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Using cryopreserved coral sperm for larval production at a restoration-relevant scale

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Abstract

Coral reefs worldwide are facing a crisis as the impacts of climate change and ocean warming increase. Cryopreservation and biobanking can play a crucial role in reef restoration by securing genetic diversity and supporting breeding programs for resilient corals, but will require upscaling of current fertilization methods using cryopreserved sperm by several orders of magnitude. We undertook fertilization experiments at increasing egg densities to better understand the fertilizing capacity of the current cryopreservation unit, and whether the increased egg densities during fertilization impacted on larval settlement and early survival of coral recruits. Overall, fertilization success using cryopreserved sperm was comparable to that of fresh sperm across all egg densities. Indexing post-thaw motile sperm concentration to that of fresh sperm enabled fertilization rates of up to 96% at an egg density of 1000 eggs/mL, potentially enabling the production of up to 960,000 larvae in one litre, with no impact on larval competence or survivorship of spat to eight weeks post-settlement. This study provides the first steps in utilising cryopreserved sperm to produce coral larvae at the scale required to support coral aquaculture production, and will help to inform the efficient use of existing cryopreserved genetic resources in coral biorepositories globally.

Introduction

Coral reefs worldwide are facing a crisis as the impacts of climate change and ocean warming increase. Current projections indicate that reefs globally will suffer significant declines in coral cover and reef function over the coming decades [1–3], with a subsequent loss of genetic and biodiversity that could impact the ability of coral to adapt to the changing climate. Biorepositories for cryopreserved germplasm can play a crucial role in reef restoration efforts by helping to maintain genetic diversity and supporting breeding programs for resilient corals. Cryopreservation enables cost-effective, long-term, storage of genetics for selective breeding, and supports meta-population management by enabling genetic crosses that would otherwise not be possible due to spatial or temporal separation of individuals [4,5]. Cryopreservation and biobanking of germplasm are used extensively for the genetic management of livestock in agriculture [6,7], especially in the dairy industry for which there is a global market for cryopreserved bull semen to facilitate genomic selection for herd improvement. A number of tools and technologies have been developed to support these commercial scale applications, including systems for sample assessment, packaging, and freezing. Biobanking programs for wildlife, including corals, often utilise these same tools and technologies, albeit on a much smaller scale, and typically focus on sperm, which are easier to collect and freeze compared to eggs and embryos.

Since the development of coral sperm cryopreservation in 2006 [8], thousands of sperm samples from over 55 coral species have been cryopreserved and banked in secure biorepository facilities around the world [9]. These samples have all been cryopreserved using a standardised approach, based on gamete collection at a ratio of five mL of gamete bundles over five mL of seawater to provide a high sperm concentration sample. Coral sperm are cryopreserved at a concentration of approximately 1×10^9/mL in

FSW with 10% dimethyl sulfoxide (DMSO) by using a custom made, 3D-printed, cryopreservation device to cool 1 mL aliquots at a rate of − 20°C/min using liquid nitrogen [10]. To date, samples have primarily been biobanked with a view to securing existing genetic diversity for the future, and experiments have shown that cryopreserved coral sperm can be used to produce larvae [11], facilitate assisted gene flow [12,13], and to produce coral spat for out planting [14].

In vitro fertilization of coral eggs is usually based on a fertilizing medium containing approximately 10 eggs/mL and a sperm concentration of 1×10^6/mL, with gametes incubated together for thirty minutes up to several hours before the eggs are washed to remove the sperm [15–17]. To date, fertilizations using cryopreserved sperm have been done in relatively small volumes (i.e., < 500 mL) in comparison to fertilizations using fresh sperm, which often utilise volumes in the range of litres to tens of litres. Although the small fertilization volume minimises the number of cryopreserved samples that need to be thawed, it also limits the number of eggs that can be fertilized if the 10 eggs/mL conventional density is maintained. Even a relatively modest fertilization quantity of 100,000 eggs in ten litres would require the use of ten cryovials to achieve a total sperm concentration of 1×10^6/mL (and likely twice as many given an expected post-thaw survival of approximately 50%), which is potentially prohibitive even with streamlining of current cryopreservation workflows for corals [18]. Larval production capacity using cryopreserved sperm will therefore require upscaling by several orders of magnitude to produce the hundreds of thousands to millions of larvae that will be required to support coral aquaculture and deployment at a restoration-relevant scale.

One option to achieve this scale would be to increase the capacity of current cryopreservation processes for coral by integrating commercial-scale technologies developed in the dairy industry, thereby increasing the number of samples that could be cryopreserved and be made available for fertilization. However, given the significant financial investment required for this approach, we first wanted to better understand the fertilizing capacity of the current cryopreservation unit to both inform future upscaling requirements and to maximise the efficiency of using cryopreserved sperm. To this end, we undertook experiments at increased egg densities of 100 and 1000 eggs/mL for fertilization, to increase the potential number of larvae while keeping the fertilization volume and sperm requirements relatively low. We also investigated the time required for fertilization to occur to minimise the duration that eggs are held at high concentrations, which could potentially cause oxidative stress and degradation of eggs with prolonged exposure. Finally, we assessed whether the increased egg densities during fertilization impacted on larval competence by assessing settlement and early survival of coral recruits. This study provides the first steps in utilising cryopreserved sperm to produce coral larvae at the scale required to support coral aquaculture production, and will help to inform the efficient use of existing cryopreserved genetic resources in coral biorepositories globally.

