Culture and Maintenance of Selected Invertebrates in the Laboratory and Classroom

Stephen A. Smith, Joseph M. Scimeca, and Mary E. Mainous

Abstract

Invertebrate species have been used for many years in the laboratory and teaching environment. We discuss some of the most commonly maintained invertebrates—the nematode (*Caenorhabditis elegans*), the California sea hare (*Aplysia californica*), the fruit fly (*Drosophila melanogaster*), terrestrial hermit crabs, the horseshoe crab (*Limulus polyphemus*), and cephalopods—and briefly describe general techniques for culturing them in captivity. The aim of this article is to give potential users an idea of the materials, methods, and effort required to maintain each type of organism in a laboratory or classroom setting.

Key Words: *Aplysia*; *Caenorhabditis elegans*; cephalopod; fruit fly (*Drosophila melanogaster*); hermit crab; horseshoe crab (*Limulus polyphemus*); invertebrate care; invertebrate culture

Introduction

Numerous species of invertebrates are used in a variety of research, teaching, and public display activities. These species have been adapted for captivity over the years for their aesthetic value, ease of culturing, and utility for a multitude of teaching and research purposes.

In the research laboratory, invertebrates have served as models for cancer, aging, immunology, endocrinology, toxicology, developmental biology, tissue regeneration, genetics, molecular biology, learning and memory, and biomimetics (Wilson-Sanders 2011, in this issue). In the primary and secondary school classroom they have been used to demonstrate comparative anatomy, developmental biology, genetics, behavior, evolution, ecology, and conservation. Public aquariums have displayed numerous species of invertebrates—sponges, coral, anemones, octopus, jellyfish, starfish, sea urchins, and crustaceans—and zoos have built ant colonies, bee hives, and butterfly houses and assembled diverse insect and arachnid collections.

In this article we discuss some of the commonly maintained invertebrate species—the nematode (*Caenorhabditis elegans*), the California sea hare (*Aplysia californica*), the fruit fly (*Drosophila melanogaster*), terrestrial hermit crabs (*Coenobita* spp.), the horseshoe crab (*Limulus polyphemus*), and cephalopods (octopuses, squid, and cuttlefish)—and describe general techniques for culturing and maintaining them in captivity. As this is only a brief introduction to these animals, readers are encouraged to acquire more detailed information by both consulting the literature cited in this review and conducting a thorough reference search for the latest information about these species and their culture and maintenance.

Caenorhabditis elegans

Caenorhabditis elegans is a free-living nematode that has been used extensively for research in developmental biology, neurobiology, behavioral biology, and cell death (Hope 1999; Riddle et al. 1997; Wood 1988) and, because it is transparent, in anatomical studies. The genome of *C. elegans* was the first to be completely sequenced for a multicellular organism. Because the nematode has only 959 cells and the position of each cell is consistent from worm to worm, researchers now know specifically which genes encode the development of each individual cell (Brenner 1974; Strange 2006) and how alteration of a gene will affect a particular cell's development (Hall and Altun 2007).

Anatomy and Biology

Caenorhabditis elegans is an unsegmented nematode that is about 1 mm in length. Its life cycle is completed in about 5 days at room temperature (22–23°C) if sufficient food is available. The worms are either male or, more often, hermaphroditic and able to self-fertilize (Byerly et al. 1976; Stiernagle 1999). The offspring of self-fertilized worms are usually genetically identical to each other and to the parent. The parent nematode

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lays eggs that hatch in about 12 hours and go through four larval stages (L1-L4) before becoming adults. However, if food is scarce, the L2 larvae enter a dauer stage, an inactive alternative form that can live without eating or reproducing. The organism can survive in this stage up to several months until food becomes available, when it enters the L4 larval stage for up to 15 days before becoming an adult.

Culturing *C. elegans*

The preferred food source for monoxenically culturing *C. elegans* is a bacterial lawn of *Escherichia coli* OP50 (Stiernagle 2006). Five ml of Luria-Bertani broth (10 g tryptone, 5 g yeast extract, and 10 g sodium chloride in 1 L of distilled water, dH_2O[1]) is inoculated with a single colony of *E. coli* OP50 and incubated for 1 to 2 days at room temperature without aeration (i.e., without shaking). A small sample (100 µl) of this culture is then spread onto a standard (60 mm) agar plate of nematode growth medium, taking care not to spread the bacteria all the way to the edges of the plate (Stiernagle 2006); the remainder of the liquid culture can be used for the next 1 to 2 weeks if stored at 4°C. Plates are incubated for 1 to 2 days until a bacterial lawn is visible; lawns can be stored at 4°C up to 1 week, but must be warmed to room temperature before use. Bacterial lawns are then inoculated with *C. elegans* by using a sterile needle or platinum wire to transfer 10 worms from an old plate, placing the worms near the center of the new plate. Worms can also be transferred by cutting a small chunk of agar from an old plate (using a sterile spatula) and placing it near the center of the new plate. This "chunking" method can transfer hundreds of worms and is commonly used to maintain a worm colony (Figure 1).

Plates with worms are maintained at between 16°C and 25°C and observed daily. Worms are transferred to new plates when the bacterial lawn has been consumed, usually about every 3 to 5 days depending on temperature (the higher the temperature the faster the lawn consumption). A recently developed chemically defined medium (*C. elegans* Maintenance Medium, CeMM) allows for a more standardized and systematic manipulation of the nutrients that the nematode receives (Szewczyk et al. 2003).

Liquid axenic cultivation of *C. elegans* enables automated culturing and experimentation with large-scale growth and screening. The nematode can also be grown in liquid media using *E. coli* OP50 as a food source, but this should be done for only one generation as keeping worms in liquid can lead to dauer formation.

For long-term storage, worms are initially frozen in liquid nitrogen or gradually cooled to −80°C (Stiernagle 2006) and then transferred to a 15% glycerol liquid freezing medium in cryovials for indefinite storage. They can be thawed as needed, but recovery rates are low, and unused portions of vials should not be refrozen.

