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Jason Hodin
*University of Washington*

Andreas Hayland
*University of Guelph*

Annie Mercier
*Marine Institute, Memorial University*

Bruno Pernet
*California State University, Long Beach*

David L. Cohen
*Anuenue Fisheries Research Center*

*See next page for additional authors*

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Hodin, J., Hayland, A., Mercier, A., Pernet, B., Cohen, D. L., Hamel, J., Allen, J. D., McAlister, J. S., Byrne, M., & George, S. B. (2019). Culturing Echinoderm Larvae Through Metamorphosis. *Methods in Cell Biology* (pp. 125-169). Elsevier. https://scholarworks.wm.edu/asbookchapters/98

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## Authors

Jason Hodin, Andreas Hayland, Annie Mercier, Bruno Pernet, David L. Cohen, Jean-Francois Hamel, Jonathan D. Allen, Justin S. McAlister, Maria Byrne, and Sophie B. George

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*running title:* Culturing echinoderm larvae

# CULTURING ECHINODERM LARVAE THROUGH METAMORPHOSIS

Jason Hodin[1,*], Andreas Heyland[2], Annie Mercier[3], Bruno Pernet[4], David L. Cohen[5], Jean-François Hamel[6], Jonathan D. Allen[7], Justin S. McAlister[8], Maria Byrne[9], Paula Cisternas[9] and Sophie B. George[10]

[1]Friday Harbor Labs, University of Washington, Friday Harbor WA, USA

[2]University of Guelph, Guelph ON, Canada

[3]Department of Ocean Sciences, Memorial University, St. John's NL, Canada

[4]Department of Biological Sciences, California State University–Long Beach, Long Beach CA, USA

[5]State of Hawai'i, Division of Aquatic Resources, Ānuenue Fisheries Research Center, Honolulu HI, USA

[6]Society for the Exploration and Valuing of the Environment (SEVE), Portugal Cove-St. Philips NL, Canada

[7]Biology Department, College of William and Mary, Williamsburg VA, USA

[8]Department of Biology, College of the Holy Cross, Worcester MA, USA

[9]School of Medical Sciences and School of Life and Environmental Sciences, University of Sydney, Sydney NSW, Australia

[10]Department of Biology, Georgia Southern University, Statesboro GA, USA

*Corresponding author: larvador@uw.edu

## 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.

## KEYWORDS

urchin, sea star, brittle star, sea cucumber, planktotrophy, lecithotrophy, feeding, non-feeding, husbandry, culture

46 **WHY CULTURE ECHINODERM LARVAE?**

48 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
50 with embryos, due to the ease of obtaining large quantities of synchronously developing, optically clear zygotes from a diversity of species. Nevertheless, when developmental studies of echinoderms
52 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);
54 the emergence of larval swimming and feeding behaviors, phenotypic plasticity, cloning, and regeneration; extensive changes in development that accompany independent evolutionary losses
56 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
58 development; and the dramatic habitat shift from planktonic life to the sea floor (Chia & Burke, 1979; Elphick & Melarange, 2001; Hodin, 2006; Lawrence, 2013; Lyons et al., this volume;
60 Strathmann, 1971, 1979; Su et al., this volume; Wray, 1996; Yaguchi et al., this volume). Indeed, early developmental processes are interesting in part because they affect the morphology of both larvae
62 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,
64 rearing larvae and juveniles is critical to our ability to implement some modern methods in genetics, as well as aquaculture.

66

Among the features that make echinoderms excellent study organisms are their remarkable

68 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 70 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 72 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 74 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.

76

There are nearly as many techniques for successfully culturing larvae as there are biologists working 78 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 80 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 82 promoting more widespread studies of the larvae and early juveniles that all too often end up down the embryologist's sink drain.

84

## General Culturing Methods

86

We here outline methods that, in our experience, apply to echinoderm larvae in general. We discuss 88 taxon-specific methods in subsequent sections.

90 **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 "$dH_2O$”).

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.

114 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 1N HCl for 10 min, followed by 116 thorough rinsing in tap water, then several rinses in $dH_2O$. The acid can be saved and used numerous times before being disposed as hazardous waste. Plasticware should never be acid 118 washed since that can compromise the surface of the plastic and thus lead to compounds being increasingly leached into subsequent embryonic and larval cultures.

120

In wet lab settings with access to flowing seawater (SW), another method for cleaning new or mildly 122 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. 124 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 126 to certain compounds that would otherwise make those containers unsuitable for standard cultures, they can be marked and maintained as such.

128

We recommend culturing larvae in high-quality natural SW deriving from offshore to minimize 130 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 132 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 134 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, 136 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
138 Strathmann (2014) for details.

140 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
142 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
144 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
146 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.

148

2) Rear larvae at low density

150

Although it is tempting to culture larvae at high density (more potential study material in a smaller
152 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,
154 Terry & 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
156 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
158 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 do not form a layer 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 below), 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 and Vaughn, 2010), which

makes cultures less synchronous. To avoid this, we recommend gradually reducing culture density
184 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
186 lowering the density about 1/3-1/2 of the way through larval development seems to yield good
results.

188

Embryos and larvae of species that do not require food to complete metamorphosis (i.e.,
190 **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
192 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
194 feeding and non-feeding larvae may not be as critical.

196 3) Stir or mix cultures gently and constantly

198 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
200 the containers. We present the specifics of mixing and stirring systems in the sections below
regarding small, medium and large-scale culturing.

202

If larvae are reared in replicate experimental vessels, after every water change the position of all
204 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

206 among-container variation (i.e., "beaker effects") during development.

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

210 Food sources for cultures of planktotrophic larvae vary, but ideally a mixture of live algal species is provided (Castilla-Gavilan, Buzin, Cognie, Dumay, Turpin & Decottignies, 2018; Ren, Liu, Dong &
212 Pearce, 2016; Scholtz, Bolton & Macey, 2013; M.F. Strathmann, 1987; Strathmann 2014). A commonly used mix for tropical and temperate echinoderm larvae includes all three or pairs of the following
214 algae: *Rhodomonas lens*, *Dunaliella tertiolecta* and *Isochrysis galbana.* A blend of *Chaetoceros*, *Chlorella*, and *Pyranimonas* works well with temperate echinoderm larvae (Zhang, Song, Hamel &
216 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,
218 but slower or less synchronous larval development may result.

220 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
222 supplies between water and food changes (usually every 1-2 d; see above); larvae should always have visible algal cells in their guts. Most echinoderm larvae cultured at low densities (≤1 per ml; see
224 above) grow rapidly at concentrations of 5000-10000 algal cells per ml (with algal cells of ~10 μm in diameter), with food replenished every 2-3 d. However, if larval size or density in cultures is high, or
226 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
228 scaled up proportionately to the volume of the individual algal cells.

230 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
232 hard a pellet during centrifugation as this will kill algae; dead algae forms a recognizable plume upon resuspension. A few trials will yield an appropriate centrifugation time and speed for
234 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
236 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
238 introduction of the algal growth medium into larval cultures.

240 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
242 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,
244 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
246 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
248 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),
250 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 & 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 & 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 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 Figure 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, upwards 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
276 microscope, or selection of a subset for fixation, biochemical analysis, etc.

278 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
280 forward filtration in small scale culture of certain robust larvae, as detailed below.

282 Frequency of water changes is dependent on a number of factors, including larval density, rearing temperature and developmental mode (planktotrophy *versus* lecithotrophy). Detailed suggestions
284 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.
286

When larvae are concentrated during water changes, the researcher may wish to select a few larvae
288 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
290 this is to use modeling clay to raise the cover glass slightly above the surface of the slide.

292 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
294 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
296 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 & 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 M.F. Strathmann (1987) and R.R. Strathmann (2014).

## 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 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 work 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 (M.F. Strathmann, 1987), 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

344 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
346 that especially fragile larvae (e.g., ophioplutei with slender arms, such as *Ophiotrix* spp., and diadematoid plutei) may not be amenable to this forward filtering technique.
348

An important distinction is whether the embryos and larvae are positively or negatively buoyant.
350 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
352 (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
354 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
356 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
358 urchin *Lytechinus variegatus* (Tom Capo, pers. comm.) with two modifications: (1) larvae are maintained under sufficient light to support continued algae growth within the culture vessels; and
360 (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
362 also provided by the algae included as food.

364 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
366 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, Dworjanyn, Hardy, Mos, Uthicke & Byrne, 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.

**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 Figure 3: a 75-L cylindrical culturing tank with gentle aeration to maintain gas exchange, prevent stratification and maintain the algae and as many as 20K larvae in suspension. At the bottom of the tank is a valve that is opened to drain the tank at

390 water changes, every 2-3 d. 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
392 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
394 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
396 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.

398

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

402

An alternative method of medium-scale culturing (which can also be modified for small or large
404 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
406 containers with perforated and meshed walls (see below) are placed inside a larger tank supplied with running ambient SW (approximately 20 L per h). This suits laboratory scale cultures in the
408 context of basic (Gianasi, Hamel & Mercier, 2018a; Montgomery, Hamel & Mercier, 2017, 2018) and applied research (Gianasi, Hamel & Mercier, 2018b). The size of the comtainers can be scaled up as
410 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
412 a constant flow of SW into the rearing vessels, holes (~40 cm$^2$) are cut out along the walls close to

the bottom of the round containers and covered with mesh (chosen to be as large as possible while
414 retaining the embryos and larvae). The parameters of the SW are left to fluctuate with ambient
conditions, including temperature.
416

The main challenge here is providing food, as food will be flushed through the system at the same
418 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
420 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
422 food is being added in any culturing system is to examine the larvae carefully throughout larval
development.
424

Overall, flow-through culture requires very little maintenance. In systems that use natural SW, the
426 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
428 regular basis to remove debris and dead embryos and larvae. Note that organisms smaller than the
mesh size will inevitably enter such cultures.
430

432 **Large-scale culturing methods**

434 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

436 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
438 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
440 behavior.

