**Standard Operating Procedures, *Euprymna scolopes* (Cephalopoda)**

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Biological Materials Protocol #SC 10-028R

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**Field collection rationale and policy**

 *Euprymna scolopes* are found only in the Hawaiian Islands, and no captive-bred source exists for them. Our research studies the unique and specific symbiotic relationship between *Euprymna scolopes* and the luminescent marine bacteria, *Vibrio fischeri*. At this time, no virtual or non-animal model exists for this relationship. *E. scolopes* is not known to be hazardous in any way to humans, and has no known toxic venom or ink as some cuttlefish and octopus species do.

 For our breeding colony, we require (and can easily house) 16 adult female and 8 adult male *E. scolopes* at one time. These adult animals live out their natural lifespan in 2-4 months, and field collections generally occur every 3 months to replace this breeding stock.

 The field collection site is in a shallow bay where fishing is legal. No permit is required for collection of *E. scolopes*, and the population is not considered threatened or endangered either locally or at the state level. We only collect adult animals (> 20 mm mantle length (ML)) for the breeding colony, although if the collection is difficult, we may collect “teenagers” (15-20 mm ML). Any additional animals caught are released unharmed on site. Occasionally additional adult, teenage, or juvenile animals are requested by researchers in the labs for specific projects, and care is taken to collect only what is needed.

**Field Collection Methods**

Collection and transportation

Animals are located in shallow (1-3 ft) waters off Oahu by spotting them (with a narrow-beam flashlight) swimming in the water column or sitting on the bottom. Long-handled dip nets are carried by field personnel. Animals are captured in the dip net, care being taken to avoid injury to the fins. If the animal is not captured with the net on the first try, it is not chased--the stress of being chased is deemed excessive and unnecessary. Animals are then transferred to a gallon-size locking plastic bag (*e.g.,* Ziploc) with 1.5 L of seawater, and air filling the remaining portion of the bag. Plastic bags are carried in a mesh “drag bag,” which keeps them in the water (and at ambient water temperature) while the field personnel continue to collect. At the end of the evening collection period (2-6 h), bags are transferred to 5-gal buckets for carrying back to the lab. Stacking the bags in the buckets allows the bags to remain relatively stable during the car ride to the lab.

Short-term holding

 Collected animals are immediately transported to Kewalo Marine Laboratory (KML), run by the University of Hawaii. Animals are sexed at the laboratory, and released from the plastic bag into 30-gal tanks. Females and males are kept in separate tanks to prevent overcrowding, aggression, and unintended breeding.

Each tank is on a flow-through system, which means that seawater pumped in from the ocean is delivered to the tank, and overflow is returned to the ocean. This ensures a constant delivery of clean oxygenated seawater to the squid. Each tank contains a 1 cm layer of local sand for the squid to bury in, and a short piece of halved PVC pipe to accommodate any females that decide to lay eggs. The tanks are also covered with a light-reducing shield. Animals are fed *ad libitum* with local marine or glass shrimp (*Palaemonetes* spp.), obtained from a local pet store.

Annual animal use

|  |  |  |
| --- | --- | --- |
| **Animal Type** | **Number** | **Use** |
| Adult female | 64 | Breeding colony |
| Adult male | 32 | Breeding colony |
| Other wild-caught | 15 | Experimental |
| Hatchlings | 65,000 | Experimental |

The facility at KML has room to house 16 adult females and 8 adult males at a time. This ratio has proved to be successful in providing 2-6 clutches daily which, in turn, release about 150 hatchlings daily for experimental procedures. We can house up to 80 clutches at a time, but usually have about 40-60 at various stages of incubation. Roughly 500 hatchlings (annually) are used for long-term experiments lasting 4 days to 6 months. All other hatchlings are used in experiments within the first 3 days of hatch.

Animal areas

 The area in which the aquaria are kept is within the McFall-Ngai laboratory, rather than the MSB vivarium. The lab employs a full-time animal husbandry manager, as well as 2 undergraduate animal-care staff who are located adjacent to the aquaria, and are able to monitor it at all times during the day.

The adult aquaria are in a light-tight hallway, in which the ceiling lights go on at 0500 h, and off at 1700 h, each day. This interval is equivalent to what the animal experiences in the wild, and provides ample time for lab maintenance and cleaning without disturbing these nocturnal animals. The air temperature is ambient for the lab space, about 24°C, which is also an environmentally natural temperature for the animals.

