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# Environmental Tipping Points for Sperm Motility, Fertilization, and Embryonic Development in the Crown-of-Thorns Starfish, *Acanthaster cf. solaris*

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**Abstract:** For broadcast spawning invertebrates such as the crown-of-thorns starfish, early life history stages (from spawning to settlement) may be exposed to a wide range of environmental conditions, and could have a major bearing on reproductive success and population replenishment. Arrested development in response to multiple environmental stressors at the earliest stages can be used to define lower and upper limits for normal development. Here, we compared sperm swimming speeds and proportion of motile sperm and rates of fertilization and early development under a range of environmental variables (temperature: 20–36°C, salinity: 20–34 psu, and pH: 7.6–8.2) to identify environmental tipping points and thresholds for reproductive success. We also tested the effects of water-soluble compounds derived from eggs on sperm activity. Our results demonstrate that gametes, fertilization, and early development are robust to a wide range of temperature, salinity, and pH levels that are outside the range found at the geographical limits of adult distribution and can tolerate environmental conditions that exceed expected anomalies as a result of climate change. Water-soluble compounds associated with eggs also enhance sperm activity, particularly in environmental conditions where sperm motility is initially limited. These findings suggest that fertilization and embryonic development of crown-of-thorns starfish are tolerant to a wide range of environmental conditions, though environmental constraints on recruitment success may occur at later ontogenic stages.

**Keywords:** fertilization; cleavage; gastrulation; sperm motility; temperature; salinity; pH; *Acanthaster* outbreaks

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## 1. Introduction

Outbreaks of the coral-eating crown-of-thorns starfish (CoTS), *Acanthaster* spp., are one of the most significant biological threats to coral reefs and account for a substantial proportion of coral mortality in the Indo-Pacific region [1–3]. CoTS are predisposed to major population fluctuations, whereby local densities may vary by several orders of magnitude [4], due to inherent features of their reproductive biology and behavior [5,6]. Reproductive success is central to explaining periodic increases and abrupt increases in local densities [7]. Understanding the critical events in the early life history of CoTS is key to identifying population bottlenecks that could be strategically targeted to improve control programs and mitigate coral mortality [8]. Despite this, environmental drivers of variation in reproductive success for *Acanthaster* spp. remain poorly understood.

Achieving high fertilization rates is vital in ensuring reproductive success [9]. Fertilization had initially been thought to be non-limiting, given that broadcast spawners, such as CoTS, release copious amounts of gametes during spawning [6,10]. Population replenishment in CoTS is believed to be largely regulated by larval provisioning, larval delivery, post-settlement competition and predation [11–14]. However, a host of factors, at the gamete, individual, and population levels, as well as prevailing environmental conditions, can influence fertilization success [9]. For example, changes in sperm swimming speeds and the proportion of motile sperm affect fertilization success in

CoTS [15] and other echinoderms [16,17]. Previous studies have shown that the number and distribution of individuals and the prevailing flow conditions during spawning dictate the local concentration of gametes [18–20]. Fertilization rates of CoTS have been reported to reach up to 83% at the peak of a major spawning event [21]. In induced spawning experiments in the field, fertilization rates can be as high as 95% when male and female starfish are in very close proximity [20]. As expected, fertilization rates drop significantly as the distance between spawning individuals increases. Nevertheless, 70% fertilization success was still achieved at distances of up to 8 m between spawning individuals and more than 20% at a distance of 60 m [20]. Fertilization success per unit distance in CoTS is higher compared to other asteroid species and significantly greater than those reported for other marine invertebrates [7]. Despite achieving high fertilization rates at given sperm concentrations, at greater distances, and at longer durations from the point of gamete release [22], very little is known on the tolerance of gametes, fertilization, and early development of CoTS to a wide range of environmental conditions.

For broadcast spawning invertebrates such as CoTS, early life history stages occur in the water column where environmental factors could disrupt the initial phases in the process of population replenishment. The persistence and success of populations require that all developmental stages be completed successfully and the variable sensitivity of planktonic stages (*i.e.* gametes, fertilization, early development) to environmental stressors (*e.g.* temperature, salinity, pH) may be a potential population bottleneck [23,24]. Evaluating the effects of environmental stress on gametes and early life history stages is important as this can result in detrimental flow-on effects where physiological performance and cellular responses of subsequent ontogeny depend on the success of preceding stages [23]. In addition, marine organisms are exposed not only to natural environmental stressors, but also the compounding effects of anthropogenic stressors, notably increasing global temperatures, pulses of decreased salinity brought about by higher frequency of cyclones and freshwater runoff, and reduced pH [24]. Climate change causes changes in baseline environmental conditions, such that inherent fluctuations of temperature, salinity, and pH, particularly in nearshore waters, may increasingly exceed tolerance thresholds, especially for populations currently living at physiological limits [25].

