Culturing echinoderm larvae through metamorphosis

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CHAPTER OUTLINE

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Abstract

Echinoderms are favored study organisms not only in cell and developmental biology, but also physiology, larval biology, benthic ecology, population biology and paleontology, among other fields. However, many echinoderm embryology labs are not well-equipped to continue to rear the post-embryonic stages that result. This is unfortunate, as such labs are thus unable to address many intriguing biological phenomena, related to their own cell and developmental biology studies, that emerge during larval and juvenile stages. To facilitate broader studies of post-embryonic echinoderms, we provide here our collective experience rearing these organisms, with suggestions to try and pitfalls to avoid. Furthermore, we present information on rearing larvae from small laboratory to large aquaculture scales. Finally, we review taxon-specific approaches to larval rearing through metamorphosis in each of the four most commonly-studied echinoderm classes—asteroids, echinoids, holothuroids and ophiuroids.

1 WHY CULTURE ECHINODERM LARVAE?

Most of the chapters in these volumes are focused on applying cell and molecular biology techniques to echinoderms. In the past, the vast majority of such studies have involved experiments with embryos, due to the ease of obtaining large numbers of synchronously developing, optically clear zygotes from a diversity of species. Nevertheless, when developmental studies of echinoderms cease during late embryonic development, they leave out an enormous amount of interesting biology: the development of diverse functioning organ systems (e.g., digestive, nervous, excretory); the emergence of larval swimming and feeding behaviors, phenotypic

plasticity, cloning, and regeneration; extensive changes in development that accompany independent evolutionary losses of larval feeding in diverse lineages; a profound shift in symmetry from bilateral to pentameral; determination of the key life-history transition of where and when to settle at the end of larval development; and the dramatic habitat shift from planktonic life to the sea floor (Chia & Burke, 1978; Elphick & Melarange, 2001; Hodin, 2006; Lawrence, 2013; chapter "The painted sea urchin, *Lytechinus pictus*, as a genetically-enabled developmental model" by Hodin et al.; Strathmann, 1971, 1978; chapter "Methods to label, isolate, and image sea urchin small micromeres, the primordial germ cells (PGCs)" by Campanale et al.; Wray, 1996; chapters "Temnopleurus as an emerging echinoderm model" by Yaguchi et al.; "Microinjection methods for sea urchin eggs and blastomeres" by Yaguchi et al.). Indeed, early developmental processes are interesting in part because they affect the morphology of both larvae and adults; as such, studying post-embryonic stages is necessary for a full appreciation of the embryonic processes themselves. Furthermore, as other authors in these volumes have argued, rearing larvae and juveniles is critical to our ability to implement some modern methods in genetics, as well as aquaculture.

Among the features that make echinoderms excellent study organisms are their remarkable diversity in adult morphology and life history, in the context of well-resolved evolutionary relationships and a robust fossil record stretching back into the early Cambrian (Smith, 1997 for review). As we describe in the sections below, one can rear large numbers of larvae at low cost through metamorphosis, consistently and in many cases synchronously, by following a few basic procedures. We hope that this chapter will pull back the curtain on echinoderm larval rearing, revealing it as an undertaking that any motivated researcher can master. Our aim is to encourage laboratories to take full advantage of the complex life histories of the organisms that they study to address the widest array of questions relevant to their particular research interests.

There are nearly as many techniques for successfully culturing larvae as there are biologists working with them, and protocols always need to be adapted to suit the available species, space and materials. Indeed we co-authors have gleaned many useful tips from one another while assembling this chapter. Therefore, we will not provide overly specific step-by-step instructions herein, but instead offer our collective experience with guidelines to follow and pitfalls to avoid in the hopes of promoting more widespread studies of the larvae and early juveniles that all too often end up down the embryologist's sink drain.

2 GENERAL CULTURING METHODS

We here outline methods that, in our experience, apply to echinoderm larvae in general. We discuss taxon-specific methods in subsequent sections.

2.1 TOP FIVE THINGS TO KEEP IN MIND FOR LARVAL CULTURING (IN ORDER OF ONTOGENY)

(1) Use clean, "embryo safe" containers and high-quality seawater

Larvae need to be cultured in containers, which either:

(a) are new;
(b) have never been used with any detergent, toxic chemicals, heavy metals, etc.; or
(c) have been cleaned in such a way as to completely remove any such compounds (see below).

We mark such containers clearly to indicate that they are safe for embryos or larvae, and are distinguished from other laboratory containers. Containers can be glass (preferred) or plastic. Note that plastic containers—particularly when new or after repeated use—could leach compounds into cultures that may impact larval development.

To clean containers between uses, wash thoroughly with tap water and never use any detergents. A brush or scrubbing pad can be used to remove adherent materials or other debris. Then, rinse with distilled, deionized or reverse-osmosis filtered water (all henceforth referred to here as "dH$_2$O").

Note that new glass or plastic containers should be washed in the manner described above before first use: the manufacturing process can leave a residue. We recommend smelling a new container before use; it should be free of any powerful odor. Likewise, some types of laboratory plasticware, when stored, will off-gas plastic compounds. We thus recommend storing containers without lids when possible, and rinsing thoroughly after extended storage.

Glassware that has either been used with potentially harmful compounds (see "b" above) or is of unknown origin can be made safe for use by acid washing with 1 N HCl for 10 min, followed by thorough rinsing in tap water, then several rinses in dH$_2$O. The acid can be saved and used numerous times before being disposed as hazardous waste. Plasticware should never be acid washed since that can compromise the surface of the plastic and thus lead to compounds being increasingly leached into subsequent embryonic and larval cultures.

In wet lab settings with access to flowing seawater (SW), another method for cleaning new or mildly compromised glassware or plasticware is to soak the containers in running SW for 1–2 weeks; a brush may be necessary to remove the biofilm that grows on the inside of the containers. Containers exposed to toxic compounds should not be rinsed in this way to avoid discharging harmful substances into the ocean. If a laboratory plans to expose larval cultures on a regular basis to certain compounds that would otherwise make those containers unsuitable for standard cultures, they can be marked and maintained as such.

We recommend culturing larvae in high-quality natural SW deriving from offshore to minimize effects of pollution and fluctuating salinity from terrestrial sources. Some degree of filtration is needed, in particular to remove potential predators. Minimally, this can involve the use of bag filters or unbleached coffee filters. Filtration through a 5-µm mesh will further remove most phytoplankton cells, which may be preferred for controlling food levels in a consistent manner, and any toxic algae (but not their dissolved products, which may affect embryos or larvae). If the researcher wishes to retain the natural bacterial biota, the 5-µm mesh is a good choice. However, many

researchers may wish to remove bacteria to reduce the likelihood of bacterial overgrowth in closed cultures, in which case we recommend filtration through a 0.45- or 0.2-μm mesh filter. See Strathmann (2014) for details.

If natural SW is unavailable, commercially available artificial seawater (ASW) yields good results when dH_2O is used to make up the ASW (see Strathmann, 2014 for protocols). We recommend ASW formulations that include a diversity of trace elements; we have observed that simple ASW recipes such as the Marine Biological Laboratory (MBL) standard ASW (Cavanaugh, 1975) do not support healthy long-term growth in echinoderms. Once the salts have gone into solution, we recommend a 0.2–0.45 μm filtration step, followed by salinity adjustment using dH_2O. It is also critical to allow freshly made ASW to off-gas CO_2 (achieved by simply leaving it in open or loosely-capped containers) for at least 12 h before use in cultures.

(2) Rear larvae at low density

Although it is tempting to culture larvae at high density (more potential study material in a smaller total volume) we strongly advise against succumbing to this temptation. Many studies have noted that larvae develop faster and more consistently when cultured at lower density (e.g., Suckling, Terrey, & Davies, 2018), particularly during late stages. Larvae in high-density, closed system cultures suffer from more rapid accumulation of waste products leading to poor water quality, frequent direct interactions with other larvae, and, for those larvae that require food to complete metamorphosis (i.e., *planktotrophic development*), less food per larva. Dying larvae in crowded conditions can create a positive feedback loop of larval death. High densities (sometimes extremely high, in excess of 10 larvae per mL) are of particular concern in studies where a stressor—such as high temperature or low pH—is applied. In such cases, the effects of the stressor may not be distinguishable from possible interaction effects with crowding. Natural zooplankton densities in the sea range from ~1 individual per 10 L to ~1 individual per 5 mL (Kacenas & Podolsky, 2018; Roman, Holliday, & Sanford, 2001). Although the lowest densities here are impractical for laboratory culture, attempts to at least approach the natural range seem prudent.

In species in which pre-hatching stages are negatively buoyant and sink to the bottom, initial culture density should be kept low enough that pre-hatching stages form a layer no more than one embryo thick to allow sufficient oxygen diffusion to all embryos and to promote synchronous development. Alternatively, cultures can be gently stirred to keep embryos suspended.

Once embryos have hatched, decant embryos swimming near the surface into a clean container, then stir and collect a few replicated aliquots to estimate the number present. Transfer embryos into natural filtered seawater (FSW) or ASW, aiming for a density of ≤1 embryo per mL for early stages, once feeding has been initiated (for those larvae requiring food). Lower densities during early larval stages may lead to more rapid and synchronous growth and better survival as larvae feed and begin excreting waste products.

At later stages, the density of larvae in culture is ideally 1 larva per 4 mL or lower. Unusually large larvae [e.g., those of diadematoid echinoids with extremely long larval arms (see Fig. 8G), some ophiuroids, giant seastar larvae in the genus *Luidia*] should be cultured at lower density still: no more than 1 larva per 10 mL. Our anecdotal observations in a diversity of echinoids indicate that rapid reductions in density can induce larval cloning (c.f., McDonald & Vaughn, 2010), which makes cultures less synchronous. To avoid this, we recommend gradually reducing culture density over the course of several days. It is difficult to propose a common benchmark for when, during larval development, to undertake this density reduction. As a rule of thumb, we have found that lowering the density about 1/3–1/2 of the way through larval development seems to yield good results.

Embryos and larvae of species that do not require food to complete metamorphosis (i.e., *lecithotrophic development*) should be cultured at low density from the outset. Such embryos and larvae are often positively buoyant, accumulating at the water surface, and crowded cultures can lead to increased mortality, with subsequent release of yolk and rapid fouling. In open culturing systems, with constant flushing of waste products (see below), the need for low density culture of feeding and nonfeeding larvae may not be as critical.

(3) Stir or mix cultures gently and constantly

Mixing of cultures is necessary for optimal larval growth. Mixing oxygenates cultures helps to maintain consistent temperatures within cultures, and keeps food and larvae evenly distributed in the containers. We present the specifics of mixing and stirring systems in the sections below regarding small, medium and large-scale culturing.

If larvae are reared in replicate experimental vessels, after every water change the position of all vessels in the culture area should be rotated. This allows larvae in the various treatments to experience all possible conditions in the sea table or environmental chamber and helps reduce among-container variation (i.e., "beaker effects") during development.

(4) High quality food in, high quality larvae out

Food sources for cultures of planktotrophic larvae vary, but ideally a mixture of live algal species is provided (Castilla-Gavilan et al., 2018; Ren, Liu, Dong, & Pearce, 2016; Scholtz, Bolton, & Macey, 2013; Strathmann, 1987a, 2014). A commonly used mix for tropical and temperate echinoderm larvae includes all three or pairs of the following algae: *Rhodomonas lens*, *Dunaliella tertiolecta* and *Isochrysis galbana*. A blend of *Chaetoceros*, *Chlorella*, and *Pyranimonas* works well with temperate echinoderm larvae (Zhang, Song, Hamel, & Mercier, 2015). *Proteomonas sulcata* has been used successfully with tropical echinoderm larvae (Wolfe, Graba-Landry, Dworjanyn, & Byrne, 2017). Each algal species can also be used individually, but slower or less synchronous larval development may result.