[1]Abbreviations that appear >3x throughout this article: dH_2O, distilled water; ppt, parts per thousand

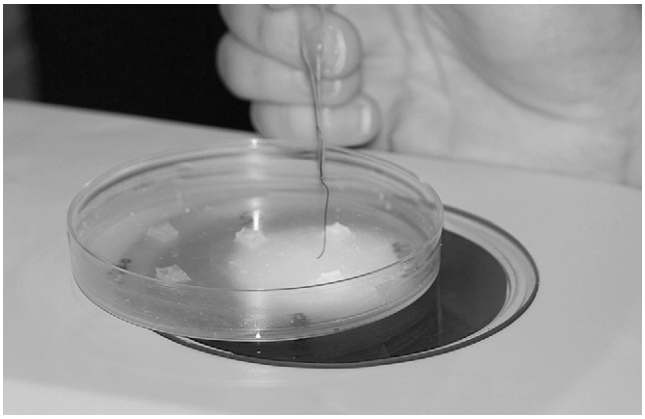

Figure 1 A nematode (*Caenorhabditis elegans*) being transferred, under a dissecting microscope (only the base shown), from a culture plate using a platinum wire technique. The nematode itself is too small to be seen, but five "chunks" are clearly visible on the plate from a previous transfer of *C. elegans* to the plate. (Photograph courtesy of the laboratory of Diya Banerjee, Virginia Tech)

Health Challenges and Treatment

Bacterial and fungal contaminants are the only potential problems with maintaining stocks of *C. elegans*. Contamination does not usually harm the worms but it can make it difficult to score phenotypes and complete transfers. Fungal contamination can be removed by repeated transfer of worms to fresh *E. coli* OP50 lawns, and bacterial contamination can be removed by treating the plate with a 5% sodium hypochlorite solution (Stiernagle 2006), which kills the bacteria on the plate without affecting worms in the egg stage. After treatment, the eggs are washed with dH_2O and seeded onto a fresh lawn of *E. coli* OP50. Contaminated plates, plates whose lawns have been consumed, and plates with surplus worms should be placed in a biohazard bag and autoclaved for disposal.

Aplysia californica

The California sea hare (*Aplysia californica*) is an opisthobranch mollusc in the order Anaspidea. *Aplysia* has long been an important animal model for developmental and neurological studies as well as for three different types of learning: habituation, sensitization, and classical conditioning (Capo et al. 2009). The organism's large and relatively few neurons facilitate the study of neuronal architecture, physiology, and control of instinctive and learned behaviors. Research on *Aplysia* has also led to a greater understanding of memory formation and developmental biology.[2]

[2]Unless otherwise indicated, information presented here is from the University of Miami/NIH National Resource for *Aplysia* at the Rosenstiel School of Marine and Atmospheric Science; available online (http://aplysia.miami.edu). This and other websites cited in this article were accessed on March 1, 2011.

Anatomy and Biology

Aplysia californica is an herbivorous marine gastropod that is found naturally in the Pacific Ocean off the coast of California. Unlike other gastropods, it does not have a large external shell but only a small vestigial shell that protects the heart and other internal organs; this lack of an obvious shell gives the animal a slug-like appearance. *Aplysia* has two large ventrally placed tentacles and, on top of the anterior portion of its head, two sensory tentacles called rhinophores, which have tactile and chemoreceptor functions.

Aplysia breathes by directing water over the gills using modified flaps, called parapodia, on its back or mantle. After the water has passed over the gills, it is expelled through a posteriorly directed tube called a siphon. When disturbed, the animal retracts its siphon into the mantle cavity, but can modify this behavior through experience. *Aplysia* also has an ink gland just under the shell and releases a purple ink when threatened. The mottled reddish-brown color of *A. californica* and the color of its ink are derived from pigments in the algae the animal consumes.

Aplysia is hermaphroditic but does not self-fertilize. Under natural conditions it alternates being male and female on different days during the spring and summer mating season. The animal lays several thousand eggs in long string masses about an hour after mating. The eggs hatch 7 to 8 days later (Hirscha and Peretza 1984; Kriegstein et al. 1974) and enter the initial free-swimming veliger phase for about 12 days, during which the veligers become larvae. The larval phase lasts until 30 days posthatch, and then the animal enters the juvenile phase. *Aplysia* reach adulthood at about 94 days and can live for an additional 6 to 10 months (Hirscha and Peretza 1984; Kriegstein et al. 1974). Adults weigh 500–1000 g (Gerdes and Fieber 2006; Michael C. Schmale, Rosenstiel School of Marine and Atmospheric Science, University of Miami, personal communication, November 24, 2010).

Housing and Diet

Aplysia may be housed in glass, fiberglass, or plastic tanks that provide for ease of cleaning and adequate circulation and/or filtration of water (Figure 2). Water quality is critical for maintaining healthy cultures of *Aplysia*. They require either natural seawater or artificial seawater at a salinity of 30 to 36 parts per thousand (ppt[1]) and a water temperature of 13–16°C. The pH of the water should be between 8.0 and 8.6 as is typical for most marine environments, and the oxygen content should be at least 95% or 8–9 mg/l.

Aplysia californica should be fed to satiation every 3 days. Its recommended diet in captivity is marine macroalgaes, principally red algae (e.g., *Gracilaria* spp., *Agardhiella* spp., and *Laurencia* spp.), which must be cultured separately from the *Aplysia*. Red algae are grown in nutrient (nitrate and phosphate)-supplemented seawater at room temperature with aeration and either direct natural light or full-spectrum fluorescent light (Schmale, personal communication, November 24, 2010).[3]

Figure 2 California sea hares (*Aplysia californica*) feeding on cultured red algae (*Agardhiella* spp.). Fresh, chilled seawater is entering the tanks at the top of the photograph and draining via the bottom of the tanks to remove detritus and fecal material in a flow-through, nonrecirculating system. (Photograph courtesy of Michael C. Schmale, National Resource for *Aplysia*)

Drosophila melanogaster

The fruit fly (*Drosophila melanogaster*) has been used as a model research organism for almost a century (Ashburn et al. 2005; Lachaise 1988; Rubin 2000), originally in studies of genetic inheritance. Today it remains the classic organism for genetic research, but more recent studies have focused on molecular and developmental biology, especially embryonic development although there is also interest in the development of adult structures in the pupal stage (Bate and Arias 1993; Goldstein and Fyrberg 1994; Markow and O'Grady 2006).

