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Cryobiology 52 (2006) 33–47
CRYOBIOLOGY
www.elsevier.com/locate/ycryo

Coral larvae conservation: Physiology and reproduction ☆

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Received 5 August 2005; accepted 13 September 2005
Available online 6 December 2005

Abstract

Coral species throughout the world's oceans are facing severe environmental pressures. We are interested in conserving coral larvae by means of cryopreservation, but little is known about their cellular physiology or cryobiology. These experiments examined cryoprotectant toxicity, dry weight, water and cryoprotectant permeability using cold and radiolabeled glycerol, spontaneous ice nucleation temperatures, chilling sensitivity, and settlement of coral larvae. Our two test species of coral larvae, *Pocillopora damicornis* (lace coral), and *Fungia scutaria* (mushroom coral) demonstrated a wide tolerance to cryoprotectants. Computer-aided morphometry determined that *F. scutaria* larvae were smaller than *P. damicornis* larvae. The average dry weight for *P. damicornis* was 24.5%, while that for *F. scutaria* was 17%, yielding osmotically inactive volumes (V_b) of 0.22 and 0.15, respectively. The larvae from both species demonstrated radiolabeled glycerol uptake over time, suggesting they were permeable to the glycerol. Parameter fitting of the *F. scutaria* larvae data yielded a water permeability $\geqslant 2$ μm/min/atm and a cryoprotectant permeability $= 2.3 \times 10^{-4}$ cm/min while modeling indicated that glycerol reached 90% of final concentration in the larvae within 25 min. The spontaneous ice nucleation temperature for *F. scutaria* larvae in filtered seawater was -37.8 ± 1.4 °C. However, when *F. scutaria* larvae were chilled from room temperature to -11 °C at various rates, they exhibited 100% mortality. When instantly cooled from room temperature to test temperatures, they showed damage below 10 °C. These data suggest that they are sensitive to both the rate of chilling and the absolute temperature, and indicate that vitrification may

☆ This work was supported by a grant to M.H. from the Friends of the National Zoo, and to L.H. from grants from the Hawaii State Biomedical Research Infrastructure Network Summer Research Scholarship (HS-BRIN; NIH/NCRR Grant RR-16467), an NSF-REU fellowship from the University of Hawaii Sea Grant Marine Science Undergraduate Research Fellowship program (NSF REU Grant 0243600) and by the University of Hawaii Investing in Multidisciplinary University Activities through Hawaii EPSCoR program (NSF RII Grant 0237065).

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[1] This author contributed to this paper in his personal capacity. The views expressed are his own and do not necessarily represent the views of the National Institutes of Health or the United States Government.

doi:10.1016/j.cryobiol.2005.09.008

be the only means to successfully cryopreserve these organisms. Without prior cryopreservation, both species of coral settled under laboratory conditions.

Keywords: Membrane permeability; Larval settlement; Chill sensitivity; Glycerol

Coral species throughout the world are facing severe environmental pressures [3]. Although they are adapted to severe natural disturbances, such as fresh-water flooding events [23], hurricanes [5], and lava flows [10], coral reefs recover quickly if chronic human impacts are absent. However, they are less adapted to human-related environmental stresses, including sedimentation, elevated nutrients, and other pollutants. These cause a reduction in the overall larval recruitment of many species [6]. Additionally, other environmental pressures, such as El Niño Southern Oscillation (ENSO) events, result in bleaching and coral mortality. As greenhouse gasses increase, atmospheric and sea-surface temperatures are also expected to increase, coupled with anthropogenic stresses, reefs will remain in crisis, threatening their existence worldwide [18,9,21].

Because of the pressing conservation needs of coral, we studied the reproduction, physiology, and settlement of coral larvae with the future goal of cryopreserving and maintaining these organisms in a genome resource bank. In this bank, the larvae would remain frozen, but alive, for many years in liquid nitrogen. These frozen populations could be thawed and released back into the reef environment once the ecological conditions for their survival had improved. These thawed populations could be reseeded with reasonable results if they matched the levels observed on unfrozen larvae observed in seeding experiments [17]. Thus, it is feasible that these genome resource banks may prevent the extinction of many coral species.

Other marine invertebrate embryos, such as oysters, sea urchins, and polycheate worms, have been successfully cryopreserved [29,40,2,8,39]. However, not all aquatic organisms have been found to be good candidates for slow-freezing cryopreservation, the type that is commonly used for most sperm. For example, starfish oocytes were found to form intracellular ice at relatively high temperatures very close to the temperature of extra cellular ice formation [26]. To circumvent this problem, Hamaratoglu et al. [15] successfully cryopreserved these organisms, using the ultra-rapid freezing technique, vitrification. Chill and/or cold shock sensitivity can also preclude slow freezing protocols. It is not known how sensitive coral larvae are to chilling temperatures.

Little attention has been focused on coral larval physiology, perhaps because many species spawn within a limited time frame each year, far away from most laboratories, thus making data collection difficult. Fortunately, corals spawn reliably throughout the summer in the warm waters off Oahu in Kaneohe Bay near the Hawaii Institute of Marine Biology [25]. To begin our studies, we chose two species of coral larvae, *Pocillopora damicornis* (lace coral), and *Fungia scutaria* (mushroom coral), because they are representative of the varying size range and physiological complexity of coral larvae. *P. damicornis* larvae are released from the adult as large, complex planulae complete with symbiotic zooxanthellae, ready to metamorphose and settle within hours [16]. In contrast, *F. scutaria* larvae are the result of broadcast spawning events, producing much smaller larvae that take 3–4 days to develop before they become infected with their zooxanthellae, metamorphose, and settle [27]. *F. scutaria* has been in culture at the Hawaii Institute of Marine Biology for many years and its larval rearing and husbandry is well developed [27,44], making it an attractive animal model for coral physiology and disease work.

To successfully cryopreserve tissue by slow freezing, intracellular water must be largely removed and, typically, a cryoprotective agent must be introduced to minimize the deleterious effects of freezing and thawing. Because coral larvae have never been successfully cryopreserved, and little is known about how water and cryoprotectants move into and out of larval coral tissue, we began by determining basic cryobiological properties of two coral species. This approach included determining morphometric variables, such as average size and fractional solids content (V_b), cryoprotectant toxicity, water (L_p), and cryoprotectant permeability (P_s), chilling injury, chilling sensitivity, and ice nucleation temperatures. These factors are essential for creating an accurate biophysical model that will

help guide the future formulation of cryopreservation protocols for coral larvae. Since glycerol is produced by coral symbionts and metabolized by coral [36], both cold and radiolabeled glycerol was chosen as the first cryoprotectant to study. Almost all of our biophysical parameters rely on morphometric measurements of the larvae. During these experiments, we found that *P. damicornis* changed shape so often, that these types of measurements were unreliable. Therefore, we present a full data set for *F. scutaria* and a representative data set on *P. damicornis* for comparison when possible. Additionally, we examined how readily the two coral species would settle in captivity. Cryopreservation can only be deemed successful if the larvae settle, grow and reproduce following freezing, storage at low temperature, and thawing.