The egg-incubation aquarium is in a separate, light- and temperature- controlled room. Lights in the incubation room go on at midnight and off at noon. This facilitates hatching and collection at a time (1200-1400 h) convenient for the researchers. The air temperature is 24-26°C.

Aquarium parameters

The total volume of each table is 200 gal of circulating seawater. Water temperature is ambient, about 24°C. Each table contains a sump with a protein skimmer and biologically active sand and rock over which the seawater passes. The seawater also runs through a UV filter before returning to the animal-containment cubes, or housing units, on the top. Seawater is prepared to a desired 34 PPT salinity from Instant Ocean salts-mix and deionized water, and allowed to sit at least 24 h before use. Ammonia and nitrite levels are monitored and kept at zero (below detection). Nitrates are kept as low as possible, and are always <10 PPM. Each of the 3 adult aquarium tables is a closed system, so should an emergency arise, not all animals are lost. In fact, each table could be used as a separate quarantine system should the need arise.

Each cube is made of dark-blue opaque acrylic. The bottom of the cube is covered with 2 cm of calcium-carbonate (marine) sand, and halved 4-in black PVC pipe are placed in the cube, within which the females lay eggs. The lids of each cube are clear acrylic and allow light to penetrate, so that the squid experiences a natural light-dark cycle.

Each adult female is housed in a separate cube, 48 cm x 38 cm x 30 cm, with a water depth of 23 cm. Each adult male is housed, individually or in pairs, in a cube of the same dimensions. When in pairs, the males are of sufficiently different size to determine individuals. Although aggression and cannibalism have been reported in the literature, we have found no evidence of it.

Should a power outage occur, the volume of water on the tables is sufficient to keep the squid immersed and oxygenated for several hours.

Feeding

 Live freshwater glass shrimp (*Palaemonetes* spp.) are fed *ad libitum* to squid each afternoon, less than 1 h before their subjective “dark.” Shrimp are enriched with a nutritional fish-flake food shortly before use. The glass shrimp are obtained through a licensed wholesale fish farm in Florida. Shrimp arrive packed in a copper sulfate solution. Because heavy metals are extremely toxic to cephalopods, the shrimp are washed 3 times in deionized water in the vivarium with dedicated equipment before they are brought into the lab for use. In the lab, the shrimp are maintained in 150-gal horse troughs with a continuous drip of deionized water. Three L of Instant Ocean are added daily to provide mineral content.

When available, or once weekly, squid are fed saltwater white shrimp, *Litopenaeus vannamei*. *L. vannamei* are considered a more nutritious food source than freshwater glass shrimp, with a higher amino acid and protein content; however, they are costlier and much more difficult to obtain regularly, as there are very few producers, and seasonal gaps in production occur.

Cleaning

On a daily basis, dead and dying feeder shrimp are removed from the tanks first thing in the morning. This prevents buildup of nitrogenous waste in the system and in the sand layer.

Each tank is cleaned once per month. The squid are transferred to a separate holding tank for the duration of cleaning (< 20 minutes). Sand is removed and washed with very hot tap water, followed by a thorough rinsing with deionized water. Algae, leftover feed, and other detritus is removed from the sand layer through this process. The sides and bottom of the tanks are also scrubbed.

Annually, the tables are completely drained, the sumps are cleaned, the UV light bulbs are changed, and deionized water is left in the tank overnight. The table is then refilled with seawater and allowed to run for at least a week before a new cohort of squid are or before new eggs are placed on the incubation table.

Breeding of adults

The mantle lengths (ML) of all squid are measured in the first week after collection. A single female is matched with a similarly sized (or slightly smaller) male, and is paired with the same male throughout her lifetime, unless that male dies. This limits some of the inherent genetic variability in offspring. During each breeding event (once every 2 weeks for each female), the male is moved into the female’s cube for 16 h overnight, and returned to its own cube the next morning for recovery. Each male has at least 2 days of recovery between mating events.