The appropriate concentration of food to add to cultures and the frequency of replenishing food depends on a variety of factors. The goal should be to prevent larvae from depleting their algal food supplies between water and food changes

(usually every 1–2 days; see above); larvae should always have visible algal cells in their guts. Most echinoderm larvae cultured at low densities (≤1 per mL; see above) grow rapidly at concentrations of 5000–10,000 algal cells per mL (with algal cells of ~10 µm in diameter), with food replenished every 2–3 days. However, if larval size or density in cultures is high, or food is changed infrequently, larvae should be provided higher concentrations of food. Likewise, if algae with diameters significantly smaller than 10 µm are used, the amount of food should be scaled up proportionately to the volume of the individual algal cells.

To feed larval cultures, briefly and lightly centrifuge algal cultures, discard the supernatant algal growth medium, and resuspend algae in FSW or ASW. Care should be taken to avoid forming too hard a pellet during centrifugation as this will kill algae; dead algae form a recognizable plume upon resuspension. A few trials will yield an appropriate centrifugation time and speed for concentrating algae using the available equipment. Estimate algal concentration in this stock solution with a hemacytometer or flow cytometer, then add the appropriate volume to each culture vessel to achieve the desired initial concentration. See Strathmann (2014) for further details. Note that a centrifugation step is not technically required, but we recommend it in order to limit the introduction of the algal growth medium into larval cultures.

Algae cultures, free of pathogens and contaminants, are imperative to the successful rearing of planktotrophic larvae. There should be regular routine screening and cleaning of stock algae cultures. As needed, new algae cultures can be obtained from a reputable source, (e.g., in the USA the UTEX Culture Collection of Algae and the National Center for Marine Algae and Microbiota, Aqualgae in Europe, and the Australian National Algae Supply Service). F/2—a medium in which SW is enriched with nutrients, trace elements and vitamins to support algal growth—is an appropriate culture medium for most microalgae (diatoms may require additional compounds, notably silica). FSW or ASW should first be sterilized either by autoclaving or microwaving, and then cooled to room temperature before adding the concentrated components of the f/2 medium (pre-mixed concentrate is available commercially, or can be made from published recipes, e.g., Guillard, 1975), and then inoculating the new medium with algal cells. Cultures typically grow well at room temperature, either under 12:12 grow lights or in indirect light near windows. Stock algae cultures of many species can be kept as cool as 10 °C (with grow lights) for long-term maintenance. Note that natural plankton can also be used as a food source if algal cultures are unavailable or if more natural feeding regimes are desired. See Strathmann (2014) for more details.

While exposure to light is essential for healthy algal cultures, it is not clear that larvae need or benefit from lighting, though this has not been well-studied. Successful larval development has been reported with a variety of light conditions, including ambient light, timed full spectrum fluorescent lights and complete darkness. Milonas, Pernet, and Bingham (2010) report some surprising differences in growth trajectory in echinoid larvae raised in 12:12 light regimes versus those in complete darkness. Montgomery, Hamel, and Mercier (2018) highlighted the role of light intensity and wavelength on larval swimming behavior.

(5) Clean cultures gently

Water changes may be accomplished by reverse filtering the water from cultures using a mesh size smaller than the size of the larvae. In very small cultures (a few hundred milliliters per container or less), equipment for reverse filtration can consist of a turkey baster and a filter basket (e.g., a plastic beaker with the bottom cut out and replaced with a Nitex mesh screen). In larger cultures (≥0.5 L), we recommend a reverse filtration apparatus like the one shown in Fig. 1A: a 50-mL conical tube with a small hole cut in the bottom, and a Nitex mesh screen affixed to the opening. A line of tubing is inserted snugly in the hole in the bottom and into the conical tube (see also Leahy, 1986). The smaller the inner diameter of the tubing, the slower and gentler will be the reverse filtering process. With this method, upward of 95% of the culture water can be exchanged at each water change (see Fig. 1B). The larvae can then be poured into a smaller vessel for examination in a binocular microscope, or selection of a subset for fixation, biochemical analysis, etc.

Forward filtering of larvae, where the entire volume of the cultures is passed through a Nitex filter basket, is often used in medium and large scale culturing systems. We have also successfully used forward filtration in small scale culture of certain robust larvae, as detailed below.

Frequency of water changes is dependent on a number of factors, including larval density, rearing temperature and developmental mode (planktotrophy versus lecithotrophy). Detailed suggestions for water change frequency can be found in the sections below on small, medium and large scale culturing methods, as well as in the sections on taxon specific approaches that follow.

When larvae are concentrated during water changes, the researcher may wish to select a few larvae for more detailed microscopic examination. To visualize live, ciliated larvae in a compound microscope, the larvae need to be immobilized but not crushed. Our favored method to accomplish this is to use modeling clay to raise the cover glass slightly above the surface of the slide.

First, one or more larvae are transferred along with a small drop of SW onto a slide. Next, the four corners of a cover glass are scraped gently through a soft piece of modeling clay (dry or brittle clay will crack the cover glass during this operation) so that very small amounts of the clay are visible in each of the four corners. This raised cover glass is then lowered gently onto the sample. If the space under the cover glass is too tall, the larvae will continue to swim. In that case, the cover glass can be carefully pressed down until the larvae are trapped but not crushed. After observations, the cover glass can be lifted and the larvae returned to their culture vessels if desired; Heyland and Hodin (2014) have shown that such "post-observation" larvae subsequently develop normally, and at the same rate as their unmanipulated siblings. For other microscopy methods for examining larvae, see Strathmann (1987a, 2014).

2.2 SMALL-SCALE CULTURING METHODS

Hundreds to thousands of echinoderm larvae can be cultured in FSW or ASW in 2–4 L containers. Even smaller scale cultures (e.g., 250 mL to 1 L) are often desirable when greater numbers of replicates or treatments are needed, though the smaller
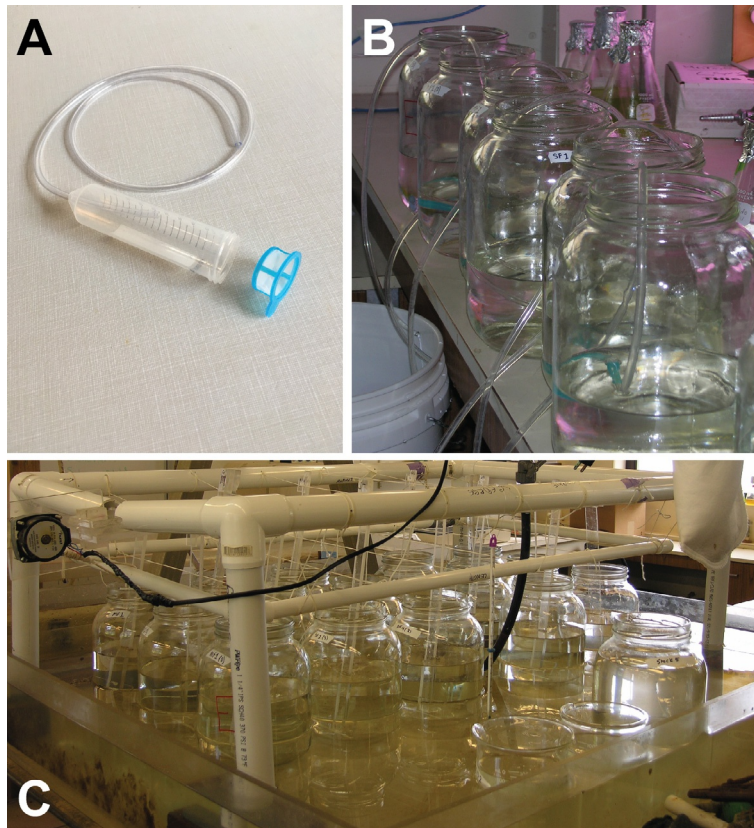
FIG. 1

Small scale culturing methods. (A) Simple and effective reverse filtration apparatus. A small hole is cut in the bottom of a 50-mL centrifuge tube, and plastic air tubing is inserted in the hole. The opening of the tube is covered with an appropriate-sized Nitex mesh. Here a commercially-available Nitex filter basket is shown which snaps in place in the 50-mL tube opening; note that not all 50-mL tubes fit these caps snugly. (B) Simultaneous reverse filtration of multiple gallon jars with reverse filtration apparatuses akin to those shown in (A). (C) Mechanical stirring rack in place with gallon jars and paddles.

Photos by P. Kitaeff (A) and S. George (B and C).

volumes mean that fewer larvae can be maintained per replicate container. Many species can be kept at room temperature (20–22 °C), but in other cases larvae require cooler or warmer temperatures. In locations where flowing SW is available at the ambient local ocean temperatures, standing culture containers in aquaria works well. Alternate approaches include standing cultures in chilling or heating water baths, or using constant temperature rooms or incubators for cultures.

Stirring in small-scale culture can be accomplished by swinging paddles (Fig. 1C), airlift-droplet stirrers, small rotating paddles (Strathmann, 1987a), or other forms of agitation, such as shaking incubators or gyratory shaker platforms

(Aronowicz, 2007). In small-scale closed cultures, we recommend that water changes generally be done approximately every other day, though daily water changes may be required if higher than ideal densities are needed. Daily water changes may also be desired in warmer temperature closed cultures, where evaporation and harmful bacterial and protist growth are a greater concern.

The following *reverse filtration* water change protocol greatly reduces the level of unwanted particles (e.g., decaying algal food) at each water change while minimizing loss of larvae. Such unwanted particles at water changes can interfere with larval feeding (Lizárraga, Danihel, & Pernet, 2017) and could be a source for bacterial and protist growth. In this protocol, the remaining water in a culture vessel after reverse filtration is first poured into one bowl. The empty culture vessel is rinsed (e.g., with a squirt bottle) thoroughly with FSW or ASW and those rinses are poured into a second bowl; the reverse filtration apparatus and screen is also rinsed into this second bowl with FSW or ASW. This second bowl will now contain the vast majority of unwanted particles and a handful of larvae, the latter of which can be transferred manually into the first bowl, and the remaining contents of the second bowl discarded. The first bowl can now be further cleaned, if desired, by manual removal of remaining unwanted particles, and then the larvae returned to their cleaned and refilled culture vessel.

An alternative protocol is *forward filtration*, which has been used successfully in small-scale culture of echinoid, asteroid, and ophiuroid larvae (Fig. 2; Leahy, 1986). This technique uses a filter basket made from a beaker with the bottom cut off and replaced with an appropriately-sized Nitex mesh (i.e., significantly smaller in mesh size than the larvae). The filter basket stands in a bowl that is wider and shorter than the sides of the basket. The contents of the culture are gently poured into the basket; the water and waste products are then flushed through the bottom of the basket and into and over the sides of the bowl; the larvae remain in suspension within the basket. The culture vessel is washed, refilled, food is added, and the larvae are returned to the culture vessel. This forward filtering protocol is rapid, and when done carefully (including daily if needed), results in few larvae being lost. It is also an effective method to lower the density of ciliates or other protists. Note that especially fragile larvae (e.g., ophioplutei with slender arms, such as *Ophiothrix* spp., and diadematoid plutei) may not be amenable to this forward filtering technique.