442 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
444 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.

446

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

452 The following routine is repeated daily:

- A known volume of water is sampled first thing in the morning to measure overnight
454 phytoplankton consumption and larval density, health and growth.
- Larvae are fed fresh food to hold them until the daily water exchange.
456 • 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
458 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.

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

482 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
484 (Hill, Berthe, Lightner & Sais, 2013).

486 Note that the example outlined above is rather modest in size relative to many aquaculture operations, which can use 1000 to 20000-L tanks (or even greater) for larval culture, for instance
488 those employed for the culture of commercial holothuroids (e.g., Mercier & Hamel, 2013; Yang, Hamel & Mercier, 2015). Either regular water changes and siphoning of the bottom of the tanks to
490 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
492 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
494 changes can be made for the remainder of the culture period (Zhang et al, 2015).

496

## TAXON SPECIFIC APPROACHES

498

In the following sections, we detail specific advice and approaches for culturing larvae of the four
500 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).
502 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 Chapter 1 in this volume on "Obtaining animals and gametes for embryological studies of echinoderms". See also Chapter 6 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, Hibino, Nakano, Yamaguchi, Kuraishi & Kiyomoto (2015), and references therein.

**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 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).

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 d 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 (R.R. Strathmann, 1971, 1987). Cells of many easily cultured micro-algae can thus be used as

food, as detailed above (see **Top Five Things** section).

528

*Cleaning cultures.* Sea star larvae are somewhat delicate and can be damaged during cleaning,
530 causing mortality and even cloning in some species. As a compromise, most larval biologists clean and feed cultures every 2-3 d, though if avoidance of mortality is critically important, longer
532 intervals are possible (Bashevkin, Lee, Driver, Carrington & George, 2016). The flow-through dripper tap system (see **Small-scale culturing methods** section, above) significantly reduces the buildup of
534 dead larvae, waste material and bacteria, and thereby reduces the need for frequent cleaning.

536 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.
538 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
540 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
542 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
544 impractical, and bulk culturing methods (as described above) can be employed.

546 Rearing lecithotrophic sea star larvae

548 *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

550 temperate species or ~1-3 dpf for tropical and subtropical species (Birkeland, Chia & Strathmann, 1971; Chen & Chen, 1992; Chia, 1966; M.F. Strathmann, 1987). These embryos are often positively
552 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
554 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
556 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
558 se, but do have problems at the surface-vessel interface, as the larvae can adhere to the sides of the vessel.

560

For medium-scale culturing of non-feeding larvae of sea stars, please refer to the general section on
562 **Medium-scale culturing methods**, above.

564 *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
566 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
568 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
570 frequent transfers.

572 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 external brooders that care for their
574 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,
576 1994).

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

580 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
582 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
584 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
586 or pieces of algae.

588 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
590 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
592 few as 3 d post-metamorphosis, even when food is provided. Therefore, maintaining juvenile
asteroids for study should be done at very low densities.

594

The ability to generate 1000's 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).

## Echinoidea

### 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 (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

618 feeding (Strathmann & Grunbaum, 2006; Wheeler, Chan, Anderson & Mullineaux, 2016), physiology and metabolism (Leong & Manahan, 1997; Marsh, Maxson & Manahan, 2001), ocean acidification
620 impacts (Dupont, Ortega-Martínez & Thorndyke, 2010), immune systems (Ho, , Buckley, Schrankel, Schuh, Hibino, Solek et al, 2016) and larval cloning (McDonald & Vaughn, 2010), among other topics.
622 Microscopic methods (preservation and various labeling methods) have improved significantly in recent years (Strickland, von Dassow, Ellenberg, Foe, Lenart & Burgess 2004), including a method
624 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
626 compound microscope with cross-polarized light (e.g., see Figs. 8C, 9D, 12A and 14C,D below; M.F. Strathmann, 1987)

628

Rearing planktotrophic echinoid larvae (echinoplutei)

630

The dominant life-history mode among echinoids is obligate planktotrophy, where adults release
632 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
634 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
636 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
638 studies ranging from larval physiology to the impact of stressors (such as ocean acidification) on larval development.

640

Planktotrophic larvae reared in low food, near starvation conditions (generally accepted to be less than 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.

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 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 "4-arm stage"), the longer postorals (PO) and the anterolaterals (AL), which is followed in most species by a 6- and then an 8-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;

664 Hart, 1991; Strathmann, 1971; Wray, 1992). Some species within the family Diadematidae have a particularly distinct larval form, termed "*Echinopluteus transversus*" (Fig. 8G; Huggett, King,
666 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
668 (PD and PR).

670 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),
672 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
674 reached at approximately 30-50% of the way through the larval period when the larvae are fed *ad libitum*.

676

Development of structures fated to form the juvenile proceed in parallel to larval development, the
678 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
680 development at the expense of larval arms. Heyland & Hodin (2014) have dissected formation of the juvenile structures in the purple sea urchin *Strongylocentrotus purpuratus* (Fig. 8C) into 8 soft tissue
682 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.,
684 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).

686

Rearing lecithotrophic echinoid larvae

688

Non-feeding (lecithotrophic) development is found in approximately 20% of extant echinoid
690 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
692 feeding morphology of planktotrophic echinoplutei, lecithotrophic larvae are highly derived,
morphologically simple and convergent across taxa (Wray, 1992; Wray & Raff, 1991). One well
694 studied species is *Heliocidaris erythrogramma*, which has a highly reduced larva that develops from
large eggs and metamorphoses in 3-6 d, quite unlike its planktotrophic cousin, *H. tuberculata* (Fig.
696 8E,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
698 corresponding gene expression (Israel, Martik, Byrne, Raff, Raff, McClay et al, 2016; Wray & Raff,
1989). There are also diverse taxa of brooding echinoids with crawl away juveniles, such as the
700 lantern urchin *Cassidulus caribbaearum* (Fig.9; Gladfelter, 1978; Hodin, 2006).

702 Induction of settlement and completion of metamorphosis

704 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
706 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*
708 *excentricus* to 120mM excess in the Hawai'ian shingle urchin *Colobocentrotus atratus* (Table 1). We
recommend that the researcher studying a species not mentioned in Table 1 expose larvae to a

710 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 712 recovery and completion of metamorphosis.

714 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, 716 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 718 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 720 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 & 722 Ruprecht).

724 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 726 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, 728 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 730 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 732 algae (Swanson, Byrne, Prowse, Mos, Dworjanyn & Steinberg, 2012). In *S. purpuratus*, histamine

exposure modulates competence to settle rather than triggering settlement per se (Sutherby,
734 Giardini, Nguyen, Wessel, Leguia & Heyland, 2012). For various sand dollars, settlement can be
triggered by sand from aquaria housing adults (Allen, 2012; Highsmith, 1982; Highsmith & Emlet,
736 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
738 of the aquarium sand is highly effective (Hodin, Ferner, Ng, Lowe & Gaylord, 2015; Hodin, Ferner, Ng
& Gaylord 2018).

740

Echinoid settlement and the drastic morphological changes associated with the completion of
742 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 &
744 Brandhorst, 2007) are completed within a few hours.

746 <u>Early juvenile rearing</u>

748 Most 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. Once their mouths are open, they are more or
750 less ready to begin feeding (Fadl & Heyland, 2017; Gosselin & Jangoux, 1998; Miller & Emlet, 1999).
At that point, in particular with juveniles deriving from planktotrophic larvae, juveniles need to be
752 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
754 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

756 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
758 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).
760 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
762 as the sea lettuce *Ulva* for 1-2 months, and then ultimately to pieces of kelp.

764

**Holothuroidea**

766

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

772

Of the approximately 1400 sea cucumber species described so far, around 80 are harvested
774 commercially (Purcell, Mercier, Conand, Hamel, Toral-Granda, Lovatelli et al, 2013). They are a valuable commodity in many countries of Asia, the Americas, and the tropical Indo-Pacific, where
776 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
778 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, Sun, Yuan, Sun, Gao, 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,B), and two other commercially harvested species, one planktotrophic (*Apostichopus californicus* Stimpson), and one lecithotrophic (*Cucumaria frondosa* Gunnerus; Fig. 10C,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).

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

802 auricularia larva metamorphoses through a doliolaria stage into a pentactula. The various developmental stages have been outlined for *I. fuscus* (Fig. 11; Mercier, Ycaza and Hamel, 2004), *H.*
804 *scabra* (Agudo, 2007; Hamel, Pawson, Conand & Mercier, 2001) and *A. japonicus* (Qiu, Zhang, Hamel & Mercier, 2015). In most hatcheries, larvae are cultured in opaque conical tanks with central
806 draining; smaller vessels are used in many laboratories (see above). Full details of commercial culture methods have been summarized previously (Mercier & Hamel, 2013; Zhang et al, 2015).
808 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
810 even embryo fusion, which among other things increases developmental asynchrony in cultures (Gianasi et al, 2018a).

