Video Article

The Barnacle *Balanus improvisus* as a Marine Model - Culturing and Gene Expression

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Abstract

Barnacles are marine crustaceans with a sessile adult and free-swimming, planktonic larvae. The barnacle *Balanus (Amphibalanus) improvisus* is particularly relevant as a model for the studies of osmoregulatory mechanisms because of its extreme tolerance to low salinity. It is also widely used as a model of settling biology, in particular in relation to antifouling research. However, natural seasonal spawning yields an unpredictable supply of cyprid larvae for studies. A protocol for the all-year-round culturing of *B. improvisus* has been developed and a detailed description of all steps in the production line is outlined (*i.e.*, the establishment of adult cultures on panels, the collection and rearing of barnacle larvae, and the administration of feed for adults and larvae). The description also provides guidance on troubleshooting and discusses critical parameters (*e.g.*, the removal of contamination, the production of high-quality feed, the manpower needed, and the importance of high-quality seawater). Each batch from the culturing system maximally yields roughly 12,000 nauplii and can deliver four batches in a week, so up to almost 50,000 larvae per week can be produced. The method used to culture *B. improvisus* is, probably, to a large extent also applicable to other marine invertebrates with free-swimminglarvae. Protocols are presented for the dissection of various tissues from adults as well as the production of high-quality RNA for studies on gene expression. It is also described how cultured adults and reared cyprids can be utilized in a wide array of experimental designs for examining gene expression in relation to external factors. The use of cultured barnacles in gene expression is illustrated with studies of possible osmoregulatory roles of Na^+/K^+ ATPase and aquaporins.

Video Link

The video component of this article can be found at https://www.jove.com/video/57825/

Introduction

Barnacles are marine crustaceans with a sessile adult and free-swimming, planktonic larvae. Most of the 1,200 species of barnacles inhabit shallow water and many are often exposed to low salinity. One species, the bay barnacle *Balanus (Amphibalanus) improvises* (*B. improvisus*), can tolerate almost freshwater and Charles Darwin described this species from a small stream in the estuary of Rio de la Plata in Uruguay[1]. The extreme tolerance tolow salinity makes *B. improvisus* a particularly relevant model for the studies of osmoregulatory mechanisms[2,3]. This barnacle prefers brackish conditions but is capable of living in waters with salinities from around 1.6 psu to as high as 40 psu[4]. It is the only barnacle species found in the brackish Baltic Sea. *B. improvisus* is believed to originate from the east coast of the American continent but today is found worldwide due to dispersal by shipping[5]. It is a major fouling organism and is commonly found on rocks, jetties, and boat hulls, and is therefore of general interest for understanding the mechanisms of biofouling on constructions in marine and brackish waters[6,7].

Similar to most other barnacles, *B. improvisus* is hermaphroditic with cross-fertilization; reproduction occurs through mating between neighboring individuals using an elongated penis and internal fertilization. The reproductive period is mainly from May to September. *B. improvisus* has seven pelagic larval stages (six nauplii followed by one cyprid stage[8]). The fertilized egg hatches into a nauplius larva which is free-swimming and feeds in the water column for up to several weeks before molting into a non-feeding cyprid larva. The cyprid uses multiple cues to find a suitable site to settle and then undergoes metamorphosis into a sessile juvenile barnacle[9]. The species can be cultured in the laboratory and has a lifespan of 1–2 years in the sea (2–3 years in laboratory culture). On average, *B. improvisus* grows to 10 mm in diameter (with a maximum of around 20 mm) and reaches a maximum height of about 6 mm (although it can grow taller under crowded conditions). The species can be identified by its smooth calcareous even shell (white or greyish), the radially patterned calcareous base of the shell plate, and the shape of the tergal plates[1,10].

The barnacle *B. improvisus* has several advantageous features as a model for studies of osmoregulation, with a focus on molecular and physiological mechanisms as well as ecological interactions and evolutionary consequences. It is also widely used as a model for the investigations of settling biology, in particular in relation to antifouling research and the mechanisms involved[7,11,12,13]. However, natural seasonal spawning yields an unpredictable supply of cyprid larvae for studies. The ability to culture this barnacle through its whole lifecycle all year round

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is, therefore, a major asset to enable various types of molecular and mechanistic studies. In addition, its presence in marine/brackish waters worldwide allows for a combination of field and experimental studies. Controlled breeding can also produce families of known pedigrees for long-term culturing[14], and a generation time of a few months may allow long-term experimental evolution. There is also a draft genome and several transcriptomes available, and these resources have been used for the cloning of several genes (*e.g.*, genes of importance in osmoregulation)[2,3].

The aim of this protocol is to describe how to establish and maintain a culture of the barnacle *B. improvisus* throughout the year in order to perform gene expression studies on adults or larvae of this organism. Rittschof *et al.*[15] briefly described a method for culturing barnacles from the release of nauplii to the settlement of cyprids for the species *Balanus amphitrite*. The protocol has been adapted for the all-year-round culturing of *B. improvisus* at Tjärnö Marine Research Laboratory (Sweden), and a detailed description of all steps in the production line is outlined, including the production and rearing of barnacle larvae, as well as the administration of feed for adults and larvae. For an overview of the complete procedure, see **Figure 1**. The use of the culturing system is exemplified with some common experimental set-ups and illustrated in functional genomics studies of Na^+/K^+ ATPase and aquaporins, elucidating their possible functions in osmoregulation[2,3]. It is sometimes essential to examine the gene expression in specific tissues, and some of the basics of barnacle dissection will be covered. With a good supply of high-quality seawater, the culturing of the barnacle *B. improvisus*, and potentially many other species, should be possible in marine laboratories throughout the world.