Results

Fertilization using cryopreserved sperm

Overall, fertilization success using cryopreserved sperm was comparable to that of fresh sperm across all egg densities and fertilization time points for *Acropora kenti* on both nights (Fig. 1d–e). On the first night, fertilization at the 60-minute timepoint in the 1000 eggs/mL treatments was 83 ± 0.1% using fresh sperm and 70 ± 0.1% using cryopreserved sperm, and on night two fertilization exceeded 90% in all treatments. On the first night, there were no significant differences between fertilization rates using fresh or cryopreserved sperm at any egg density at the 15-minute time point ($p > 0.05$). At a fertilization density of 1000 eggs/mL, the fertilization rate with fresh sperm was significantly higher than with cryopreserved sperm at the 30 min ($p = 0.000$) and 60-minute ($p = 0.000$) timepoints, but no other significant differences were observed between fertilization rates using fresh or cryopreserved sperm at any of other egg densities or time points (Fig. 1d). Fertilization rate using cryopreserved sperm did not differ over time at 10 eggs/mL ($p = 0.564$), 100 eggs/mL ($p = 0.420$), or 1000 eggs/mL ($p = 0.507$), indicating that most fertilization occurred early in the 60-minute fertilization period. In contrast, fertilization using fresh sperm at 1000 eggs/mL showed significantly higher fertilization at 30 minutes ($p = 0.000$) and 60 minutes ($p = 0.000$) compared to the 15-minute timepoint, whereas fertilization at 10 eggs/mL ($p = 0.534$) and 100 eggs/mL ($p = 0.352$) did not differ over the 60-minute fertilization period. At the 60-minute timepoint, fertilization in the 1000 eggs/mL treatments was 83 ± 0.08% in the fresh sperm treatment and 70 ± 0.05% using cryopreserved sperm. On the second night, fertilization rates exceeded 90% across all treatments (Fig. 1e), with a background fertilization rate of 18 ± 4%, and there were no significant differences between fertilization rates using fresh or cryopreserved sperm at any egg density or at any time point ($p > 0.05$). Fertilization rates using cryopreserved sperm did not differ over time at the 10 eggs/mL ($p = 0.888$) and 100 eggs/mL ($p = 0.657$); however, at 1000 eggs/mL, the fertilization rate using cryopreserved sperm showed a slight but significant increase at the 60-minute time point compared to 30 minutes ($p = 0.027$).

Settlement and early post-settlement performance

Larvae produced using cryopreserved sperm at a density of 1000 eggs/mL developed normally and were able to successfully settle, metamorphose, and take on algal photo endosymbionts at similar rates to larvae produced at a density of 1000 eggs/mL with fresh sperm, and to control larvae produced using standard larval production methods. Immediately after settlement, the number of spat per settlement plug was significantly higher in the control compared to the cryo treatment ($p = 0.020$), and this difference was maintained at eight weeks post-settlement ($p = 0.006$) despite a significant decline in the number of spat per settlement plug in all treatments (fresh $p < 0.0001$; cryo $p < 0.0001$; control $p < 0.0001$) (Fig. 2b). Despite this, the number of settlement plugs with at least one spat in the cryo treatment did not differ significantly from the number of plugs with spat in the fresh sperm or control treatments either immediately after settlement (fresh $p = 0.900$; control $p = 0.329$) or after eight weeks (fresh $p = 0.737$; control $p = 0.262$) (Fig. 2c). At eight weeks post-settlement, a significant decline was observed in the number of settlement plugs with spat in the cryo ($p = 0.007$), fresh ($p = 0.007$), and control ($p = 0.013$) treatments, which was consistent with expected early juvenile mortality across all treatments (Fig. 2d–f).

Discussion

While threats to coral reefs continue unabated, the need for coordinated biobanking efforts and the development of biorepositories is increasing as scientists, reef managers, and communities seek ways to secure a future for their local reefs. Key to this will be the development of methods to integrate cryopreserved germplasm into aquaculture production of coral recruits to enable the transfer of resilient genomes and phenomes back out onto reefs at scale as part of strategic restoration efforts. Fertilization experiments undertaken in the present study indicate that existing systems for cryopreserving coral sperm (i.e., 1 mL samples in cryovials) may in fact be suitable for use at production scale, assuming that appropriate quantities of high-quality gametes can be obtained for cryopreservation. While future commercial scale applications for coral aquaculture will likely still require upscaling of cryopreservation processes, this is an important outcome as it indicates for the first time that it will be possible to use existing cryopreserved sperm samples at a restoration relevant scale.