Collection Sharing

 Requests for eggs or squid are evaluated individually based on our needs in lab at the time and our knowledge of the requestee’s system and ability to care for the animals. Occasionally, when eggs are shipped to other labs, a clutch is selected which is 7-10 days old. This gives the embryos a chance to experience critical early development, but reduces the chance of hatching en route. Adults may be shipped at any time. Eggs and adults are prepared for shipping as described above (**Long-term handling and transportation**). If juveniles are shipped, they are collected on the day of hatch and are placed in 20-25 mL of seawater in a 50 mL conical tube. The tube is placed in a Styrofoam holder within a box. All shipments are next day delivery, so eggs, adults, or hatchlings spend no more than 24 hours in the shipping setup.

Outreach

On occasion, we are asked to bring juveniles or adults to another location for a demonstration. If an adult is needed, a male is selected—it is in some ways less valuable to the breeding collection, and is larger and “hardier” than an adult female. A 25-gal tank is set up the night before the event, and is filled with salt water and sand. A bubbler and a filter unit are attached to the aquarium. The male is added to the tank about an hour before the event, to allow it time to acclimate and bury. Only a trained animal-care technician is allowed to touch or manipulate the squid. This may mean rousing the squid from the sand in order to demonstrate swimming behavior. A turkey baster is brought along to suck out any ink that may be expelled during the demonstration. Generally, this type of outreach is less than 1 hour, and does not seem to affect the long-term behavior or health of the squid.

When juveniles are used in outreach programs, they are kept in large shallow dishes (with a lot of surface area) for demonstration. After the demonstration, they may be used in experiments or euthanized.

Record Keeping

### We recognize the importance of records over the long-term for measurements of laying/hatching success, trends, and consistency when memory fails. Electronic animal records are maintained in a relational database for ease of input and access. Facility records, such as water parameters and purchasing receipts, are maintained in paper form.

 Each new cohort (“field collection”) of animals belongs to the same alphabet letter. Clutch identifiers and animal names are associated with that letter. Animals in the breeding colony are individually identified with a unique number and given an alphabetical name. Each animal record includes the date of capture, the initial size (ML), the sex, the number, and the name of the animal. In addition, if there are any distinguishing characteristics of the animal, that information is noted as well. Animal death dates are recorded, and if the animal is euthanized, that is recorded as well. Mean longevity in captivity can thus be calculated.

 Each clutch is assigned a unique identifier, which includes the cohort alphabet designation. For each clutch, the lay date, the approximate number of eggs, the female, and the PVC cave identifier are recorded.

 From each clutch, the number of hatchlings is recorded. This information includes the number of hatchlings that hatched overnight (“earlies”) and the number of hatchlings that hatched at known times (“normals”). Any dead, sickly, or premature hatchlings are also noted. From these data, we can compute the number of hatchlings per clutch total, the number of hatchlings per time period, the number of hatchlings per female, and the clutch’s range of incubation time.

**Incubation and hatching**

Aquarium parameters

When a clutch is laid in an adult tank, it is recorded and moved (while immersed in a glass bowl filled with seawater) to the incubation aquaria. At no time is the clutch exposed to air.

The incubation aquaria house egg bowls, each of which may contain 1-2 clutches. The egg table has 150 gal of circulating seawater. The water temperature is kept at 23-24° C through the use of a chiller attached to the system. Levels of ammonia, nitrite, and nitrate are in the water are maintained at zero (*i.e*., below detection), and salinity is kept at 34 PPT. The circulating water is exposed to UV illumination. Each egg bowl is housed under a spigot delivering the circulating seawater, so aeration and water movement are very high. The egg table undergoes a water change twice weekly, at which time old clutches are removed from the table.

Because constant aeration of the eggs is essential for their normal development, should a power outage occur, the egg table pump is attached to an outlet that receives power from the building’s emergency generator.

Hatching

Most eggs (70-80%) hatch within 2 h of dark, that is, between noon and 1400 h. Animals are collected regularly from the egg table using a 5 mL disposable plastic pipette to transfer them to a glass dish. During the day, animals are collected within 30 min of hatch and are placed into filter-sterilized (0.45 µM pore-sized filter membrane) Instant Ocean water (FSIO). Animals are washed 3 times in this filtered water to prevent any contamination from the egg table water. Overnight animals (the “earlies”), may remain on the table as long as 16 h, and are used for experiments that do not require either aposymbiosis (no colonization of the symbiotic light organ) or knowledge of the exact time of hatch.

