10.1086/656387

Montgomery, M. K., and McFall-Ngai, M. (1994). Bacterial symbionts induce host organ morphogenesis during early postembryonic development of the squid *Euprymna scolopes*. *Development* 120, 1719–1729. doi: 10.1242/dev.120.7.1719

Moynihan, M. (1983). Notes on the behavior of *Euprymna scolopes* (Cephalopoda: Sepiolidae). *Behaviour* 85, 25–41. doi: 10.1163/156853983x00020

Nabhitabhata, J. (2014). "Sepiella inermis", in *Cephalopod culture*. Eds. J. Iglesias, L. Fuentes and R. Villanueva (Dordrecht: Springer Netherlands), 225–240. doi: 10.1007/978-94-017-8648-5_13

Nabhitabhata, J., Nilaphat, P., and Promboon, P. (2005). Life cycle of cultured bobtail squid, *Euprymna hyllebergi* Nateewathan. *Spec. Publ. Phuket Mar. Biol. Cent* 66, 351–365.

Nabhitabhata, J., and Nishiguchi, M. K. (2014). "*Euprymna hyllebergi* and *Euprymna tasmanica*," in *Cephalopod culture*. Eds. J. Iglesias, L. Fuentes and R. Villanueva (Dordrecht: Springer Netherlands), 253–269. doi: 10.1007/978-94-017-8648-5_15

Naughton, L. M., and Mandel, M. J. (2012). Colonization of *Euprymna scolopes* squid by *Vibrio fischeri*. *J. Vis. Exp.* 61, e3758. doi: 10.3791/3758

Navet, S., Buresi, A., Baratte, S., Andouche, A., Bonnaud-Ponticelli, L., and Bassaglia, Y. (2017). The pax gene family: Highlights from cephalopods. *PloS One* 12, e0172719. doi: 10.1371/journal.pone.0172719

Norman, M. D., and Lu, C. C. (1997). Redescription of the southern dumpling squid *Euprymna tasmanica* and a revision of the genus *Euprymna* (Cephalopoda: Sepiolidae). *J. Mar. Biol. Assoc. United Kingdom* 77, 1109–1137. doi: 10.1017/s0025315400038662

O'Brien, C. E., Roumbedakis, K., and Winkelmann, I. E. (2018). The current state of cephalopod science and perspectives on the most critical challenges ahead

from three early-career researchers. *Front. Physiol.* 9. doi: 10.3389/fphys.2018.00700

Ogden, B. E., Pang William, W., Agui, T., and Lee, B. H. (2016). Laboratory animal laws, regulations, guidelines and standards in China mainland, Japan, and Korea. *ILAR J.* 57, 301–311. doi: 10.1093/ilar/ilw018

Okutani, T., and Horita, E. (1987). Identity of *Euprymna berryi* Sasaki, 1929 (Cephalopoda: Sepiolidae). *Venus* 46 (2), 91–107. doi: 10.18941/venusjjm.46.2_91

Peyer, S. M., Pankey, M. S., Oakley, T. H., and McFall-Ngai, M. J. (2014). Eye-specification genes in the bacterial light organ of the bobtail squid *Euprymna scolopes*, and their expression in response to symbiont cues. *Mech. Dev.* 131, 111–126. doi: 10.1016/j.mod.2013.09.004

Pichon, D., Gaia, V., Norman, M. D., and Boucher-Rodoni, R. (2005). Phylogenetic diversity of epibiotic bacteria in the accessory nidamental glands of squids (Cephalopoda: Loliginidae and idiosepiidae). *Mar. Biol.* 147, 1323–1332. doi: 10.1007/s00227-005-0014-5

Raj, P. J. S., and Kalyani, N. (1971). *Euprymna morsei* (Verril) (Sepiolidae: Cephalopoda) from the Indian coast. *J. Mar. Biol. Assoc. India* 131, 135–375.

Reid, A., and Jereb, P. (2005) *Family sepiolidae, in cephalopods of the world* (Rome: FAO). Available at: http://www.fao.org/3/ac479e/ac479e00.htm (Accessed May 8, 2020).