An important distinction is whether the embryos and larvae are positively or negatively buoyant. For species with negatively or neutrally buoyant larvae the shaking or mixing methods described above can be applied; modifications are needed for species with positively buoyant larvae (Aronowicz, 2007). For example, yolky embryos and larvae that do not require algal food can be kept in plastic tea infusers where oxygen can enter through the mesh if kept in a larger aquarium or flow-through SW system. Alternatively, embryos and larvae can be enclosed in roller bottle culture systems filled to the brim, and thus without air volume. The larvae will be protected from capture or rupture in the surface tension and from damage by paddles. Longer culture periods between water changes using the roller bottle system have been successfully employed for the planktotrophic sea urchin *Lytechinus variegatus*
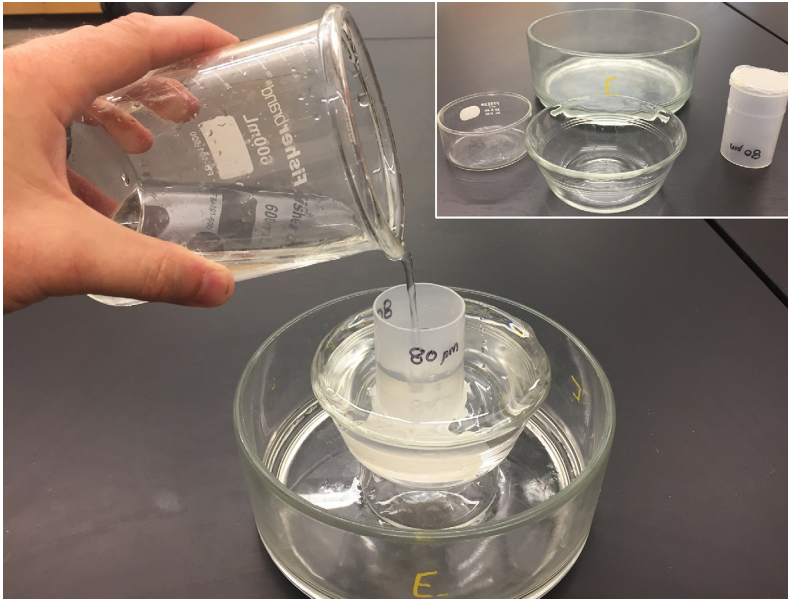
FIG. 2

Forward filtration set-up as an alternative water change method for small scale culturing. First, clean SW is poured into the filter basket to cover the filter mesh. Then, as pictured, the culture is gently poured into the filter basket, allowing the water to fill (and spill over the sides of) the small glass bowl and into the larger retention bowl. The small glass bowl and filter basket can then be moved directly to a dissection microscope to observe the concentrated larvae. The filter basket is then lifted out of the small glass bowl, inverted over a clean culture vessel containing clean sea water, and rinsed into that vessel with clean SW. The components of this system are shown in the *inset*. Note that, when assembled, the small glass bowl sits atop the large Petri dish shown in the *inset at left* so that the rim of the small glass bowl is above the rim of the larger retention bowl.

Photos by B. Pernet.

(T. Capo, Personal Communication) with two modifications: (1) larvae are maintained under sufficient light to support continued algae growth within the culture vessels; and (2) the openings of the bottles are secured with parafilm followed by plastic wrap and a rubber band. This latter presumably allows sufficient gas exchange to avoid oxygen depletion, something also provided by the algae included as food.

Short-term culturing (a few days at most) can also be done in multi-well plates, shot glasses or Petri dishes. This approach is particularly useful when individual larvae need to be monitored (e.g., for larval cloning; McDonald & Vaughn, 2010). It is recommended to provide a minimum of 1 mL of water with algae per larva and change water daily. If possible, such containers should be agitated, for example, with a shaker platform set to fewer than 80 rotations per minute or with a plankton wheel. For tropical species, water changes using this method may need

to be conducted more than once per day to limit harmful bacterial or protist growth. Note that tissue culture-treated multi-well plates should be washed thoroughly before first use, especially taking care to remove the tissue culture treatment from the surface: treated well plates have been shown to be biologically active (Herrmann, Siefker, & Berking, 2003). As above, to avoid negative interactions with plasticware, we recommend glass for long-term cultures when possible.

Finally, if flowing SW is available, larvae can be reared in small containers with filter windows to prevent loss of larvae. The larvae are supplied with SW though a dripper tap system. The dripper valves used in garden watering systems are very useful in this context (Kamya et al., 2014). This works well for both planktotrophic and lecithotrophic larvae. For the former, the system can be periodically turned off to allow larvae to feed on added phytoplankton.

2.3 MEDIUM-SCALE CULTURING METHODS

If more than a few thousand late stage larvae are required, a medium-scale culturing method may be useful. One such system is pictured in Fig. 3: a 75-L cylindrical culturing tank with gentle aeration to maintain gas exchange, prevent stratification and maintain the algae and as many as 20 K larvae in suspension. At the bottom of the tank is a valve that is opened to drain the tank at water changes, every 2–3 days. The water is drained slowly through a Nitex mesh basket of a mesh size significantly smaller than the size of the larvae. The mesh basket (e.g., a large plastic beaker with the bottom cut off and replaced with the appropriately-sized Nitex mesh, or a 30-cm wide plumbing tubing with a Nitex mesh similarly affixed at one end) is taller and narrower than a bucket in which it is sitting. As the water is drained from the tank, it passes through the mesh and into and overflowing the sides of the bucket; the larvae are always maintained suspended in water inside the mesh basket. Once recovered, the larvae can be examined while the tank is cleaned if needed, refilled with FSW or ASW and re-stocked with algae. Then, the larvae are returned to the tank.

Because late-stage larvae of some species might settle on the sides of the column, the researcher employing this system might consider moving late-stage larvae into smaller vessels for settlement and subsequent study.

An alternative method of medium-scale culturing (which can also be modified for small or large scale applications) is a constant flow-through system. Here, there is no need to change water, as clean SW is being constantly circulated through the system. In one design, round 4-L plastic containers with perforated and meshed walls (see below) are placed inside a larger tank supplied with running ambient SW (approximately 20 L per hour). This suits laboratory scale cultures in the context of basic (Gianasi, Hamel, & Mercier, 2018a; Montgomery, Hamel, & Mercier, 2017; Montgomery et al., 2018) and applied research (Gianasi, Hamel, & Mercier, 2018b). The size of the containers can be scaled up as needed; black containers provide a darker environment, which may be beneficial both for encouraging settlement in some larvae and for locating recently settled juveniles. In order to ensure a constant flow of SW into the rearing vessels, holes ($\sim$40 cm^2) are cut out along the walls close to the bottom

FIG. 3

An example medium scale larval culturing set-up. Larvae and food are kept in suspension in this 75-L cylindrical tank by gentle aeration. For water changes, the valve at the bottom drains the tank; larvae are retained on a Nitex mesh by a forward filtration method similar to that pictured in Fig. 2 but at a larger scale. To maintain the larvae below ambient temperatures in this set-up, the tank is kept within a temperature controlled, insulated cabinet.

Photo by C. Lowe.

of the round containers and covered with mesh (chosen to be as large as possible while retaining the embryos and larvae). The parameters of the SW are left to fluctuate with ambient conditions, including temperature.

The main challenge here is providing food, as food will be flushed through the system at the same rate as the water flow. Two solutions that have proven effective are: (1) a drop-by-drop system where food is provided continuously; and (2) punctuated pulses of food several times a day, with or without temporary cessation of

water flow through the cultures during feeding bouts (see Mercier & Hamel, 2013). As mentioned previously, the best way to determine that an appropriate amount of food is being added in any culturing system is to examine the larvae carefully throughout larval development.

Overall, flow-through culture requires very little maintenance. In systems that use natural SW, the meshed windows must be cleaned weekly to maintain optimum water circulation, and the bottom of the vessels must also be vacuumed (i.e., by siphoning through a narrow diameter tubing), on a regular basis to remove debris and dead embryos and larvae. Note that organisms smaller than the mesh size will inevitably enter such cultures.

2.4 LARGE-SCALE CULTURING METHODS

In an aquaculture context, or if hundreds of thousands of larvae or juveniles are otherwise required, a large-scale culturing technique should be employed. In many ways, larger tanks are more forgiving and easier to work with than smaller tanks: temperature and pH tend to be more stable, and there are fewer replicates and tanks to monitor and clean. However, when we move to larger cultures the stakes are higher, with "more eggs in the same basket." As tanks get larger, flow dynamics change, which could be a source of stress on larvae or otherwise lead to changes in larval behavior.

Simply put, scaling up requires more of everything: food, water, electricity, tanks, space, labor and patience. It is important that pumps, compressors, blowers, pipes, filters and sieves be sized correctly as you increase scale. It is vital that the broodstock is healthy, and that the researcher be conscious of the "Top Five Things" suggested above (Section 2.1).

We will give an example here based on the Ānuenue Urchin Hatchery in Oahu, Hawai'i, where the collector urchin *Tripneustes gratilla* is cultured from egg to juvenile in about 23 days. Larvae are housed in 200-L conical bottom tanks provided with steady but gentle aeration (see Section 2.3).

The following routine is repeated daily:

- A known volume of water is sampled first thing in the morning to measure overnight phytoplankton consumption and larval density, health and growth.
- Larvae are fed fresh food to hold them until the daily water exchange.
- 200-L tanks (Fig. 4A) are drained down to 100 L with a Banjo screen filter (Fig. 4B).
- Water is exchanged—using clean, pH adjusted, UV sterilized FSW—at a rate of 5 L per min for 60 min (i.e., a 300% water exchange).
- Tanks are refilled; larvae are fed a base quantity of cultured phytoplankton.
- A water sample is taken 2–4 h later to determine phytoplankton density and estimate feeding rate.
- If necessary, larvae are fed again at the end of the day.

Care must be taken during water exchanges and tank changes to insure that larvae do not get injured or stuck to sieves or screens. Therefore, if higher draining rates are required, a greater surface area of the sieve or mesh is needed.

FIG. 4

An example large scale culturing set up. (A) Interior of climate controlled larval rearing room. The black cylinders are 200-L cone bottom larval rearing tanks where larvae and phytoplankton are suspended with gentle aeration. (B) Banjo screens are used for daily water exchanges by reverse filtration; this is a larger version of the reverse filtration apparatus shown in Fig. 1A. (C) When larvae need to be moved, or if the tanks are in need of thorough cleaning, forward filtration is carried out with a screened bucket and tote system. The principle is the same as the set-up shown in Fig. 2, but at a larger scale.

Photos by D.L. Cohen.

Large-scale culture vessels need to be cleaned and sanitized regularly, while ensuring that larvae never come into contact with cleaning agents. Chlorine, iodine or acid may be used to sanitize equipment. Any of these or ozone may be used to clean plumbing. Viricides such as Virkon Aquatic may be used in footbaths and to sanitize walls and floors in larval labs as well as phytoplankton labs. One effective protocol is to use 12.5% sodium hypochlorite (bleach) for most disinfecting procedures: it is inexpensive, easily acquired, approved for use in agriculture and food

systems, can be neutralized with sodium thiosulfate, and is quite effective. Appropriate protective equipment should be worn.

With larger systems, it is important to implement "clean in place" methodologies, including benchtops and the 200-L tanks. When possible, the entire system should be sanitized from end to end. Saltwater plumbing and aeration supply plumbing should be cleaned frequently. Saltwater supply lines can be flushed with freshwater daily and should be sterilized between larval cycles, as should aeration systems. Smaller, unattached parts of the system should be broken down and placed in a chlorine bath, including sieves, beakers, pitchers, Banjo screens, and siphon tubes. It is just as vital to make sure that all equipment is free from bleach before putting it back into service (Hill, Berthe, Lightner, & Sais, 2013).

Note that the example outlined above is rather modest in size relative to many aquaculture operations, which can use 1000–20,000-L tanks (or even greater) for larval culture (e.g., Lawrence, Zhao, & Chang 2019; Mercier & Hamel, 2013; Yang, Hamel, & Mercier, 2015). Either regular water changes and siphoning of the bottom of the tanks to remove dead larvae and debris are performed every other day (with similar methods as described above), or a flow-through system of SW exchange is used. To minimizes manipulations and decreases stress to the larvae, some laboratories start the culture in vessels that are only one-third full and simply add more FSW or ASW at regular intervals until the vessel is full, after which water changes can be made for the remainder of the culture period (Zhang et al., 2015).

3 TAXON SPECIFIC APPROACHES

In the following sections, we detail specific advice and approaches for culturing larvae of the four most-studied classes of echinoderms: the Asteroidea (sea stars), Echinoidea (sea urchins, sand dollars and kin), Holothuroidea (sea cucumbers) and Ophiuroidea (brittle stars and basket stars). Note that many of the methods outlined below in a given section might apply equally well to larvae from the other three classes. For information on collection, spawning, and fertilization of each of these four classes, see "Procuring animals and culturing of eggs and embryos" by Adams et al., in this volume. See also chapter "Culturing echinoderm larvae through metamorphosis" by Hodin et al., in this volume for more details on the sea urchin *Lytechinus pictus*. Because of the dearth of studies (especially recent studies) on Crinoidea (feather stars and sea lilies; the fifth extant echinoderm class), and in the interest of space, we here refer the interested reader to Holland (1991), Balser (2002), and Amemiya et al. (2015), and references therein.