812

Please refer to the **General Culturing Methods** above for recommended larval densities and water
814 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
816 flow-through conditions, siphoning the bottom to remove dead larvae, detritus and bacterial mats is crucial.

818

We recommend gentle aeration to maintain oxygen levels and to provide mixing of the cultures
820 (Mercier & Hamel, 2013; Zhang et al, 2015); one should avoid fine bubbles that may obstruct the digestive tract of some larvae. The optimal adjustments for pH, temperature, salinity, and dissolved
822 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.

824

*Feeding.* All planktotrophic species need to be fed micro-algae until they reach the doliolaria (non-
826 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
828 monocultures. The most common species of micro-algae used are *Dunaliella euchlaia*, *Chaetoceros*
*gracilis*, *C. muelleri*, *C. calcitrans*, *Phaeodactylum tricornutum*, *Rhodomonas salina* and *Tetraselmis*
830 *chuii*.

832 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,
834 Pickering, Meo, Vereivalu, Hunter, Cavakiqali et al, 2011). Exact mixes and densities of food deemed
to optimize larval health and growth are species-specific (Mercier & Hamel, 2013). Researchers
836 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
838 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
840 (open) conditions, cultures can be maintained with a continuous drop-by-drop supply of algae or
via daily meals (Mercier & Hamel, 2013).

842

*Health.* Diseases can develop in embryos and larvae, particularly when culturing at larger scales. *A.*
844 *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
846 problems. Central to culture issues is the accumulation of excess food, feces, and harmful
microorganisms. Symptoms dubbed ‘rotting edges’, ‘stomach ulceration’ (auricularia stage), and

848 ‘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 850 treatments when available (Mercier & Hamel, 2013).

852 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 854 culprit identified in lesions are *Vibrio* sp., *Bacteroides* sp. and α-Proteobacterium (Becker, Gillan, Lanterbecq, Jangoux, Rasolofonirina, Rakotovao et al, 2004). In the case of *I. fuscus*, infestation of the 856 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 858 disappears, which is usually fatal. Although antibiotics and chemicals [e.g., ethylenediaminetetraacetic acid (EDTA)] have been used to mitigate these problem in commercial 860 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 862 directly damage the larvae.

864 Rearing lecithotrophic sea cucumber larvae

866 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 **Medium-scale** 868 **culturing methods**, above) 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 870 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%
872 (typical of closed culture) to 15-40% (under flow-through), depending on the other culture
parameters (Mercier & Hamel, 2013).
874
*Culture.* The major peculiarity of lecithotrophic development, apart from the larger size of the non-
876 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
878 studied lecithotrophic sea cucumber, and can thus serve as a case study (Fig. 10C,D; Hamel &
Mercier, 1996). This species is found on hard substrates in cold waters across the North Atlantic and
880 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
882 meshed; see **Medium-scale culturing methods**, above), which are placed inside a larger tank
supplied with running ambient SW (20 L per h). With *C. frondosa* the rearing temperature generally
884 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
886 Aronowicz (2007).

888 Brooding species

890 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
892 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) makes them ideal organisms

894 for whole mount observations (Hoekstra, Moroz & Heyland, 2012).

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

898 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 20-25°C, settlement occurs after about 12-16 dpf in *A.*
900 *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 stumulation, conditioned
902 surfaces can increase the number of larvae that successfully settle (Mercier & Hamel, 2013); similar observations have been made in *A. californicus* (approximately 30 d to settlement at 14°C; C. Lowe,
904 pers. comm.) 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
906 pentactula stage.

908 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.,
910 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
912 *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
914 promote health in early juveniles (Gianasi et al, 2018b).

916

**Ophiuroidea**

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

<u>Rearing planktotrophic ophiuroid larvae (ophioplutei)</u>

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

940 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

942 break easily, we suggest 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

944 reduced to every four 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

946 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.

948

*Water changes*. For reverse filtration (see above) the size of the mesh should be at least 50 μm less

950 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

952 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

954 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

956 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

958 forming.

960 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

962 exception of the two posterolateral arms, reduction in the 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., *Tetraselmis chuii* and diatoms like *Chaetoceros calcitrans, C. gracillis, C. muelleri, Pavlova viridis* and *Phaeodactylum 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 d, with suggested densities (based on 5-10 µm algal cells) of approximately $7.5 \times 10^{3}$ cells per ml every two days for ophioplutei of tropical species (Allen and Podolsky 2007) and $2 \times 10^{4}$ cells per ml every four days for larvae of temperate species (Selvakumaraswamy & Byrne, 2000).

Rearing lecithotrophic ophiuroid larvae

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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.

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996 Notes on ophiuroids that brood embryos

998 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).

1000

1002

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

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swimming vitellaria larva (Fig. 15B; Byrne, Cisternas & O'Hara, 2008).

1010

The embryos, larvae and early juveniles of southern tropical *O. spinosa* (Fig. 15 A,B; Byrne et al, 2008)

1012 and the cosmopolitan temperate and warm temperate brooder *A. squamata* (Fig. 15C; M.F.

Strathmann, 1987) can be dissected out of the bursae and transferred individually with a pipette to

1014 dishes (e.g., 6-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

1016 the disc to the skeletal elements above each arm. The bursae, which can be seen inter-radially and

on both sides of the base of each of the arms, can gently be teased apart with fine forceps to reveal

1018 the embryos and juveniles (Fig. 15). FSW or ASW can be replaced every two days as indicated for

other larval cultures until metamorphosis into juveniles is complete.

1020

Encapsulated embryos

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A number of ophiuroids have embryos that develop within capsules such as *O. hexactis* (Mortensen,

1024 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

1026 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

1028 fertilization envelopes. Water should be replaced every two days until development is completed

(Hendler, 1977).

1030

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,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 onwards as needed. Juveniles have been cultured for a short period of time (a few weeks) in these conditions (P. Selvakumaraswamy, pers. comm.).

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 d and until the mouth opens.

## Acknowledgements

We thank Richard Strathmann and Chris Lowe for advice and discussions. We dedicate this chapter to Dr. Susie Balser, who looked at the same larvae that many had before her, and yet saw things that nobody else had.

1054

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## TABLES

1416

| Order: Family<br>*Species* | KCl concen-tration | References |
|---|---|---|
| **Clypeasteroida: Clypeasteridae** | | |
| *Clypeaster rosaceus* Linnaeus | 80 mM | Heyland, Reitzel, Price & Moroz, 2006b |
| **Clypeasteroida: Dendrasteridae** | | |
| *Dendraster excentricus* Eschscholtz | 40 mM | Heyland & Hodin 2004, Hodin et al, 2015 |
| **Clypeasteroida: Mellitidae** | | |
| *Leodia sexiesperforata* Leske | 40 mM | Heyland Reitzel & Hodin, 2004 |
| *Mellita tenuis* Clark | 40 mM | Heyland et al, 2004 |
| **Diadematoida: Diadematidae** | | |
| *Diadema antillarum* Philippi | 40 mM | A.H and J.H unpublished* |
| **Echinoida: Echinometridae** | | |
| *Colobocentrotus atratus* Linnaeus | 120 mM | J.H. unpublished |
| *Echinometra lucunter* Linnaeus | 70 mM | J.H. unpublished |
| *Echinometra viridis* Agassiz | 70 mM | J.H. unpublished |
| *Heterocentrotus mammillatus* Linnaeus | 120 mM | J.H. unpublished |
| **Echinoida: Strongylocentrotidae** | | |
| *Mesocentrotus franciscanus* Agassiz | 100 mM | Carpizo-Ituarte, Salas-Garza & Parés-Sierra, 2002; Salas-Garza, Carpizo-Ituarte, Parés-Sierra, Martínez-López & Quintana-Rodríguez, 2005 |
| *Strongylocentrotus droebachiensis* Müller | 70 mM | J.H. unpublished |
| *Strongylocentrotus fragilis* Jackson | 100 mM | J.H. unpublished |
| *Strongylocentrotus pallidus* Sars | 70 mM | J.H. unpublished |
| *Strongylocentrotus purpuratus* Stimpson | 70 mM | Gaylord, Hodin & Ferner, 2013 |
| **Echinoida: Toxopneustidae** | | |
| *Lytechinus pictus* Verrill | 40 mM | Cameron Tosteson & Hensley, 1989 |
| *Lytechinus variegatus* Lamarck | 40 mM | Heyland, Price, Bodnarova-Buganova & Moroz, 2006a |

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**Table 1. Minimum effective KCl concentrations for induction of settlement** (complete transformation from larva to juvenile) with a 1 h exposure in various echinoids. Note that these concentrations are the excess potassium added to SW (which already contains potassium at approximately 90 mM). 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 12 h (e.g., in some *S. fragilis* larvae; note that '12 h' here is 1 h of exposure and 11 h of recovery). Asterisks refer to limited data – in such cases, a higher concentration than the one listed could be more effective.

## FIGURE LEGENDS

**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,C).

**Fig. 2. Forward filtration set-up as an alternative water change method for small scale**

1444 **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

1446 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

1448 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

1450 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.

1452 Photos by B. Pernet.

1454 **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

1456 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

1458 tank is kept within a temperature controlled, insulated cabinet. Photo by C. Lowe.

1460 **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

1462 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

1464 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-

1466 up shown in Fig. 2, but at a larger scale. Photos by D.L. Cohen.

1468 **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
1470 stage larva. B) Early brachiolaria stage larva. C) Advanced brachiolaria. Note the juvenile rudiment with 5-fold symmetry apparent (*white arrowhead)*, as well as closeups of the juvenile skeleton (*upper*
1472 *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)–
1474 300 µm. Main panel photos by S. George; inset photos in (C) by J. Hodin.