Materials and methods

Animal collection

Two species of coral larvae were collected during spawning events in Kaneohe Bay, Hawaii during the summer months of 2002, 2003, and 2004. Adult *P. damicornis* colonies were harvested in shallow water reef flats off of Coconut Island (21°26′ N, 157°46′ W) at the Hawaii Institute for Marine Biology, University of Hawaii. They were immediately transferred to an outdoor seawater table. Following the protocols of Jokiel et al. [22], each colony was immersed in running seawater from the reef while held in individual 2 L plastic bowls with a spout. Plastic beakers (400 ml) with plankton mesh sides were placed at the outflow of the bowls to collect the planulae as they were released from the colony over time. These beakers were checked daily for new larvae. After 7 days, the colonies were returned to the reef. Collected this way, these planulae could be maintained in the laboratory for many days.

Fungia scutaria adults, collected from various shallow reef flats in Kaneohe Bay, have been maintained in a flow-through seawater system at the marine station for many years and the methods for obtaining gametes are well published [27,44,49]. One to two days before the full moon, male and female corals are placed in individual glass dishes receiving 50 μm filtered seawater (FSW) and the level of the seawater was adjusted to about 10 cm above the level of the culture dishes. All FSW used in this paper was made by passing it through a 0.45 μm Millipore filter.

At 4 PM on the night of an anticipated spawning event (*F. scutaria* spawns 1–4 nights following the full moon, between 5 and 7 PM during the months of June through October), the water level in the seawater tables was lowered to isolate the individuals in their bowls. Individuals are monitored as they begin to spawn over the next few hours. Eggs collected from individual females were gently transferred to 2 L plastic bowls and fertilized with 50–100 ml of a sperm suspension collected from a minimum of three males. The bowls of fertilized gametes were then transferred to shaded seawater tables receiving a continuous supply of seawater at ambient seawater temperatures (27–29 °C during the spawning months). The egg–sperm mixture was gently stirred every 30 min for 4 h following fertilization. Thousands of larvae were raised in 2 L plastic bowls filled with FSW surrounded by flowing seawater from the seawater system. The following morning (approximately 12 h after spawning) and every day thereafter for 10 days, the bowls were cleaned by filtering the larvae through 40 μm mesh filters and resuspending in FSW. This husbandry yielded very low mortality, and massive numbers of larvae could be raised for up to 10 days.

The *F. scutaria* larvae do not have zooxanthellae when spawned and must take up their symbionts from their environment. In the laboratory, this can be achieved using the zooxanthellae of an adult [49]. *P. damicornis* larvae were measured within 1–2 days after being released (receiving zooxanthellae before release from the parent), whereas *F. scutaria* larvae were measured 3–6 days after fertilization. This period encompasses the time when *F. scutaria* larva would normally ingest their symbionts. However, for most of these experiments, the *F. scutaria* larvae were not infected with zooxanthellae.

Toxicity experiments

Larvae of *P. damicornis* were exposed to 5, 10, or 15% solutions (v/v) one of four cryoprotectant solutions (i.e., glycerol, methanol, propylene glycol, or dimethyl sulfoxide, in FSW) for 15 min at room temperature (2–6 larva/trial with a total of 4 trials/solution). The results of these experiments guided the design of the experiments for *F. scutaria* (7–100 larva/trial with a total of 6 trials/solution). *F. scutaria* larvae were placed into a 10% solution of one of the four cryoprotectant solutions for 30 min at room temperature. To move larvae easily

from solution to solution, the larvae were transferred into 40 μm mesh cell-straining baskets (FalconFisher Scientific) that were placed in a 35 mm petri dish containing 4 ml FSW. The basket containing the larvae was removed from the FSW, the excess liquid was blotted dry, and then the basket was placed into a 35 mm petri dish containing 4 ml of one of the test solutions. After the allotted period of time, the basket was removed, blotted dry, rinsed once in 4 ml of FSW, and then immediately placed into fresh FSW. Survival was assessed by the presence of normal swimming movement within a few minutes after being returned to FSW. At these stages, coral larvae are covered with active cilia that keep them in constant motion in FSW. These experiments were repeated 4–6 times per concentration and cryoprotectant for each coral species.

Morphometric analysis

To calculate the volume and surface area of each larva, its cross sectional area (30–40 larvae of each species) was measured in FSW using a computer-aided microscopy. A 20 μl drop of FSW with tens of larvae was placed onto a depression slide with a cover slip to prevent drying. Digital images (Olympus BX41 microscope with an attached digital camera Sony DFWV300) of a single larva were digitized onto a G4 Macintosh computer, and then cross sectional area determined using NIH Image software and assuming cylindrical symmetry ($V = \pi r^2 L$, $A = 2\pi\,(r^2 + rL)$).

Drying experiments (V_b)

We used a microbalance with 0.01 mg readability to determine the larval wet/dry weight ratio. To obtain wet weights, larval samples ($N = 12$/basket a total of three baskets of *P. damicornis* larvae; $N =$ thousands/basket for a total of five baskets of *F. scutaria* larvae) were transferred into pre-weighed 40 μm mesh cell-straining baskets that were blotted dry and reweighed. Six baskets were measured for each species. To obtain dry weights, the baskets containing the larvae were placed in a 50 °C oven to dry for 12–24 h and then reweighed. Triplicate measurements were taken to reduce variability, and an average value was used for further calculation. The mean larval wet/dry weight ratio was calculated as (mean dry weight − mean basket weight)/(mean wet weight − mean basket weight).

We used the following formula and assumptions to find the fractional solids volume (V_b). We assumed the gastrovascular cavity had a negligible volume during these developmental periods (ca. less than 10% for *Fungia*, Hagedorn et al., unpublished) and take no account of it in these formulas

$$V_b = W_b/[W_b + (\rho_s/\rho_w)(1 - W_b)],$$

where

$$W_b = \text{wt fraction of solids} = W_s/(W_s + W_w)$$

and W, weight; V, volume; ρ, density; subscripts s, solids; w, water. The density of the solids and water were assumed to be 1.15 and 1.0 g/cm^3, respectively.

Permeability experiments—cold glycerol

Because coral zooxanthellae produce glycerol that can be used by corals, glycerol seemed a good choice of cryoprotectant to begin the examination of permeability parameters. We determined the water permeability (L_p) and cryoprotectant permeability (P_s) by measuring the time course of volume changes induced in coral larvae after exposure to a 5% (0.685 M) or 7% (0.960 M) glycerol solution made up in FSW (v/v). Hundreds of *F. scutaria* larvae were placed into a 40 μm mesh cell-straining baskets (Falcon) in FSW, the basket was removed from the FSW, patted dry, and then placed into the cryoprotectant solution. A 20 μl sample of solute with tens of larvae was placed onto a depression slide with a cover slip. Digital images (Olympus BX41 microscope with an attached digital camera, Sony DFWV300) of a single larva were collected every minute for 15 min, and then the volume change was measured with NIH Image software. Data were modeled as in previous studies [14] by numerical integration and least squares parameter fitting of the two coupled transport equations for L_p and P_s [24]. Dimensional data and fitting parameters are shown in Table 1. The larval surface area (A_0) was considered fixed and given by the area of the initial volume.