3.1 ASTEROIDEA

Asteroid echinoderms typically develop via either feeding or non-feeding planktonic stages, or in benthic broods protected by a parent. All species with feeding (planktotrophic) larvae develop through a bipinnaria stage; most also have a subsequent
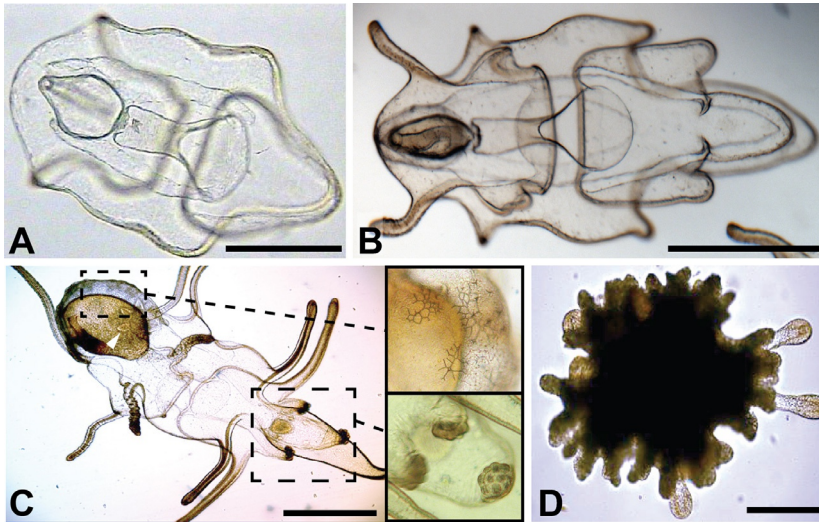
FIG. 5

Asteroid planktotrophic larval development through metamorphosis, as exemplified by the NE Pacific ochre sea star *Pisaster ochraceus* Brandt at approximately 14 °C. (A) Early bipinnaria stage larva. (B) Early brachiolaria stage larva. (C) Advanced brachiolaria. Note the juvenile rudiment with fivefold symmetry apparent (*white arrowhead)*, as well as closeups of the juvenile skeleton (*upper inset*) and brachiolar arms (*lower inset*). *N.b.*, the inset photos are from different larvae than the one pictured in the main panel in (C). (D) Juvenile. Scale bars: (A): 200 μm; (B): 400 μm; (C): 1000 μm; (D): 300 μm.

Main panel photos by S. George; inset photos in (C) by J. Hodin.

brachiolaria stage (McEdward & Miner, 2001; Fig. 5). Non-feeding (lecithotrophic) planktonic stages are diverse in developmental pattern, form, and nomenclature (McEdward & Janies, 1993; McEdward & Miner, 2001).

3.1.1 Rearing planktotrophic sea star larvae

Establishing cultures. The embryos of sea stars with feeding larvae are ready to be distributed into culture vessels when they hatch (as swimming blastulae or gastrulae, depending on species), approximately 2–4-day post fertilization (dpf) for tropical and temperate species.

Providing larval food. Sea star larvae can capture and ingest particles of a wide range of shapes and sizes (Strathmann, 1971, 1987b). Cells of many easily cultured micro-algae can thus be used as food, as detailed above (see Section 2.1).

Cleaning cultures. Sea star larvae are somewhat delicate and can be damaged during cleaning, causing mortality and even cloning in some species. As a compromise, most larval biologists clean and feed cultures every 2–3 days, though if avoidance of mortality is critically important, longer intervals are possible

(Bashevkin, Lee, Driver, Carrington, & George, 2016). The flow-through dripper tap system (see Section 2.2) significantly reduces the buildup of dead larvae, waste material and bacteria, and thereby reduces the need for frequent cleaning.

Cleaning may be achieved by reverse or forward filtration (see above), always being careful to minimize turbulence and contact of larvae with filtering surfaces to reduce damage to the larvae. Once concentrated, larger larvae can be picked out of the container one by one with a large-bore pipette and transferred to fresh FSW or ASW in a clean culture vessel (i.e., a 100% water exchange). If larvae are sufficiently large and swim to the surface when stirring is paused, then the reverse/forward filtration step can be skipped, and larvae picked out from the surface with a pipette, as above. Direct transfer is an excellent way to keep track of the total numbers of larvae. Nevertheless, for cultures of hundreds or more larvae per container, direct transfer is likely impractical, and bulk culturing methods (as described above) can be employed.

3.1.2 Rearing lecithotrophic sea star larvae

Establishing cultures. Like feeding larvae, the embryos of sea stars with non-feeding larvae can be distributed into culture vessels when they hatch, typically as swimming blastulae, ~4–5 dpf for temperate species or ~1–3 dpf for tropical and subtropical species (Birkeland, Chia, & Strathmann, 1971; Chen & Chen, 1992; Chia, 1966; Strathmann, 1987a). These embryos are often positively buoyant, so proximity to the water surface is not a good indication that they have hatched. The larvae can be maintained in small volumes of FSW or ASW in glass bowls or plastic tissue culture dishes with culture temperature carefully controlled. Gentle stirring may help to keep delicate larvae out of the surface tension, as can frequent dripping of water on to the surface of the culture vessel (Aronowicz, 2007), as well as the addition of small amounts of cetyl alcohol (Hurst, 1967). Note that some lecithotrophic sea star larvae do not encounter problems when at the surface per se, but do have problems at the surface-vessel interface, as the larvae can adhere to the sides of the vessel.

For medium-scale culturing of non-feeding larvae of sea stars, please refer to Section 2.3.

Cleaning cultures. If cultures of non-feeding larvae are low in larval density, cleaning should be carried out quite infrequently (1–2 times per week) to minimize the risk of damaging larvae. Instead of allowing them to contact filtering meshes, it is best to either gently transfer these larvae one by one by wide-bore pipette (easily done, as they are typically quite large and brightly colored) or use beakers or other vessels smaller than the size of the culture vessel to gently scoop up the buoyant larvae and then transfer them to clean FSW or ASW. Higher temperature cultures require more frequent transfers.

There are also diverse taxa of brooding asteroids that offer easy access to all developmental stages through the crawl away juvenile. These species range from
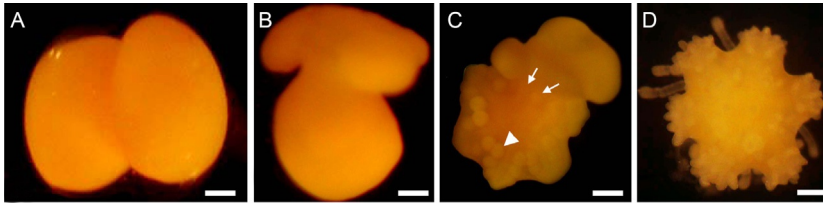
FIG. 6

Asteroid benthic larval development through metamorphosis in the six-armed brooding sea star *Leptasterias hexactis* Stimpson, from the NE Pacific. Dissected oocytes will mature with 1-methyladenine by standard methods (See chapter "Procuring animals and culturing of eggs and embryos" by Adams et al., in this volume); embryos can thus be cultured in vitro apart from the mother through the juvenile stage (see Hodin, 2006). (A) Two-cell stage. (B) Modified non-feeding brachiolaria larva. (C) Brachiolaria larva undergoing metamorphosis, oral view, with podia forming (*arrowhead*) along each ambulacrum (arm) in the juvenile portion of the larva (*lower left*). At this stage, the primordia of five of the six juvenile arms are well-formed—with three pairs of podia each. The primordium of the sixth arm is the last to form, here with only one pair of podia just visible (*white arrows*). (D) Aboral view of six-armed juvenile, with spines and tube feet visible. Scale bars: (A): 260 μm; (B): 100 μm; (C and D): 120 μm.

Photos by J. Hodin.

external brooders that care for their young under the oral surface (e.g., *Leptasterias* spp.; Fig. 6) to the viviparous asterinids that give birth to juveniles (e.g., *Parvulastra vivipara* and *Cryptasterina hystera*) (Byrne, 1996, 2005; George, 1994).

3.1.3 Induction of settlement, completion of metamorphosis and early juvenile growth

Competent larvae of asteroids with planktotrophic and lecithotrophic development settle readily in response to a biofilm on the surface of the culture container or other surfaces such as shell fragments placed in the containers. The competent larvae of many species appear to settle particularly well in response to the addition of coralline algae, including the crustose form on small rocks and geniculate algal fragments (Fig. 7). These substrates are useful as the newly metamorphosed juveniles can be transferred to new containers by picking up the shells, small rocks or pieces of algae.

Asteroid juveniles start life as predominantly herbivores feeding on biofilms and algae, with some becoming predators with time (Martinez, Byrne, & Coleman, 2017). Thus, to rear juvenile asteroids, it is important to renew the algae or shell fragments with biofilm regularly to ensure enough food. However, as in adult asteroids, we have noticed that cannibalism by juveniles can occur within as few as 3 days post-metamorphosis, even when food is provided. Therefore, maintaining juvenile asteroids for study should be done at very low densities.
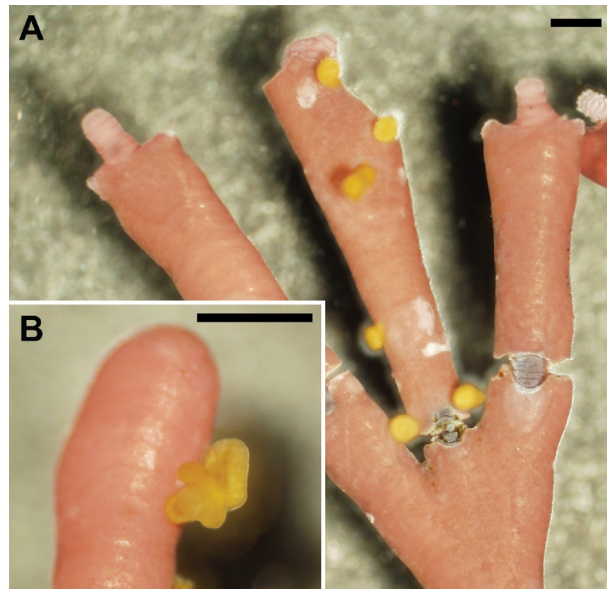
FIG. 7

Asteroid settlement on coralline algae (*Amphiroa* sp.), in the six-armed cushion star *Meridiastra gunnii* Gray, from subtropical and temperate Australian waters. (A) Several settled *M. gunnii* larvae and juveniles (orange) on an *Amphiroa* frond. (B) Close-up of a single settled *M. gunnii* larva. Scale bars: approx. 1 mm.

Photos by M. Byrne.

The ability to generate 1000s of juveniles by rearing the benthic larvae of direct developing species such as *Leptasterias hexactis* Stimpson and *Parvulastra exigua* Lamarck provide the easiest way to access asteroid juveniles for study (Fig. 6; Byrne, 1995; George, 1994; Hodin, 2006).

3.2 ECHINOIDEA

3.2.1 History of echinoid larval culturing

Echinoids have perhaps the richest history of marine invertebrate larval culturing. The first microscopic observation of fertilization was in sea urchins (Derbès, 1847), and Müller (1846) coined the term "pluteus" (*L*: easel) to describe the larval form. Echinoids serve as the stereotype for deuterostome development (Pearse & Cameron, 1991). Mortensen published extensive observations of larval development on tropical taxa (1921, 1931, 1937, 1938) and summarized (Mortensen, 1921) prior echinoderm larval rearing accomplishments, mainly using European, Scandinavian or Mediterranean taxa. Mortensen reared larvae without providing food, transferring larvae to fresh SW (thus containing the natural assemblages of food) every day to few days, which he described as quite laborious, and he recounts an experience in

Mauritius in which his cultures died due to the use of harbor water that was polluted with sewage (Mortensen, 1931). His culturing experiences appear to have hinged on the same factors as those of modern researchers: frequent water changes with fresh, clean SW, and abundant food (Leahy, 1986).