Rocha, F., Guerra, A., and González, A. F. (2001). A review of reproductive strategies in cephalopods. *Biol. Rev. Camb. Philos. Soc* 76, 291–304. doi: 10.1017/s1464793101005681

Rodrigues, M., Garcí, M. E., Troncoso, J. S., and Guerra, A. (2010). Burying behaviour in the bobtail squid *Sepiola atlantica* (Cephalopoda: Sepiolidae). *Ital. J. Zool.* 77, 247–251. doi: 10.1080/11250000903282899

Rodrigues, M., Garcí, M. E., Troncoso, J. S., and Guerra, Á. (2011). Spawning strategy in Atlantic bobtail squid *Sepiola atlantica* (Cephalopoda: Sepiolidae). *Helgol. Mar. Res.* 65, 43–49. doi: 10.1007/s10152-010-0199-y

Rosas, C., Gallardo, P., Mascaró, M., Caamal-Monsreal, C., and Pascual, C. (2014). "Octopus maya", in *Cephalopod culture*. Eds. J. Iglesias, L. Fuentes and R. Villanueva (Dordrecht: Springer Netherlands), 383–396. doi: 10.1007/978-94-017-8648-5_20

Ruby, E. G. (1996). Lessons from a cooperative, bacterial-animal association: the *Vibrio fischeri-Euprymna scolopes* light organ symbiosis. *Annu. Rev. Microbiol.* 50, 591–624. doi: 10.1146/annurev.micro.50.1.591

Ruby, E. G., and Lee, K. H. (1998). The *Vibrio fischeri-Euprymna scolopes* light organ association: Current ecological paradigms. *Appl. Environ. Microbiol.* 64, 805–812. doi: 10.1128/AEM.64.3.805-812.1998

Sanchez, G., Fernández-Álvarez, F.Á., Taite, M., Sugimoto, C., Jolly, J., Simakov, O., et al. (2021). Phylogenomics illuminates the evolution of bobtail and bottletail squid (order sepiolida). *Commun. Biol.* 4, 819. doi: 10.1038/s42003-021-02348-y

Sanchez, G., Jolly, J., Reid, A., Sugimoto, C., Azama, C., Marlétaz, F., et al. (2019). New bobtail squid (Sepiolidae: Sepiolinae) from the Ryukyu islands revealed by molecular and morphological analysis. *Commun. Biol.* 2, 465. doi: 10.1038/s42003-019-0661-6

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., et al. (2012). Fiji: an open-source platform for biological-image analysis. *Nat. Methods* 9, 676–682. doi: 10.1038/nmeth.2019

Schmidbaur, H., Kawaguchi, A., Clarence, T., Fu, X., Hoang, O. P., Zimmermann, B., et al. (2022). Emergence of novel cephalopod gene regulation and expression through large-scale genome reorganization. *Nat. Commun.* 13, 2172. doi: 10.1038/s41467-022-29694-7

Sinn, D. L., Apiolaza, L. A., and Moltschaniwskyj, N. A. (2006). Heritability and fitness-related consequences of squid personality traits. *J. Evol. Biol.* 19, 1437–1447. doi: 10.1111/j.1420-9101.2006.01136.x

Sinn, D. L., Gosling, S. D., and Moltschaniwskyj,. N. A. (2008). Development of shy/bold behaviour in squid: context-specific phenotypes associated with developmental plasticity. *Anim. Behav.* 75, 433–442. doi: 10.1016/j.anbehav.2007.05.008

Sinn, D. L., and Moltschaniwskyj, N. A. (2005). Personality traits in dumpling squid (*Euprymna tasmanica*): context-specific traits and their correlation with biological characteristics. *J. Comp. Psychol.* 119, 99–110. doi: 10.1037/0735-7036.119.1.99

Smith, J. A., Andrews, P. L. R., Hawkins, P., Louhimies, S., Ponte, G., and Dickel, L. (2013). Cephalopod research and EU directive 2010/63/EU: Requirements, impacts and ethical review. *J. Exp. Mar. Bio. Ecol.* 447, 31–45. doi: 10.1016/j.jembe.2013.02.009