Protocol

1. Collection of Adult Barnacles in the Field to Start a New Broodstock

1. **Deploy thermoplastic (poly-methyl methacrylate) transparent panels (110 x 110 x 1.5 mm^3) at ≈ 1–3 m depth in calm waters in order to collect adult barnacles from the field. Use frames that can take several panels (Figure 2A), drill 2 holes (with a diameter of 6 mm) in the top corners of each panel and attachthepanelstotherackusingcableties. Thus, thepanelswillhangverticallyin the water.**
    1. When the settlement of barnacles has occurred (identified as small white hard dots on the panels), leave the panels for at least 2–3 weeks to make sure that the barnacles are large enough (5 mm) to be transferred to the lab without getting dried or damaged.
2. Bring the panels into the laboratory when they are covered with settled *B. improvisus* that have reached a size of *ca*. 5 mm indiameter.
NOTE: The time for adults to develop to 5 mm in the field is highly dependent on the availability of food and the temperature. Normally, this takes about 3 weeks at the Tjärnö Marine Research Laboratory (58.87 °N, 11.14 °E). Most commonly, panels for the new broodstock are generally deployed in late June and collected at the end of August.
3. Before introducing the panels into the culture facility, clean other organisms from the panels. It is most important to remove other barnacle species. Please note that it is important to frequently clean the panels to get rid of contaminating species during the cultivation since this will significantly increase the chances of survival of the broodstock.
4. Place the panels vertically on edge in a stand with milled grooves in a polyethylene tray (400 x 400 x 20 mm^3) (**Figures 2C** and **2E**). The grooves in the stand are 15 mm apart.
5. In order to prepare the trays, make an entrance port at the base and an exit port at the top on the opposite side of each tray. Connect the entrance port of each tray to a 100 L reservoir containing about 30 L of water with an inlet of open seawater at 20 °C to allow a flow-through at a rate of *ca*. 2 L per min.
NOTE: Up to 8 trays were connected to a single 100 L reservoir.
6. Connect the 100 L reservoir to temperature-controlled seawater (20 °C) (*e.g.,* provided by a heat pump exchanging heat from the incoming seawater).
NOTE: The barnacle *B. improvisus* can grow and reproduce at a full marine salinity (30–35 psu); however, reducing the salinity to *ca*. 25 psu by adding freshwater to the 100 L reservoir increases the output of larvae from the culture.

2. Starting New Generations of Adults from Cultured Cyprids

1. Construct cubes of thermoplastic panels open at the top by taping 5 panels together using a non-toxic, water-resistant tape.
2. Place the cube in a small tray (in case of leakage) and fill it with seawater (25 psu). Keep the water at room temperature (*ca*. 20 °C). Add roughly 200 cyprids at the top of the cube.
3. Feed the juveniles with 100 mL of *Skeletonema marinoi* every other day (see step 5) while in the cube.
NOTE: Juvenile barnacles can have difficulties with ingesting *Artemia salina* (see step 3) since they are big and, hence, difficult to swallow for a newly settled barnacle.
4. Change the water 1x a week.
5. After 2 weeks, when the panels contain enough established juveniles of at least 5 mm in diameter, take the cube apart and move the panels with the juveniles to trays with flow-throughseawater and, thereafter, feed the barnacles with *A. salina* nauplii.

3. Culture of *Artemia salina* Nauplii as Feed for Adult Barnacles

1. Set up a culturing system for *A. salina* by using a 1.5 L plastic bottle where the bottomis cut off and the bottle is placed upside-down in a stand and illuminated from the side. Fit the bottleneck to a silicon tube with a clamp and attach it to an aeration pump (**Figure 2D**).
2. In order to hatch the eggs, add about 15 mL of dry resting eggs of *Artemia* to 1 L ofseawater.
NOTE: *Artemia* eggs hatch into nauplius larvae after 24–48 h. To delay the hatching of *A. salina* nauplii (*e.g.*, during weekends), the light can be turned off to reduce the temperature slightly.
3. To harvest *Artemia* nauplii, turn off the aeration and darken the upper part of the bottle with aluminium foil (or similar *e.g.*, a can).. Illuminate the lower part of the bottle for 10 min. Hatched *Artemia* nauplii will swim toward the light. Non-hatched cysts will sink to the bottom, and cyst shells will float at the surface.
4. Open the clamp at the bottom. Collect the intermediate fraction as a dense population of the swimming nauplii.

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NOTE: Every day (except on weekends), 1 L of the dense *Artemia* nauplii suspension was manually added to the 100 L reservoir connecting to the trays with the adult barnacles. Avoid any feeding with empty cyst shells, since they do not provide nutrition and mainly result in needing to clean the culture at more frequent intervals.

4. Collection and Rearing of Barnacle Larvae

1. Clean the panels with adult barnacles by gently spraying them with freshwater and, if necessary, remove any fouling from the barnacle shells and panels using a soft toothbrush. Also, clean the trays (without barnacles) and tubes in hot (75 °C) freshwater.
2. Place a sieve made of a 90 µm plankton-net glued to the end of a cut PVC pipe (16 cm in diameter, 15 cm in height) into a polyethylene tray (30 x 20 x 10 cm^3) with an overflow port. Collect the *B. improvisus* larvae in the sieve overnight (**Figure 2C**).
   NOTE: The sieve was positioned just below the exit port of the tray of the barnacle panels to receive the outflowing water and to filter out the nauplii. The larvae remained in the sieve while the seawater overflowed the small tray. This small tray ensured that the sieve never driedout.
3. Place a 30 L bucket in a large water bath where the temperature is maintained at 26 °C using aquarium fish tank heaters (**Figure 2E**). Aeration and agitation are assured by air-bubbling through the system.
4. Fill the bucket with 20 L of filtered seawater (0.2 µm; 25 psu). Add 1 L to the bucket of a 60/40 mix of the 2 diatom microalgae, *S. marinoi* and *C. gracilis* (see step 5). This will give an initial density of about 5 x 10^4 diatoms per mL in the bucket.
   NOTE: Barnacle nauplii larvae are positively phototactic. The buckets were made of opaque white plastic with lids that let through some light. During the winter, the room was lit during the daytime (8:00–17:00), but dark during the nighttime. During the summer, the outside light came into the room during most of the day.
5. Transfer the collected *B. improvisus* nauplii to a crystallizing dish (300 mL; 90 mm in diameter; 50 mm in height).
6. Illuminate the dish from the side, which will attract the larvae to the light source. Collect the barnacle nauplii that gather at the incoming light with a pipette and transfer them to another crystallizing dish. Remove any remaining *Artemia* larvae.
7. Transfer the *B. improvisus* nauplii larvae to a beaker with 1 L of filtered seawater.
8. Count the number of nauplii by stirring the beaker gently to get an even suspension of larvae and then taking five 1 mL samples from different places in the beaker. Transfer each sample into a microplate for a visual inspection with a stereomicroscope. Count the number of nauplii in each of the five samples and add them up.
9. Multiply the counted number by 200 (1,000 mL/5 mL) to estimate how many thousands of larvae are in the whole beaker. If there are too many nauplius larvae in the beaker, dilute the sample until the density is at most 14,000 larvae/L (usually in the range of 11,000–12,000 larvae/L).
10. Add 1 L of *B. improvisus* nauplii to one bucket, thus adding roughly 11,000–12,000 larvae per bucket.
    NOTE: Make sure not to add more nauplii, since this will result in too little food in relation to the larvae and, hence, increase mortality.
11. After 3 days, collect barnacle nauplii on a 90 µm sieve. Then clean the bucket (with 75 °C freshwater), fill it with filtered seawater, add a new diatom feed (the same amount as at the start), and finally add the nauplii again. Cyprids start to appear after about 6 days (± 1 day).
12. Collect cyprids first with a 90 µm sieve (designed as in point 4.2 above). Then separate non-moulted barnacle nauplii and cyprids with a 320 µm sieve on top of a 160 µm sieve. *B. improvisus* nauplii will collect in the 320 µm sieve and cyprids in the 160 µm sieve.
    NOTE: The cyprid larvae may be stored at 10 °C in a crystallizing dish in darkness for later use, up to *ca*. 6 days. However, the storage can affect the quality and performance of the barnacle larvae, so experiments comparing different treatments should ideally use larvae from the same batch (and similar storage time) to avoid confounding effects[16].