More recently, echinoid larvae have emerged as useful subjects for studies of swimming and feeding (Strathmann & Grünbaum, 2006; Wheeler, Chan, Anderson, & Mullineaux, 2016), physiology and metabolism (Leong & Manahan, 1997; Marsh, Maxson, & Manahan, 2001), ocean acidification impacts (Dupont, Ortega-Martínez, & Thorndyke, 2010), immune systems (Ho et al., 2016) and larval cloning (Eaves & Palmer, 2003; McDonald & Vaughn, 2010), among other topics. Microscopic methods (preservation and various labeling methods) have improved significantly in recent years (Strickland et al., 2004), including a method that maintains intact skeletal elements during long-term storage (Emlet, 2010; Turner, 1976). Larval and juvenile skeleton (in all echinoderms) can be visualized by an inexpensive modification of any compound microscope with cross-polarized light (e.g., see Figs. 8C, 9D, 12A, and 14C and D; Strathmann, 1987a).

3.2.2 Rearing planktotrophic echinoid larvae (echinoplutei)

The dominant life-history mode among echinoids is obligate planktotrophy, where adults release many small eggs (<250 μm diameter, averaging ∼100–125 μm) that give rise to pluteus larvae (Fig. 8) that must feed on phytoplankton for a time in order to attain sufficient energy to complete metamorphosis (reviewed in McEdward & Miner, 2001; Soars, Prowse, & Byrne, 2009). This obligate feeding period can last from days to a year or longer, depending on egg size, food levels and developmental temperature. Planktotrophic echinoid larvae tend to develop quite synchronously to the stage at which they are competent to settle, making them a good choice for a variety of studies ranging from larval physiology to the impact of stressors (such as ocean acidification) on larval development.

Planktotrophic larvae reared in low food, near starvation conditions (generally accepted to be <500–1000 algal cells per mL), respond by growing relatively longer feeding structures—the skeleton-supported larval "arms" that extend and support a continuous ciliated feeding band. This response is one of the best-studied examples of phenotypic plasticity among marine invertebrates (reviewed in McAlister & Miner, 2018) and may be associated with the evolutionary transition to lecithotrophic development in some groups (Strathmann, Fenaux, & Strathmann, 1992). The relative ease of measuring arm length in these larvae offers precise quantitative measures of larval growth and quality, and makes echinoid larvae a beneficial choice for a wide range of manipulative experiments.

3.2.3 Developmental staging in echinoplutei

Development proceeds post-fertilization through identifiable embryonic stages, hatching as blastulae, followed by gastrulation, prism (body skeletal rods present), and the early pluteus capable of feeding (Kumé & Dan, 1968). The timing of
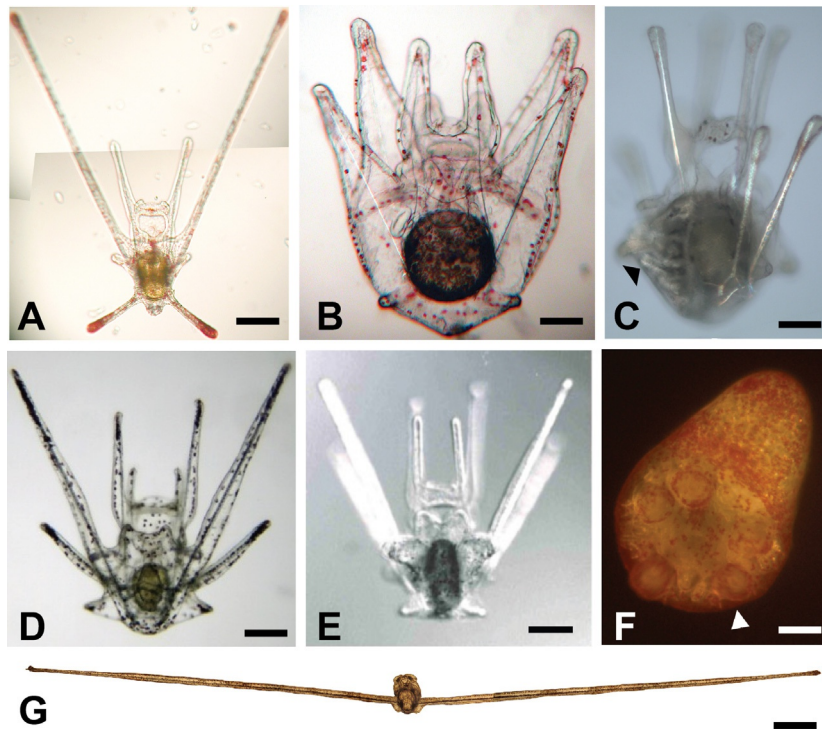
FIG. 8

Larval diversity in echinoids. (A) Ten-arm feeding pluteus of the NW Atlantic purple-spined sea urchin *Arbacia punctulata* Lamarck (Arbaciidae), pieced together from two photographs. (B) Eight-arm feeding pluteus of the NE Pacific purple sea urchin, *Strongylocentrotus purpuratus* Stimpson (Strongylocentrotidae). (C) Advanced eight-arm pluteus of the NE Pacific sand dollar, *Dendraster excentricus* Eschscholtz (Clypeasteridae). Well-developed adult spines (*arrowhead*) and other juvenile structures are visible in the large rudiment of this competent larva. (D) Eight-arm feeding pluteus of the tropical E Pacific urchin *Echinometra vanbrunti* Aggasiz (Echinometridae). (E and F) Larvae from two sister species of Australian urchins from the genus *Heliocidaris* (Echinometridae) with radically different developmental modes. (E) Eight-arm feeding pluteus of *H. tuberculata* Lamarck. (F) Non-feeding (lecithotrophic) larva of *H. erythrogramma* Valenciennes; juvenile rudiment clearly visible (one of the five primary podia indicated with *white arrowhead*). (G) Feeding pluteus larva, resulting from a hybrid cross between two Indian Ocean long-spined urchins (Diadematidae): *Diadema savignyi* Audouin (male) and *Diadema setosum* Leske (female). Effectively, this advanced larva has only one pair of enormous arms remaining; the three other arm pairs formed transiently earlier in ontogeny. Scale bars: (A, D, E): 65 μm; (B): 90 μm; (C): 110 μm; (F): 60 μm; (G): 190 μm.

Photos by J. Hodin (A-C), J. McAlister (D), R. Emlet (E), P. Cisternas (F) and S. Dautov (G). Panel (E): Reproduced by permission from Byrne, M., Emlet, R., & Cerra, A. (2001). Ciliated band structure in planktotrophic and lecithotrophic larvae of Heliocidaris *species (Echinodermata:Echinoidea): conservation and change.* Acta Zoologica, 82, *189–199.*
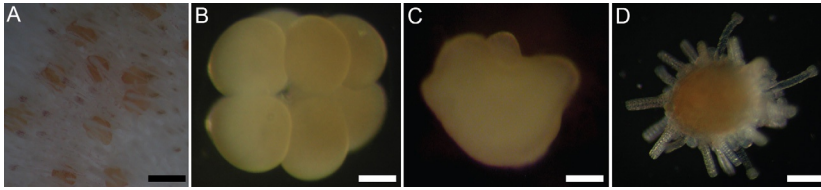
FIG. 9

Echinoid benthic larval development through metamorphosis in the brooding lamp urchin, *Cassidulus caribaearum* Lamarck (Cassidulidae), from the Eastern Caribbean. (A) Larval brood among the oral spines of the mother. (B) Eight-cell stage. (C) Non-feeding reduced pluteus larva (at approximately the same stage as the brooded larvae in (A). (D) Post-metamorphic juvenile, at which point the offspring crawl away from the mother. Scale bars: (A): 500 μm; (B)- 70 μm; (C): 120 μm; (D)-200 μm.

Photos by J. Hodin.

development to each stage varies by species and rearing temperature, but most species will attain the early pluteus stage at about 2–5 dpf.

Early pluteus larvae have two pairs of arms (the "four-arm stage"), the longer postorals (PO) and the anterolaterals (AL), which is followed in most species by a six- and then an eight-arm stage (see Smith, Smith, Cameron, & Urry, 2008). There is considerable evolutionary diversity of echinopluteus morphology with some species possessing 10 arms (e.g., Fig. 8A) or even 12 arms, single or dual posterior arms, terminal spines that may assist in defense, as well as fleshy, ciliated vibratile lobes or epaulettes, that develop late in development and increase swimming speed (Fig. 8; Emlet, 1988; Hart, 1991; Soars & Byrne, 2015; Strathmann, 1971; Wray, 1992). Some species within the family Diadematidae have a particularly distinct larval form, termed "*Echinopluteus transversus*" (Fig. 8G; Huggett, King, Williamson, & Steinberg, 2005; Mortensen, 1921, 1937; Soars et al., 2009), and characterized by strikingly long PO arms (up to several mm's long) with all other arms highly reduced (AL) or absent (PD and PR).

One benchmark that can be used to determine the stage at which larval density should be lowered is the first appearance of juvenile specific structures. In most echinoids (cidaroids are an exception), this stage is visible as the timing of invagination of the echinus rudiment (rudiment soft tissue *stage i* in Heyland & Hodin, 2014), which will contribute to the ectoderm of the juvenile, and is generally reached at approximately 30–50% of the way through the larval period when the larvae are fed ad libitum.

Development of structures fated to form the juvenile proceeds in parallel to larval development, the timing of which varies by species and with amount of food provided. This is a second aspect of the phenotypic plasticity alluded to above: high food causes larvae to proceed with juvenile structure development at the expense of larval arms. Heyland and Hodin (2014) have dissected formation of the juvenile structures in the purple sea urchin *Strongylocentrotus purpuratus* (Fig. 8C) into 8 soft tissue

stages and 10 juvenile skeletal stages. Subsequent studies have revealed that this basic scheme applies to a wide diversity of echinoids, although there are interesting variations among taxa (e.g., Kitazawa, Kobayashi, Kasahara, Takuwa, & Yamanaka, 2012) as well as heterochronies during larval development (J. Hodin, Unpublished), and once juveniles have emerged (Burke, 1980; Emlet, 2010).

3.2.4 Rearing lecithotrophic echinoid larvae

Non-feeding (lecithotrophic) development is found in approximately 20% of extant echinoid species; including extinct taxa it has been estimated to have evolved independently in a minimum of 14 lineages (Wray, 1992; Wray & Bely, 1994; Wray & Raff, 1991). Compared to the elaborate feeding morphology of planktotrophic echinoplutei, lecithotrophic larvae are highly derived, morphologically simple and convergent across taxa (Wray, 1992; Wray & Raff, 1991). One well studied species is *Heliocidaris erythrogramma*, which has a highly reduced larva that develops from large eggs and metamorphoses in 3–6 days, quite unlike its planktotrophic cousin, *H. tuberculata* (Fig. 8E and F; Byrne, Emlet, & Cerra, 2001). This change in life history is accompanied by apparent shifts in the embryonic fate map, cleavage patterns, process of formation of juvenile structures and corresponding gene expression (Israel et al., 2016; Raff & Byrne, 2006; Wray & Raff, 1989). There are also diverse taxa of brooding echinoids with crawl away juveniles, such as the lantern urchin *Cassidulus caribbaearum* (Fig. 9; Gladfelter, 1978; Hodin, 2006).

3.2.5 Induction of settlement and completion of metamorphosis

As far as we are aware, all echinoids that have been tested respond to "excess" potassium in SW by completing their transformation from larva to juvenile (n.b., excess potassium is potassium added above the background concentration). The concentration of potassium chloride (KCl) that elicits this effect does vary among taxa from 40 mM excess KCl in the NE Pacific sand dollar *Dendraster excentricus* to 120 mM excess in the Hawai'ian shingle urchin *Colobocentrotus atratus* (Table 1). We recommend that the researcher studying a species not mentioned in Table 1 exposes larvae to a range of KCl concentrations to determine the minimum effective dose for that species. We recommend a 1 h exposure to excess potassium followed by transfer into clean FSW or ASW for recovery and completion of metamorphosis.