By indexing the post-thaw motile sperm concentration to that of the fresh sperm samples, fertilization success using cryopreserved sperm was comparable to that of fresh sperm across all egg densities and fertilization time points for *Acropora kenti* (Fig. 1). At the highest density tested (i.e., 1000 eggs/mL) the fertilization rate at 60 minutes was 70.4 ± 0.1% on night one and 96.1 ± 0.01% on night two, which would correspond to the production of approximately 700,000–960,000 larvae when scaled to a 1 L fertilization volume, sufficient to stock large-scale rearing systems [19]. The downside to this approach of indexing post-thaw sperm additions against motility rate in the control is that the number of motile sperm used was relatively low in all treatments when fresh sperm quality was poor. Future experiments may be able to address this by increasing the amount of sperm added (both fresh and cryopreserved) to meet a pre-determined threshold (e.g., $1 \cdot 10^6$ motile sperm/mL). An interesting finding of this experiment was that the highest egg density (1000 eggs/mL) generally corresponded to the highest fertilization rates, in contrast to what might be expected when the egg:sperm ratio is reduced and consequently there are fewer sperm available per egg. Further experiments will be required to better understand the fertilization dynamics of using cryopreserved sperm together with high egg densities, and to determine the optimal motile sperm:egg ratios for this new approach.

Another component of the present study that will require refinement for upscaling the use of cryopreserved sperm is the preparation of eggs for fertilization. One of the primary benefits of using cryopreserved sperm as part of breeding programs is the ability to generate specific crosses between individuals. For corals, this requires the isolation of individual colonies for spawning and the cleaning of eggs to remove homologous sperm. To maximise the chances of successful fertilization, gametes need to be combined as soon as possible after spawning, ideally within two hours of sample collection. The methods used to clean eggs in the present study were effective but were time consuming and required multiple personnel to ensure that fertilization could be achieved within this time window. Future use of cryopreserved sperm for larval production will therefore require an automated approach similar to that used by the coral autospawner system [19] to minimise the number of personnel required and ensure efficient preparation of eggs for fertilization. The pooling of eggs from multiple colonies is unlikely to be

a common approach for using cryopreserved sperm due to the potential for background fertilization, and was used in the present study primarily to achieve the egg quantities required for the highest egg densities while minimising colony-to-colony variation across the treatments. Nonetheless, assessment of the pooled eggs on the second spawning night showed a background fertilization rate of 18 ± 4%, which is a relatively small proportion of the > 90% fertilization rates observed on that night. Although it was not assessed on the first night there is no reason to expect that background fertilization would have been any higher on night one than on night two, given the overall lower fertilization rates observed on that night. An approach that may work is to treat each 5 mL of bundles collected for sperm cryopreservation as a fertilization unit, with each set of eggs cleaned and fertilized separately following isolation of the sperm. This would equate to approximately 25,000–30,000 eggs in the fertilization vessel, but could potentially be scaled by increasing the number of bundles collected while maintaining the 1:1 ratio with FSW to obtain samples for cryopreservation.

In addition to egg density, we also looked at fertilization time, i.e., the duration of sperm exposure before eggs were assessed for fertilization. Incubation times for coral fertilization protocols range from 30 minutes up to several hours before the eggs are washed [15–17]; however, it is unclear how long this exposure time actually needs to be to achieve fertilization when using high egg densities. Defining fertilization time course would potentially improve embryo health, especially at higher egg densities, by enabling them to be cleaned and moved into optimal culture conditions sooner. By removing eggs from the fertilization mixture at 15, 30, and 60 minutes after sperm addition and rinsing in SDS and seawater to inactivate and remove sperm, we found that the majority (> 75%) of fertilization occurs within 15 min of sperm addition, with a further slight increase to 30 minutes (Fig. 1d–e). Although in some instances there were further increases in fertilization up to 60 minutes, we also noted a higher incidence of abnormal eggs at this time point (i.e., elongated or otherwise misshapen) likely caused by the constant air bubbling required to prevent eggs from forming a thick layer at the water surface and potentially becoming anoxic. These observations indicate that eggs fertilized using this approach would likely need to be transferred into culture after around 30 minutes to minimise the risk of damage to the eggs while providing sufficient time for fertilization to occur. The development of fertilization vessels that more efficiently maintain egg health during fertilization would be beneficial to enable greater workflow flexibility while maintaining egg and embryo quality.

An important requirement for generating coral recruits using cryopreserved sperm is that the offspring produced are able to achieve key early developmental milestones including settlement, metamorphosis, and acquisition of photosynthetic endosymbionts [11,13,14]. Outcomes from settlement experiments in the present study indicated that fertilization of *Acropora spathulata* eggs at high density with cryopreserved sperm did not affect larval settlement rates or survival of coral spat over an eight-week period. Although there was a decline in the number of coral spat over the eight-week period across all treatments, this was not unexpected since coral typically show high rates of mortality in the first weeks and months following settlement [20,21]. Despite a higher number of spat per plug in the control compared to the high-density fertilization treatments, there was no difference in the number of plugs with spat at 8 weeks

across the treatments (Fig. 2). This is important because it means that cryopreserved sperm in combination with high-density fertilization could potentially be used to generate coral recruits at a restoration relevant scale with outcomes equivalent to using traditional fertilization methods. Moreover, studies on the effects of settlement density in *Acropora* species indicate that survival of coral spat at one month is not always density dependent [22], and a higher initial settlement density may actually result in a lower longterm colony abundance at two-years post-settlement compared to low or moderate settlement rates [23]. Future studies will need to determine whether the methods developed and outcomes at eight weeks observed in the present study translate into longterm survival either in *ex situ* grow-out systems or, ideally, following deployment onto the reef.