5. Culture of Microalgae as Feed for Barnacle Nauplius Larvae

NOTE: Algae were grown in 3 different types of cultures: (i) stock cultures, which are for the long-term maintenance of the strains that were being used for the inoculation of the scaling-up; (ii) start cultures, which are the first step in the scale-up; and finally (iii) the production culture, which is the final production scale of large quantities of algae as barnacle feed.

1. Order diatom species from the Culture Collection of Algae and Protozoa (CCAP) to be used as feed for the barnacle nauplius larvae. The 2 species *Skeletonema marinoi* (CCAP strain 1077/5) and *Chaetoceros simplex* var. *gracilis* (CCAP strain 1085/3) both give good results as feed.
2. Filter all seawater to be used in the algal cultures, using a cartridge filter system with a nominal pore size of 0.2 µm. (The filtered seawater is also to be used for the hatching of *A. salina* eggs, and the culturing of barnacle nauplius larvae.) Autoclave the filtered seawater for the algal cultures (at 105 °C for 5 min).
3. Prepare a medium for the culture of microalgae, using autoclaved seawater enriched with Guillard's f/2 solution containing inorganic nutrients, trace metals, and vitamins (see Guillard 1975[17] for the detailed recipe). In addition, prepare a solution of silicate (Na_2SiO_3), but keep this separate from the f/2 enrichment to prevent a solid precipitation.
   NOTE: The concentrations of the enrichment stock solution and the stock solution of silicate were prepared to be used as a 1 mL addition of each to 1 L of seawater.
4. Autoclave all equipment used in the algal culture at 120 °C for 20 min. Autoclave screw-capped test tubes with 1 mL of f/2 enrichment and 1 mL of silicate solution. Add the enrichment and silicate solutions to theseawater when the autoclaved glassware and liquids have cooled down to room temperature.
5. **Grow stock cultures of microalgae in 40 mL test tubes with screw caps.**
   1. Fill the test tubes with about 30 mL of the enriched seawater. Inoculate the cultures with a sterile Pasteur pipette using about 1 mL of a 2 week old stock culture. The screw cap is then fitted but not tightened,to allow some gas exchange. If available, the inoculation should be carried out in a laminar flow cabinet to reduce the risk of contamination.
      NOTE: The aim of the stock cultures is to maintain algal cultures on a long-term basis and to serve as inoculum for start cultures. Stock cultures were re-inoculated every 2 weeks.
   2. Expose the new stock culture to a white light with an intensity of *ca*. 25–50 µmole $m^{-2}s^{-1}$ (with a light-dark cycle of 16:8 h). Move the old stock cultures to a low light intensity as a back-up. Discard any 2 week old stock cultures.

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6. **For the scaling up to the production culture of algae, inoculate the start cultures from the stock culture and grow them in 500 mL Erlenmeyerflasks.**
    1. Autoclave 4 Erlenmeyer flasks, pipettes and cotton stoppers with 300 mL of filtered (0.2 µm) seawater, and 4 test tubes with 0.3 mL of f/2 enrichment and 4 with silicate solutions. Cool down the f/2 enrichment and the silicate to room temperature, and add the solutions to the Erlenmeyer flasks. Inoculate them with *ca*. 1 mL of the 2-week old stock culture.
    NOTE: Prepare 2 flasks with *S. marinoi* and 2 with *C. simplex*.
    2. Put the flasks on a shaking table in a light intensity of 50 µmole $m^{-2}s^{-1}$. When the start cultures have acquired dense algal populations (yellow-brown in color), they are ready to be used as inoculum for the production cultures that will provide the barnacle larvae with food.

7. **Grow production cultures in 4 L polycarbonate bottles with silicone stoppers with 2 drilled ports fitted with glass tubes.**
    1. Connect 1 glass tube reaching to the bottom of the bottle to an air pump *via* a silicone tube fitted with a 0.2 µm air filter. Make sure the second glass tube ends just below the silicone stopper and is filled with cotton to allow the exit of injected air.
    2. Each week, fill four 4 L bottles with filtered seawater and autoclave them together with the stoppers. In addition, autoclave 4 test tubes with 4 mL of f/2 enrichment and silicate solutions.
    3. After cooling, add the f/2 enrichment and silicate solutions to the bottles and then inoculate them with half of the volume of the start-up cultures in the Erlenmeyer flasks.
    4. Place the four 4 L bottles at a light intensity of 50–100 µmole $m^{-2}s^{-1}$.
    5. Harvest the production cultures after *ca*. 1 week; they are then sufficiently dense to be used for feeding the barnacle nauplius larvae.
    NOTE: Production cultures have a lifespan of about 2 weeks, which means that there are 8 active production bottles at any time.