One disadvantage of excess potassium exposure is that it stuns the larvae, so they do not exhibit the normal progression of pre-settlement behaviors (see Bishop & Brandhorst, 2007); in that sense, the process seems somewhat short-circuited. We therefore recommend that whenever possible, researchers attempt to find "natural inducers": namely, the kinds of compounds or substrates that larvae might respond to in the field. Nevertheless, our unpublished observations indicate that there is little to no difference in growth and survival of *S. purpuratus* juveniles during the week after recovery from KCl exposure (70 mM excess) when compared to those settling in response to a natural inducer (in this case, fronds of the coralline alga *Calliarthron tuberculosum* Postels & Ruprecht).

Table 1 Minimum Effective KCl Concentrations for Induction of Settlement (Complete Transformation From Larva to Juvenile) With a 1 h Exposure in Various Echinoids

Order: Family *Species*	KCl Concentration (mM)	References
Clypeasteroida: Clypeasteridae		
Clypeaster rosaceus	80	Heyland, Reitzel, Price, and Moroz (2006)
Clypeasteroida: Dendrasteridae		
Dendraster excentricus	40	Heyland and Hodin (2004) and Hodin, Ferner, Ng, Lowe, and Gaylord (2015)
Clypeasteroida: Mellitidae		
Leodia sexiesperforata	40	Heyland, Reitzel, and Hodin (2004)
Mellita tenuis	40	Heyland et al. (2004)
Diadematoida: Diadematidae		
Diadema antillarum	40	A.H. and J.H., Unpublished[a]
Echinoida: Echinometridae		
Colobocentrotus atratus	120	J.H., Unpublished
Echinometra lucunter	70	J.H., Unpublished
Echinometra viridis	70	J.H., Unpublished
Heterocentrotus mammillatus	120	J.H., Unpublished
Echinoida: Strongylocentrotidae		
Mesocentrotus franciscanus	100	Carpizo-Ituarte, Salas-Garza, and Parés-Sierra (2002)
Strongylocentrotus droebachiensis	70	J.H., Unpublished
Strongylocentrotus fragilis	100	J.H., Unpublished
Strongylocentrotus pallidus	70	J.H., Unpublished
Strongylocentrotus purpuratus	70	Gaylord, Hodin, and Ferner (2013)
Echinoida: Toxopneustidae		
Lytechinus pictus	40	Cameron, Tosteson, and Hensley (1989)
Lytechinus variegatus	40	Heyland, Price, Bodnarova-Buganova, and Moroz (2006)

Note that these concentrations are the excess potassium added to SW (which already contains potassium at approximately 90mM). Our experience is that there are significant differences among species in the rate at which they show signs of settlement following KCl exposure, from minutes (e.g., D. excentricus*) to 12h (e.g., in some* S. fragilis *larvae; note that "12h" here is 1h of exposure and 11h of recovery).*
[a]*Limited data—in such cases, a higher concentration than the one listed could be more effective.*

Natural inducers tend to be quite species-specific; therefore reviewing the entire spectrum of natural inducers for echinoids is beyond the scope of this chapter. In some cases, larvae settle readily in response to microbial biofilms (reviewed in Hadfield, 2011), sometimes grown in the presence of conspecifics. In other cases, the triggers appear to be specific types of benthic algae, often coralline algae (though in most cases the possibility that the trigger is a specific biofilm growing on the surfaces of these algae has not been excluded; Hadfield, 2011). Histamine, a compound released by macroalgae, appears to be an effective settlement trigger in a variety of echinoids, in some cases at concentrations that have been measured in the field near to the source algae (Swanson et al., 2012). In *S. purpuratus*, histamine exposure modulates competence to settle rather than triggering settlement per se (Sutherby et al., 2012). For various sand dollars, settlement can be triggered by sand from aquaria housing adults (Allen, 2012; Highsmith, 1982; Highsmith & Emlet, 1986; Vellutini & Migotto, 2010). For the NE Pacific sand dollar, *D. excentricus*, a specific peptide released from adults into the sand, appears to be the trigger (Burke, 1984); but a simple SW extract of the aquarium sand is highly effective (Hodin, Ferner, Ng, & Gaylord, 2018; Hodin et al., 2015).

Echinoid settlement and the drastic morphological changes associated with the completion of metamorphosis can be observed in real time under a dissecting or compound microscope after settlement has been induced. In most echinoids the major events of this process (Bishop & Brandhorst, 2007) are completed within a few hours.

3.2.6 Early juvenile rearing

Most planktotrophic echinoids settle a few days to a week or so before their juvenile mouths open, during which time they use up endogenous reserves from the larva; the time to mouth formation can be a few weeks or longer in lecithotrophic species (e.g., Olson, Cameron, & Young, 1993). Once their mouths are open, they are more or less ready to begin feeding (Fadl, Mahfouz, El-Gamal, & Heyland, 2017; Gosselin & Jangoux, 1998; Miller & Emlet, 1999). At that point, in particular with juveniles deriving from planktotrophic larvae, juveniles need to be transferred onto natural biofilms or cultures of benthic diatoms such as *Navicula* or *Nitzschia* species, or they will soon die of starvation (Xing, Wang, Cao, & Chang, 2007). Pure cultures of a variety of benthic diatoms can be obtained, for example, from the UTEX Culture Collection of Algae (TX, USA), the National Center for Marine Algae and Microbiota (ME, USA), the Australian National Algae Supply Service and Aqualgae (Spain/Portugal). Either flowing sea water or frequent water exchanges appear to be required for good growth and survival during the critical first weeks following settlement, during which time juveniles can grow quite rapidly. Eventually juveniles can be transitioned from biofilm/diatom cultures to small pieces of adult food (macroalgae and kelp). Leahy (1986) recommends feeding diatoms to *S. purpuratus* juveniles for 2 weeks, then transitioning the juveniles to rocks covered with natural biofilm for 1 month, then to pieces of thin seaweed such as the sea lettuce *Ulva* for 1–2 months, and then ultimately to pieces of kelp.

3.3 **HOLOTHUROIDEA**

Holothuroid echinoderms, commonly called sea cucumbers, exhibit a diversity of reproductive strategies from small, poorly-provisioned, unprotected pelagic propagules to large, abundantly-provisioned, brood-protected benthic propagules, and nearly everything in between. However, the majority of species can be classified as either planktotrophic (producing small, feeding larvae) or lecithotrophic (producing large, non-feeding larvae).

Of the approximately 1400 sea cucumber species described so far, around 80 are harvested commercially (Purcell et al., 2013). They are a valuable commodity in many countries of Asia, the Americas, and the tropical Indo-Pacific, where they are mainly processed for trade on Chinese markets (Hamel & Mercier, 2008a; Toral-Granda, Lovatelli, & Vasconcellos, 2008; Yang et al., 2015). Today, many sea cucumber populations are overexploited and some species are listed as endangered on the IUCN Red List (Purcell, Polidoro, Hamel, Gamboa, & Mercier, 2014). Apart from their commercial importance, sea cucumbers are ecologically significant members of benthic communities (Purcell, Conand, Uthicke, & Byrne, 2016) and they are a promising subject for studies of their remarkable regenerative abilities (Zhang et al., 2017). Therefore, interest in the development of breeding programs for sea cucumber is trending upward, and knowledge of larval development has been gathered from a diversity of tropical and temperate species in the context of scientific research and hatchery production.

Here we summarize the rearing protocols that have been developed to date, chiefly for commercial sea cucumbers, including the top three planktotrophic aquaculture species (*Apostichopus japonicus* Selenka, *Holothuria scabra* Jaeger and *Isostichopus fuscus* Ludwig; Fig. 10A and B), and two other commercially harvested species, one planktotrophic (*Apostichopus californicus* Stimpson), and one lecithotrophic (*Cucumaria frondosa* Gunnerus; Fig. 10C and D). Additional information will relate to an unusual lecithotrophic species with no commercial interest (*Psolus chitinoides* Clark) and a brooding species that does not produce dispersing larvae (*Leptosynapta clarki* Heding).

3.3.1 *Rearing planktotrophic sea cucumber larvae*

Larval cultures may follow two main protocols: most use closed conditions and water changes at regular intervals, although some use flow-through (open) conditions. Ultimately, the quality of the water supply is the primary determinant of success.

Culture. The developmental biology of planktotrophic sea cucumber species is very consistent: the auricularia larva metamorphoses through a doliolaria stage into a pentactula. The various developmental stages have been outlined for *I. fuscus* (Fig. 11; Mercier, Ycaza, & Hamel, 2004), *H. scabra* (Agudo, 2007; Hamel, Pawson, Conand, & Mercier, 2001; Ramofafia, Byrne, & Battaglene, 2003) and *A. japonicus* (Qiu, Zhang, Hamel, & Mercier, 2015). In most hatcheries, larvae are cultured in opaque conical tanks with central draining; smaller vessels are used in many laboratories (see above). Full details of commercial culture methods
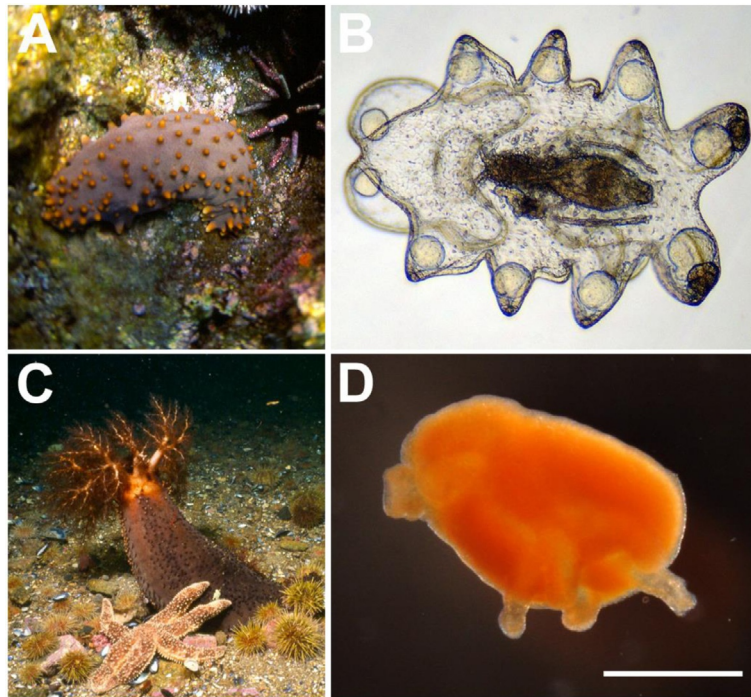
FIG. 10

Holothuroid adults and larvae of two commonly-cultured species. (A) Adult of the brown sea cucumber, *Isostichopus fuscus* Ludwig, an endangered species from the Tropical Pacific and Latin America. (B) Auricularia larva of *I. fuscus*. (C) Adult of the orange-footed sea cucumber, *Cucumaria frondosa* Gunnerus, from the North Atlantic. (D) Pentactula larva of *C. frondosa*. Scale bar in (D) applies to all four panels but at different magnifications as follows: (A): 10 cm; (B): 440 μm; (C): 12 cm; (D): 350 μm.

Photos by A. Mercier and J.-F. Hamel.

have been summarized previously (Mercier & Hamel, 2013; Zhang et al., 2015). Generally, early embryos are stocked at densities that do not exceed 1–5 individuals per mL due to density-dependent mortality (Sui, 1990), asynchrony and delayed growth in high densities, and even embryo fusion, which among other things increases developmental asynchrony in cultures (Gianasi et al., 2018a).

Please refer to Section 2 for recommended larval densities and water changes in closed cultures, and from small to large scale. Good results can also be obtained under flow-through (open) cultures with 200% or more water renewal every day. Under both closed and flow-through conditions, siphoning the bottom to remove dead larvae, detritus and bacterial mats is crucial.