Collectively, these results demonstrate that high-density fertilization using cryopreserved sperm can provide an effective method for producing large numbers of competent coral larvae capable of settlement, metamorphosis, and photo endosymbiont acquisition. While further work is required to improve the efficiency of egg preparation for fertilization and to optimise egg:sperm ratios, the refinement of this approach will enable the use of cryopreserved sperm for genetic management and production in restoration aquaculture to support reef resilience.

Methods

Coral collection and spawning

Experiments were undertaken over two spawning seasons: in November 2021 using *Acropora kenti* (formerly *Acropora tenuis* [24]) (Fig. 1a), and in December 2022 using *Acropora spathulata* (Fig. 2a). Both species were collected in the week prior to coral spawning by divers from the Australian Insitute of Marine Sciences (AIMS) and brought to the National Sea Simulator facility (SeaSim) at AIMS in Townsville, where they were maintained in flow-through aquaria with shaded natural light (~ 200 µmol quanta m−2s − 1) at temperature profiles matching the reefs from which they were collected (~ 27 ºC). *Acropora kenti* and *Acropora spathulata* were collected from Davies Reef in the central GBR under Great Barrier Reef Marine Park Authority permits G21/45348.1 and G23/49085.1, respectively, and with free prior and informed consent (FPIC) from the Bindal Traditional Use of Marine Resources Agreement (TUMRA) committee.

On nights when spawning was expected, colonies were transferred individually to 50-L plastic tubs containing 1-µm filtered seawater (FSW) prior to the expected spawning time (19:00, *A. Kenti*; 21.00, *A. spathulata*) and monitored for signs of ‘setting’ (when gamete bundles move into the polyp mouth in readiness for release). Gamete bundles were collected from the water surface with plastic cups, then sub-sampled into 50-mL tubes using a transfer pipette at a ratio of five mL of gamete bundles over five mL of FSW (10 mL total volume). The 50 mL tubes were gently agitated to assist bundle break up then the sperm samples were filtered through 100 µm cell strainers (Corning, United States) into new 50 mL tubes to exclude eggs. The filtered sperm samples were then taken for quality assessment and cryopreservation, and the eggs were transferred to a separate station for cleaning.

Gamete preparation and cryopreservation

Sperm quality metrics in each sample were estimated by loading 4 µL of activated sperm [18] into the chamber of a fixed-depth well slide (Leja, 20 µm) and analysing at least 200 individual sperm across a minimum of five microscope fields using Computer Assisted Sperm Analysis (CASA) [18,25]. Total concentration and the concentration of motile sperm were recorded for each sample, and samples were combined in approximately equal amounts by volume to create a pooled sample (n = 4 colonies). Motility and concentration of the pooled sample were assessed using CASA as described then the pooled sample was split, with approximately two-thirds of the sample prepared for cryopreservation and the remainder retained for use as a fresh sperm control. Sperm were cryopreserved at a concentration of approximately 1×10^9 cells/mL in FSW with 10% (v/v) DMSO by cooling at a rate of 20°C/min with liquid nitrogen (–196°C) as described in [18]. Immediately prior to use for fertilization experiments, cryopreserved sperm were thawed by gentle agitation of the cryovial in a water bath containing FSW at 30°C for approximately two and a half minutes until the sample was completely thawed.

For cleaning, eggs from an individual colony were transferred into a filter made from a short (~ 10 cm) piece of 100 mm diameter PVC pipe with a 106 µm mesh screen over one end. The filter with eggs was gently swirled in a 2 L bowl containing FSW, then carefully transferred into a clean 2 L bowl with FSW and gently swirled again. The process of swirling the filter with eggs in FSW then transferring into a clean bowl was repeated at least five times to ensure that as much sperm as possible was removed from around the eggs. Cleaned eggs were transferred into a labelled 2 L bowl containing FSW with gentle aeration (~ 2 bubbles/s), and the process was repeated for the remaining colonies, with each batch of eggs maintained in a separate bowl.