6. Designing Experimental Studies Using Barnacles

1. **Place panels with juvenile or adult barnacles in controlled aquaria where they can be grown under identicalconditions. This is called a common-garden experiment, which, for instance, can be used to understand local adaptions or phenotypic plasticity[18], or to study gene expression changes in relation to external factors (*e.g.*, salinity, temperature, or pH).**
NOTE: It is also possible to use barnacles directly collected from the field on panels. An advantage of using laboratory-bred individuals is that maternal effects can be avoided when using the next generation of offspring.
    1. Expose barnacles to the specific environmental conditions during a chosentime interval, followed by the harvesting of adults (*e.g.*, for studies on gene expression)[2].

2. **For experiments with cyprid larvae to study gene expression[3], place the cyprids in controlled aquaria where larvae can be cultivated under identical conditions.**
    1. Harvest the cyprids after specified time periods by filtering with sieves (as described in step 4.7), and extract RNA according to the protocols below (step 8).

7. Dissection of Barnacles

1. Clean the lab space where the dissection and DNA sampling are performed, bothbefore and between individuals, including all dissection tools. This is done using chlorine for the bench (or 96% ethanol for the forceps).
NOTE: Labelled tubes containing fixation media (ethanol or an RNA stabilization solution) are prepared in advance.
2. Select large and short-term starved individuals (do not feed them for 2 days prior to the dissection). Clean the barnacle shell with a toothbrush to minimize the risk of contamination from other species (*e.g.*, bacteria, algae) and rinse it off with water.
NOTE: The reason for the short-term starvation of individuals before the dissection is to avoid any DNA contamination (*e.g.*, from *Artemia* cysts in the gut).
3. **Place the individual barnacles on an even surface, either attached to a panel or loose in a dish. Dissect the respective tissues from the adult. There are several ways to dissect barnacles, depending on the purpose of the study.**
    1. Dissection method A: fixing the whole barnacle as quickly as possible.
        1. Remove the whole barnacle from the panel by using a scalpel, inserting it under the barnacle and close to the panel surface.
        NOTE: In most cases, this manipulation leaves the basal plate almost intact, thus not affecting the barnacle inside.
        2. Carefully crack the outer shell on one side by inserting forceps (**Figure 3**). This is done to facilitate the intrusion of a fixating medium inside the shell.
        NOTE: If the shell is not broken at all before placing the barnacle in a test tube, it may lead to a slower fixation process and an inferior quality of DNA later.
        3. Put the broken barnacle in the test tube containing either an RNA storage solution or ethanol. Leave the barnacle in the solution for at least 24 h for its fixation. When the barnacle is fixed, the animal can be taken out of the fixation media and placed on a dissection tray. The cirri, mantle, and soma (body) can be separated from each other and placed in separate tubes for further extractions of DNA or RNA.
        NOTE: It is important to observe if egg lamellae with fertilized eggs are present inside the barnacle. If present, these should be removed before proceeding with the DNA extractions to avoid finding multiple genotypes in the sample.

    2. Dissection method B: removing the barnacle from the shell before its fixation.
    NOTE: This method may not always result in the mantle being sampled since it is attached to the inside of the calcareousshell. But it can be a quick method for sampling DNA from an individual.
        1. Carefully remove, by using dissection forceps, the tergal and scutal plates (**Figure 3**) by inserting the tip of the forceps between the tergal and scutal plates, grabbing hold of a plate, and pulling gently to remove them.

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2. Grab hold of the cirri with the forceps and pull the barnacle straight out and place it directly in 96% ethanol or RNA stabilizing solution for fixation..
NOTE: Sometimes, the mantle will also be sampled at the same time, as seen as a thin epithelium with dark pigmentation (in *B. improvisus*). Otherwise, the mantle can be removed from the inside of the shell using a scalpel and, thereafter, placed in ethanol or RNA stabilizing solution.
NOTE: The tergal and scutal plates (if intact) can be dried and saved since they are useful for the species' identification[10].
A general recommendation, if there is any doubt of species, is to also photograph the whole barnacle prior to initializing the dissection.

8. RNA Extraction for Quantitative PCR

1. Put adults to be used for RNA extractions into an RNA stabilizing solution, incubate them overnight (up to 24 h) at 4 °C, and then store them at -80 °C.
NOTE: Cyprid larvae are preferentially dry-frozen, without RNA later directly in -80 °C, by placing them in cryotubes and then submerging the tubes briefly (30 s) in liquid nitrogen before storing them at -80 °C.
2. At the time of the RNA extraction, thaw the barnacles on ice and use them intact or dissect out the tissues to be used (see step 7).
NOTE: Due to high genetic diversity between individuals (≈ 3–5% variation in coding regions; Alm Rosenblad *et al.*, unpublished data) it is advisable to poola number of adult individuals, which minimizes the effect of sequence variation between individuals in the later qPCR step.
3. For the RNAextraction, add 350 µL of lysis buffer provided with an RNA preparation kit into homogenization tubes containing 2.8 mm of ceramic beads.
NOTE: Ceramic beads provide a better yield of RNA compared to sonication (**Representative Results**).
4. Take an adult barnacle (whole or tissues) or collect a minimum of 20 cyprid larvae using forceps and put them into homogenization tubes.
5. Proceed directly to the disruption and homogenization with a bead mill.
6. Put the homogenization tubes with the sample and beads in the bead mill holder. Shake them with a frequency of 4.0 m/s for 20 s. Cool the sample for 1 min on ice. Repeat 2x.
7. Prepare the RNA according to the protocol of the commercially available RNA isolation kit.
NOTE: A risk assessment (including a reading of the safety data sheets) should be performed prior to using RNA extraction methods, to identify hazardous chemicals that require the use of a fume hood and other protective clothing, including gloves (*e.g.*, the addition of β-mercaptoethanol to the lysis buffer during the RNA extraction should be performed in a fume hood).
8. Quantify the RNA. Prepare a working solution and add a standard and samples to a total volume of 200 µL. Vortex them for 2–3 s and incubate them for 2 min. Insert the tubes into a fluorometer and take readings.
9. Check the RNA samples for any protein contamination with a spectrophotometer, and check their RNA integrity with an automated electrophoresis system (**Representative Results**).
NOTE: For good quality preparations, RNA is recommended to have a ratio of 260/280 nm of 2–2.2 and a DNA of around 1.8. Lower values indicate protein contaminations and that the extraction procedure has to be optimized.