Anatomy and Biology

Drosophila, like other insects, have an exoskeleton to protect their internal body parts, and their bodies are divided into three segments: the head, the thorax, and the abdomen. The head comprises compound eyes, a mouth, antennae, and ocelli. The thorax is the middle segment, where the wings and legs are attached, and the rearmost part of the fly is the abdomen. The body is covered with small hairs, called sensilla, which act as sense organs and provide information about touch, taste, smell, and sound to the fly's nervous system.

The typical life cycle of *Drosophila* lasts about 12 to 14 days (Matthews 1994). Flies are fertile and begin mating

[3]As distinct from the other species discussed in this article, the scientific literature does not include reports of diseases or treatments for *Aplysia*.

within 12 hours of eclosure (emergence of an adult insect from a pupal case). Females can store sperm deposited internally by multiple males. Fertilized eggs are deposited directly on a food source. Within a day, they hatch and the larva emerges. The first instar larva stage lasts about 24 hours, then the organism molts to a second instar larva and, after about a day, the third instar larva stage, which lasts about 3 days, after which the larva molts and forms a pupa. The pupal stage lasts 5 to 7 days, during which the larva undergoes metamorphosis to become an adult fly.

Drosophila are sexually dimorphic: females (about 2½ mm in length) tend to be larger than males and have a pointed, banded abdomen; the abdomen of the male is more rounded and generally darker than that of the female. Males also have a darkened area on their front legs called the sex comb. Virgin females are generally larger than mature females.

Housing and Diet

Drosophila are typically maintained in commercially available fly culture vials or small glass jars containing a commercial fly growth medium (Figure 3). The medium is mixed with tap water and allowed to solidify and dry before flies are added to the vial and the mouth of the container closed with a cotton or foam plug (Dahmann 2010). Flies are incubated at room temperature (22–23°C) and then transferred as necessary, for example to new vials containing fresh medium as the old medium is consumed or in the case of overcrowding by adult flies.

Culturing and Genetic Breeding

One method of propagating *Drosophila* is to transfer adult flies from the old vial to a new one as the food source is consumed. Gently tapping the old vial causes the flies to fall to the culture medium at the bottom, at which point the foam plug is removed and the new vial quickly placed so that the two vials open into each other. Gentle tapping will prompt the flies to fall from the old vial into the new. When all flies are in the new vial, the two vials are separated and a foam plug is quickly placed into the new vial.

If it is necessary to separate adult flies based on sex or phenotypic characteristics, they must first be anesthetized, using a commercially available anesthetic (e.g., chloroform or ether), CO_2, or chilling (Dahmann 2010). They can then be separated using a dissecting microscope and transferred onto a white card or into a Petri plate for observation and separation. Chilled flies must be kept on a chilled glass Petri plate (on ice) or they will start to wake up. A small paintbrush is an effective tool for gently separating the flies, after which the desired populations (usually 5 to 10 flies) can be placed for mating in vials containing fresh medium.

Genetic crosses require the collection of virgin females (which must be collected within 8 hours of eclosure), which are anesthetized and placed in separate vials for 2 to 3 days of observation to verify their virginity (virgin females can lay eggs but the eggs will not be fertile and will not hatch). The virgins are then placed in a fresh vial with an equal number of males and allowed to mate. Breeding flies are removed from the vial when the larvae begin to pupate. The adult offspring can then be anesthetized, examined, and enumerated using a dissecting microscope. Surplus flies can be disposed of by first anesthetizing them and then placing them in a jar or vial containing 70% ethyl alcohol, isopropyl alcohol, or mineral oil.

Figure 3 The fruit fly (*Drosophila melanogaster*) being cultured in bottles with sponge plugs to prevent escape. Note the larvae (white), pupae (dark), and empty pupal cases (transparent) on the sides of the bottle where the larval stages undergo metamorphosis to the adult fly. (Original photograph taken by Robert Cudmore)

Health Challenges and Treatment

There are few health challenges associated with *Drosophila* cultures. Contamination with bacteria or fungus and infestation with mites are the most common problems. Bacterial and fungal contaminations are prevented or eliminated by using only freshly made fly medium and by frequently transferring flies to clean vials containing fresh food. Bacteria can also be eliminated by adding a small amount (1%) of a 1:100 penicillin-streptomycin solution to the surface of the food and allowing it to dry completely before flies are added.[4] Mites are difficult to eradicate; in most cases cultures contaminated with mites must be destroyed to eliminate the infestation.

Hermit Crabs

Hermit crabs have been used for scientific research in the fields of behavior, competition, and population studies, but they are more often purchased as pets or display animals for school classrooms (Fox 2000).

[4]Detailed information about *Drosophila* culture media is available from the Harvard Medical School *Drosophila* RNAi Screening Center (www.flyrnai.org/DRSC-PRC.html).

There are two broad categories of hermit crabs: marine (or aquatic) and terrestrial (Giwojna 2009; Nash 1976). There are four families of aquatic hermit crabs: Diogenidae, Paguridae, Pylochelidae, and Pylojacquesidae; and terrestrial hermit crabs are classified into two families: Coenobitidae and Parapaguridae. Of the more than 600 species of hermit crabs, most are marine species; the most commonly maintained are species of *Clibanatus*, *Paguristes*, and *Calcinus*. There is only one known species of true freshwater hermit crab, *Clibanarius fonticola*, which lives in a small lake on Espiritu Santo Island, Vanuatu, in the South Pacific Ocean. Land hermit crabs are more commonly used as laboratory animals or kept as pets than marine hermit crabs, and the most common types are various species of *Coenobita* (e.g., *C. clypeatus*, *C. compressus*, and *C. rugosus*).