Permeability experiments—hot glycerol

Radiolabeled cryoprotectant experiments provided an independent means to measure cryoprotectant permeability and permitted some tests for the presence of gastrovascular cavity-volume within each coral larva. Larvae were placed into a 1.54 molal glycerol solution consisting of radiolabeled

Table 1
Fungia scutaria morphology and modeling parameters

Parameter	Value	Units
Length, L	0.205	mm
Radius, R	0.065	mm
Volume, V_0 (cylinder)	0.00274	mm^3
Solids volume fraction, V_b	0.15	—
Area, A_0 (cylinder)	0.111	mm^2
Water permeability, L_p	fitted	μm/min/atm
Solute permeability, P_s	fitted	cm/min
Partial molar $V_{glycerol}$	0.071	L/mol
FSW concentration	0.995	osmol/kg
Cell isosmolality	0.995	osmol/kg
5% Glycerol concentration	0.685	molar
	0.728	molality
7% Glycerol concentration	0.960	molar
	1.04	molality
Radiolabel glycerol	1.37	molar
Concentration (total)	1.54	molality
Temperature	292	K

The length and radius were measured microscopically in filtered sea water (FSW). The volume (V_0) and area (A_0) in FSW are computed from these assuming a cylindrical shape.

[^{14}C]glycerol (Sigma Aldrich, St. Louis, MO) and cold glycerol was made up in FSW. The hot glycerol comes mixed in water at a sub-millimolar concentration and thus, for the purpose of calculating solution concentrations, can be consider as pure water. The activity of the hot glycerol in counts per min (CPM) was determined via a series of dilution curves (1:1–1:1000) in FSW. The larval test solution contained a small amount of labeled glycerol, FSW, and cold glycerol at the desired concentration (1.54 molal). Thus, we were able to prepare any desired concentration of test glycerol for the larvae. Knowing the CPM of the labeled glycerol and proportions of labeled and cold glycerol in the final solution, we can convert CPM in the larva to moles of total glycerol that crossed the membrane of the larva.

Slightly different procedures were used for the two species because of their size differences. For the larger *P. damicornis* larvae, samples of 20–30 planulae were pipetted into the radiolabeled solution, and subsamples of larvae ($N = 5$–10) were removed at fixed time intervals (1, 5, 15, 30, and 60 min). Larvae were rinsed three times in cold glycerol (1.54 molal in FSW), blotted onto filter paper, and then the filter paper with larvae was immersed in 8 ml of ScintiSafe Econo 1 scintillation fluid (Fisher Scientific).

For the smaller *F. scutaria* coral larvae, thousands of planulae were placed into a 40 μm mesh cell-straining baskets in FSW. These were blotted dry, then placed into 4 ml of a 1.54 molal glycerol solution consisting of radiolabeled [^{14}C]glycerol and cold glycerol in FSW for 1–30 min. At fixed time intervals (1, 5, 15, and 30 min), a basket was removed from the labeled solution, blotted, and then rinsed by placing the basket into three aliquots of cold glycerol solution (1.54 molal in FSW). The whole basket was then placed into a scintillation vial containing 12 ml of ScintiSafe Econo 1 scintillation fluid. The counts per minute were measured with a Beckman LS 3801 (Fullerton, CA) scintillation counter. These experiments were repeated at least 5 times at each time point for both species. Data were fit as above, for cold glycerol.

Pocillopora damicornis larvae have a large gastrovascular cavity. Thus, radiolabel uptake might occur across the larval membrane into the cytoplasm or through the pharynx into the gastrovascular cavity. To distinguish between these two possibilities, we immersed *P. damicornis* larvae, as before, in the radiolabeled cryoprotectant for 60 min. At this time, the sample was split and one group ($N = 5$ larvae/trial, repeated three times) were processed as above, while the larvae in the second group ($N = 5$ larvae/trial, repeated three times) were surgically cut in half with micro scissors to expose the gastrovascular cavity to the cold glycerol in the final rinse. Larvae were placed on blotting paper and processed as before. If the radiolabeled cryoprotectant were present in the gastrovascular cavity, we expected the counts from the surgical group to be lower than those from the intact group. Student's t test was used to distinguish between the means of the radiolabel uptake of the intact and bisected groups.

Ice nucleation temperature

It is usually easier to develop a slow-freezing protocol for cryopreservation, rather than an ultra-fast, vitrification protocol. The objective of a slow-freezing protocol is to remove intracellular water before it freezes intracellularly. During slow cooling, cells supercool while the extracellular solution freezes and concentrates. This leads to an osmotic gradient which dehydrates the cells. However, the cells must be held above their ice nucleation temperature for this dehydration to occur (intracellular ice formation is lethal).

Fungia scutaria larvae in FSW were transferred into the cooling chamber of a Linkam BSC 196 cryostage mounted on an Olympus BX41

microscope, and cooled at 10 °C/min from RT to −40 °C (20 μl samples/tens of larvae per sample). The image of the larva was displayed with a computer-aided video (described above). Intracellular ice formation was noted as a rapid optical darkening of the cells called "black flashing." These experiments were repeated five times.

Chilling damage

Cells respond to chilling temperatures in a number of ways. Two distinct types of chilling exist [33]. First, cold shock (where the rate of chilling is damaging) and, second, chilling injury (which results from cooling below a critical temperature). To examine these two types of chilling damage, we rapidly chilled *F. scutaria* larvae, or gradually cooled them to a particular temperature.

To examine cold shock, two types of slow chilling experiments were performed. In one, five 10 ml samples of Day 4 *F. scutaria* larvae (N = thousands) in FSW contained in polystyrene sealed tubes were placed into styrofoam cups filled with 250 ml of RT water. These were placed in a cold room (4 °C) for 4 h. Then, the tubes were warmed rapidly to 20 °C in air and three 100 μl samples were taken from each tube to assess viability under a microscope. In a second procedure, 20 μl samples (N = tens of larvae/sample) *F. scutaria* larvae in FSW were placed onto a cryomicroscope stage (Linkam BSC 196, Surrey, UK) and cooled at a very low rate (0.1–2 °C/min), an intermediate rate (10 °C/min) and a high rate (40 °C/min) from 19 to −11 °C, held for 1 min, then warmed at 20 °C/min. Images were taken during the cooling phase and after the larvae returned to room temperature. These studies were carried out on Day 3 to Day 7 larvae and repeated three times at each rate.

To examine chilling injury, samples of *F. scutaria* larvae were placed into 5 ml of FSW in glass scintillation vials with caps (N = 5 vials/time at each temperature). These vials were transferred into a controlled-rate freezer (Bio Cool III-80/SR-36, FTS Systems Missisauga, ON) and held at a constant chilling temperature. The freezer was filled with 95% ethanol, just covering the level of solution within the vials. This dropped the temperature of the vials containing the larvae to the holding temperature of the bath within seconds. The temperatures we tested were 10, 5, 0, and −5 ° C, and the vials were held at these temperature for 5, 15 or 30 min, then returned to RT and examined. The total number of larvae in each vial was counted under a dissecting microscope and the number of abnormal larvae assessed. Larvae were considered abnormal if they had blebbing membranes or ceased swimming (larva constantly swim until they settle and attach). Different developmental stages often have different susceptibilities to chilling; therefore, these same tests were carried out on *F. scutaria* larvae from Day 2 to Day 7.