1476 **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-
1478 methyladenine by standard methods (See Chapter 1 in this volume); embryos can thus be cultured *in vitro* apart from the mother through the juvenile stage (see Hodin, 2006). A) 2-cell stage. B)
1480 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
1482 (*lower left*). At this stage, the primordia of 5 of the 6 juvenile arms are well-formed– with 3 pairs of podia each. The primordium of the 6th arm is the last to form, here with only one pair of podia just
1484 visible (*white arrows*). D) Aboral view of 6-armed juvenile, with spines and tube feet visible. Scale bars: (A)–260 µm; (B)–100 µm; (C,D)–120 µm. Photos by J. Hodin.

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**Fig. 7. Asteroid settlement on coralline algae** (*Amphiroa sp.*), in the six armed cushion star
1488 *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*
1490 larva. Scale bars: approx. 1 mm. Photos by M. Byrne.

1492 **Fig. 8. Larval diversity in echinoids.** A) 10-arm feeding pluteus of the NW Atlantic purple-spined sea urchin *Arbacia punctulata* Lamarck (Arbaciidae), pieced together from two photographs. B) 8-
1494 arm feeding pluteus of the NE Pacific purple sea urchin, *Strongylocentrotus purpuratus* Stimpson (Strongylocentrotidae). C) Advanced 8-arm pluteus of the NE Pacific sand dollar, *Dendraster*
1496 *excentricus* Eschscholtz (Clypeasteridae). Well-developed adult spines (*arrowhead*) and other juvenile structures are visible in the large rudiment of this competent larva. D) 8-arm feeding
1498 pluteus of the tropical E Pacific urchin *Echinometra vanbrunti* Aggasiz (Echinometridae). E,F) Larvae from two sister species of Australian urchins from the genus *Heliocidaris* (Echinometridae) with
1500 radically different developmental modes. E) 8-arm feeding pluteus of *H. tuberculata* Lamarck. F) Non-feeding (lecithotrophic) larva of *H. erythrogramma* Valenciennes; juvenile rudiment clearly
1502 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):
1504 *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
1506 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). E reproduced
1508 by permission from Byrne et al (2001).

1510

1512 **Fig. 9. Echinoid benthic larval development through metamorphosis** in the brooding lamp urchin, *Cassidulis caribaearum* Lamarck (Cassidulidae), from the Eastern Caribbean. A) Larval brood
1514 among the oral spines of the mother. B) 8-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
1516 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.

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**Fig. 10. Holothuroid adults and larvae** of two commonly-cultured species**.** A) Adult of the brown
1520 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,
1522 *Cucumaria frondosa* Gunnerus, from the North Atlantic. D) Pentactula larva of *C. frondosa*. Scale bars: (A)–10 cm; (B)–440 µm; (C)–12 cm; (D)–350 µm. Photos by A. Mercier and J.-F. Hamel.

1524

**Fig. 11. Holothuroid planktotrophic larval development through metamorphosis** in *I. fuscus*. A)
1526 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.

1528

**Fig. 12. Main larval types in ophiuroids.** Fully developed planktotrophic ophioplutei of A)
1530 *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
1532 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).

1534

**Fig. 13. Developmental patterns of ophiuroids** with larval development (Type I and Type II)
1536 classified according to mode of larval feeding (planktotrophy *versus* lecithotrophy) and larval form during metamorphosis.

1538

**Fig. 14. Ophiuroid planktotrophic development though metamorphosis and post-settlement**
1540 **cloning** in the daisy brittle star, *Ophiopholis aculeata* Lyman, a species with circumpolar distribution in the North. A) Initiation of 5-fold symmetry in the rudiment; at this stage, the primordia of the 5
1542 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
1544 arms. E) Still image from a video showing discarded posterolateral arms swimming away from a just-settled juvenile. Scale bars: 100 µm. Photos by J. Hodin.

1546

**Fig. 15. Brooding ophiuroids.** A,B) *Ophiopeza spinosa* Ljungman. A) The disc and some of the
1548 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*
1550 *squamata* Delle Chiaje partially attached to the bursal wall. Scale bars: (A)–600 µm; (B)–300 µm; (C)–100 µm. Photos by M. Byrne (A, B) and P. Cisternas (C). B reproduced by permission from Byrne et al
1552 (2008).

**FIGURE AND TABLES**

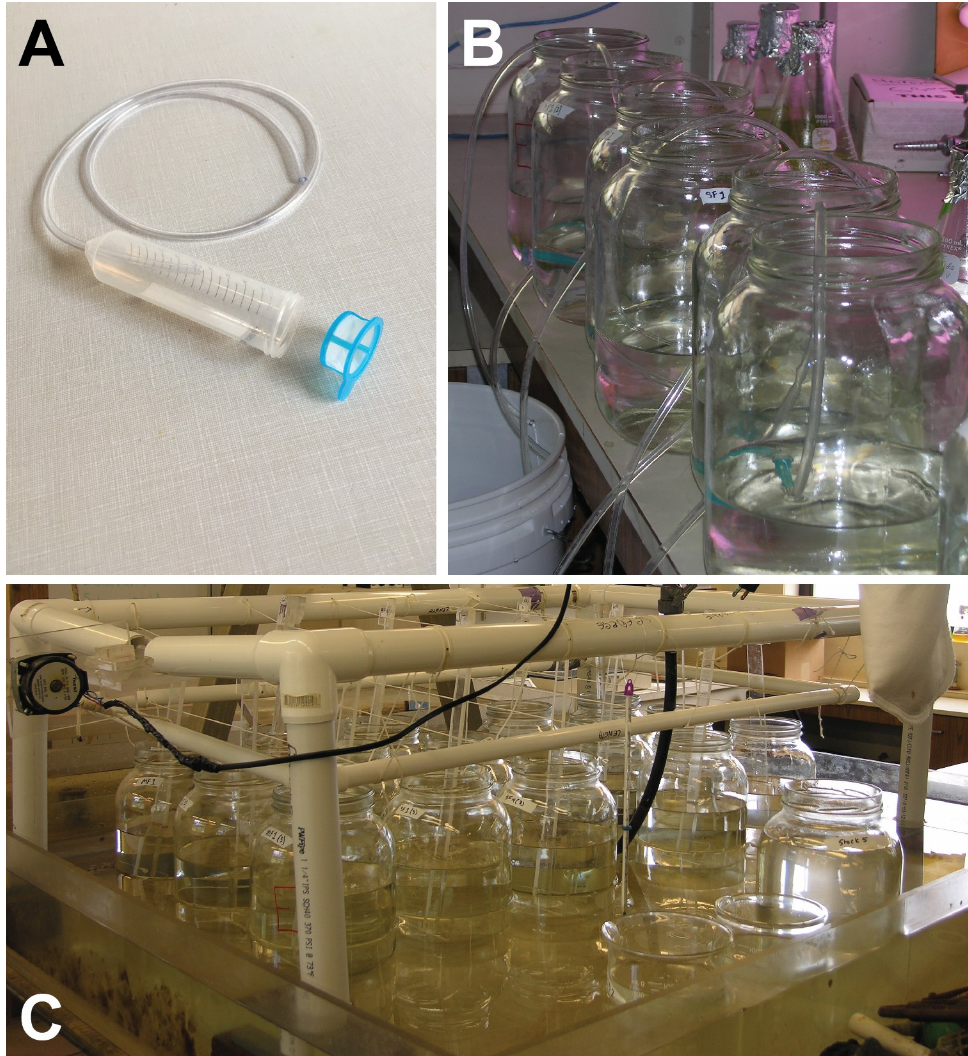



**Fig. 1. Small scale culturing methods.** A) Simple and effective reverse filtration apparatus. A small hole is cut in the bottom of a 50-ml centrifuge tube, and plastic air tubing is inserted in the hole. The opening of the tube is covered with an appropriate-sized Nitex mesh. Here a commercially-available 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 using 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,C).