Anatomy and Biology

Hermit crabs are not true crabs, as they lack a complete exoskeleton. Only the front portion of the animal's body is covered with an exoskeleton; the rearmost portion is soft tissue that is protected by a shell. Similar to other crabs, hermit crabs have five pairs of legs, one of which has a large pinching claw. The innermost fifth pair of legs is small and serves to help keep the crab inside its borrowed shell. Hermit crabs molt their exoskeletons periodically as they develop, and move to successively larger shells for protection of the soft body as they grow in size.

Hermit crabs are most active nocturnally. They are also social animals, preferring to be in groups of three or more rather than singly housed. They live for up to 30 years in their natural habitats and as long as 15 years in captivity.

Terrestrial Hermit Crabs

Housing and Husbandry

Both tropical and subtropical land hermit crabs are commonly kept in a glass aquarium with a ventilated lid and a substrate of sand, wood chips, or coconut fiber (Figure 4). The substrate should be deep enough that the crab can bury itself when it needs to molt.

Land crabs require water, and their habitat ("crabitat") should contain two nonmetallic water dishes, one with fresh, chlorine-free water and the other with saltwater. The water dishes should be deep enough for the crab to bathe but not so deep that it cannot get back out of the dish, and the water in each bowl should be changed daily. The habitat should be cleaned out weekly to remove waste and any food that the crabs may have hidden, and dishes and "toys" should be cleaned and rinsed well to completely remove any detergent residue.

Land hermit crabs are omnivores and scavengers, and may be fed fruits, vegetables, and meats as well as commercially available pelleted food. Fruits and vegetables should be washed thoroughly to remove any pesticide or detergent residue. Hermit crabs may also occasionally eat their own exoskeleton after shedding.

Figure 4 A typical classroom habitat for terrestrial hermit crabs. Note the wood bark substrate used for burrowing by the hermit crab.

Temperature and humidity levels are critical (Provenzano 1962). Land hermit crabs have modified gills that allow them to breathe air, but these gills must be kept moist. Humidity in the crabitat should be between 70% and 80%. It should never be allowed to drop below 70%, and above 90% it can cause unacceptable bacterial and fungal growth. A natural sponge in a dish of freshwater can help to maintain humidity. Temperature can be maintained with an undertank heater.

Hermit crabs like "toys" such as driftwood and stones; items made of metal should be avoided. Empty shells of varying sizes should be provided for the crabs to use after molting.

Health Challenges and Treatment

One of the primary problems with land hermit crabs is suboptimal husbandry. Environmental stressors such as incorrect temperature or humidity can cause a crab to discard its shell and go naked, rendering it vulnerable to injury and infection. Crabs may also lose limbs due to aggression from tank mates, traumatic injuries, or improper environmental conditions, but with proper husbandry the limbs generally regenerate when the crab molts.

Of particular concern is infestation by various species of mites. To prevent or remove them, the aquarium and substrate should be thoroughly cleaned and sterilized on a regular basis (e.g., once a week). Infestations of dust (*Dermatophagoides* sp.), grain (*Acarus* sp.), and house (*Liponyssoides* sp.) mites can be prevented by removing uneaten food daily and by cleaning the tank weekly. Substrate and pieces of wood may be baked in an oven to sterilize them, and a small vacuum should be used to remove all sand and grit from the corners of the habitat.

Infestation by fungus gnats (*Bradysia* spp.) can also be a problem for terrestrial hermit crabs. In addition to a thorough cleaning of the habitat and replacement of the substrate, fresh gnat traps made with a small jar containing apple cider vinegar or a similar solution can be added to the crabitat.

There are also predatory mites, *Hypoaspis* sp. (Cloyd 2010), that attack gnats and other mites and are effective for eradicating such infestations on hermit crabs.

Marine Hermit Crabs

Marine hermit crabs breathe through gills and require a saltwater aquarium (Bookhout 1964; Dawirs 1979; Harms 1992; Roberts 1971; Young and Hazlett 1978). The salinity of the water should be between 30 and 40 ppt, with a temperature of 22–28°C. The aquarium should be large enough that the crabs have space to roam, and there should also be rocks or corals for the crabs to climb. As with land hermit crabs, extra shells of various sizes should be provided to accommodate molting.

Aquatic species are omnivores and scavengers and will eat detritus, algae, shed exoskeletons, and dead organic matter. There are also several commercially available pelleted foods for marine hermit crabs.

Horseshoe Crab (*Limulus polyphemus*)

Horseshoe crabs have survived essentially unchanged for more than 200 million years, with fossil records of members of the family Limulidae dating back as far as 500 million years (Shuster et al. 2003; Walls et al. 2002). They are the closest living relatives of the ancient trilobites and are more closely related to modern-day scorpions and spiders than to true crabs. Today only four species of horseshoe crabs remain in various regions of the world. The "American" horseshoe crab, *Limulus polyphemus*, occupies the Atlantic coast of North America from Maine to the Yucatan peninsula (Shuster 1990). The other three species—*Tachypleus tridentatus*, *T. gigas*, and *Carcinoscorpius rotundicauda*—are found in coastal waters of Asia from India to Japan to the Philippine Islands (Shuster 1990). Because limited information is available about the culture of these latter species, the remainder of this section concerns the North American species of horseshoe crab.

Limulus polyphemus is a unique marine invertebrate with multiple commercial uses. Once an important resource for fertilizer and livestock feed, the horseshoe crab is now a bait in commercial whelk and eel fisheries; a common exhibit animal in public aquaria and classrooms to teach conservation and environmental issues; a laboratory research animal model to study the embryology, physiology, and function of marine invertebrates; and the primary source of *Limulus* amebocyte lysate, which is widely used to detect endotoxins on or in medical devices, implants, and vaccines (Berkson and Shuster 1999; Botton and Ropes 1987; Walls et al. 2002).