Recent studies on the response of early life history stages of marine invertebrates to ocean warming and acidification, have improved our knowledge on environmental thresholds of several species [24]. Generally, temperature affects everything an organism does through its pervasive physiological impact on all biological functions [26]. Ocean acidification has negative impacts on development due to direct pH effects and hypercapnic suppression of metabolism, and is a major threat to marine calcifiers because acidification decreases carbonate saturation with a negative impact on skeleton formation [23,27]. Pulses of reduced salinity brought by heavy rainfall or freshwater lenses of river plumes have been reported to result in decreased growth and reproduction rates in some invertebrates [28] and affect the cellular osmoregulation in gametes and embryos [29]. The response of echinoderms to these environmental stressors are stage- and species-specific, but gametes and fertilization appear to be robust to a wide range of temperature, salinity, and pH levels [30–32]. Environmental tolerances of echinoderm embryos are generally narrower than for gametes and fertilization [16,31,33].

Spermatozoa of free-spawning marine organisms remain immobile at the time of gamete release but become motile spontaneously upon dilution in seawater. Evaluating the response of spermatozoa to environmental factors is important since activation is influenced by seawater temperature, osmotic pressure, extracellular pH, and the concentration of specific ions relative to that in the seminal plasma in echinoderms [34,35]. Sperm swimming speeds in the polychaete, *Galeolaria caespitosa*, have been reported to be enhanced under increased water temperatures [36], but comparable research is yet to be undertaken for most echinoderms [23,27]. Decreased motility and inactivation of sperm at low salinities has also been reported in sea urchins [29,37]. Previous studies on echinoids also show reductions in the percentage of motile sperm at decreased pH and ultimately reproductive success [18–20]. There is also evidence that oocytes from conspecifics release attractants that induce chemotaxis toward the egg [38–40]. In some marine invertebrates,

chemoattractants may not only change the direction of sperm swimming but also increase sperm swimming speeds and the proportion of motile sperm [41–43]. The interactive or additive effects of environmental stressors and egg-derived chemoattractants warrant further attention, especially given potential impacts of climate change on fertilization success.

The purpose of this study is to compare sperm behavior and rates of fertilization, as well as early development under a range of environmental variables to identify environmental tipping points and thresholds for reproductive success. As fertilization immediately follows spawning, existing environmental conditions during gamete release could potentially limit fertilization rates and early development even when sperm-to-egg ratios are optimal, which is expected when spawning individuals are aggregated, gamete release is synchronized, and flow conditions are low to moderate [7]. Here, we examine temperature, salinity, and pH thresholds of sperm motility, fertilization, cleavage, and gastrulation. Reproductive failure in echinoderms has been reported at different levels of these environmental parameters, but few have looked whether this is due to the sensitivity of gametes, failure of fertilization, or failure of fertilized eggs to cleave or hatch [44,45]. We also tested the excitatory effect of water-soluble egg extracts on sperm behavior to add a maternal dimension to the characterization of sperm motility. Sperm swimming speeds and proportion of motile sperm are discussed in relation to fertilization rates. Previous studies on the impacts of these environmental variables on marine invertebrates have mostly set experimental conditions with respect to IPCC [46] projections for temperature rise (2°C to 4°C above ambient), pulses of decreased salinity (regionally variable), and ocean acidification (0.2 to 0.4 pH units below ambient) [47]. Here, we included extreme environmental stressor treatments to determine how far gametes, fertilization, and early development can be pushed to identify tipping points and thresholds for deleterious effects. Developmental arrest in response to multiple environmental stressors at the earliest stages can be used to define lower and upper limits for normal development. Quantifying environmental regulation of initial elements of reproductive success is important in understanding the spatial and temporal dynamics of populations of *Acanthaster* spp., as well as understanding vulnerability to environmental changes.