**Rearing**

Roughly 500 hatchlings annually are used for experiments lasting longer than 3 days. In these cases, they are taken care of by staff dedicated to the rearing of *E. scolopes*. Beyond 3 days, the hatchlings must be fed. Further, they must learn to hunt successfully within the first few critical days, or will not perish within one week. Animals have been reared and maintained in the laboratory through an entire life cycle and up to 9 months at the longest.

Aquarium parameters

 Animals are raised in round black tubs containing a 1 cm layer of finely crushed coral sand. Each tub can hold 25-50 hatchlings. The dimensions of the tubs are 16 cm in diameter and a height of 8 cm. The tubs each contain an aeration stone and clear plastic cover to prevent evaporation. A water temperature of 23° C is constantly maintained through adjusting air temperature. For the first 7 days after being introduced into the rearing tubs, water volume is maintained at 4 L. During days 8-21, the water volume is increased to 6 L and the maximum stocking density is decreased to 20 squid per tub to accommodate the growing squid. After day 21, a water volume of 8 L is used for the remainder of the animal’s life. After week 6, the maximum squid per tub is decreased to 10 and eventually to 5 per tub after 3 months.

Feeding

 Feeding begins as soon as possible, typically within 24 hours of hatching and 16 hours of inoculation. During the first week, the squid are fed fully-grown mysids, *Mysidopsis bahia* (1-1.5 cm in length) and 4-week-old brine shrimp, *Artemia franciscana* (1-1.5 cm in length). The feedings consist of using fine-mesh nets to place the mysids and brine shrimp into the rearing tubs and then removing any that are uneaten at the end.

 To maximize the chances for success during the critical first 3 days, numerous short feedings are used. For the first week, 5 feedings of 30 minutes each are performed daily. The feedings are spread out so that there is at least 1 hour in between. To better stimulate the squid, the lights are dimmed during the first 25 minutes of each feeding.

 As the squid become more proficient at hunting, feedings are lengthened and performed less frequently. For week 2, feedings are 3 times a day, 1 hour in length, the lights are dimmed for the first 45 minutes, and the feedings are separated by at least 2 hours. In addition to mysids and brine shrimp, postlarval marine shrimp *Litopenaeus vannamei* (1-1.5 cm length) are now fed.

 At week 3, the squid are fed twice daily, once in the morning after lights-on and once just before lights-off. The feedings last 1 hour with the lights dimmed for the first 30 minutes.

 For week 4, the transition to nocturnal behavior has occurred and feedings are performed partially at night. Approximately 30 minutes before lights-off, the squid are fed and left to hunt overnight. Anything uneaten is removed the next morning.

 After week 4, the squid have reached mature behavior and are fed only overnight and the fine coral sand is replaced with coarse coral sand. At week 6, freshwater shrimp *Palaemonetes* spp.(2 cm length) are fed in increasing amounts as the mysids and brine shrimp are decreased. Marine shrimp of 2 cm length or longer can also be fed. As the squid increase in size larger shrimp are fed.

Cleaning

 Full water and sand changes are performed daily throughout the life of the squid. Rearing tubs with clean water and sand are prepared at the end of the day prior to use. Every morning, the squid are transferred using a pipette or plastic tumbler into the new tub. The used water is discarded and the used sand is rinsed three times with reverse osmosis purified water (RO) and dried completely before being used again. The used tubs are rinsed three times with RO and allowed to dry completely. Because of the daily water changes, testing for ammonia and nitrates is not performed unless signs of stress are exhibited by the squid. Upon this occurring, Tetra drop kits (United Pet Group, Cincinatti, OH) are used to test for each individually.

**Live-animal experiments**

Experiments not requiring anesthesia

All experiments are performed within the first 3 days after hatching when the squid still have egg yolk nutrient reserves on which to survive.

To maintain squid in the aposymbiotic state after collection (non-colonized), the animals are transferred to individual 20 mL scintillation vials containing 3 mL FSIO or filtered seawater.

Hatchlings used in colonization experiments are transferred and pooled (50 squid maximum) in a plastic cup containing 100 mL of FSIO with 3,000-100,000 CFU/mL of *Vibrio fischeri*. After 24 h, the animals are transferred to individual scintillation vials as described above. Scintillation vials are placed in racks and loosely covered with shrink-wrap plastic film to limit evaporation. Racks are stored in the incubation room, so that the hatchlings experience the same light-dark cycle and temperature as they did as eggs during incubation.