Anatomy and Biology

The body of the horseshoe crab is dorsoventrally flattened and divided into three sections: a frontal prosoma (cephalothorax) with an anterior flange; a hindbody opisthosoma (abdomen), and a posterior telson (tail). The dorsal surface of the prosoma has a pair of laterally located compound eyes, a centrally located ocellus, a raised anterior-to-posterior midline keel, and a hinge connecting the prosoma and opisthosoma. The opisthosoma protects the soft internal organs and book gills, and the animal uses the highly mobile telson to right itself. Ventrally the horseshoe crab has a single, anterior pair of modified chelicera, followed by five pairs of segmented legs, and then posteriorly the brachial appendages. The latter are attached to the underside of the opisthosoma and bear five pairs of book gills that contain the gill leaflets used for respiration, osmoregulation, and propulsion during swimming. Like most invertebrates, the horseshoe crab has an open circulatory system with hemolymph containing a copper-based hemocyanin for oxygen transport (Bolling et al. 1976).

When horseshoe crabs in the wild reach sexual maturity they mate during an annual spring migration to inshore spawning areas. Females may lay multiple clusters of 20,000 to 30,000 eggs on each spawning visit to the beach, after which multiple males pass over the clusters and release sperm to fertilize the eggs. Horseshoe crabs also readily spawn in the laboratory—females release eggs on the bottom of the tank and, as in the wild, males externally fertilize the eggs (Brown and Clapper 1981; French 1979; Gonzalez-Uribe et al. 1991).

The small, green, round eggs have a relatively opaque shell (chorion) and take approximately 1 month to hatch at room temperature. A day or two before hatching, the outer shell splits open, revealing a transparent membrane surrounding the developing larva. First-stage larvae resemble adult horseshoe crabs except for the lack of a telson, which shows up after the first molt. In the wild, juvenile horseshoe crabs molt several times the first year and thereafter once annually, approximately 16 to 18 times over the next 10 to 12 years as the animal eventually reaches sexual maturity. (In the laboratory, where nutrition can be optimized and water temperatures can be held constant or elevated above natural cyclic temperatures, juvenile horseshoe crabs may have a higher number of molts annually.) The lifespan of horseshoe crabs is not well documented, but it is estimated at 18 to 22 years (Shuster et al. 2003).

During the molting process, the horseshoe crab's external carapace splits along the cranial peripheral margin of the prosoma, allowing the soft-bodied crab to crawl out between the dorsal and ventral portions of the old outer shell. Until the new shell hardens, the horseshoe crab is susceptible to predation by horseshoe crabs and other marine species.

It is not possible to morphologically distinguish the sexes until the horseshoe crab reaches the final (terminal) molt. Sexually mature males are generally smaller than adult females and are readily identifiable by their first pair of legs, which are modified during the last molt into large bulbous claspers for grasping the female's opisthosoma during mating.

Husbandry

Horseshoe crabs have been maintained in captivity in a wide range of systems from small glass aquaria to large fiberglass

tanks depending on the size and number of animals (Figure 5; Smith 2006; Smith and Berkson 2005). As with all aquatic organisms held in captivity, adequate mechanical and biological filtration is essential for maintaining the appropriate environmental quality.

Mechanical filtration is provided by flosslike material, static sumps, sand filters, or other devices that filter out particulate matter, and biological filtration is provided by in-tank box filters, outside power filters, canister filters, external trickle columns, or suspended bead columns, all of which provide expanded surface area for bacterial growth, which in turn removes nitrogenous waste products. A sand or crushed coral substrate on the bottom of the tank or aquarium allows for the natural burrowing activity of the horseshoe crab and may even help during the animal's molting process. However, depending on the type of filtration, a substrate may or may not be appropriate as it increases the difficulty of cleaning the systems and may harbor harmful pathogens. Some systems may also have protein skimmers, ultraviolet (UV) filtration, and ozonation that reduce the presence or risk of potentially harmful bacteria and other compounds in the water column.

Horseshoe crabs are extremely tolerant of a wide range of environmental conditions, but it is nonetheless important to conduct adequate water testing to evaluate various water quality parameters on a regular basis (weekly or more often depending on the number of horseshoe crabs or other biomass being maintained). Horseshoe crabs have been reported to exist in natural waters ranging in temperatures from −5°C to 35°C, and they can tolerate salinities ranging from 5 ppt to 35 ppt, although larval stages do not survive well at low salinities (Nolan and Smith 2009; Smith 2006; Smith and Berkson 2005). In the laboratory, juvenile and adult horseshoe crabs have been maintained long-term with water temperatures between 15°C and 21°C and salinity around 27 ppt (Brown and Clapper 1981; French 1979; Gonzalez-Uribe et al. 1991; Laughlin 1982; Smith and Berkson 2005).

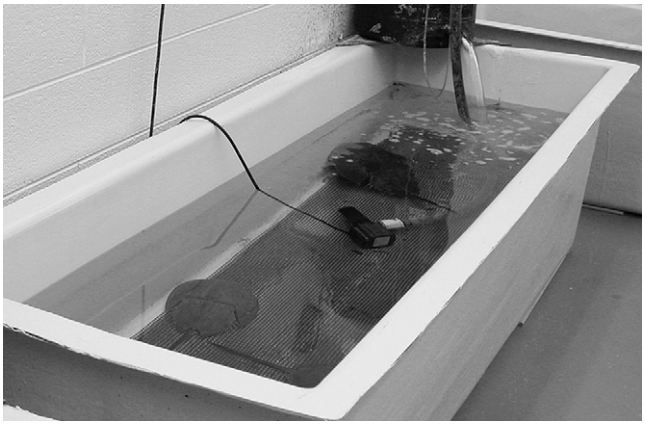

Figure 5 A simple recirculating aquaculture system for maintaining horseshoe crabs (*Limulus polyphemus*) in captivity. Note the filtration unit at the far end of the fiberglass holding tank, the submersible pump in the tank for circulating water through the filter, and the plastic screening on the bottom of the tank to assist the horseshoe crab in righting itself (originally published in Smith and Berkson 2005).

Both natural seawater and commercially prepared synthetic marine salts can be effective, but with a natural marine water source appropriate disinfection is necessary before use to reduce the risk of introducing any potential infectious or parasitic pathogens into the system. With recirculation systems, 25–30% of the marine water should be replaced every 3 to 4 weeks to reduce the amount of accumulated nitrates and to replenish any ions or minerals removed from the water by the crab's osmoregulatory processes (Nolan and Smith 2009; Smith 2006).