We recommend gentle aeration to maintain oxygen levels and to provide mixing of the cultures (Mercier & Hamel, 2013; Zhang et al., 2015); one should avoid fine bubbles that may obstruct the digestive tract of some larvae. The optimal adjustments
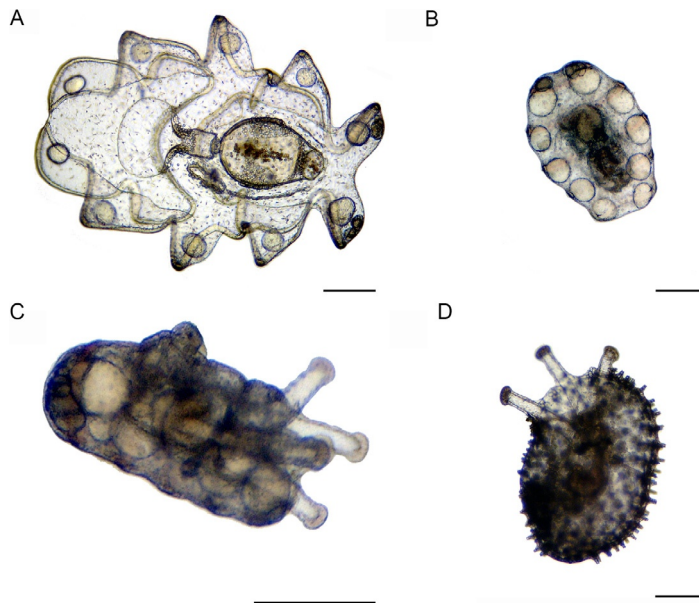
FIG. 11

Holothuroid planktotrophic larval development through metamorphosis in *I. fuscus*. (A) Fully developed auricularia larva. (B) Doliolaria larva. (C) Pentactula undergoing settlement. (D) Newly settled juvenile. Scale bars: 200 μm.

Photos by A. Mercier and J.-F. Hamel.

for pH, temperature, salinity, and dissolved oxygen are species-specific, but typically, water pumped in from offshore locations in the regions where the adults are found is sufficient to promote good larval cultures.

Feeding. All planktotrophic species need to be fed micro-algae until they reach the doliolaria (non-feeding) stage. The algal cultures should be of high quality and free of bacteria and copepods; locally available strains provided as a mix of species may yield better results in some cases than monocultures. The most common species of micro-algae used are *Dunaliella euchlaia*, *Chaetoceros gracilis*, *C. muelleri*, *C. calcitrans*, *Phaeodactylum tricornutum*, *Rhodomonas salina* and *Tetraselmis chuii*.

A number of investigators and hatcheries use alternative sources of food when live algae are not available or deemed too costly. Commercial marine micro-algae have been used successfully (Hair et al., 2011). Exact mixes and densities of food deemed to optimize larval health and growth are species-specific (Mercier & Hamel, 2013). Researchers should monitor larvae to ensure that algal cells are found in their digestive tract, at which point, adding more algae is not required; larvae of sea cucumbers collected from the field rarely exhibit full digestive tracts, and thus probably do not require much food to survive. Indeed, overfeeding can quickly lead to fouling of the culture vessels under closed conditions. Under flow-through (open)

conditions, cultures can be maintained with a continuous drop-by-drop supply of algae or via daily meals (Mercier & Hamel, 2013).

Health. Diseases can develop in embryos and larvae, particularly when culturing at larger scales. *A. japonicus* is by far the most extensively cultivated species with billions of larvae produced annually (Yang et al., 2015) and is (unsurprisingly) also the species with the highest number of reported problems. Central to culture issues is the accumulation of excess food, feces, and harmful microorganisms. Symptoms dubbed "rotting edges," "stomach ulceration" (auricularia stage), and "gas bubble disease" are the most common. The best way to avoid or mitigate these problems is prevention, careful monitoring to remove the first affected individuals, or use of antibiotic and other treatments when available (Mercier & Hamel, 2013).

The most pathogenic agents reported in cultures of *H. scabra* are bacteria causing ectoderm ulceration (Eeckhaut, Parmentier, Becker, Gomez Da Silva, & Jangoux, 2004). The most common culprit identified in lesions are *Vibrio* sp., *Bacteroides* sp. and α-proteobacterium (Becker et al., 2004). In the case of *I. fuscus*, infestation of the digestive system of early larvae by protozoan parasites is the most common (Mercier, Ycaza, Espinoza, Haro, & Hamel, 2012). In the worst cases, the digestive tract completely shrivels up and disappears, which is usually fatal. Although antibiotics and chemicals [e.g., ethylenediaminetetraacetic acid (EDTA)] have been used to mitigate these problem in commercial settings, they are not generally recommended (Zhang et al., 2015). Larger larval cultures of sea cucumber can be plagued by copepod infestations, which are presumed to compete for food and directly damage the larvae.

3.3.2 Rearing lecithotrophic sea cucumber larvae

While it may be achieved under closed conditions with regular water changes (see above), the culture of sea cucumber propagules is optimal in a flow-through system (see Section 2.3) with 200% or more daily turnover of ambient SW. Exposing propagules early in their development to natural conditions not only increases survival rates, but also yields healthier propagules that are strong enough to be released back in the ocean or in pens for grow out. For sea cucumbers, survival rates between fertilization and settlement may increase from 1–3% (typical of closed culture) to 15–40% (under flow-through), depending on the other culture parameters (Mercier & Hamel, 2013).

Culture. The major peculiarity of lecithotrophic development, apart from the larger size of the non-feeding larvae, is that the typical auricularia larval stage of planktotrophic sea cucumber species is absent, and the doliolaria stage is often replaced by a vitellaria. *C. frondosa* is very likely the most studied lecithotrophic sea cucumber, and can thus serve as a case study (Fig. 10C and D; Hamel & Mercier, 1996). This species is found on hard substrates in cold waters across the North Atlantic and Arctic (Hamel & Mercier, 2008b). Embryos of *C. frondosa* are typically incubated at densities of ~0.4 embryos per mL in round 4-L plastic containers with black bottom and walls (perforated and meshed; see Section 2.3), which are placed inside a larger tank supplied with running ambient SW (20L per h). With

C. frondosa the rearing temperature generally varies between ∼1 °C during the late winter spawning to ∼5 °C at the time of settlement. For an effective small-scale method of culturing of lecithotrophic sea cucumbers in multiple replicates, see Aronowicz (2007).

3.3.3 Brooding species
Embryos and juveniles of the burrowing sea cucumber *L. clarki* from the NE Pacific have been used to study the developing nervous system. After collection by sifting sediment, this species can be easily maintained in the lab and broods can be collected and studied during their reproductive season. Their size, optical clarity and relatively fast development (days) make them ideal organisms for whole mount observations (Hoekstra, Moroz, & Heyland, 2012).

3.3.4 Induction of settlement, completion of metamorphosis and early juvenile growth
The goal of larval cultures is often to rear them to settlement; in the case of hatcheries, this is the main goal. At temperatures between 20 °C and 25 °C, settlement occurs after about 12–16 dpf in *A. japonicus* and *H. scabra* (Hamel et al., 2001; Qiu et al., 2015), and 21–27 dpf in *I. fuscus* (Mercier et al., 2004). While some of the larvae in the three species will settle without stimulation, conditioned surfaces can increase the number of larvae that successfully settle (Mercier & Hamel, 2013); similar observations have been made in *A. californicus* (approximately 30 days to settlement at 14 °C; C. Lowe, Personal Communication) and other NE Pacific species. When planktotrophic sea cucumber larvae reach the doliolaria stage, they are approaching competence to settle, which occurs at the subsequent pentactula stage.

In both lecithotrophic and planktotrophic species, early juveniles do require a food source. If the system supplying SW to the rearing tanks does not have any filtration, then natural plankton (e.g., small ciliates, flagellates, diatoms and copepod nauplii) and suspended organic material present in ambient SW will be available. Attempts to mimic the natural habitat are a good idea; in the case of *C. frondosa*, small rocks often covered with coralline algae can be spread evenly inside the rearing tanks to provide shelter and substrate that will favor settlement (Hamel & Mercier, 1996) and promote health in early juveniles (Gianasi et al., 2018b).

3.4 OPHIUROIDEA
There are two main larval types found among ophiuroids with larval development: the ophiopluteus and the vitellaria (compare Fig. 12A and D; Brooks & Grave, 1899; Byrne & Selvakumaraswamy, 2002; Fenaux, 1969; Hendler, 1975, 1991; McEdward & Miner, 2001; Mortensen, 1921, 1938; Stancyk, 1973). The ophiopluteus is primarily a feeding (planktotrophic) larva, but in several species, a non-feeding (lecithotrophic) ophiopluteus is present (Fig. 12C; Allen & Podolsky, 2007; Hendler, 1991; Mladenov, 1979) and there is one report of a
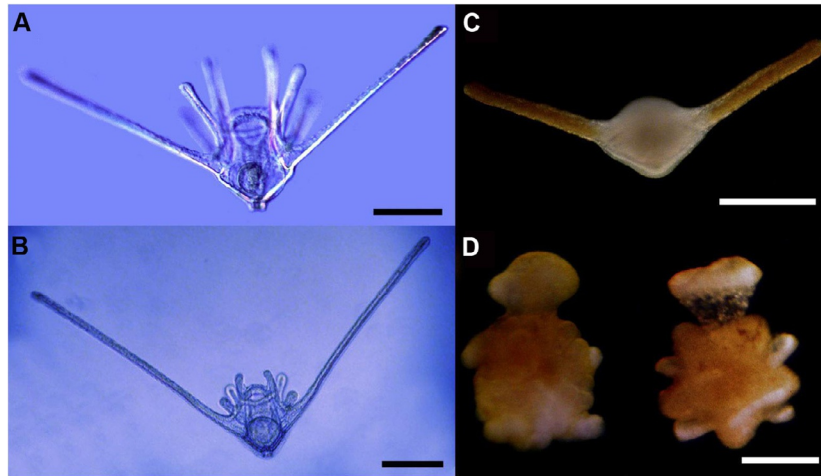
FIG. 12

Main larval types in ophiuroids. Fully developed planktotrophic ophioplutei of (A) *Ophiactis resiliens* Lyman from Oceania and (B) the spiny brittle star *Ophiothrix spiculata* Le Conte from the Eastern Pacific. (C) Lecithotrophic ophiopluteus of *Macrophiothrix caenosa* Hoggett from Western Australia. (D) Vitellaria larva of *Clarkcoma canaliculata* Lütken from Oceania. Scale bars: 100 μm.

*Photos by P. Cisternas (A, B, D) and J.D. Allen (C).*

facultatively-feeding ophiopluteus (Allen & Podolsky, 2007). The vitellaria larva is obligately lecithotrophic and it is the more common non-feeding larval form (Cisternas & Byrne, 2005).

Developmental patterns in ophiuroids with larval development are broadly categorized based on the predominant larval form (ophiopluteus versus vitellaria), feeding type (feeding versus non-feeding larva) and the pattern of metamorphosis that the larva undergoes to give rise to the juvenile (Fig. 13; Byrne & Selvakumaraswamy, 2002; Cisternas & Byrne, 2005; Hendler, 1975, 1991; McEdward & Miner, 2001). Ophiuroids that do not develop through a free-living (pelagic, benthic or encapsulated) larva instead brood embryos internally which are later released from the parent as advanced juveniles (Hendler, 1991).