Egg density for fertilization

Egg density and fertilization time experiments were undertaken in November 2021 using gametes from *Acropora kenti*. Fertilization experiments were performed in 1 L Imhoff cones containing 100 mL of FSW at egg densities of 10, 100, and 1000 eggs/mL, corresponding to 1000, 10,000, and 100,000 eggs respectively in the fertilization volume. Eggs at all three densities were fertilized using fresh (control) or cryopreserved sperm in triplicate, for a total of 18 fertilization cones (nine fresh sperm, nine cryopreserved sperm). Given the time required to prepare the gametes and the large numbers of eggs required, it was decided that manually counting eggs for each treatment was not feasible. Instead, the packed cell volume of eggs needed for each fertilization density was calculated by multiplying the volume of an individual egg by the number of eggs required (Table 1). For *Acropora species*, which have egg diameters ranging from 450–600 µm[26], we chose the upper end of the egg size range to avoid underestimating egg densities and calculated a packed cell volume of 113 µL for 1000 eggs as follows:

$$V_{egg} = \frac{4}{3}\pi r^3 \approx 4.18879 \times 0.3\, mm^3 \approx 0.113\, \mu L$$

$$0.113\, \mu L \times\ 1000 = 113\, \mu L$$

In practice, this volume was rounded up to 120 µL for ease of measurement and to allow for a small amount of water space between eggs.

Table 1
Packed cell volumes to achieve the required egg densities for fertilization.

Egg density (cells/mL)	Fertilisation volume	Total number of eggs	Packed cell volume (mL)
10	100	1000	0.12
100	100	10000	1.2
1000	100	100000	12

Immediately prior to fertilization, cleaned eggs from each colony (n = 4–6 colonies) were combined in a 2 L measuring cylinder containing FSW and allowed to settle into a thick layer at the water surface. Eggs were gently pipetted from this layer using either a P1000 micropipetter (120 µL, 10 eggs/mL treatment) or with an electronic autopipetter fitted with a 10-mL serological pipette (1.2 mL, 100 eggs/mL treatment; 12 mL, 1000 eggs/mL treatment) (Fig. 1b) and the required packed cell egg volumes were transferred to 1 L Imhoff cones containing approximately 800 mL of FSW with gentle aeration (~ 5 bubbles/s) (Fig. 1c). For fertilization, aeration was removed from the fertilization cones, and the eggs were allowed to float to the surface, then the water volume was reduced to 100 mL.

Eggs in the control treatments were fertilized using fresh (i.e., not cryopreserved) sperm at a total concentration of $1{\cdot}10^6$/mL, as per traditional fertilization methods for coral. The volume of sperm added to each fertilization cone was calculated based on the total concentration of the pooled fresh sperm sample. A timer was started immediately after addition of sperm to the first cone in the control treatment, with subsequent sperm additions taking place at 30 second intervals. While the fresh sperm additions were taking place, cryopreserved sperm were thawed and assessed as described. The volume of cryopreserved sperm added to each cone for fertilization was calculated by indexing the motile sperm concentration of the post-thaw sample against the motile concentration of the pooled fresh sperm sample. As such, the volume of sperm added and the total sperm concentration in the cryopreserved sperm treatments were higher than in the fresh sperm treatments due to a lower overall motility post-thaw, but the concentration of motile sperm was the same across all treatments. Addition of cryopreserved sperm proceeded at 30 second intervals as described for the control treatments, with a timer started immediately after addition of sperm to the first cone. Gentle aeration (1 bubble/s) was provided throughout the fertilization period to help keep eggs suspended in the water column and prevent them from forming a thick layer at the surface, to minimise the chances of anoxia.

To determine whether there was an impact of egg density on the time required for fertilization to occur, a small sub-sample of approximately 100 eggs was removed from each fertilization cone at 15, 30, and 60 minutes after the addition of sperm using a 3-mL transfer pipette. To immobilise sperm and halt fertilization, each batch of sub-sampled eggs was placed into a 100-µm cell strainer and transferred to a small dish containing FSW with 0.01 g/L sodium dodecyl sulphate (SDS) [27]. The cell strainer with eggs was gently swirled in the SDS solution for approximately 10 seconds then rinsed twice in clean FSW to remove any remaining sperm and SDS. The cell strainer with eggs was then placed into the well of a 6-well plate containing 10 mL of FSW until assessment for fertilization. Fertilization rate was assessed two hours after the 60 min time point (i.e., three hours after sperm addition) by counting approximately 100 eggs and determining the proportion showing cell division.

Larval production and rearing

To determine whether fertilization at a density of 1000 eggs/mL impacted on the ability of the larvae to settle and take on their algal photosymbionts, larval competence experiments were undertaken in December 2022 using gametes from *Acropora spathulata*. Gamete bundles were collected and sperm and eggs were separated and processed as described for the egg density experiments. Sperm samples were combined in approximately equal amounts by volume to create a pooled sample (n = 4) and assessed for motility and concentration using CASA, then approximately two-thirds of the pooled sample was cryopreserved as described. Cleaned eggs (n = 8 colonies) were pooled in a 2 L measuring cylinder and allowed to settle at the water surface. Due to fewer eggs being available from *A. spathulata* compared to previous experiments using *A. kenti*, the packed cell volume of eggs added to each cone was reduced to 10 mL and the fertilization volume was reduced to 80 mL to maintain the approximately 1000 eggs/mL fertilization density. Eggs were fertilized as described with fresh and cryopreserved sperm in triplicate, for a total of six fertilization cones (three fresh sperm, three cryopreserved sperm). Eggs were allowed to fertilize for 30 minutes, then were washed to remove excess sperm by filling the fertilization cones with FSW to approximately 800 mL, allowing the eggs to float to the surface, then draining the water via a tap at the bottom of the cone. This process was repeated three times for each fertilization cone, then the fertilized eggs were allowed to float to the surface and triplicate sub-samples of approximately 100 eggs each were removed from each cone for assessment of fertilization as described.