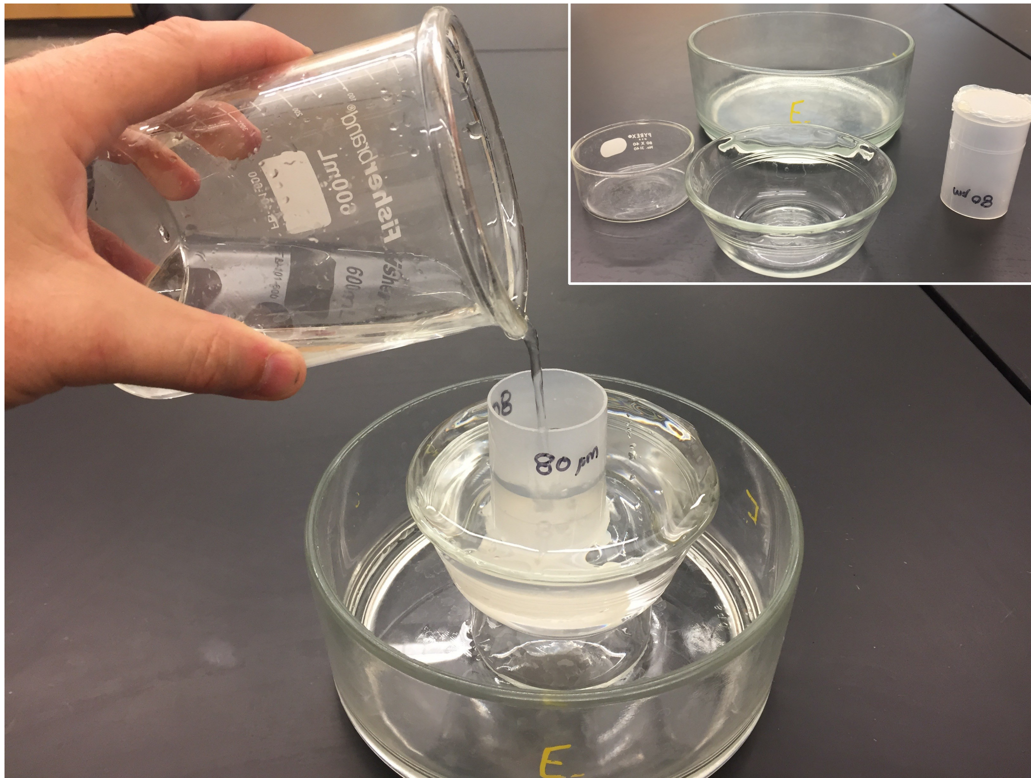



**Fig. 2. Forward filtration set-up as an alternative water change method for small scale culturing**. First, clean seawater is poured into the filter basket to cover the filter mesh. Then, as pictured, the culture is gently poured into the filter basket, allowing the water to fill (and spill over the sides of) the finger bowl and into the larger retention bowl. The finger 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 finger bowl, inverted over a clean culture vessel containing clean sea water, and rinsed into that vessel with clean seawater. The components of this system are shown in the inset. Note that, when assembled, the finger bowl sits atop the large Petri dish shown in the inset at left so that the rim of the finger bowl is above the rim of the larger retention bowl. Photos by B. Pernet.

**Fig. 3. An example medium scale larval culturing set-up**. Larvae and food are kept in suspension in this 75-liter 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.

**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. B) When larvae need to be moved, or if the tanks are in need of thorough cleaning, forward filtration is carried out using a screened bucket and tote system as described in the legend to Fig. 3. The principle is the same as the set-up shown in Fig. 2, but at a larger scale. Photos by D.L. Cohen.

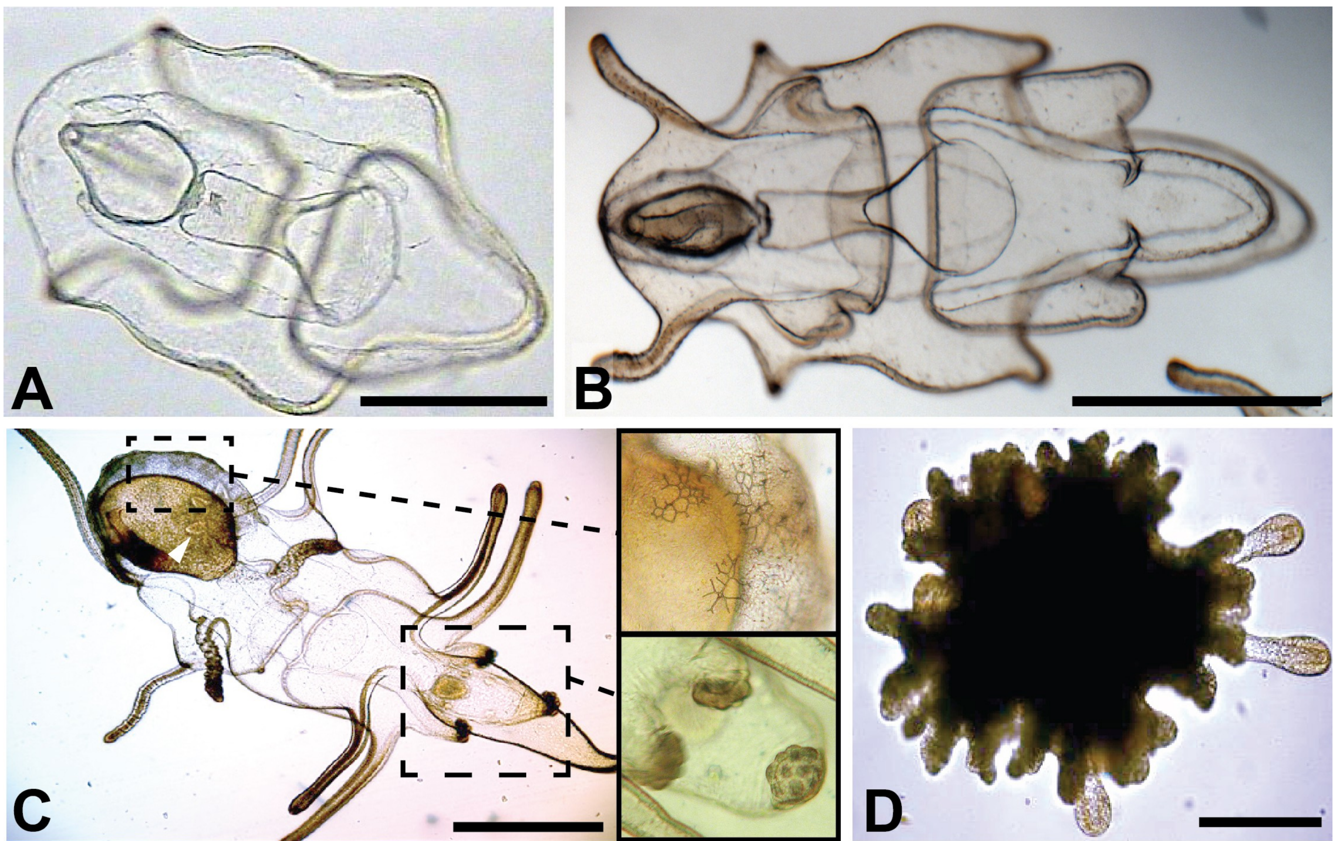



**Fig. 5. Asteroid planktotrophic larval development through metamorphosis**, as exemplified by the NE Pacific ochre sea star *Pisaster ochraceus* Brandt at approximately 14°C. A) Early bipinnaria stage larva. B) Early brachiolaria stage larva. C) Advanced brachiolaria. Note the juvenile rudiment with 5-fold 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)–200µm; (B)–400 µm; (C)–1000 µm; (D)–300 µm. Main panel photos by S. George; inset photos in (C) by J. Hodin.

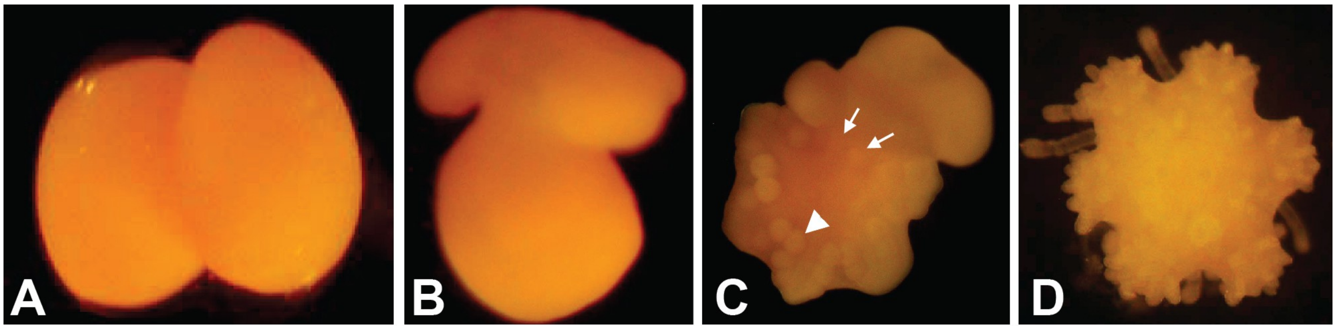



**Fig. 6. Asteroid benthic larval development through metamorphosis** in the six-armed brooding sea star *Leptasterias* hexactis Stimpson, from the NE Pacific. Dissected oocytes will mature by 1-methyladenine using standard methods (See Chapter 1 in this volume), and in this way, embryos can be cultured *in vitro* apart from the mother through the juvenile stage (see Hodin 2006). A) 2-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 5 of the 6 juvenile arms are well-formed– with 3 pairs of podia each. The primordium of the 6th arm is the last to form, here with only one pair of podia just visible (*white arrows*). D) Aboral view of 6-armed juvenile, with spines and tube feet visible. Scale bars: (A)–260µm; (B)–100 µm; (C,D)–120 µm. Photos by J. Hodin.