Larval settling

Experiments to determine the feasibility of larval settlement were conducted. Eight-day-old *F. scutaria* larvae that had been infected at Day 3 to 4 with homologous zooxanthellae extracted from adult *F. scutaria* according to the techniques of Schwarz et al. [44] and Weis et al. [49] were utilized in the settling experiments. Four floating settlement chambers constructed from plastic rings (approximately 20 cm diameter × 10 cm deep) with 40 μm plankton mesh bottoms and small pieces of styrofoam glued to sides such that the top of the chambers were not submerged in the flow-through seawater table. Prior to the start of the settling experiment, six microscope slides were placed into each floating settlement chamber for approximately 24 h to allow a bio-film to develop on the surface of the slides. Then two settlement chambers were filled with thousands of Day 8 larvae, and the other two chambers were left undisturbed as controls. Approximately every other day, the slides were moved into a seawater-filled glass bowl to observe the settled larvae. Encroaching algae was removed with fine needles under a dissecting microscope. The containers were maintained for over 30 days as the larvae settled, metamorphosed, and secreted calices. In contrast, *P. damicornis* larvae, possessing maternally derived zooxanthellae, did not require any special treatment to promote settlement and metamorphosis. Larvae were placed in glass dishes filled with FSW on the laboratory bench. Within hours to days, settled larvae were observed in the dish. These were maintained by periodic changes of the FSW.

Statistical analysis

To determine whether the median radiolabeled cryoprotectant uptake differed significantly between the cut and intact *P. damicornis* larva, a nonparametric, Mann–Whitney U test was done using

GraphPad Instat 3.0 B software for the Macintosh (San Diego, CA).

Results

Toxicity

Toxicity experiments are designed to understand which cryoprotectants are most useful in designing a cryopreservation protocol. Both species of coral larvae tolerated the four cryoprotectant solutions very well at solutions equal to or less than 10% (v/v; Fig. 1). However, for *P. damicornis*, as the solutions approached 15% (v/v) their survival after 15 min diminished (Fig. 1). The *F. scutaria* were only exposed to 10% solutions, but for twice as long as *P. damicornis*, and all the solutions resulted in excellent survival in *F. scutaria* larvae (Fig. 1). This wide tolerance of cryoprotectants gives a great deal of flexibility in designing solutions for freezing protocols.

Morphometric analysis

The volume of *F. scutaria* larvae could be determined because they held a relatively constant shape as they moved in FSW. This was not true of *P. damicornis* (see below). Generally, coral larvae are cylindrically shaped. *F. scutaria* have a mean length and width of 0.205 ± 0.001 and 0.130 ± 0.002 mm, respectively, yielding a mean cylindrical volume of 0.00274 mm^3 ($N = 5$ larvae were measured every min over at least 10 min in FSW). *P. damicornis* move and change shape quite rapidly, therefore, we took single-image measurements of more larvae. These larvae are larger than *F. scutaria* having a mean length and width of 2.5 ± 0.1 and 1.31 ± 0.04 mm ($N = 17$).

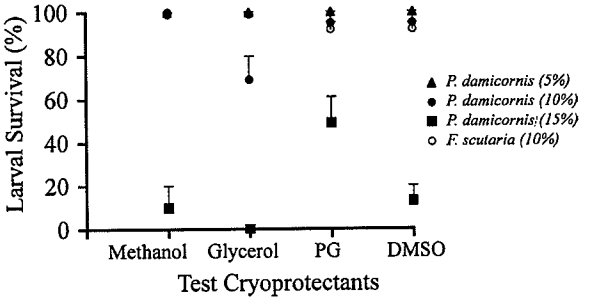

Fig. 1. Survival of coral larva exposed to four cryoprotectant solutions in FSW over time. *Pocillopora damicornis* larvae (represented by the filled symbols) were exposed to three different concentrations of four different cryoprotectant for 15 min. Survival was reduced for larva exposed to 15% solutions. *Fungia scutaria* larvae (represented by the open symbols) were exposed to a single concentration (10%) of four different cryoprotectant for 30 min. All the larvae survived well in all the cryoprotectants.

Drying experiments (V_b)

The osmotically inactive fraction, i.e., solids volume fraction, (V_b) can be estimated by measuring the dry/wet weight ratio of the organism. The average dry weight of *P. damicornis* ($N = 3$ baskets/12 embryos/basket) was 24.5% of wet, while the average dry weight of *F. scutaria* was 17% of the wet ($N = 5$ baskets/thousands of larvae per basket). These values yield V_b's = 0.22 and 0.15 for *P. damicornis* and *F. scutaria*, respectively, assuming a solids density of 1.15 g/cm^3. Because the gastrovascular cavity volume of *P. damicornis* was not taken into account, $V_b = 0.22$ may be an underestimate.

Membrane permeability experiments—cold glycerol

No data on the L_p for *P. damicornis* were reported for cold glycerol because these larvae did not stop swimming and they changed shape so rapidly in cryoprotectant solutions that it was impossible to get accurate kinetic dimensional measurements. Therefore, we reported cold glycerol permeability data only for *F. scutaria.*

When we immersed *F. scutaria* larvae in 5% (v/v; i.e., 0.73 molal or 0.68 M), and 7% (v/v; i.e., 1.04 molal or 0.96 M) glycerol solutions in FSW, we noted a gradual increase in volume over time (Fig. 2A, average of five runs each). Typically, under such test conditions, a shrink–swell curve is expected in which the water rapidly leaves the cell because of the high external osmolality of glycerol, followed by a gradual reswelling as glycerol enters the cell and water follows. However, here, the first volume measurement at 1 min was consistently smaller than the morphometrically measured mean volume in FSW. Two possibilities were considered for this apparent rapid decrease in volume during the first minute of exposure to glycerol: (i) a rapid gastrovascular cavity response or (ii) a high L_p resulting in a very rapid efflux of cell water during the first minute. We knew from histological examinations (Hagedorn et al., unpublished data) that no large volume existed in the *F. scutaria* gastrovascular cavity at this time that might rapidly expel water. Therefore, this suggested a high L_p. Fitting the data, however, is something of