Aeration of the system is important for both the animal's respiration and the nitrification process of the holding system. Most aquaculture systems use water movement or supplemental air stones to ensure adequate aeration.

Horseshoe crabs in the wild feed on a variety of bivalve molluscs, marine snails, marine worms, and other benthic invertebrates. In captivity, larval horseshoe crabs can be fed newly hatched live brine shrimp, and juveniles and adults are commonly fed dead fish, squid, small crabs, clams, and frozen brine shrimp (Botton 1984; Smith and Berkson 2005). The horseshoe crab also readily consumes commercially prepared artificial shrimp and fish diets but their nutritional value for the horseshoe crab is not known (Smith 2006).

Health Challenges and Treatment

There are only a few descriptions of the diseases and syndromes that affect horseshoe crabs. Noninfectious challenges in captive horseshoe crabs include water quality problems (e.g., ammonia toxicity, pH extremes, gas supersaturation, and high turbidity), developmental problems (e.g., molting problems of the shell, legs, or telson), and traumatic injuries caused during collection, transport, or overcrowding during captivity (Nolan and Smith 2009; Smith 2006). Physical trauma can result in puncture wounds, crushing, and fractures of the exoskeleton. Hemorrhage from traumatic lesions often appears significant but is rarely fatal.

Infectious challenges include algae, fungus, colonial and filamentous cyanobacteria, Gram-negative bacteria, and a variety of parasites (Smith 2006; Nolan and Smith 2009). Lesions of the shell due to external pathogens are probably the most common problem seen in horseshoe crabs and are usually evident in discoloration or erosion of the carapace. Algal, fungal, and bacterial infections may colonize and penetrate the carapace, eyes, and gill surfaces and go on to become systemic, involving the deeper tissues of the organs, gills, and circulatory sinuses and resulting in extensive tissue necrosis and death. Specific bacteria species isolated from external lesions of the horseshoe crab include *Oscillatoria*, *Leucothrix*, *Vibrio*, *Flavobacterium*, *Pseudomonas*, and *Pasteurella*.

In addition, a variety of internal and external parasites have been reported, including protozoans, a larval digenetic

trematode, a few nematodes, and several turbellarid worms. Probably the most significant parasites are the turbellarid worms, which reside between the gill leaflets, on the ventral surface of the carapace, or on the appendages. These ectoparasites lay stalked cocoons on the external surfaces of the gill leaflets, causing superficial lesions on the surface of the gill tissue.

The most noticeable organisms are the ectocommensals—bryozoans, sponges, barnacles, blue mussels, lady slippers, snails, oysters, and whelks—that frequently occur on the external surfaces of the exoskeleton but seldom cause any harm to the horseshoe crab. Freshwater baths (3–12 min), acetic acid baths (3–5% for up to 1 hour), and formalin baths (1–1.5 ppt for up to 12 hours) have been used to remove external parasites and ectocommensals from the carapace of the horseshoe crab (Bullis 1994; Landy and Leibovitz 1983; Nolan and Smith 2009).

The pharmacokinetic profiles of oxytetracycline and itraconazole have been reported in the American horseshoe crab (for a full discussion of anesthesia and analgesia in invertebrates, see Cooper 2011, in this issue). A single oral or intravenous (i.v.) dose of oxytetracycline (25 mg/kg or 50 mg/kg) yielded pharmacokinetic data suggesting that i.v. would be the route of choice for continued maintenance of drug-serum concentrations (Nolan et al. 2007). Data from a study involving i.v. administration of a single dose of itraconazole at 10 mg/kg suggested that such a dose would be necessary every 24 hours to maintain an effective treatment level (Allender et al. 2008). Fluconazole, injected i.v. into the cardiac sinus at a dose of 2 mg/kg body weight every 4 days for 6 treatments, has been reported for the treatment of *Aspergillus niger* fungal infections (Tim Tristan, Texas State Aquarium, personal communication, November 22, 2010).

Unfortunately, most treatments for bacterial or fungal infections are generally ineffective, as the horseshoe crabs become lethargic and anorectic before eventually dying from the infection. Humane and rapid euthanasia is possible with the injection of 1 to 2 ml of pentobarbital solution directly into the dorsal cardiac sinus (Smith 2006).

Cephalopods

Cephalopods (the name originates from the Greek meaning "head foot") are an ancient group of animals that has been developing through the ages with many earlier species now extinct. They have significant commercial value both for human and animal consumption and for basic and biomedical research. Research has largely focused on neurobiology and behavior, with studies on sensory perception, angular acceleration, vision, central and peripheral nerve conduction, neurotransmitters, and cells associated with the skin function.[5] Cephalopods have been successfully maintained and cultured for over 30 years at the National Resource Center for Cephalopods (NRCC), a laboratory supported by the National Institutes of Health's National Center for Research Resources. The squid (*Sepioteuthis lessoniana*) has been cultured through seven successive generations (Walsh et al. 2002); the pharaoh cuttlefish (*Sepia pharaonis*) through five consecutive generations in the laboratory (Minton et al. 2001) using closed, recirculating water filtration systems; and *S. officinalis* through seven generations in a recirculating marine system (Forsythe et al. 1994). However, as explained under Husbandry below, cephalopods are not the easiest of animals to maintain and care for unless the user is both knowledgeable about species-specific aquatic environment needs and experienced with water quality parameters essential for marine invertebrates.

Scientists believe ancestors of modern cephalopods (subclass Coleoidea) diverged from the primitive externally shelled Nautiloidea very early, perhaps in the Ordovician period some 438 million years ago, before the existence of mammals and fish on this planet. Cephalopods were once one of the dominant life forms in the world's oceans, but today there are only about 800 living species.[6] The class Cephalopoda (phylum Mollusca) has two very distinct subclasses: Coleoidea (octopuses, squid, and cuttlefish) and Nautiloidea (nautilus). This section focuses on octopuses, squid, and cuttlefish (nautilus can be raised in captivity but the author [JMS] is most familiar with the Coleoidea).