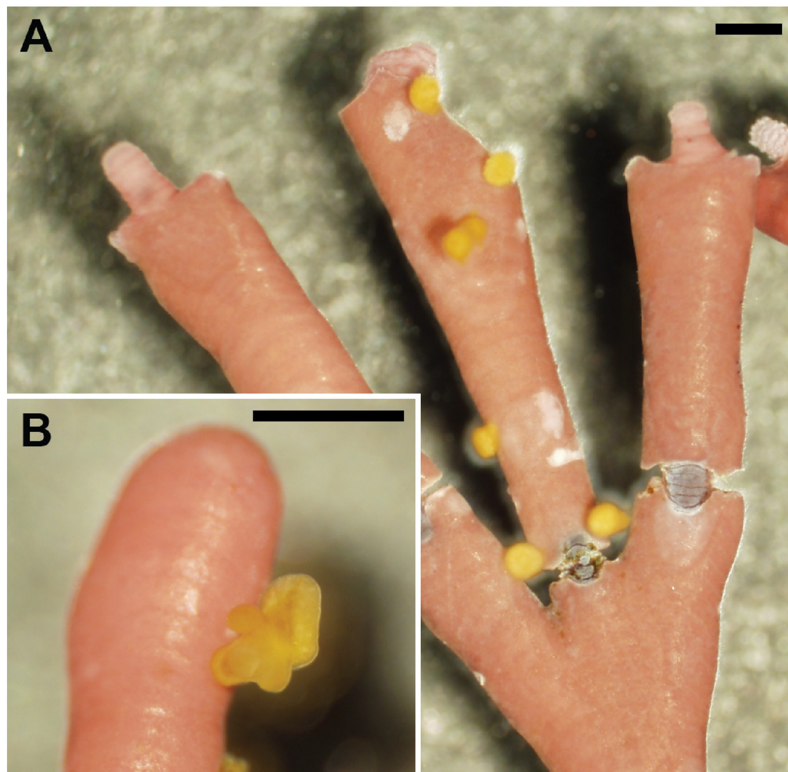



**Fig. 7. Asteroid settlement on coralline algae** (*Amphiroa sp.*), in the six armed cushion star *Meridiastra gunnii* Gray, from subtropical and temperate Australian waters. (A) Several settled *M. gunnii* larvae and juveniles (orange) on an *Amphiroa* frond. (B) Close-up of a single settled *M. gunnii* larva. Scale bars: approx. 1 mm. Photos by M. Byrne.

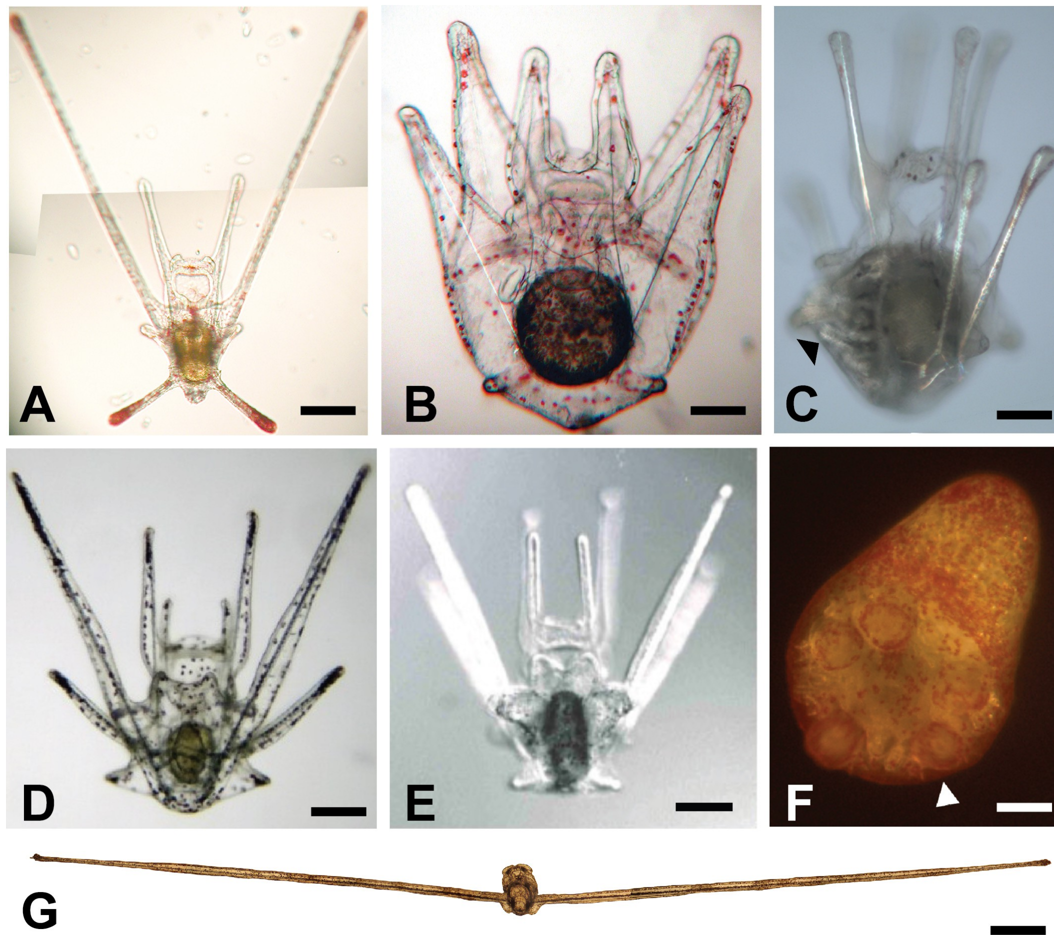



**Fig. 8. Larval diversity in echinoids.** A) 10-arm feeding pluteus of the NW Atlantic purple-spined sea urchin *Arbacia punctulata* Lamarck (Arbaciidae), pieced together from two photographs. B) 8-arm feeding pluteus of the NE Pacific purple sea urchin, *Strongylocentrotus purpuratus* Stimpson (Strongylocentrotidae). C) Advanced 8-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) 8-arm feeding pluteus of the tropical E Pacific urchin *Echinometra vanbrunti* Aggasiz (Echinometridae). E,F) Larvae from two sister species of Australian urchins from the genus *Heliocidaris* (Echinometridae) with radically different developmental modes. E) 8-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). E reproduced by permission from Byrne et al. (2001).

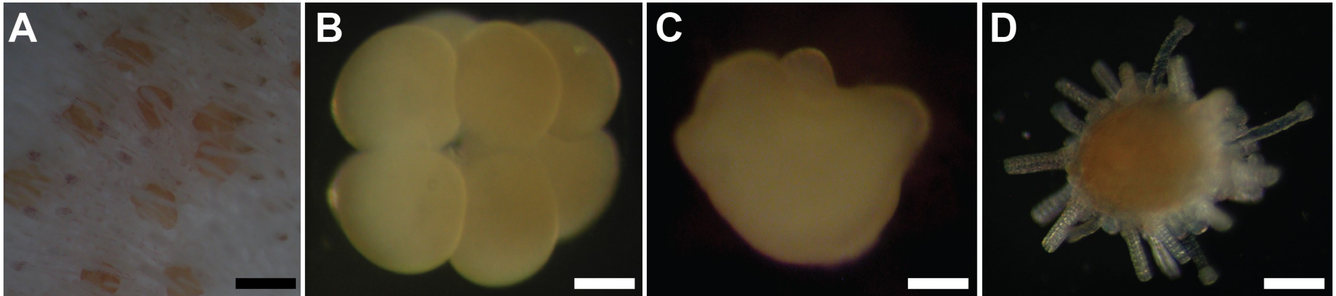
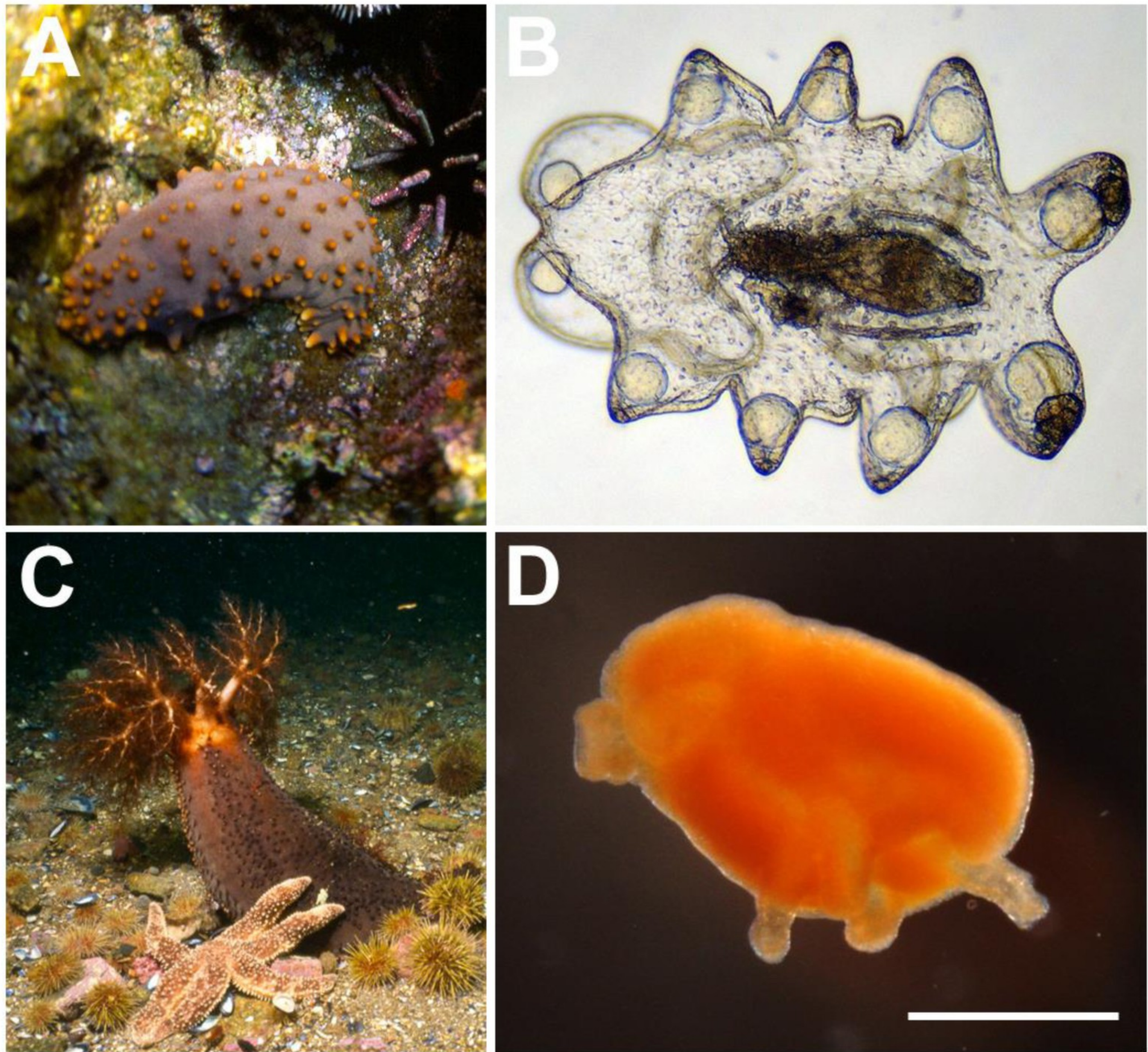