For stocking of the larval rearing cones, the volume of water in each of the fertilization cones was reduced to approximately 200 mL and the fertilized eggs were combined into a single 2 L jug per treatment. Fertilized eggs were allowed to float to the water surface of the jug, then an electronic autopipetter fitted with a 10 mL serological pipette was used to transfer 2 mL packed cell volume of eggs into each of three 15 L rearing cones per treatment (i.e., three fresh, three cryo) fitted with 212 µm outlet filters, for a stocking density of approximately 1.1 fertilized eggs/mL. An additional two 15 L rearing cones were stocked at the same density with fertilized eggs produced from the same colonies by AIMS colleagues. These eggs were fertilized at a density of ~ 10 eggs/mL in a 50 L plastic tub with

approximately 1x106 sperm/mL, and were included as an additional control for comparison with the high-density fertilization treatments. Rearing tanks were maintained in a temperature-controlled aquarium room (27–28°C) with minimal water flow and no aeration overnight. The following morning (approximately 10 hours post-fertilization) larval rearing tanks were cleaned to remove accumulated lipids from non-fertilized eggs that had broken down. To clean the larval rearing cones, water flow was turned off and the water volume was carefully reduced by approximately 50% via a tap at the bottom of the cone. The insides of the tanks were wiped with paper towel to remove lipids, then the outlet filters were replaced and the tanks were allowed to refill with FSW. Gentle aeration (~ 4 bubbles/s) was provided via a rigid airline, which was positioned at the bottom of the tank so that bubbles passed over the outlet filter to prevent larvae from sticking. After gastrulation (approximately 24-hours post-fertilization) aeration was gradually increased to provide circulation, and water flow was increased to approximately 15 L/h. Larvae were maintained under these conditions for the remainder of the rearing period.

Larval settlement and symbiont infection

Larvae were settled on aragonite plugs that were pre-conditioned with crustose coralline algae (CCA) in outdoor tanks at SeaSim for approximately 10 weeks leading up to coral spawning. For settlement, the conditioned plugs were mounted in small plug trays (52 plugs/tray) in 5 L flow through aquaria with gentle aeration. On day 5 after fertilization, larvae from the same treatment were combined in 2 L plastic jugs and larval density was estimated via manual counts using a dissecting microscope. For each fertilization treatment (i.e., 1000 eggs/mL with fresh sperm, 1000 eggs/mL with cryopreserved sperm, 10 eggs/mL with fresh sperm) approximately 600 larvae were added to the 5 L settlement tanks in triplicate, for a total of nine settlement tanks. Larvae were allowed to settle for 48 h at 28 ºC with slow flow through FSW and gentle aeration to maintain circulation, after which the settlement plugs were randomised across three large plug trays (165 plugs/tray) and maintained in 50 L flow-through acrylic aquaria.

Eleven days after fertilization, settled coral spat were infected with symbionts from the genus *Cladicopium* that were grown in culture at the AIMS Symbiont Culture Facility (culture ID SCF055-01.10). Symbiont culture density was confirmed by manual counts on a haemocytometer, and coral spat in each of the 50 L tanks were exposed to approximately $1.2{\cdot}10^8$ symbiont cells in a volume of 10 L. Spat were maintained at this volume under static conditions for 24 h, after which a further $1.2{\cdot}10^8$ symbiont cells were added. After a total of 48 h exposure to the symbionts, the aquaria were slowly refilled to 50 L with FSW, and the spat were reared in flow-through FSW at 28 ºC under a 12:12 light:dark regimen. Spat were reared with minimal husbandry for eight weeks, during which they were fed once daily with a mixture of microalgae (*Tisochrysis lutea*, *Chaetoceros muelleri*, *Dunaliella spp., Nannochloropsis oceania*, and *Proteomonas sulcata*) and rotifer nauplii.

To assess settlement and symbiont infection, the large plug trays were imaged immediately following the 48-hour infection period, and again after the eight-week rearing period. Images were taken in batches

of 5 plugs along a coordinate grid using a Nikon D810 DSLR with two Ikelite DS160 substrobes, using Camera Control Pro software. Images were subsequently viewed on a computer and the number of coral spat on each plug were counted manually and recorded into an Excel spreadsheet.

Statistical analyses

Triplicate fertilization data were pooled for analysis by Fisher's exact test of independence and pairwise nominal independence analysis with 'fdr' adjustment using R (R Core Team 2019), with the "rcompanion" package [28]. Settlement data were analysed using GraphPad Prism version 11 for MacOS. The number of individual settlers per plug at settlement (t = 0) and after 8 weeks (t = 8) were compared across treatments using the Kruskall-Wallis test with Dunns multiple comparisons post-hoc test. The number of plugs with settled larvae were compared across treatments at t = 0 and t = 8 by one-way ANOVA with Tukey's post-hoc test. Within each treatment, the number of plugs with settled larvae at t = 0 and t = 8 was also compared by paired T-test. All data are presented as mean ± S.E.M.