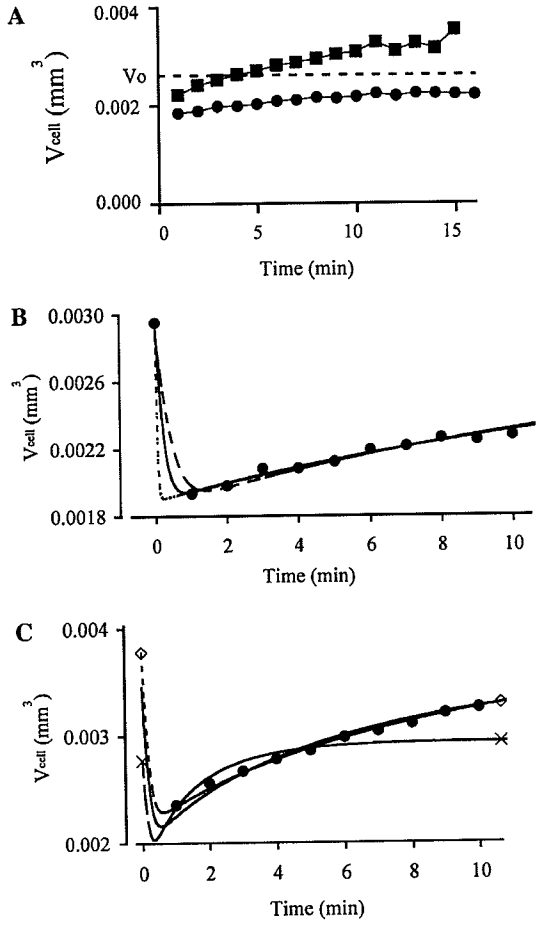

Fig. 2. Measured and modeled physiological data for *F. scutaria* larvae. (A) Showed the morphometric, mean volume kinetics of *F. scutaria* larvae immersed in 5% (shown as filled squares) and 7% (shown as filled circles) glycerol (v/v) solutions in FSW at 19 °C. These data indicated only a slight increase in the volume of the larvae over time. However, the dotted line indicated the initial volume (V_0) of the larvae while in FSW, suggesting there was a rapid decrease in volume of the larvae within the first minute of the experiment. (B) Modeled fits demonstrated the effects of varying the L_p for the 5% glycerol data (indicated by the filled symbols). The fits were for $L_p = 1.0$ μm/min/atm (represented by a dashed line), $L_p = 2.0$ μm/min/atm (represented by a solid line), and $L_p = 10.0$ μm/min/atm (represented by a dotted line). The corresponding P_s values were 1.28, 1.07, and 1.05×10^{-4} cm/min, respectively. These fits suggested an $L_p \geqslant 2.0$ μm/min/atm. (C) Modeled fits demonstrated the effects of varying the V_0 for the 7% glycerol data (indicated by the filled symbols) with L_p fixed at 2.0 μm/min/atm. The fits were for $V_0 = 0.00274\ mm^3$ (represented by a dashed line bounded by x's), $V_0 = 0.00343\ mm^3$ (represented by a solid line), and $V_0 = 0.00375\ mm^3$ (represented by a dashed line bounded by ◇'s). The largest V_0 yielded the smallest sum of squared errors fit. The corresponding P_s were 26.4, 5.01, and 2.95×10^{-4} cm/min, respectively. Restricting the fitted V_0 to ± 25% of the microscopically measured mean value of $0.00274\ mm^3$ yields the solid line fit with $L_p \geqslant 2.0$ μm/min/atm, $P_s = 5.01 \times 10^{-4}$ cm/min, and $V_0 = 0.00343\ mm^3$.

an art. Too many adjustable parameters will yield a good fit with very little specificity of individual parameters. Typically, we let V_0, L_p, and P_s vary during a fit. V_0 is allowed to vary, because the initial volume of an individual larva may differ from the mean measured volume in seawater ($0.00274\ mm^3$). How much V_0 should be allowed to vary from the mean is a matter of judgment, as illustrated below. Furthermore, the data may not tightly constrain all the parameters or may only place limits on some of the parameters. The later is the case for the 5 and 7% glycerol test solutions, where the data only placed a lower limit on L_p. This point is illustrated in Fig. 2B. The experimental data and three fitting curves are displayed with $L_p = 1.0$, 2.0, and 10.0 μm/min/atm. The data placed a lower limit of two on L_p. As L_p drops below two, the fitted curve increasingly missed the first data points at 1 and 2 min. Conversely, any value of L_p greater than two yields a fit that passed through all the data points. (For clarity, a point is placed at time zero on the graph, but it was not part of the experimental data set nor used in fitting.) Although L_p was not tightly constrained, P_s and V_0 were. The glycerol permeability (P_s) is 1.1×10^{-4} cm/min and varied by less than 2% as L_p increases from 2 to 10 μm/min/atm. Similarly, the best fit V_0 value was $0.00295\ mm^3$ for $L_p \geqslant 2$ μm/min/atm. This is only 8% higher than the FSW mean value of $V_0 = 0.00274\ mm^3$. We judged this difference to be within the range of sample to sample variation and experimental error. Thus, we reported the fitting parameters for the $L_p = 2$ fit in Table 2, as elaborated in Discussion.

The 7% glycerol data presented a somewhat different set of problems (Fig. 2A). As above, L_p values $\geqslant 2.0$ μm/min/atm yielded good fits. However, the best fit (dashed line between diamonds) was obtained with $V_0 = 0.00375\ mm^3$ which is 37% above our FSW mean of $0.00274\ mm^3$. We judged this an unacceptably large excursion in V_0 and unlikely to reflect a real variation in larval size. Thus, we chose to arbitrarily limit the fitted V_0 to within 25% of the FSW mean value. This yielded the solid fitting curve in Fig. 2C for which $L_p = 2.0$ μm/min/atm and $P_s = 5.0 \times 10^{-4}$ cm/min. The last fit in Fig. 2C (dashed line between the X's) illustrated the consequence of forcing V_0 to the FSW mean of $0.00274\ mm^3$. This fit was unacceptable.

In summary, the 5 and 7% glycerol data yielded a water permeability (L_p) of $\geqslant 2.0$ μm/min/atm and a

Table 2
Permeability results

Coral (species)	Glycerol (molal)	L_p (μm/min/atm)	P_s (10^{-4} cm/min)	V_0[a] (mm^3)
F. scutaria	0.73 (cold)	≥2.0	1.1	0.00295 (fitted)
	1.04 (cold)	≥2.0	5.0	0.00343 (limited)
	1.54 (hot)	2.0 (assumed)	0.83	0.00206 (limited)
	Best estimate values	2.0	2.3	0.00274
P. damicornis	1.54 (hot)	2.0 (assumed)	0.12	3.37 (fixed)

[a] V_0 values marked as (limited) were constrained by the criterion that the fitted V_0 not deviate by more than ±25% from the mean FSW value (0.00274 mm^3).

glycerol permeability (P_s) of 1.1×10^{-4} and 5.0×10^{-4} cm/min, respectively. These results were collected in Table 2.

Membrane permeability experiments- hot glycerol

The radiolabeled cryoprotectant experiments provided an attractive way to test the cold glycerol permeability values in an independent fashion. Both species of coral larvae demonstrated an uptake of labeled glycerol over time (Figs. 3A and B). After reduction, the hot glycerol data yield a plot of N_s (moles of glycerol per larva) versus time. These could be fit with the transport equations used previously. Because we were working with N_s, rather than the larval volume, the results were quite insensitive to the value of L_p chosen. We used an $L_p = 2.0$ μm/min/atm given by the cold glycerol experiments. As was the case for the 7% cold glycerol data, the best fit was given by an unreasonable value of V_0, namely 0.0011 mm^3 in contrast to the morphometric mean in FSW of 0.00274 mm^3 (dashed-line fit, Fig. 3C). Applying our previous criterion that the fitted V_0 be within 25% of the FSW mean yields $P_s = 0.83 \times 10^{-4}$ cm/min (solid-line fit, Fig. 3C). Forcing V_0 to 0.00274 mm^3 reduced P_s by 33%. We attributed the low, unconstrained fit V_0 of 0.0011 mm^3 to having only four data points (containing experimental noise).

A similar analysis of the *P. damicornis* isotopic data yielded $P_s = 0.12 \times 10^{-4}$ cm/min if we assume their water permeability is comparable to that of *F. scutaria*; namely $L_p = 2.0$ μm/min/atm. We also tested for the effect of an appreciable gastrovascular cavity volume. None was found based on no difference in the mean uptake of *P. damicornis* larvae that were intact, immersed in labeled glycerol, rinsed in cold glycerol, and measured compared to those that were bisected prior to being rinsed in cold glycerol ($P > 0.05$).