**Fig. 9. Echinoid benthic larval development through metamorphosis** in the brooding lamp urchin, *Cassidulis caribaearum* Lamarck (Cassidulidae), from the Eastern Caribbean. A) Larval brood among the oral spines of the mother. B) 8-cell stage. C) Non-feeding reduced pluteus larva (at approximately 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.

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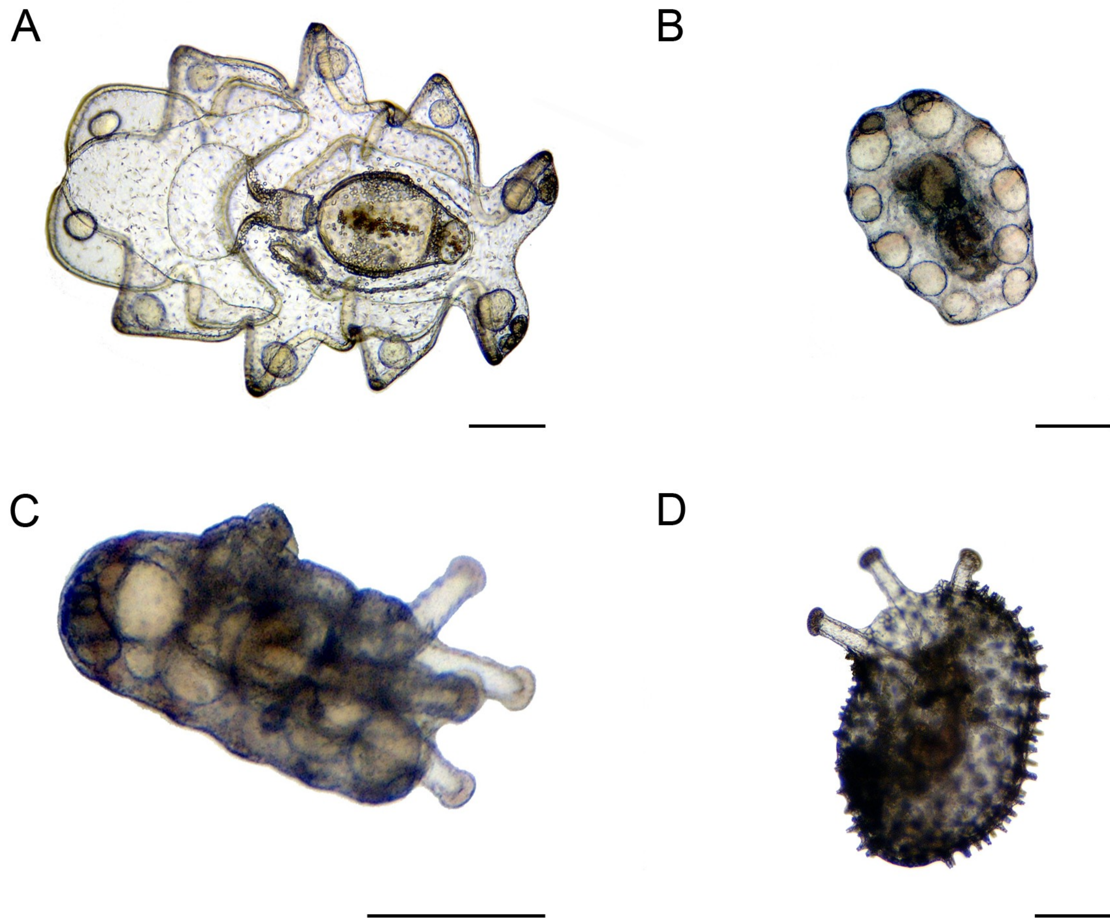


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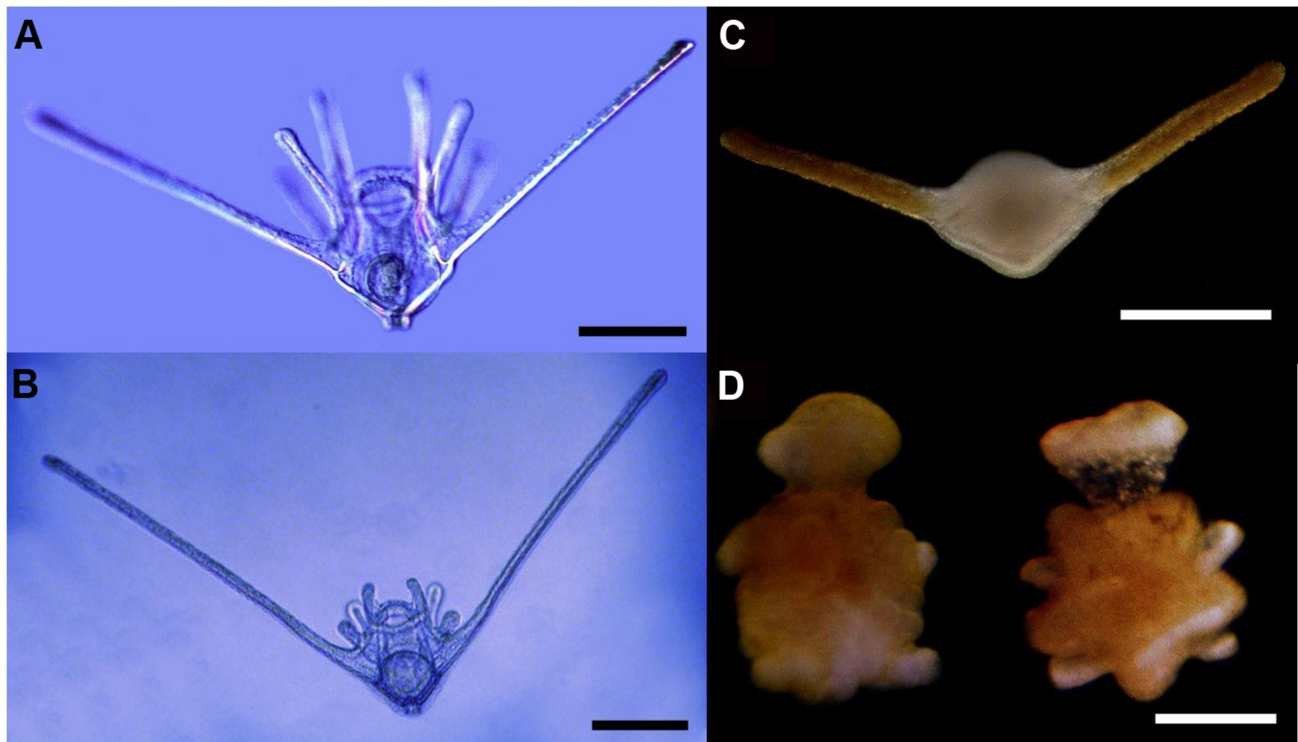