9. Gene Expression: cDNA Synthesis and qPCR

1. **Treat the obtained RNAwith DNase to remove any remainingDNA before making the cDNA for qPCR.**
    1. Check for contaminating genomic DNA by adding an RNA sample but no reverse transcriptase when preparing the cDNA. If there is a PCR amplification of the selected gene on the cDNA made without reverse transcriptase, there is DNA contamination in the RNA.
    NOTE: For samples to be used for RNA-seq (RNA-sequencing using next-generation sequencing technology), the DNase step is less critical. In particular, for samples with low levels of RNA, the DNase step might be omitted in order not to lose too much of the RNA in the process.

2. Perform cDNA synthesis on DNase-treated RNA using an amount of RNA within the range specified in the commercial cDNA synthesis kit, but usually, use at least 50 ng.
3. Design qPCR primers so that they anneal to parts of the gene of interest where the sequence identity between individuals is as high as possible, but the sequence identity between gene paralogs is as low as possible. Please see the corresponding publications for the specific primer-pairs used for gene expression studies of Na^+/K^+ ATPase[3] and aquaporins[2] (**Figure 5**).
NOTE: For this purpose, RNA-seq sequence data from hundreds of cyprids or several adults can be used.
4. Design primers for a gene to be used for the normalization of the expression levels (*e.g.*, actin). The primers used successfully for actin are, forward: 5'-CATCAAGATCAAGATCATCGC-3', and reverse: 5'-ATCTGCTGGAAGGTGGAC-3'.
NOTE: Actin is a commonly-used control gene. However, others have also been suggested as a reference and tested out on barnacles[19]. Besides actin, the use of RPL8, 36B4, EF1, and NADHd1 as a reference has been tested[3]. It was concluded that there are no large differences in the expression levels of the respective reference genes between soma, cirri, adult, or cyprids.
5. Optimize the annealing temperature for the designed primers by adding increasing amounts of cDNA (typically in the range of 0.25–50 ng) to a mix containing SYBR Green, dNTP, polymerase, and 0.3 µM each of forward and reverse primer.
6. Run qPCR protocols at different annealing temperatures as follows: an initial denaturation temperature of 95 °C for 3 min, a denaturation step at 95 °C for 20 s, annealing temperatures of 55–63 °C for 20 s, and an elongation at 72 °C for 30 s. In total, run 40 PCR cycles.
NOTE: Primer efficiencies should lie in the range of 90–105% and are calculated as E = (10^(-1/slope)-1) x 100 where the slope is the slope of the curve obtained when plotting the log value of the cDNA concentrations against their cycle threshold (Ct) values.
7. Perform qPCR using an appropriate amount of cDNA, usually 1–10 ng, using the PCR protocol described in step 3 with the optimum annealing temperature found.
NOTE: The higher amount of cDNA is only used for genes that are expressed in extremely low amounts.

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Representative Results

With the described procedure for the culturing of adult barnacles of *B. improvisus*, up to four batches of nauplius larvae can be produced per week. It would be possible to collect nauplius larvae almost every night, but this requires more people and infrastructure (with many barnacles in the broodstock, a culture will release larvae continuously). An additional limiting factor for the larvae production appears to be the availability of feed of high quality, in particular regarding the diatom *Skeletonema*. Maximally, each batch from the culturing system consists of roughly 12,000 nauplii, so up to 50,000 nauplii per week can be cultured. However, some weeks there may be up to tenfold fewer larvae produced. A single adult can produce up to 7,000 larvae per day[14], which means that 1–2 adults are releasing larvae for each batch. Within a week, about 70–90% of the collected nauplii will develop into cyprids (yielding roughly 30,000 cyprids per week, maximally) that can be used for settlement assays and molecular studies.

It should be stressed that there are variations in cyprid features between batches, and in general, there are larger variations *between* batches than *within* batches. For example, the settling success in settlement assays varies between 30 and 70% for different batches. Most likely, this is caused by the individual genetic variation between the specific pairs of adults releasing larvae during the different sampling periods. It is, of course, recommended that repeated experiments (biological replicates) should include cyprids from a number of batches if more general statements about the results are to be made. The batch-to-batch variation puts demands on the experimental design, where proper controls and normalizations in gene expression studies should be applied. However, even after several statistical normalization procedures have been implemented that considerably reduce between-batch variation, some effects of the batch are usually still apparent (unpublished data).

Following the provided protocol, it is possible to obtain, on average, 500 ng of high-quality RNA from as little as 20 cyprids, irrespective of the stage of barnacle settlement (**Table 1**). The quality of the RNA is usually measured as the ratio between the 18S and 28S peaks (the expected position of the two peaks are indicated in **Figure 4**). However, in the case of barnacles and many other arthropods, the 28S rRNA breaks down when heated (as part of the analysis method) and migrates together with the 18S peak[20]. This is why there is, in principle, one single rRNA peak in this type of analysis for barnacles. It is clear from this test (**Figure 4**) that a homogenization by ceramic beads provides the RNA with the highest integrity and is, therefore, the method of choice. The RNA is sufficient in amount and quality to generate high-quality sequencing libraries for sequencing, resulting in an average of 70 million reads per sample (the number of reads, of course, depends on the level of multiplexing during the sequencing). The amount of RNA is also sufficient for cDNA synthesis and qPCR expression analysis of a large number of genes.

Figure 5 shows the result from qPCR analyses of aquaporins and of Na^+/K^+ ATPase (NAK1) splice variants, where expression changes were investigated in response to changes in environmental cues[2,3]. A comparison of the relative expression of the long and short splice variants of NAK1 shows a twofold increase for the long NAK1 mRNA in low salinity in relation to the short NAK (**Figure 5A**). Thus, the data indicate that alternative splicing makes the long form predominant under low salinity conditions. In the case of aquaporins, it is apparent that the two water-transporting paralogs AQP1 and AQP2 display differential expression (**Figure 5B**). In particular, in the mantle tissue, it is apparent that the AQP1 is substantially down-regulated at lower salinities, which is not seen for AQP2. Instead, AQP2 shows a slightly increased expression at lower salinities, but in the soma. These findings provide a base for investigations of the functional roles of the different *B. improvisus* ion transporters and aquaporins in barnacle osmoregulation.