Ice nucleation temperature

During a two-step slow freezing protocol, cells are held at a subzero temperature and the extra cellular solution is seeded with ice. This removal of water in the form of ice dehydrates the cells, concentrating any cryoprotectants that entered the cells. Once they have achieved approximately 90% dehydration, the cells can be safely plunged into liquid nitrogen [33]. If intracellular ice forms within a cell, it is generally lethal, so the temperature at which ice spontaneously forms within a cell must be determined (and avoided in the two-step freezing). Although the freezing point of seawater is −1.9 °C, we found that we could form and propagate visible ice crystals at around −11 °C when seeded. *F. scutaria* in FSW formed spontaneous intracellular ice at a mean temperature of −37.8 ± 1.4 °C. Therefore, in a two-step slow freezing method, we would ideally load the larvae into a cryoprotectant solution in FSW into straws, nucleate the straws, and hold the larvae about 5 °C or more above −37 °C until they were sufficiently dehydrated (e.g., 90%) to avoid the formation of spontaneous intracellular ice. However, none of this ice nucleation information may be relevant to a successful cryopreservation protocol for coral, because the chilling information below suggests that vitrification may be necessary.

Chilling sensitivity

At each step of the slow freezing process, damage should be assessed. Since seawater can be seeded at −11 °C and we determined that *F. scutaria* larvae in FSW spontaneously formed intracellular ice at −37 °C, we tested to determine whether the larvae were damaged by merely chilling them to −11 °C (without any formation of ice in the surrounding FSW). When small samples of Day 4 *F. scutaria* larvae (20 μl containing tens of larvae) were put onto

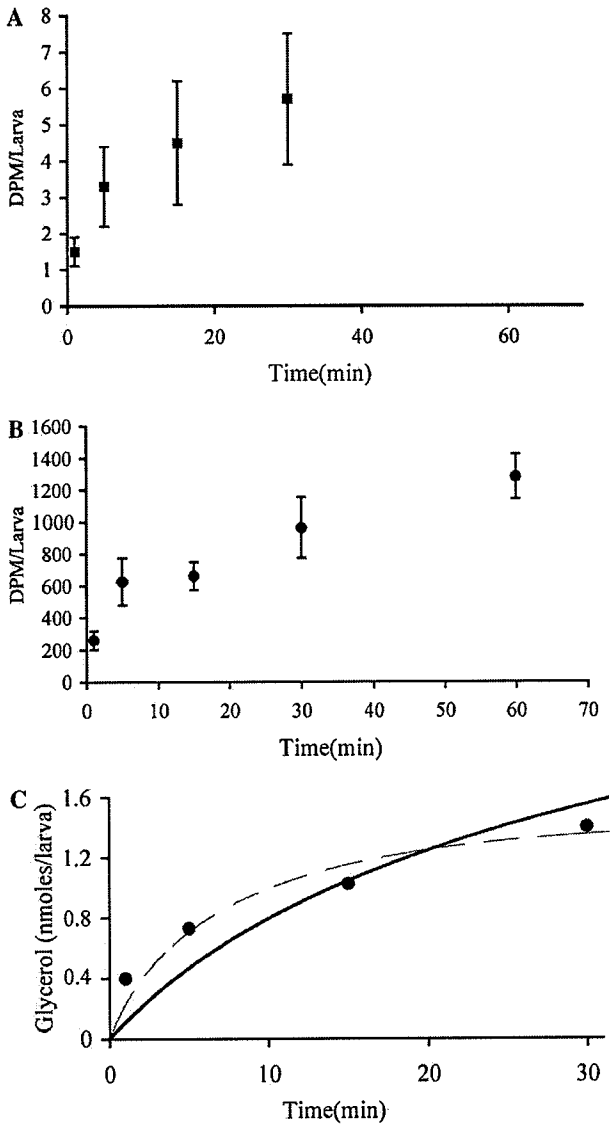

Fig. 3. Coral larva were immersed in 1.54 molal radiolabeled glycerol solutions at 19 °C in FSW. Both species (A) *F. scutaria* (data indicated by filled symbols) and (B) *P. damicornis* (data points indicated by filled symbols) were permeable to the external glycerol as indicated by the uptake of hot glycerol. (C) A model was fit to the *F. scutaria* glycerol uptake data (indicated as filled symbols). The figure demonstrated the effect of varying V_0 with L_p fixed at 2.0 μm/min/atm. The dashed line corresponded to a $V_0 = 0.0011$ mm^3, and yielded the smallest sum of squared error. The solid line corresponded to a $V_0 = 0.00206$ mm^3 with the fitted V_0 limited to ± 25% of the microscopically measured mean value of *F. scutaria* in FSW $V_0 = 0.00274$ mm^3. The corresponding P_s values are 3.2 and 0.83×10^{-4} cm/min, respectively.

the cryomicroscope and chilled from RT to −11 °C at low (0.1–2 °C/min), intermediate (10 °C/min) or high (40 °C/min) rates, held for 1 min, then warmed to RT, they exhibited 100% mortality. This suggested severe cold shock sensitivity. To test this further,

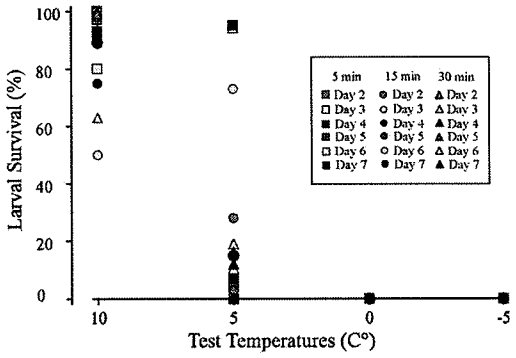

Fig. 4. Coral chilling sensitivity of *F. scutaria* larva show a decrease in survival when the larva were exposed to temperatures below 5 °C, especially for periods longer than 5 min. Although several different developmental stages (Day 2 to 7) were tested they all show the same sensitivity.

another procedure used for many types of chill sensitive cells, such as boar semen, was performed. Polystyrene tubes were filled with a 10 ml sample of Day 4 *F. scutaria* larvae in FSW, loosely sealed, and immersed in 250 ml water in a styrofoam cup. These were chilled slowly from RT in a cold room to 4 °C for several hours. Upon warming, all of the samples were damaged and dead.

Many organisms exhibit chill injury dependent on the developmental stage; however, that was not the case for *F. scutaria* larvae. All developmental stages tested had identical chilling injury (Fig. 4). Larvae held at 10 °C for 5, 15, or 30 min remained relatively robust, but those held at 5 °C began to become damaged, especially at holding times beyond 5 min. All larvae held at 0 or −5 °C were damaged, no matter how briefly they experienced the temperature. Thus, the extreme chill sensitivity for *F. scutaria* larvae seems to be supported by these experiments. Although there may have been some slight difference in the ability of the various developmental stages to handle the 10 or 5 °C chilling, because it was a binary decision (i.e., they were chill sensitive or not), we did not submit the data to statistical analysis to pull out the possible variation in the developmental patterns. Most of the chilling damage was in the form of membrane damage which was present during the chilling phase but became more severe upon rewarming (Fig. 5).