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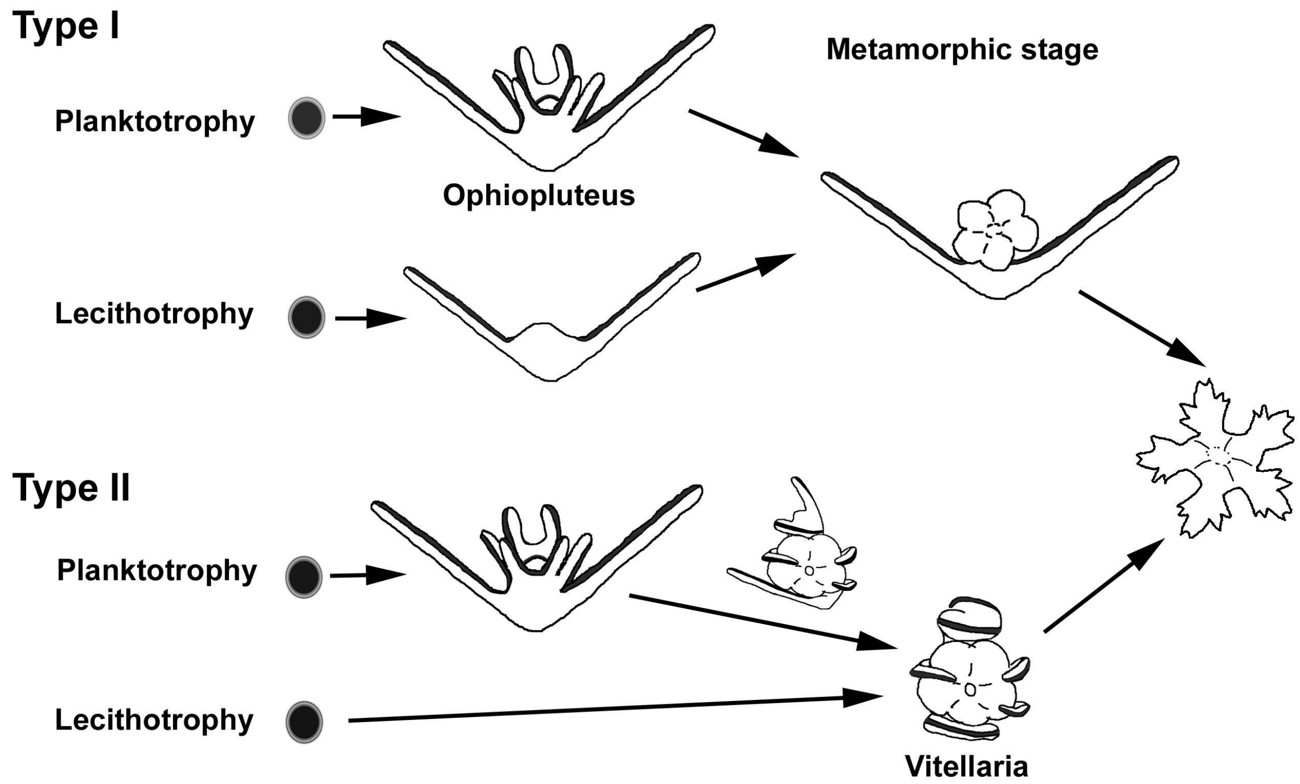


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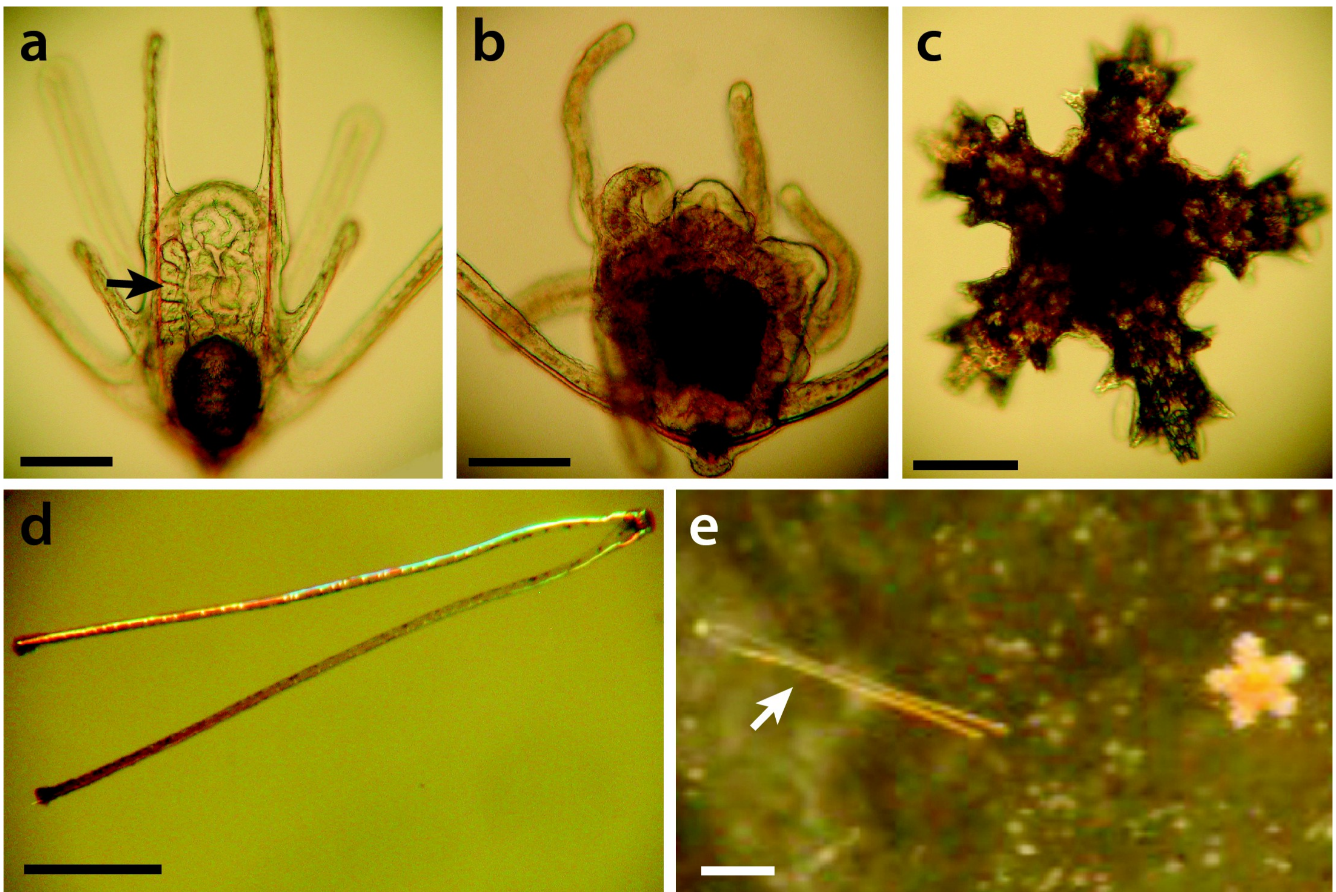


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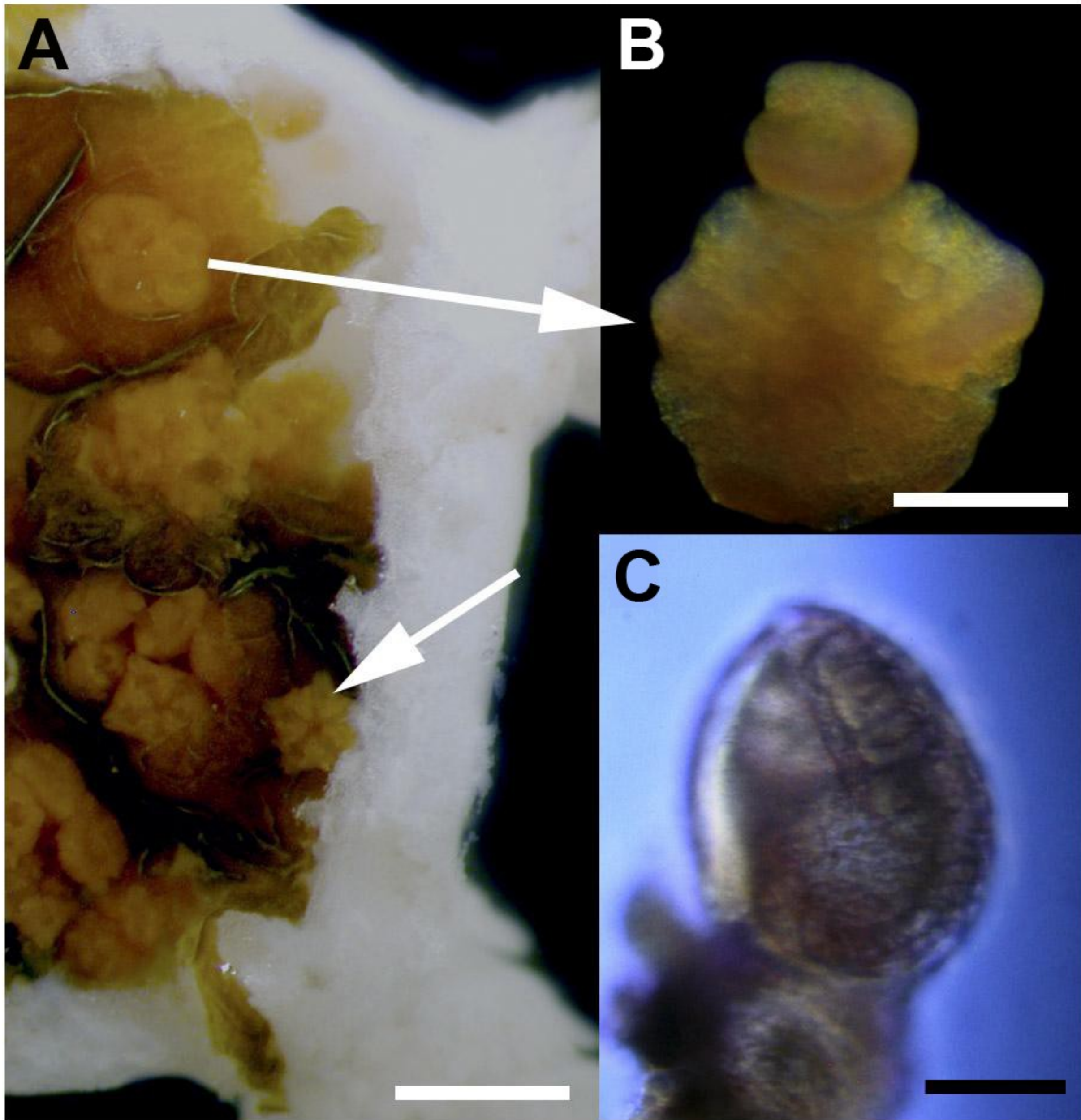


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