Squires, Z. E., Norman, M. D., and Stuart-Fox, D. (2013). Mating behaviour and general spawning patterns of the southern dumpling squid *Euprymna tasmanica* (Sepiolidae): A laboratory study. *J. Molluscan Stud.* 79, 263–269. doi: 10.1093/mollus/eyt025

Steer, M. A., Moltschaniwskyj, N. A., Nichols, D. S., and Miller, M. (2004). The role of temperature and maternal ration in embryo survival: using the dumpling squid *Euprymna tasmanica* as a model. *J. Exp. Mar. Bio. Ecol.* 307, 73–89. doi: 10.1016/j.jembe.2004.01.017

Sugimoto, C., Yanagisawa, R., Nakajima, R., and Ikeda, Y. (2013). Observations of schooling behaviour in the oval squid *Sepioteuthis lessoniana* in coastal waters of Okinawa island. *Mar. Biodivers. Rec.* 6, e34. doi: 10.1017/S1755267213000067

Sundaram, S., and Sreeram, M. P. (2008). First record of sepiolid squid, *Euprymna berryi* Sasaki, 1929 from the west coast of India. *J. Mar. Biol. Assoc. India* 50, 221–223.

Sykes, A. V., Baptista, F. D., Gonçalves, R. A., and Andrade, J. P. (2012). Directive 2010/63/EU on animal welfare: a review on the existing scientific knowledge and implications in cephalopod aquaculture research. *Rev. Aquac.* 4, 142–162. doi: 10.1111/j.1753-5131.2012.01070.x

Takayama, K., and Okutani, T. (1992). Identity of *Sepiola parva* Sasaki, 1913 and *S. birostrata* Sasaki, 1918 in the Northwest Pacific (Cephalopoda: Sepiolidae). *Venus* 51, 203–214. doi: 10.18941/venusjjm.51.3_203

Tarazona, O. A., Lopez, D. H., Slota, L. A., and Cohn, M. J. (2019). Evolution of limb development in cephalopod mollusks. *Elife* 8, 1–19. doi: 10.7554/eLife.43828

Toriyama, M., Kudo, S., and Kuroki, T. (1970) *On the small - trawl fishery in the nobeoka - wan - II*. Available at: http://feis.fra.affrc.go.jp/publi/bull_nansei/bull_nansei0402.pdf.

Vidal, E. A. G., Villanueva, R., Andrade, J. P., Gleadall, I. G., Iglesias, J., Koueta, N., et al. (2014). Cephalopod culture: current status of main biological models and research priorities. *Adv. Mar. Biol.* 67, 1–98. doi: 10.1016/B978-0-12-800287-2.00001-9

Villanueva, R. (1995). Experimental rearing and growth of planktonic *Octopus vulgaris* from hatching to settlement. *Can. J. Fish. Aquat. Sci.* 52, 2639–2650. doi: 10.1139/f95-853

Villanueva, R., Perricone, V., and Fiorito, G. (2017). Cephalopods as predators: A short journey among behavioral flexibilities, adaptions, and feeding habits. *Front. Physiol.* 8. doi: 10.3389/fphys.2017.00598

Villanueva, R., Vidal, E. A. G., Fernández-Álvarez, F.Á., and Nabhitabhata, J. (2016). Early mode of life and hatchling size in cephalopod molluscs: Influence on the species distributional ranges. *PloS One* 11, e0165334. doi: 10.1371/journal.pone.0165334

Walsh, L. S., Turk, P. E., Forsythe, J. W., and Lee, P. G. (2002). Mariculture of the loliginid squid *Sepioteuthis lessoniana* through seven successive generations. *Aquaculture* 212, 245–262. doi: 10.1016/S0044-8486(02)00126-6

Zepeda, E. A., Veline, R. J., and Crook, R. J. (2017). Rapid associative learning and stable long-term memory in the squid *Euprymna scolopes*. *Biol. Bull.* 232, 212–218. doi: 10.1086/693461