Settling

Pocillopora damicornis larvae, possessing maternally derived zooxanthellae, settled on laboratory glassware within hours to days of being released

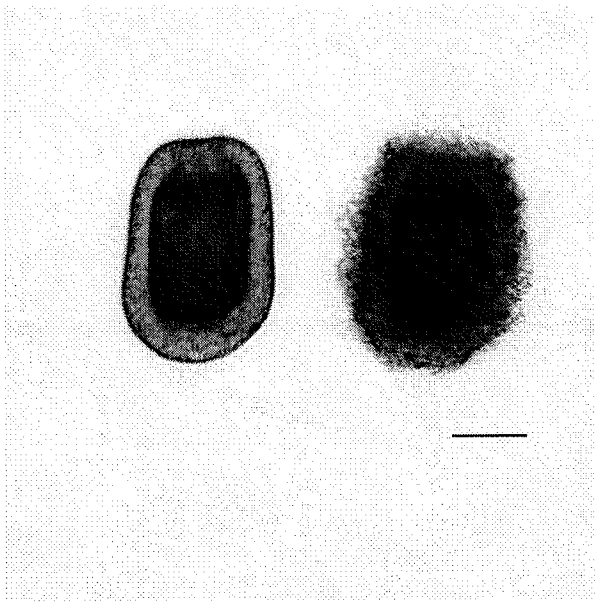

Fig. 5. *Fungia scutaria* larvae were slowly chilled from RT (left image) to 4 °C (right image), then rewarmed on a cryomicroscope at 0.1 °C/min. Membrane damage to the chilled larvae was extensive, resulting in 100% mortality. Bar = 50 μm.

from the adult. There was no problem growing these larvae in FSW in the laboratory.

Eight-day-old *F. scutaria* larvae settled on the glass slides within a few days after being placed in the settlement chambers. However, these larvae required more attention. *F. scutaria* produces gametes that lack zooxanthellae, therefore the larvae required experimental infection with homologous zooxanthellae extracted from adult corals prior to commencing the settling experiment. Furthermore, the newly settled polyps are extremely small (approximately 100 μm diameter) and very susceptible to algal overgrowth. Since settlement and culture occurred in a flow-through seawater table receiving unfiltered seawater, encroaching algal growth required constant removal using fine needles under a dissection microscope. In spite of these difficulties, we were successful in maintaining larvae that had settled on the glass slides for over 30 days (Fig. 6). Previous efforts to culture *F. scutaria* larvae in glass dishes containing FSW in the laboratory (to eliminate the difficulties associated with algae removal) have not been successful (Krupp and Hollingsworth, unpublished). Larvae cultured in the laboratory readily settled on glassware or slides and began to secret calices, but after several days, the polyps "bailed out" of their calices and did not resettle. It is unclear what aspect of the settlement chambers

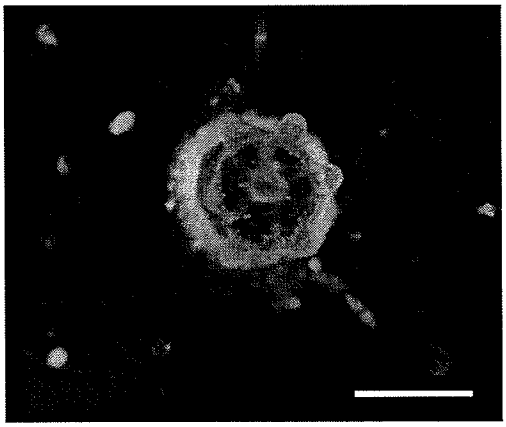

Fig. 6. *Fungia scutaria* larva can be successfully settled under laboratory conditions. This image shows the beginnings of tentacles and the calcium calyx. Bar = 100 μm.

(light, water motion, dissolved oxygen concentrations, zooplankton prey, etc.) enhances survival. No larvae settled on the control slides.

Discussion

We conducted physiological experiments on coral larvae to reveal their tolerance to cryoprotectants, their membrane permeability to water and cryoprotectants, their intracellular nucleation point, and their tolerance of chilling temperatures. Coral larvae demonstrated similarities and differences from other invertebrate larva in these physiological properties. For example, coral larvae are widely tolerant to many cryoprotectants, as are many other invertebrate embryos and oocytes, such as sea urchins [2,8], oysters, clams, and scallops [20,41,42, 28,11,40], starfish [26,15], polychaete worms [39], fruit flies [46,34], and flies [47]. Like starfish oocytes [26,15], coral larvae share a great sensitivity to chilling temperatures. However, coral larvae permeability to water and cryoprotectants seem to be very different from other invertebrates. Permeability parameters at 20 °C have been measured in a few other marine species, including sea urchin [1] and hard clam eggs [28]. In fertilized sea urchin eggs, *Evechinus chloroticus*, $L_p = 0.65$ μm/min/atm and the P_s of DMSO, ethylene glycol, and propylene glycol averages 8.6×10^{-4} cm/min. In small abalone eggs, *Haliotis diversicolor*, $L_p = 0.38$ μm/min/atm and in hard clam eggs, *Meretrix lusoria*, $L_p = 0.14$ μm/min/atm. These other marine data are consistent with our observation that L_p is relatively high and P_s

relatively low in *F. scutaria*. The *F. scutaria* permeability data were collected in Table 2. These data were assessed to come up with 'best estimate' values for *F. scutaria*. The 5 and 7% glycerol data indicate that L_p is a least 2 μm/min/atm, but possibly several times larger. We preferred to err on the conservative side and used a best-estimate value of 2 μm/min/atm. The failure of the data to pin down L_p was not unexpected. It is well understood that for a shrink swell response, the shrink response is dominated by L_p and the swell response by P_s. Here, the rapid response of the *F. scutaria* yielded no data from the shrink part of the curve and, therefore, limited information, other than a lower bound, about L_p.

The glycerol permeability varied by a factor of six between the smallest and largest fit values with the volume kinetic data, yielding larger values and the radiolabel experiments, smaller values. We took a simple average yielding $P_s = 2.3 \times 10^{-4}$ cm/min. In fitting these data, the initial larval volume (V_0) was made a fitting parameter. It has been our experience that this typically leads to better fits. The consequences of forcing V_0 to have the presumed value are clearly shown in Fig. 2C, where this value (0.00274 mm^3) yielded a fit which clearly did not 'fit' the data. However, most fitting parameters required some form of constraint. For instance, V_0 obviously can not be negative. Just how much to constrain V_0 was a matter of judgment depending on numerous factors. The choice was somewhat arbitrary and driven by the following considerations: (i) too much freedom for the fitting parameters can easily leads to 'good' fits that yielded meaningless numbers; (ii) the difficulty of accurately measuring the FSW volume (V_0) of *F. scutaria* because of movement and modest shape changes while moving; and (iii) the desire to allow enough freedom in V_0 to obtain sensible fits to the data. With these considerations in mind, we chose to constrain the fitted V_0 to ±25% of the mean value in FSW. Had V_0 not been constrained, the average Ps would have been 2.4×10^{-4} cm/min, fortuitously, only a modest 4% increase.

The volume kinetic data suggested that the isotonic volume may be larger than the mean value in FSW of 0.00274 mm^3 while the radiolabel data suggested V_0 is less than that. Therefore, we took the middle value ($V_0 = 0.00274$ mm^3), the microscopic volume measurement in FSW, as the best measure of V_0.