Declarations

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Author contributions: JD, AS, RH, and JO planned and designed the experiments; JD, LB, RH, JO, and FMN ran the experiments and collected data, JD analysed the data and wrote the manuscript; all authors reviewed the manuscript.

Data availability statement: Data are available on reasonable request.

Competing interests statement: The authors declare no competing interests.

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Figures

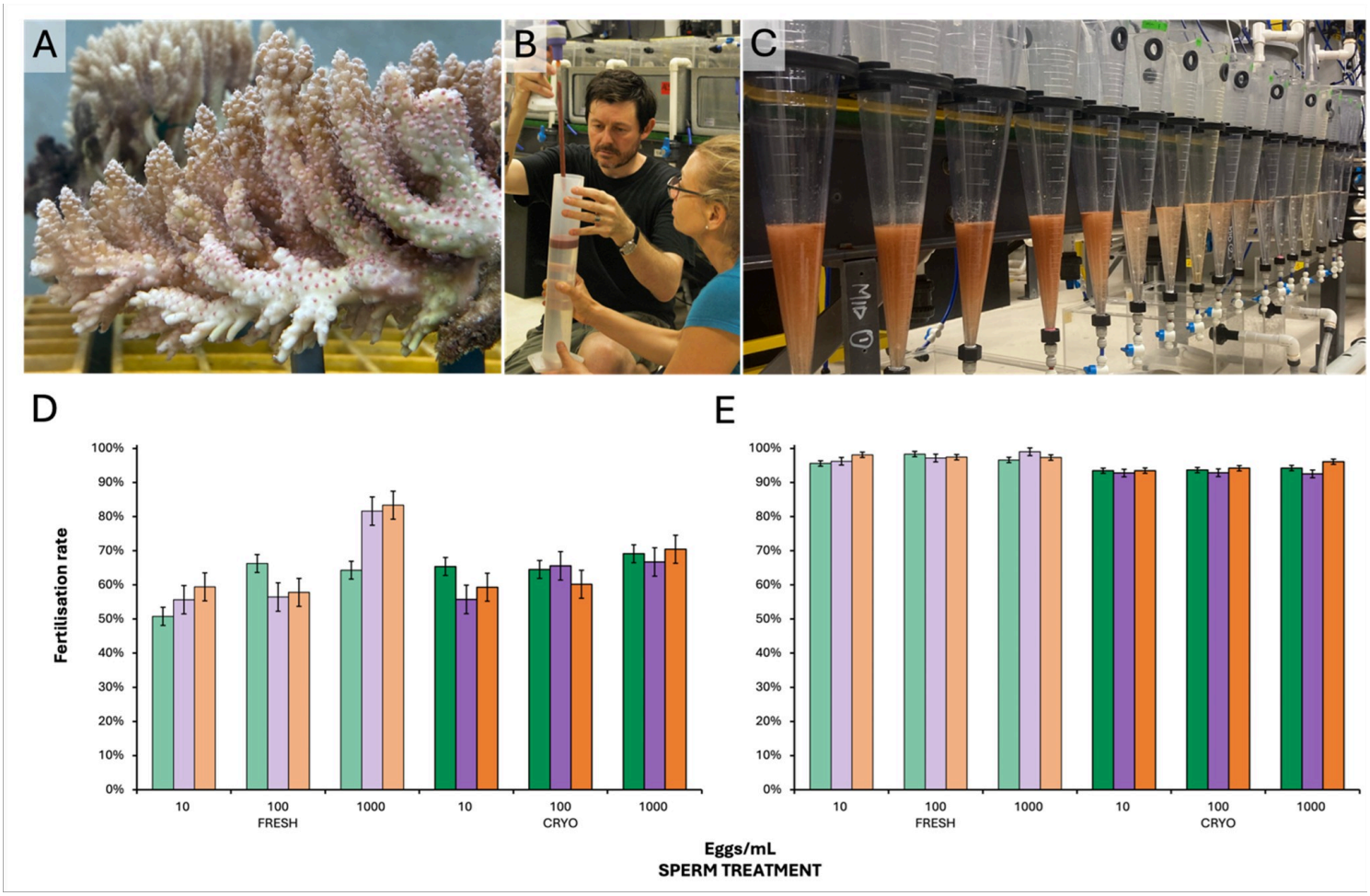


Figure 1

Fertilization of *Acropora kenti* eggs at different egg densities using fresh and cryopreserved sperm. **A)** *Acropora kenti* colony showing 'setting' of gamete bundles in the polyp mouths. **B)**The packed cell volume of eggs was measured using a serological pipette to estimate egg densities. **C)** Fertilization experiments were performed in 1 L Imhoff cones. **D–E)** Comparison of fertilization rates (mean ± S.E.M.) at densities of 10, 100, and 1000 eggs/mL assessed at 15 minutes (turquoise and green), 30 minutes (mauve and purple) and 60 minutes (peach and orange) after addition of fresh (pale columns) and cryopreserved (dark columns) sperm. On night one **(D)**fertilization at the 60-minute timepoint in the 1000 eggs/mL treatments was 83±0.08% using fresh sperm and 70±0.05% using cryopreserved sperm. On night two **(E)** fertilization exceeded 90% in all treatments.