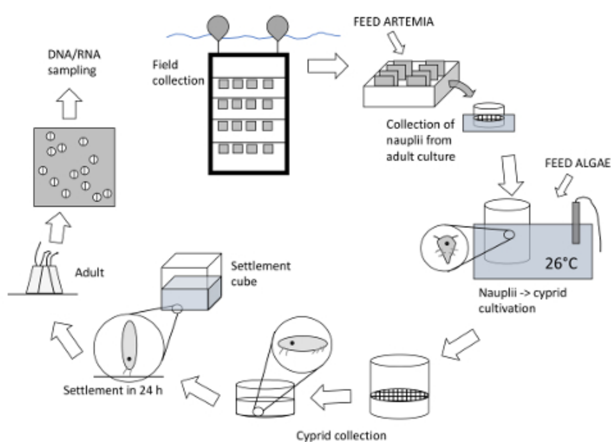

Figure 1: Overview of the whole culturing procedure and the RNA extraction for gene expression studies in adults. To initiate a new culture, panels are attached to a frame and deployed in the sea at 1 - 3 m depth. After several weeks, the panels with adults/juveniles are placed vertically in racks in trays in the laboratory. Each tray holds about 40 panels with adults. With roughly 100 adults per panel, in total ≈ 4,000 adult individuals are cultured per tray. The adult barnacles are fed with *Artemia* and can be kept year-around. Nauplius larvae are collected several times per week from the trays *via* a filtering through a sieve. The collected nauplii are transferred to buckets kept at 26 °C in a water bath and fed with microalgae. Nauplii are reared until they molt into non-feeding cyprids, which are collected by filtering. New panels can be established in the laboratory by the settling of cyprids on panels, either to provide new panels for the year-around culture or to be used for specific experimental set-ups with altered external conditions. RNA is then extracted from juveniles/adults at the end of the experiment or at specific time-points. Please click here to view a larger version of this figure.

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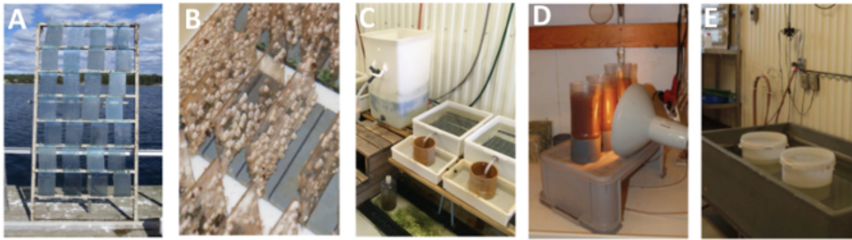

Figure 2: Images of some important steps in the culturing procedure. (**A**) This image shows the frames with panels for collecting new populations from the field. (**B**) This image shows the panels with adult barnacles of *B. improvisus* in racks that are placed in trays. The panels are placed about 2 cm apart. (**C**) This image shows the trays with the barnacle panels and the feeding tank to the left. From each tray, there is an outlet where the sieves are placed for the collection of nauplius larvae. (**D**) This image shows the production of *Artemia* feed for the adult barnacles. (**E**) This image shows the rearing of nauplii in buckets placed in a water bath set to 26 °C. Please click here to view a larger version of this figure.

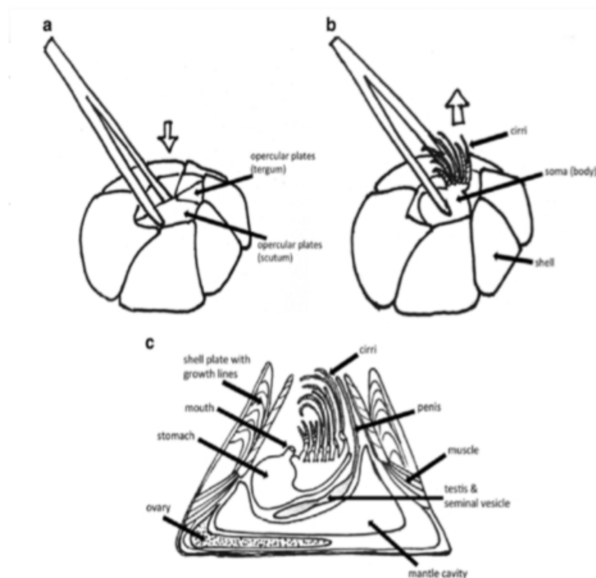

Figure 3: Description of the initial dissection steps of adult barnacles: removing the body from its shell. (**A**) Grab one of the opercular plates by inserting forceps gently through the aperture. Pull gently to remove the plate and expose the animal. (**B**) Pull out the animal by grabbing the soma part just below the cirri. (**C**) This panel shows the overall anatomy of acorn barnacles. The mantle and potentially fertilized eggs (in the ovary) stay in the shell cavity when the body is pulled out. A note on barnacle anatomy (for a more in-depth account, see Anderson)[9]: the wall plates of a barnacle slope inward, and together, they form a volcano-like cone. An opening, the aperture, is covered by the two opercular plates, which form a door, or operculum, to close the aperture. Acorn barnacles generally have a calcareous basal plate that is glued firmly to the substratum; however, some species of barnacles lack this calcareous plate (*e.g.*, *S. balanoides*). Barnacles secrete the exoskeleton from the darkly pigmented mantle (the carapace). The outer surface of the double-layered mantle is calcified to become rigid, while the inner surface of the mantle is not calcified and is therefore flexible. Inside the aperture, the cirri are present in a retracted position. These are the thoracic appendages that barnacles use for suspension feeding. The ovaries are located close to the base of the barnacle, while the testes lie in the soma. This figure has been adopted from Panova *et al.*[21] and has been published with permission from Springer. Please click here to view a larger version of this figure.

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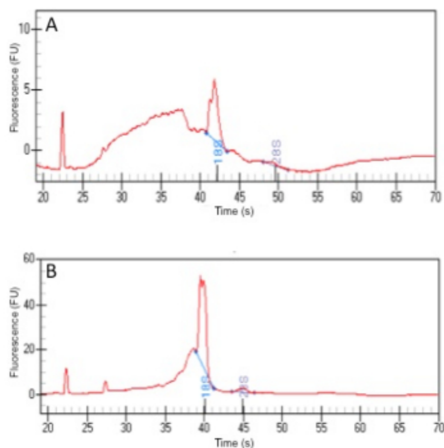

Figure 4: Determination of rRNA integrity by capillary gel electrophoresis. RNA was prepared by two different homogenization methods: (**A**) sonication and (**B**) ceramic beads. The unit on the y-axis, FU, stands for fluorescence units. Please click here to view a larger version of this figure.