Using these permeability data for *F. scutaria*, we modeled their intracellular glycerol concentration, as a function of time, on exposure to 1.54 molal external glycerol (Fig. 7). The model suggested that the internal glycerol reaches 90% of its final concentration in 25 min; not an unreasonable time for cryoprotectant loading. Using our preliminary parameter estimates for *P. damicornis* (Table 2) yielded a glycerol concentration of only 27% of final after 4 h. Since the V_0/A_0 ratio of *P. damicornis* was ten times greater than that of *F. scutaria,* and because *P. damicornis* appears to have an order of magnitude lower permeability to glycerol, this result was not surprising. However, it suggested that larger species of coral larva may be much harder to cryopreserve because of their long loading time for cryoprotectant.

Comparing coral larvae permeability with other cell types, *F. scutaria* larvae have a relatively high water permeability and relative low glycerol (solute) permeability. In Kaneohe Bay, where these larvae were harvested, the high water permeability may help protect against large variations in sea water salinity due to flood water run off into the bay [27].

All the developmental stages of *F. scutaria* coral larvae that we tested showed a step chill sensitivity below 10 °C with no larvae surviving at or below 0 °C. Many organisms show some type of chilling

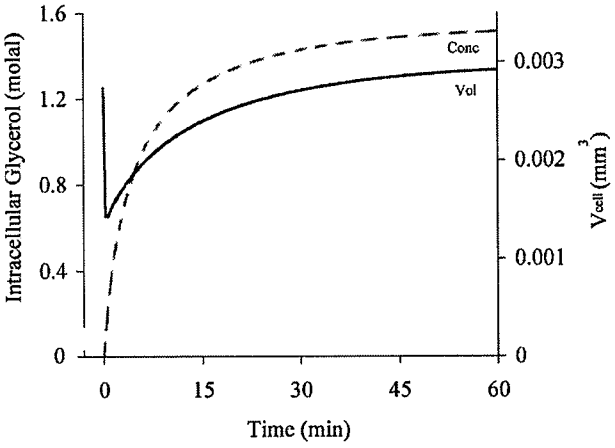

Fig. 7. Using the best estimate parameters, this model demonstrated that the intracellular glycerol of *F. scutaria* in a 1.54 molal glycerol solution in FSW at 20 °C reached 90% of its final value within 25 min. The rapid initial decrease in larva volume was as the result of the high water permeability. Kinetic response of the glycerol molality was represented by a dashed line and larva volume was represented by a solid line. The best estimate parameters used were: $L_p = 2.0$ μm/min/atm, $P_s = 2.3 \times 10^{-4}$ cm/min, and $V_0 = 0.00274$ mm^3.

sensitivity. Insects have varying chill sensitivities, depending on the season of the year and their "cold-hardiness" [50,38]. In one of the most thoroughly investigated species, Mazur and colleagues [33] found that *Drosophila* embryos show sensitivity to chilling, and die in increasing numbers when exposed to temperatures between 0 and −25 °C in the absence of ice formation. Early-stage embryos (before 12 h) were the most sensitive [33]. Myers and Steponkus [37] found that conditioning *Drosophila* embryos to 0 °C for 1 h reduced their sensitivity. Stage-dependent chill sensitivity has also been reported for many species of fish embryos by Maddock [31] (for brown trout), Haga [12] (for rainbow trout), Cloud et al. [4] (for fathead minnows), and Zhang and Rawson [51] and Hagedorn et al. [13] (for zebrafish). Before 50% epiboly in zebrafish, the embryos are extremely sensitive to chilling, and this sensitivity decreases somewhat with development. However, both Zhang et al. [52] and Liu et al. [30] found ways to mitigate this sensitivity in older zebrafish embryos by exposing them to methanol or reducing their yolk lipids. Oocytes of many mammals are extremely chill sensitive, as well. For example, rhesus monkey oocytes chilled to 0 °C for as little as 1 min showed tubulin depolymerization, however this could be partially reversed by culturing at 37 °C for 1 h [45]. GV-staged bovine oocytes are very sensitive to chilling and show damage at 10 °C where only 6% develop after exposure and almost none developed after exposure to 0 °C [32]. Clearly, in some species the chilling sensitivity can be mitigated as long as extensive damage is not evident. This ability to mitigate or reduce the sensitivity in coral is unlikely, because membrane damage becomes evident even with short exposures to chilling temperatures.

Another type of chilling injury is cold shock. Generally, cryoprotectants are added to the cells at room temperature and then the solution with cells is slowly cooled to minimize cold shock. Therefore, it is the rate of cooling which is critical. Ram, bull, rabbit, and human spermatozoa are sensitive to cold shock stress [48,19,7]. Coral larvae chilled very slowly did not show any improvement in their survival, in fact all the larvae chilled at 0.1 °C/min to 4 °C died. This suggests that slow freezing will not be an option and that vitrification will be necessary to achieve cryopreservation.

As cryopreservation techniques are developed for coral, elucidating the factors that promote larval settlement and metamorphosis become increasingly important. Successful cryopreservation requires freezing, thawing, and settling larvae that grow and reproduce normal offspring. Coral larvae from spawning species become competent to settle between 18 and 72 h following fertilization, depending on species and egg size [43]. Smaller Favid embryos develop cilia and become competent more quickly that larger Acropora embryos. However, Acropora and Goniastrea larvae are very selective, and previous experiments indicate several species are highly specific, settling only on particular species of crustose coralline algae (*Hydrolithon reinboldii*) [35].

Our work indicates that the brooded, zooxanthellae planulae of *P. damicornis* are fairly non-specific and will readily settle on laboratory glassware that can be easily maintained in the laboratory. While this aspect makes *P. damicornis* an attractive species for cryopreservation as the larvae do not require experimental infection with symbionts and no special care is necessary to promote settlement and metamorphosis, the larva's rapid shape changes hinder collecting accurate morphological measurements. Furthermore, our work suggests that the larger *P. damicornis* larvae may require a longer cryoprotectant loading time, thus making *P. damicornis* larvae more difficult to cryopreserve.

In contrast, morphological measurements of *F. scutaria* larvae were considerably easier to obtain and the larvae required a shorter loading time for cryoprotectant. However, *F. scutaria* produces gametes that lack zooxanthellae. These larvae must take up their zooxanthellae from their environment at around Day 3 or 4 [27,49]. In experimental conditions, these zooxanthellae can be extracted from a few adult corals infecting millions of larvae. Unfortunately, this process damages the adults, and seems incongruent with our ultimate conservation objectives. However, nondestructive cultures of zooxanthellae are currently under development (Hollingsworth et al., unpublished) and may provide a reliable means of infecting *F. scutaria* larvae with homologous zooxanthellae for future cryopreservation studies. Depending on the stage needed for vitrification, the *F. scutaria* larvae will either be frozen with their symbionts, or the symbionts will have to be frozen separately.

In spite of the difficulties associated with maintaining newly settled polyps, *F. scutaria* was relatively easy to settle, making it an attractive species for cryopreservation. Combined with the fact that *F. scutaria* reliably spawns on multiple nights from

June to October, we believe that *F. scutaria* will provide an excellent model system for understanding the physiology, reproduction and cryobiology of coral larva.

Acknowledgments

We thank Dr. Gordan Grau, Hawaii Institute for Marine Biology, for use of his equipment and hosting us in his laboratory during the summer on Coconut Island, and Dr. Lee Anne Hyak, Smithsonian Institution, for her assistance with statistical analysis.

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