Anatomy and Biology

Cephalopods have some unique anatomical features and should be considered highly specialized animals that have successfully evolved through the ages. Cuttlefish, octopuses, and squids are semelparous, meaning they grow rapidly to sexual maturity, spawn once, and die (Hanlon 1987). The animals' lifespan in laboratories, depending on temperature and nutrition, is usually 1 year (the nautilus, in contrast, may live up to 20 years; Scimeca 2006). Cephalopods (excluding the nautilus) are primarily top predators and very active in hunting their prey. They are not good community tank inhabitants and the various species are best housed separately.

Most cephalopods have only a shell remnant that is either greatly modified or completely absent. In the subclass Coleoidea, the shell is internal and reduced in size. In the order Sepioidea (the cuttlefishes and bottle-tailed squids), the calcareous chambered shell is present internally, functioning as a buoyancy organ. Representatives of this subclass include *Sepia* and *Euprymna*; *S. officinalis* is the common or European cuttlefish. In the order Teuthoidea (the shallow-water and oceanic squids), the shell is reduced to a chitinous "pen" (or gladus) that lies dorsally in the body. The body is elongated and usually finned, with eight suckered arms and two long tentacles; the Atlantic brief squid (*Lolliguncula brevis*) belongs to this group. The order Octopoda

[5]Information Resources on Amphibians, Fish and Reptiles Used in Biomedical Research; available online (www.nal.usda.gov/awic/pubs/amphib.htm).

[6]Information from the Cephalopod Page (www.thecephalopodpage.org).

(the octopuses) have a shell that is markedly reduced and split into two lateral rods, and a globular body with or without fins; the California two-spot octopus (*Octopus bimaculoides*) is an example of this group and the most popular species among aquarists that keep octopuses.

Organisms in the other cephalopod subclass, Nautiloidea, have a heavy, external, chambered shell that may be straight or coiled; nephridia; two pairs of gills; and numerous non-suckered appendages. There are six living species worldwide in two genera, the most commonly known of which is *Nautilus pompilius*, the chambered nautilus.

The integumentary system is composed of a delicate 1-cell-layer-thick epidermis consisting of columnar epithelial cells with a microvillous border and a deeper dermis containing chromatophores, iridophores, and leucophores (Hanlon and Messenger 1996). Because of the fine nature of the skin, utmost care and careful handling of cephalopods are mandatory to prevent tears or other trauma to the skin membrane.

The respiratory system of cephalopods consists of well-vascularized gills suspended in the mantle cavity. The water flows over the gill lamellae in the opposite direction of blood flow, producing a countercurrent system that maximizes the exchange of gases (Wells and Wells 1982). Cephalopods are the only molluscs with a closed circulatory system, comprising a complex arrangement of vessels and cardiovascular tissues, a single systemic heart, two branchial hearts, vena cavae, and auricles, all of which have a contractile function for movement of the hemolymph. The blue-colored hemolymph is similar to that of the horseshoe crab and other invertebrates in that the oxygen-carrying pigment is the copper-based hemocyanin.

Cephalopods have a large and well-developed nervous system that is the most evolved of all invertebrates. Octopuses, cuttlefish, and squids are often considered the most "intelligent" invertebrate species in part because of their mating and social behaviors and their social recognition and display patterns (Boal 2006; Hanlon and Messenger 1996).

Husbandry

Creating a laboratory environment for cephalopods that promotes species-typical behavior, reproduction, and optimal health conditions is challenging. There is a wide variety of seawater systems suitable for maintaining these animals in captivity and there is considerable variation in their design to accommodate laboratory location and species (Figure 6). Tanks and holding systems are highly variable and adaptable; both open and closed seawater systems are available. Piping should be of PVC, not copper, which is toxic to all cephalopods. With any type of support system, close monitoring is necessary to maintain optimal water quality because animal densities in captivity are often higher than those in the wild.

There is a wide range of suitable housing composition and substrates depending on the proposed use (e.g., culture or display) (Forsythe and Hanlon 1980; Forsythe et al. 1991; Hanlon and Forsythe 1985; Yang et al. 1989), and several companies custom design and produce tanks to suit different needs. Fiberglass composite works well and tanks can be fitted with viewing windows (acrylic is strong but scratches fairly easily). Large animal water troughs have also been modified for use with cephalopods. Tanks with exposed metal should be avoided.

Figure 6 Numerous octopuses (*Octopus bimaculoides*) in a fiberglass tank with short lengths of PVC piping for housing and enrichment. Note the live shrimp (upper left) provided for food.

Creating an environment suitable for cephalopods is essential to successful culture. The temperature range for temperate species of cephalopods is 15–22°C and for tropical species 25–32°C. In general, salinity should be 27–36 ppt, pH 7.7–8.2, ammonia and nitrites <0.1 mg/l, and nitrates <20.0 mg/l (Hanlon 1987; Lee et al. 1994a; Oestmann et al. 1997; Sherrill et al. 2000). Water outside these parameters will likely cause cephalopods to become stressed and result in greater susceptibility to disease. Therefore, routine monitoring of water quality cannot be overemphasized.

Water should be conditioned and adjusted for the correct salinity, and, in the case of open water systems, filtered and contaminants removed. Care should be taken when using potable municipal water, which may be treated with potentially toxic chlorines and chloramines. Evaluating makeup water is essential for the culture of cephalopods and sending water out for analysis for metals, organics, hardness, and possible toxins is well worth the time and effort. Such analysis enables more precise adjustment of the animal's holding water and thus results in a suitable environment that both supports the animal's health and minimizes variability in research outcomes.

After analysis of the makeup water, water filtration should occur as follows: from the holding tank water passes through (1) a protein skimmer (or foam fractionator) that strips dissolved organic compounds (this equipment is essential as cephalopods produce and often expel abundant ink when alarmed or startled); (2) a mechanical filter that removes particles down to 100 µm; (3) a high-grade activated carbon and a biological filter, where ammonia is eventually broken down to nitrites and nitrates; and (4) a UV sterilization unit before the water is returned to the animal holding

tank. Experience in correct sizing of the biological filter will help as some cephalopods produce large amounts of ammonia that need to be reduced to the relatively nontoxic nitrates. Systems with anaerobic filters to remove nitrates reduce the amount of water that needs to be exchanged on a regular basis. Water conditioning and treatment equipment may well take up more space than the holding tanks themselves.