3.4.1 Rearing planktotrophic ophiuroid larvae (ophioplutei)
For planktotrophs, low densities are recommended, in particular at late stages of development. Larval cultures require either very gentle stirring with paddles (as described above) or gentle rocking; we recommend the latter for ophioplutei that have long and slender arms such as those of *Ophiothrix* species (e.g., Fig. 12B). Because most ophioplutei are quite fragile and the arms may break easily, we suggest
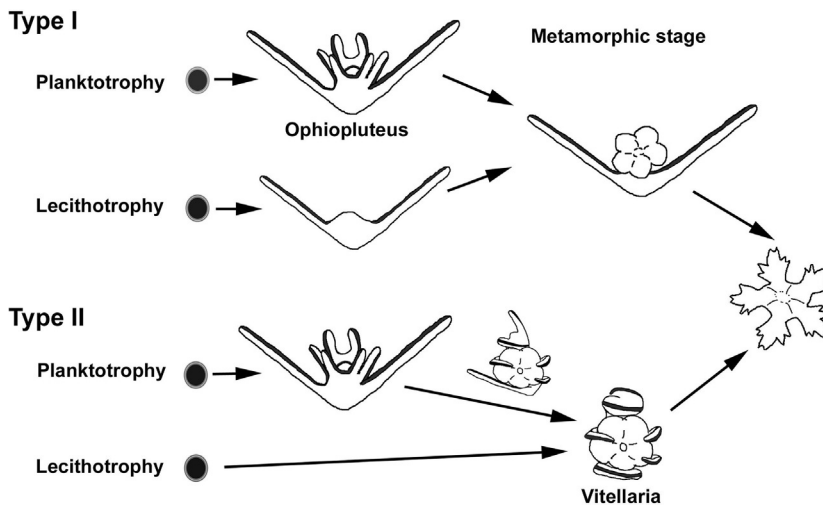
FIG. 13

Developmental patterns of ophiuroids with larval development (Type I and Type II) classified according to mode of larval feeding (planktotrophy versus lecithotrophy) and larval form during metamorphosis.

that densities be reduced to 1 larva per mL or lower as development progresses and lower still as larvae near metamorphic competence. Similarly, water changes can be reduced to every 4 days maximum or once a week to reduce any damage caused by handling. Depending on the amount and frequency of food added, these water changes may need to be adjusted accordingly. When larvae are subject to high food regime, more frequent water changes are necessary to prevent a build-up of ciliates in the culture.

Water changes. For reverse filtration (see above) the size of the mesh should be at least 50 μm less than the maximum diameter of the larval body and arms so as to avoid passing the larvae through the mesh. Before aspirating water through the filter (and after removal from the stirring mechanism), one should let the larvae sink down from the top of the water surface to about 1/3 down the water column. This will reduce damaging the larvae in the filtration process and minimize the chance of larvae being caught on the underside of the mesh. For fragile larvae, we recommend retaining a greater proportion of the water in the vessel so that larvae are not damaged by excessive concentration and contact with the filters. Ideally, containers, paddles and other equipment should be washed in hot tap water at least once a week during the culturing process to avoid biofilms from forming.

Advanced ophioplutei metamorphose while suspended in the water column and these can be easily identified by changes in the shape of the larval body: degeneration of larval arms, with the exception of the two posterolateral arms, reduction in the
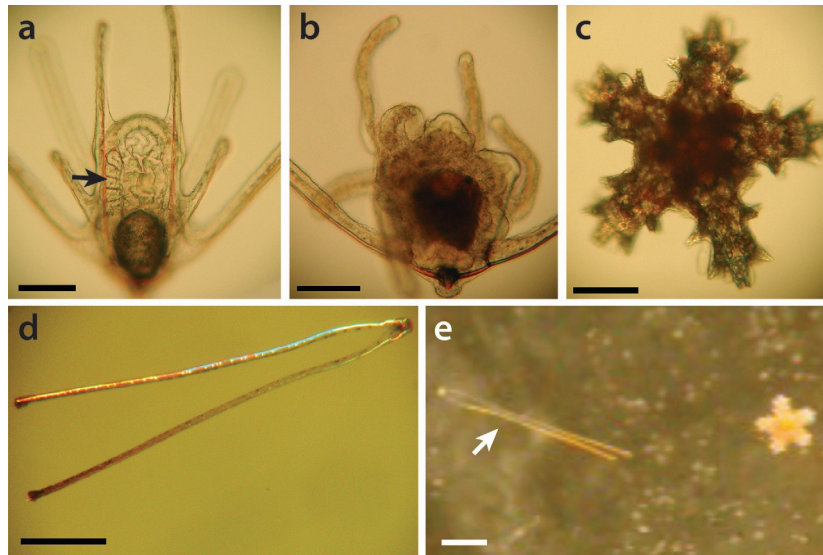
FIG. 14

Ophiuroid planktotrophic development though metamorphosis and post-settlement cloning in the daisy brittle star, *Ophiopholis aculeata* Lyman, a species with circumpolar distribution in the North. (A) Initiation of fivefold symmetry in the rudiment; at this stage, the primordia of the five arms (one indicated with *arrow*) are visible on the left side. (B) Disintegration of the larval arms as the larva nears the settlement stage. (C) Settled juvenile. (D) Cloning via discarded pair of posterolateral arms. (E) Still image from a video showing discarded posterolateral arms (*white arrow*) swimming away from a just-settled juvenile. Scale bars: 100 µm.

Photos by J. Hodin.

oral hood and mouth opening, increasing opacity of the central portion of the larval body, and appearance of the star-shaped juvenile rudiment (Fig. 14; Byrne & Selvakumaraswamy, 2002; Selvakumaraswamy & Byrne, 2006). At this stage larvae are usually located in the mid-water column, and care should be taken when removing water as the posterolateral arms are easily broken.

Cloning by ophioplutei. A new ophiopluteus can develop by cloning from the tissue suspended between the posterolateral arms that are released by the juvenile at settlement (Fig. 14; Balser, 1998), as has been recorded in *Ophiopholis aculeata* and unidentified ophioplutei found in the Gulf Stream off Florida.

Food sources and feeding regime. A mixed diet containing microalgae and diatoms is more commonly used for feeding ophioplutei but single strain diets may also be used (Mladenov, 1985; Podolsky & McAlister, 2005; Selvakumaraswamy & Byrne, 2000; Yamashita, 1985). The most commonly used algae (in the 2–15 µm diameter range) include *Dunaliella tertiolecta*, *Amphidinium*

earterae, Isochyrsis galbana, Isochyrsis sp., *Rhodomonas* spp., *T. chuii* and diatoms like *Chaetoceros calcitrans, C. gracillis, C. muelleri, Pavlova viridis* and *P. tricornutum*. The optimal diet including a mix of strains, densities, particle sizes and other factors are as yet to be determined. Cultures are typically fed every 2 days, with suggested densities (based on 5–10 μm algal cells) of approximately 7.5×10^3 cells per mL every 2 days for ophioplutei of tropical species (Allen & Podolsky, 2007) and 2×10^4 cells per mL every 4 days for larvae of temperate species (Selvakumaraswamy & Byrne, 2000).

### 3.4.2 Rearing lecithotrophic ophiuroid larvae

For ophiuroid species with non-feeding larvae, embryos can be distributed into containers with FSW or ASW after fertilization. The embryos and larvae need gentle agitation (rocking or bubbling from a pipette) to break the surface tension and to prevent damaging embryos. They can also be maintained at low densities (1 larva per mL) in small culture dishes (∼250 mL); these larvae are large and often buoyant, and can easily be transferred individually with a wide-bore pipette. Similarly, for negatively buoyant larvae, water can be removed from the surface through a filter mesh as they swim close to the bottom of the dish. Water changes can be carried out twice a week and containers cleaned once a week as previously described for planktotrophs.

3.4.3 Notes on ophiuroids that brood embryos

Ophiuroids that brood embryos are often small-bodied hermaphroditic species that produce large eggs, retained in the ovaries after fertilization until an advance juvenile stage (Fig. 15; Byrne, 1991; Hendler, 1975, 1991). Embryos are brooded internally in the bursae at the base of the arms and the juveniles emerge through the bursal slit (Byrne, 1991; Hendler, 1975).

Some brooding ophiuroids are known to be matrotrophic (i.e., they support embryos with maternally derived extraembryonic nutrition) including *Amphipholis squamata* (Fig. 15C; Fell, 1946), *Amphiura magellanica* Ljungman (Mortensen, 1920), *Ophiactis kroeyeri* Lütken (Mortensen, 1920), *Ophiophycis gracilis* Mortensen (1933) and *Ophionotus hexactis* Smith (Mortensen, 1921). Few species are known to brood pelagic-type lecithotrophic larvae. A vitellaria larva that lacks ciliary bands has been reported in *Ophionereis olivacea* Clark (Byrne, 1991) and *Ophiopeza spinosa* has a swimming vitellaria larva (Fig. 15B; Byrne, Cisternas, & O'Hara, 2008).

The embryos, larvae and early juveniles of southern tropical *O. spinosa* (Fig. 15A and B; Byrne et al., 2008) and the cosmopolitan temperate and warm temperate brooder *A. squamata* (Fig. 15C; Strathmann, 1987a) can be dissected out of the bursae and transferred individually with a pipette to dishes (e.g., six-well culture dishes) with 10 mL FSW or ASW. The aboral side of the parent's disc can be removed by cutting along its edge with scissors and severing the connective tissue that connects
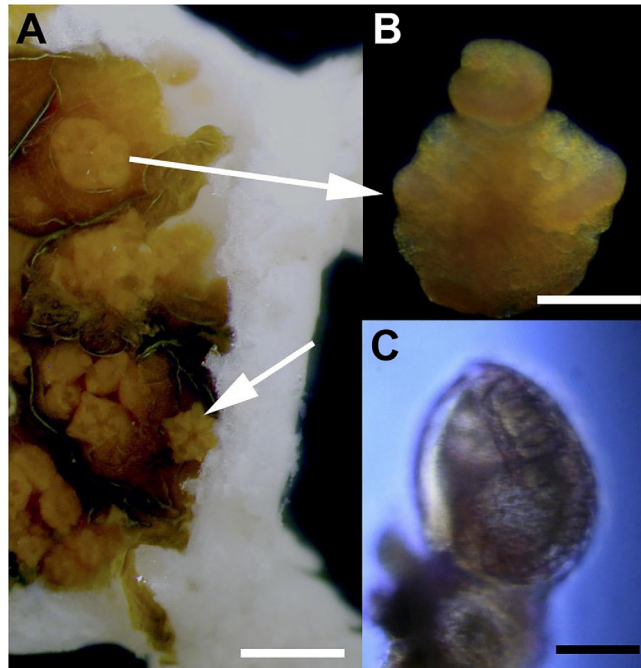
FIG. 15

Brooding ophiuroids. (A and B) *Ophiopeza spinosa* Ljungman. (A) The disc and some of the digestive system has been removed to show developing offspring inside the bursae (*arrows*). (B) Vitellaria larval stage from brood clutch in *O. spinosa*. (C) Developing embryo of *Amphipholis squamata* Delle Chiaje partially attached to the bursal wall. Scale bars: (A): 600 μm; (B): 300 μm; (C): 100 μm.

the disc to the skeletal elements above each arm. The bursae, which can be seen interradially and on both sides of the base of each of the arms, can gently be teased apart with fine forceps to reveal the embryos and juveniles (Fig. 15). FSW or ASW can be replaced every 2 days as indicated for other larval cultures until metamorphosis into juveniles is complete.

3.4.4 Encapsulated embryos

A number of ophiuroids have embryos that develop within capsules such as *O. hexactis* (Mortensen, 1921), *Amphioplus abditus* Verrill (Hendler, 1977) and *Amphiodia occidentalis* Lyman (Emlet, 2006). Of these, only the embryos of *A. abditus* have been cultured. After spawning, fertilized eggs can be transferred with

pipettes to glass dishes at densities of 2–5 embryos per mL in 50 mL of FSW or ASW. Water can easily be aspirated as the embryos are attached to the bottom of the dish via their fertilization envelopes. Water should be replaced every 2 days until development is completed (Hendler, 1977).

3.4.5 Induction of settlement, completion of metamorphosis and early juvenile growth

Unlike other echinoderms, ophiuroid larvae appear to settle naturally and without the need for specific exogenous settlement cues (Allen & Podolsky, 2007; Hendler, 1991; Selvakumaraswamy & Byrne, 2006). They may be seen sinking to the bottom of the container, testing the surfaces with their juvenile podia. The larval arms appear to be released from the juvenile body in mid-water or upon attachment to a surface (Fig. 14D and E; Hendler, 1991; Selvakumaraswamy & Byrne, 2006). Once the larvae have metamorphosed and settled, the developing juveniles are usually found attached to the sides or bottom of the container. Water can be gently decanted out and replaced once a week from here onward as needed. Juveniles have been cultured for a short period of time (a few weeks) in these conditions (P. Selvakumaraswamy, Personal Communication).

For juveniles deriving from lecithotrophic (vitellaria) larvae, we have continued the larval culturing techniques for vitellaria outlined above with good success after settlement for ~10 days and until the mouth opens.

ACKNOWLEDGMENTS

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