On a daily basis until the experiment is complete, hatchlings are checked for colonization and luminescence. The scintillation vial is put into a Turner Design Luminometer TD 20/20, and the light produced by the animal is recorded.

Hatchlings or eggs/embryos may be used for photography through a dissecting microscope. Such use is not lethal, and does not require anesthetic.

Experiments commonly expose squid to:

* *Vibrio* species in FSIO
* Chemical reagents/inhibitors/antibodies diluted in FSIO
* 4% PFA in marine PBS (after anesthesia)
* RNAlater (after anesthesia)

**Anesthesia and euthanasia**

Recognition of stress or illness

 Adult squid are observed at least twice daily (first thing in the morning, and during the evening feeding). Signs of illness or distress include failure to bury in the sand during daytime hours, failure to eat, and failure to respond to gentle touch. Animals deemed to be ill are euthanized (see below). Lesions and scars may appear on females immediately after mating, but do not appear to affect the short- or long-term overall health of the animal, and usually disappear within a few days.

 During cleaning and breeding, it is necessary to rouse the squid from its sand cover. This may evoke a temporary stress response (inking, jetting around), but care is taken to minimize these occurrences, and to proceed as carefully and gently as possible. Because the viscous ink may clog gills, it is removed immediately from the water with a turkey baster. Unlike some other cephalopod species, *E. scolopes* do not exhibit the end-of-life stage of senescence.

Anesthesia

Many experiments (live imaging, fixed-animal imaging [confocal and epifluorescence microscopy], collection of light-organ contents, collection of hemolymph, *etc*.) require prior anesthesia of the squid. Animals are transferred to a vessel containing salt water containing either 2% ethanol or 0.12M MgCl2. Anesthesia is considered complete when the animal begins to drift or become ataxic.

Squid used for live imaging are anesthetized as described, and then their central nervous system is disrupted by applying pressure with forceps quickly between the eyes. The mantle can then be dissected open, and the animal is mounted in FSIO for imaging.

Hemolymph can be collected from adult squid. Adult animals are anesthetized in FSIO containing 2%, and hemolymph is withdrawn from the cephalic artery located between the eyes using a sterile 1-cc syringe and 28½-gauge needle. Animals subsequently revived from this procedure are able to survive well.

The following procedures are conducted in the dark under a dim red light during the animal’s nocturnal period. To collect light-organ contents *in situ*, adult animals are anaesthetized either in FSIO containing 2% ethanol or 0.12 M MgCl2, or in 250 mL FSIO on ice. When the animal is pale and cannot correct its position when oriented dorsally, the animal is transferred to a dissecting tray, and covered in sufficient FSIO (cold, 2% ethanol, or 0.12 M MgCl2) to ensure ventilation by the gills. A small primary incision is made in the animal’s mantle. If, at this point, the squid does not respond by moving tentacles or dilating pupils, then a medial incision is made through the ventral mantle tissue to expose the animal’s light organ. A medial incision is also made in the funnel to fully expose the light organ. To induce expulsion of the light-organ contents, incandescent white light is directed at the squid’s eyes. The expelled material is extruded out of the lateral openings of the light organ, and is collected with a 50-µl capacity Gilson Microman negative-pressure pipet. Expulsion of the crypt contents may occur as much as 30 min following initial light exposure, depending on the method of anesthesia used, among other factors.

Termination of experiments and euthanasia

Adult animals die either naturally, at the termination of an experiment, or are euthanized after 3 months in captivity. Hatchlings and reared animals are euthanized at the termination of an experiment. Euthanasia is performed through over-anesthetization followed by a quick-freeze method. Anesthetization is performed as described above: exposure to either 2% ethanol, 0.12M MgCl2, or FSIO on ice. Anaesthetized animals may be put into a dry, sealed container (a plastic bag or vial), and dropped into a bath of ethanol and dry ice. Within a few seconds, the animal is completely frozen, and the tissue stored at -80˚ C.

Carcass disposal and storage

After an experiment, or at the end of an adult’s life, the tissues (hemolymph, light organ, testes, eyes, *etc.*) are often harvested, and frozen for future use. Otherwise, after experiments and euthanasia, hatchlings are thrown out with trash or flushed into the sink; similarly, after experiments and euthanasia, the entire carcass of adult/reared animals is put into labeled and sealed plastic bags, and stored in a dedicated freezer for future use or reference.

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