All cephalopods actively catch and eat a variety of live prey items (Boletzky and Hanlon 1983; Castro and Lee 1994; Lee 1994; Nixon 1987). Squids and cuttlefish maneuver so they can strike their prey with extension of their tentacles and rapid body propulsion. Once trapped in the tentacles, the prey is drawn toward the mouth and bitten by the chitinous beak or "mandibles" and swallowed. Cephalopods, depending on their growth stage, can be fed live mysid shrimp, grass shrimp, or fish fry of a nonoily fish species. Experience in the laboratory has shown that live or fresh frozen prey produces better growth and survival than artificial diets, although careful evaluation of live or fresh frozen food is important as it can sometimes transmit unwanted pathogens.

Health Challenges and Treatment

Most health problems in cephalopods result from either poor water quality or traumatic injuries. Wild-caught animals are particularly prone to physical and mechanical damage. As mentioned above, the epidermal microvillous skin layer is easily injured, and cuts and abrasions of the skin from nets and handling are a frequent cause of morbidity and eventual mortality. Captive cuttlefish can jet across the tank and damage the skin, eventually leading to septicemia and death (Scimeca and Oestmann 1995). Mantle lesions are typically preceded by abnormal swimming behavior and commonly lead to secondary bacterial infections that affect multiple organ systems and rapidly lead to death (Sherrill et al. 2000). Furthermore, if the force of the animals' propulsion against the side of the tank is great, as is often the case with cuttlefish, the result may be fractures of the internal cuttlebone.

Bacterial infections and septicemias in cephalopods are commonly reported to be caused by various species of the bacterium *Vibrio* (Hanlon and Forsythe 1990; Scimeca 2006). Common infections secondary to abraded or traumatic skin lesions have been summarized in different cephalopod species (Scimeca 2006).

Cannibalism is well documented in captive octopuses, cuttlefish, and squids (Budelmann 2010). Stocking densities and limited food supplies have been suggested as possible etiologies for this problem. Autophagy has been reported in *Octopus vulgaris* and there is some evidence that it may be due to a released substance or possibly a virus or bacteria (Budelmann 2010).

Various anesthetic compounds are effective for cephalods; as with other animals, the age, sex, size, and species should be considered closely before performing anesthesia. Magnesium chloride ($MgCl_2$) is the anesthetic agent of choice for cephalopods because it is easy to obtain, inexpensive, stable, nontoxic, and easy to prepare (Messenger et al. 1985). A standard solution of 75 g of $MgCl_2$ dissolved in 1 L of dH_2O and mixed with 1 L of seawater has worked well (Scimeca and Forsythe 1999). This one to one (1:1) mixture can be used for surgical anesthesia and some invasive clinical procedures, a 1:3 or 1:4 dilution is adequate for handling and examinations, and a 1:10 dilution induces mild sedation for stress reduction and shipping.

Preliminary evaluation of $MgCl_2$ in five different genera of cephalopods showed effective anesthesia in all individuals (Scimeca 2006). The site of action for $MgCl_2$ is thought to be the central nervous system because stimulation of the fin nerve in anesthetized cuttlefish elicits a motor response. Thus, it is believed that $MgCl_2$ works at the postsynaptic membrane of the nerve-muscle junction in crustaceans and vertebrates (Scimeca 2006).

The $MgCl_2$ preparation can also produce a surgical plane of anesthesia in octopuses with the addition of an ethanol "push" consisting of a 1% solution (by volume) of ethanol (e.g., 10 ml of ethanol is added to 1 L of the 1:1 seawater/$MgCl_2$ mixture). And researchers have described the use of ethanol for anesthetic induction and maintenance during an excisional biopsy and surgical repair of a mycotic skin lesion on a European cuttlefish (Harms et al. 2006). The authors reported an induction time of less than 1 minute with 3% ethanol (30 ml/l) and successful anesthetic maintenance at 1.5% ethanol in seawater.

Benzocaine has been used in the giant Pacific octopus for effective anesthesia at concentrations above 1,000 mg/l and also as a compound for euthanasia. This method of euthanasia was reported to be a relatively rapid and humane method at 3,500 mg/l compared to other euthanasia practices (Barord and Christie 2007).

Treatment with antibiotics such as enrofloxicin (Baytril), gentocin, nitrofuran, and metronidazole has been reported (for a review of suggested dosages and routes of administration, Scimeca 2006). Because cephalopod skin is just 1 cell layer thick and the animals have a large surface area, care in the dosage and time of exposure for water baths is essential—it is easy to overdose these animals with water bath antibiotics. An alternative technique used with cuttlefish involves injecting live shrimp with 10 mg/kg enrofloxicin and immediately feeding this medicated food to the cuttlefish. If the cuttlefish are not eating, i.v. injection using a 25 g or smaller needle of 5 mg/kg enrofloxicin into the muscular portion of the cephalic vein every 8 to 12 hours is also effective (Gore et al. 2005).

As is true for all species of laboratory animals, training of staff and research personnel is essential for successfully maintaining cephalopods in the research laboratory. Appropriate care and husbandry are imperative, as is clinical observation, especially given the semelparous life cycle challenges in the management of cephalopods' 12- to 14-month lifespan (Sherrill et al. 2000). Experience in the necropsy and pathology of cephalopods is also a key component to success when problems arise related to colony health.

Aquarium personnel, research investigators, and pathologists need to work together in seeking answers related to health problems or deaths of unknown etiology.

Conclusions

Invertebrates have been used for many years in various research, teaching, and public display settings. As with other laboratory animals, the proper culturing and care of invertebrates in captivity entails specific housing, environmental, nutritional, and management requirements. A thorough investigation of these requirements is always advisable together with implementation of the specific recommendations to ensure the humane care and welfare of these organisms.

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