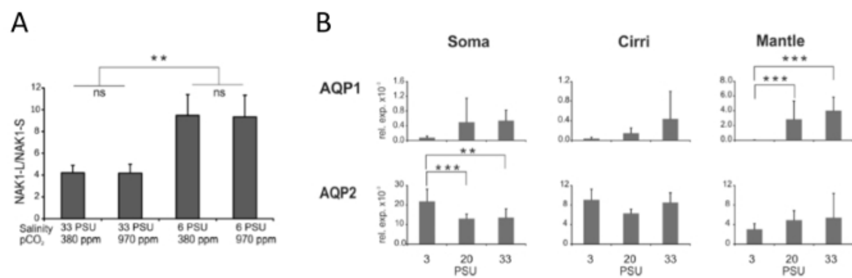

Figure 5: Gene expression results from two different studies of genes important for osmoregulation in *B. improvisus*. (**A**) This panel shows the differential expression as measured by qPCR of splice variants of the Na^+/K^+ ATPase *NAK1* in response to various salinities and pCO_2 levels[3]. In the low salinity treatment, the expression of the long isoform (NAK1-L) is increased relative to the short (ANOVA, $P < 0.001$). (**B**) This panel shows the expression of aquaporins in adults of *B. improvisus* during their exposure to various salinities[2]. qPCR was used to determine the aquaporin expression levels relative to actin. Adult individuals were incubated at 3 different salinities (3, 20, and 33 PSU) for 14 days. For the RNA preparation, the soma, cirri, and mantle of the adults were separated. In both figures, error bars indicate the standard deviation. The ** and *** indicate the level of significance (ANOVA), 0.01 and 0.001, respectively. These figures have been modified from Lind *et al.*[2,3]. Both figures are published with permission from PLoS ONE. Please click here to view a larger version of this figure.

Settlement Phase	Total amount of RNA (ng)
Free swimming	512
Exploration: Close search	518
Attached cyprid	550
Newly metamorphosed juvenile	832

Table 1: Yields of RNA. This table shows the RNA quantity extracted with an RNA preparation kit from pools of 20 cyprid individuals collected at different stages during the settlement process.

Discussion

The barnacle culture at Tjärnö Marine Research Laboratory (Sweden) has been running over 20 years and has been used for studies in many different research areas. Over 30 scientific papers have been published that have utilized the culturing system during the past years, including studies in antifouling[13,22], hydrodynamics[23], chemical ecology[24], climate change[16], evolutionary biology[5], and molecular biology[2].

To avoid the selection of certain individuals that are more adapted to the laboratory environment (individuals that might not be representative of the wild population), it is recommended to collect a new broodstock from the field each year. In addition, it is also good practice to rejuvenate the culture annually, since there is roughly 50 - 80% of mortality in adults during a normal year. However, if the aim is to produce inbred lines or to set up studies of experimental evolution, only laboratory-reared families are to be used.

A good time to collect *B. improvisus* on panels at the Tjärnö Marine Research Laboratory is in June–August because at that time, there is a good supply of cyprid larvae in the sea. Check the panels weekly to see when the barnacle settlement starts and manually remove other settled species than *B. improvisus* (*e.g.*, mussels, tunicates, bryozoans, hydroids, nemerteans/tubeworms, and other barnacle species) from the panels

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(*e.g.*, with a toothbrush). Around Tjärnö, there are three shallow-water barnacle species present (*B. improvisus*, *Semibalanus balanoides*, and *Balanus crenatus*). However, *B. improvisus* is the dominant fouler of smooth hard surfaces during July–August. *S. balanoides* has its settlement period during early spring and prefers mainly natural substrates (*e.g.*, stones). *B. crenatus* can occur at low numbers on the panels during the summer.

It is also possible to start new adult barnacle generations from cultured cyprids, which would be essential if certain linages with specific traits have been established, or in studies of experimental evolution. The most convenient way to start new generations of adults is to settle cyprids on thermoplastic panels in the laboratory. These panels with newly settled cyprids could also be used in experimental treatments or for exposure in the field. In emergency cases, one can also use adults on boulders from a nearby site (*e.g.*, Idefjorden in the case of the Tjärnö Marine Research Laboratory) where *B. improvisus* is common. These already established adults are treated in the same way as the adults on the panels, thus being placed in trays and fed *via* the flow-through system. Flow cells can also be used to establish panels with barnacles[25]. These are flow-through chambers with plankton net on the sides on which the cyprids do not settle, with panels as the only settlement surface for the larvae.

There are several steps that are critical for setting up a long-term functioning barnacle culture including all life stages. The methods used to culture *B. improvisus* are probably, to a great extent, also applicable to other marine invertebrates with free-swimming, planktotrophic larvae. Culturing procedures for some species are already well described (*e.g.*, for blue mussels and different species of oysters)[26], while for other marine invertebrates, there are only a few examples of long-term cultures spanning their whole life cycle. One of the first successful attempts to culture barnacles (*B. amphitrite*) was done by Rittschof *et al.*[15]. Long-term financial and personal resources should be in place before considering setting up a barnacle culturing facility. The maintenance of this kind of year-around barnacle culture requires at least one person working half-time. There may be some potential for the future automation of some steps in the production line, mainly the culturing of microalgae[27]. In addition, in order to succeed, it is essential to have access to large quantities of high-quality seawater. The culturing of microalgae, *Artemia,* and barnacles does not involve any particular safety procedures. However, tests of some antifouling substances or toxic chemicals may need special precautions.