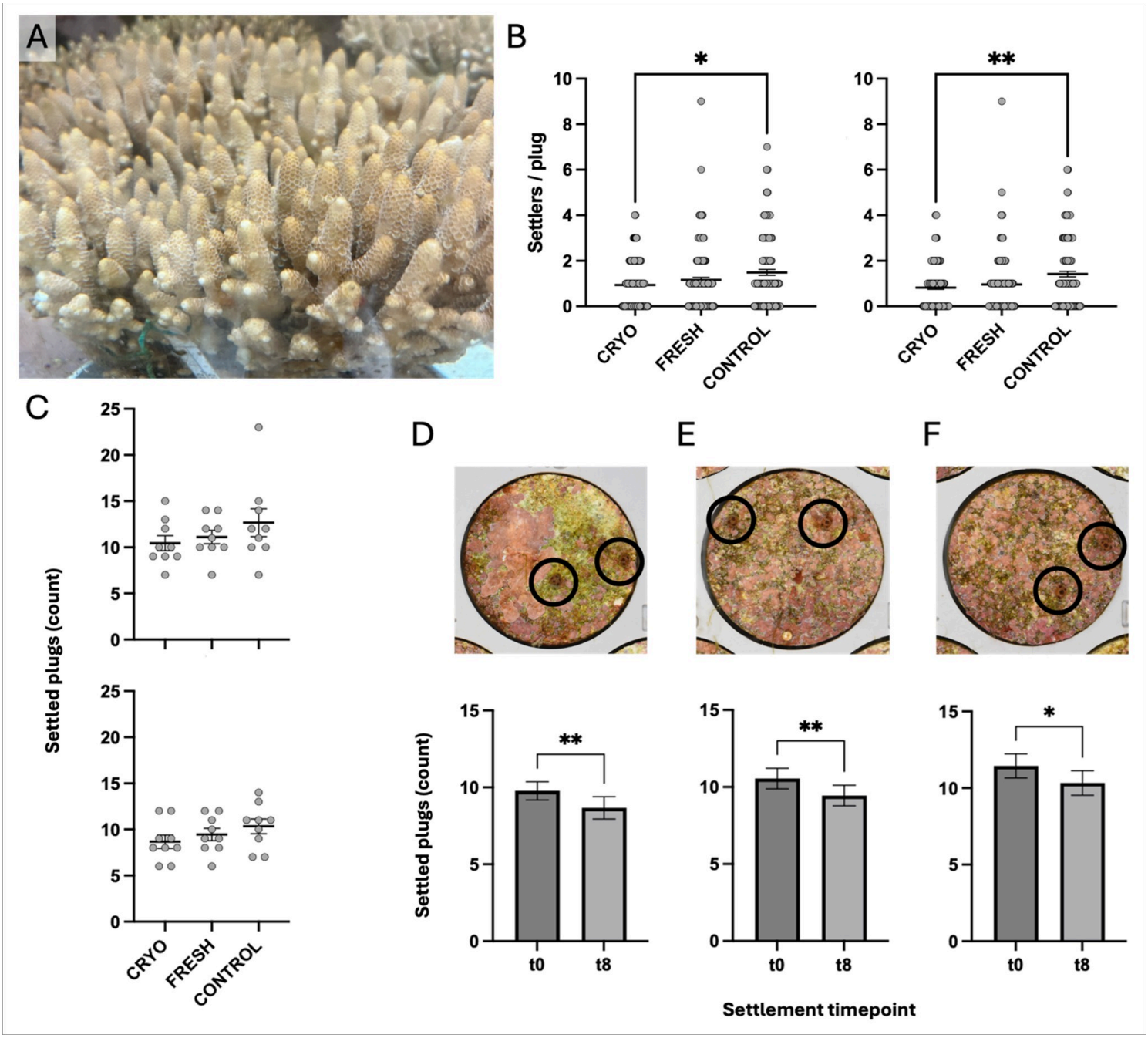


Figure 2

Settlement of larvae from *Acropora spathulata* produced using the high-density fertilization approach (approximately 1000 eggs/mL) with fresh (FRESH) and cryopreserved (CRYO) sperm compared to larvae produced using standard low density fertilization methods (CONTROL). **A)** *Acropora spathulata* colony in a holding tank at AIMS SeaSim. The number of spat per plug differed ($p<0.05$) between the CRYO and CONTROL treatments **(B)** but there was no difference in the overall number of plugs with spat across any of the treatments **(C)**. By 8-weeks post-settlement, spat (black circles) had begun to develop secondary polyps **(D–F)**. A slight but significant ($p<0.05$) decline in the number of plugs with spat was observed at the 8-week timepoint in the CRYO **(D)**, FRESH **(E)**, and CONTROL **(F)** treatments consistent with expected early juvenile mortality for coral.