The panels were checked several times a week for contaminations. The seawater used in the culture was pumped up from a 40 m depth in the Koster Fjord outside the Tjärnö Marine Research Laboratory and was passed through two sand filters before entering the lab water system. If no filtering of the water had been done, there would be much more contamination in the culture. It is essential to regularly clean the panels in the culture from detritus and other invertebrates (*e.g.*, stolon-building hydroids and predatory nemerteans) that enter the system through the supply of seawater from the field. For example, if no larvae are produced despite the fact that the culture has been well-fed and otherwise seems to be in good condition, the problem might be the presence of nemerteans that appear to inhibit mating. Naturally, many of the contaminating organisms in the culture at the Tjärnö Marine Research Laboratory were specific for the Swedish west coast, and other types of contaminating organisms will be prevalent and be more of a challenge in other geographic areas. On the west coast of Sweden, it is unusual to find contamination by other barnacle species on the panels. Occasionally, the establishment of *S. balanoides* has been found, but this is a very marginal problem (at the most, one *S. balanoides* contaminant for 10,000 *B. improvisus* samples). The lack of contaminating species was most likely dependent on the regime to establish new cultures during the summer, when larvaefrom *B. improvisus* were highly dominant. In addition, there was also a clear enrichment of *B. improvisus* on the panels since this species is selective for smooth surfaces[13].

It is essential to remove dead adult barnacles. If the empty shells are left on the panels, they can become a shelter both for *Artemia* nauplii as well as for various contaminating species. In addition, it has been noticed that dead individuals influence the well-being of neighboring individuals, probably with the release of toxic compounds during the decomposition. An additional consequence of adult mortality is that some individuals will be left alone and too far away from any other adults to allow mating (even though barnacles have the longest penis in the animal world in relation to its size)[28]. These individuals will survive but are non-productive for larvae. However, these solitary adult individuals can gently be removed without harming the base-plate and be placed horizontally close to others to enable mating. Barnacles can also be mated by placing panels with one adult on each but close enough so that cross-fertilization can occur. In this way, genetic lines can be produced[14].

It is critical to produce a feed of high quality and to feed cultures almost every day. Even a few days without food can result in a decreasing release of larvae. Previous tests of diet composition have shown that diatoms are essential for the growth and survival of barnacle nauplii. Several diatom species seem adequate as feed, although small or solitary cells (less than 10 µm in diameter) may be necessary for the nauplii's ingestion. The species *S. marinoi*, *C. simplex*, and *T. pseudonana* have all proved to be adequate feed for *B. improvisus* nauplii, as well as easy to cultivate. In addition, the feed quality is generally higher for exponentially growing algae. It has also been reported that diatoms are essential for establishing productive cultures of *B. amphitrite*[15]. A theory of the importance of diatoms is that they have a unique fatty acid profile and are particularly rich in the highly polyunsaturated 20:5 fatty acid[29]. It has been shown that certain fatty acids are important for the successful development of oyster larvae[30].

Over the years, there have been no incidences of detrimental diseases in the barnacle culture. In many commercial invertebrate aquacultures, like oysters and mussels, diseases are rather common and may be very harmful. Detrimental effects of viruses have also been reported from wild populations. The native oyster in France was replaced by the Portuguese oyster *Crassostrea angulata* in 1925, but this species was wiped out by an iridovirus around 1970[31]. More recently, there have been massive mortality events in the Pacific oyster *Crassostrea gigas* in cultures worldwide, which appears to be associated with the ostreid herpesvirus 1[32]. No reports on pathogens, bacteria, or viruses on barnacles have been published so far. However, in the ongoing genome-project on *B. improvisus*, virus sequences were found (Alm Rosenblad *et al.*, unpublished data) but with no apparent link to symptoms of diseases. Mixtures of antibiotics have previously been applied to the cultures to minimize the risk of bacterial infections; however, this procedure is currently abandoned and so far, this has not caused any contamination problems.

If seawater is heated (as described above), overheating may be the most serious risk in the culture production line. It is, of course, difficult to safeguard against overheating, although sensors and appropriate alert systems may be used (*e.g.*, sending e-mail or text messages to responsible persons). Incidences of this kind in the past have resulted in the substantial killing of adults in the culture. This can, of course, be devastating and ruin long-term investments of time and money. In particular, this would be catastrophic if inbred genetic lines have been established. To ensure the longevity of such lines and secure them from accidental loss, it would be desirable to develop a cryopreservation methodology for barnacles. It has been reported that larvae from the Pacific oyster can be frozen down and revived with partial success[33]. Cryobanking has also been a valuable tool to preserve the genetic resources of a wide range of species[34]. Even nauplii from *B. amphitrite* are

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reported to survive freezing[35], and it was found that 20% of the frozen-down individuals successfully metamorphosed into cyprids[36]. However, applying freezing for the long-term sustainability of cultures has so far not been adopted, but this would indeed be needed for the maintenance of selected lines; this would be an essential step to firmly establishing *B. improvisus* into a potent marine modelsystem.

Here, a protocol was presented for the dissection of various tissues from adults of *B. improvisus* (*i.e.*, cirri, soma, and mantel). However, it should be stressed that other tissues can also be extracted. For instance, the soft tissue between the exterior and internal mantle of the membranous-base species *Tetraclita japonica formosana* has been carefully isolated and used for an RNA extraction and RNA-seq analysis of gene expression[37]. The outlined optimized extraction protocol described here provides sufficient amounts of high-quality RNA for sequencing from a minimal amount of starting material. First, the collection of individual larvae directly into the homogenization tubes minimizes any loss during the transfer from one tube to another. Furthermore, among the different methods tested, the homogenization with ceramic beads proved to be the most efficient in terms of RNA yield and integrity, compared to sonication or pestle homogenization. When planning for gene expression or genomics experiments, one has to keep in mind the challenge of the high genetic variation in barnacles, at least for *B. improvisus*. Barnacle has a genetic diversity in the range 3–5%, even in coding regions (Alm Rosenblad *et al.*, unpublished data). This, of course, puts specific demands on the design of primers for qPCR analysis, where more conserved regions should be identified and used as templates for primers in order to get consistent expression results betweenbatches. Conserved regions for target genes, like aquaporins and Na+/K+ ATPases, can be identified by studying the sequence variability of these genes in RNA-seq data obtained from populations of cyprids containing hundreds of individuals. For a genome analysis, DNA will be sampled. However, obtaining high-quality DNA from *B. improvisus* can be challenging[21].

In conclusion, the established barnacle culture has proven to be instrumental in different kinds of experimental studies. In particular, the all-year-around larval production allows us to conduct experiments without being limited to the naturally occurring spawning period (for *B. improvisus*, this is during the summer). Obtained larvae can be used to perform a wide set of experimental studies, including settlement assays, behavior assays, expression studies on specific genes, as well as genome-wide transcriptome studies.

Disclosures

The authors have nothing to declare.

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