## 2. Materials and Methods

### 2.1. Collection and maintenance of animals for experiments

Adult individuals of the Pacific species of crown-of-thorns starfish (*Acanthaster* cf. *solaris*) were collected from aggregations in reefs around Puntan Dos Amantes (13° 32.346' N, 144° 48.200' E) on the northwest coast of the island of Guam, Micronesia in October 2013. Starfish were immediately transported to the University of Guam Marine Laboratory and allowed to acclimatise to ambient conditions (28.79 ± 0.23°C; 34.19 ± 0.04 psu; pH 8.23 ± 0.02) in 1000-L concrete tanks with flow-through seawater. Individuals were sexed by drawing contents from gonads along the arm junction using a syringe with a large-bore biopsy needle [7]. Male and female CoTS were placed in separate tanks prior to experiments. Gametes from gravid individuals were examined under a compound microscope to generally assess reproductive maturity of oocytes and sperm motility.

### 2.2. Preparation of experimental seawater

#### 2.2.1. Water-soluble egg extracts

Water-soluble egg extracts and seawater solutions (ESW) were prepared by incubation of unfertilized eggs from five females (standardized to 100 egg ml<sup>-1</sup>) for 60–90 min [41,43] under different levels of temperature, salinity, or pH as described below. Eggs were filtered through a 0.22-µm syringe filter (Millipore, Darmstadt, Germany) and immediately used in experiments. Filtered seawater (0.2-µm) was used as controls and incubated under different levels of environmental treatments. Experimental seawaters were kept in sealed Nalgene® glass containers prior to experiments.

### 2.2.2. Temperature

Preliminary pilot studies have shown that temperature below 20°C resulted in zero fertilization and cleavage. Temperatures ranging from 20°C to 36°C, at 2°C intervals, were tested in this study. This experiment was done inside a temperature-controlled room set at 16°C. Parafilm®-sealed beakers with 0.2-µm filtered seawater were placed in water baths with aquarium heaters (Eheim Jäger, Deizisau, Germany) connected to digital controllers (Aqua Logic Inc., CA, USA) to maintain set temperatures.

### 2.2.3. Salinity

Initial rangefinder experiments showed zero fertilization at 18 psu. Eight salinity levels were tested in this study: 20, 22, 24, 26, 28, 30, 32, and 34 psu. Salinity treatments below ambient conditions (<34 psu) were prepared by adding distilled freshwater to 0.2-µm filtered seawater until set levels are reached. This experiment was done in an incubator (VWR International, PA, USA) set at 28°C. Beakers were fitted with plastic lids that had a 12-rpm synchronous motor attached to a plastic stirrer to maintain set conditions and prevent the formation of artificial haloclines within beakers. .

### 2.2.4. pH

This experiment was conducted to test the tolerance of fertilization and cleavage in CoTS to different pH<sub>NBS</sub> levels: 7.4, 7.6, 7.8, 8.0, and 8.2. Experimental seawater pH levels (below ambient pH 8.2) was achieved by gently bubbling CO<sub>2</sub> into reservoir overhead tanks, using a pH computer (Aqua-Medic of North America, CO, USA) connected to a solenoid valve, until programmed levels were reached. Experimental 0.2-µm filtered seawater was gravity-fed to containers with 45-mm mesh windows enclosed by a plastic jacket placed in water baths set at 28°C.

## 2.3. Sperm speed and motility

Sperm speed (sperm point-to-point velocity = total distance traveled per second) and sperm motility (percentage of motile sperm) were measured from five male starfish, using techniques described for crown-of-thorns starfish [15] and sea urchins [17]. Experimental seawater treatments were prepared as described in the previous section. For each dilution, 2 µl of dry sperm were diluted with 4 ml of experimental seawater. One drop (~100 µl) of this sperm suspension was placed on an albumin-coated microscope slide and a coverslip, which were separated by a 0.75mm thick O-ring and focus set midplane to minimise wall effects on sperm swimming speed [16]. Sperm behaviour was captured using a Canon EOS 60D single lens reflex camera coupled with a Zeiss Axio Scope A1 (A-Plan ph1 10×/0.25 objective). The videocamera was remotely controlled using Canon EOS Utility and set to take 25 frames per second over a two second period. All recordings were made within 10s of the sperm suspension being placed on the slide. For each male, three replicate observations (slides) were made for three independent sperm dilutions under each temperature, salinity, or pH level and water-soluble egg extract treatment combination. Video recordings were post-processed with Sony Vegas Movie Studio HD (Sony Creative Software Inc., Middleton, WI), and 1s video clips from each slide (replicate) were analyzed using computer-assisted sperm analysis (CASA) plugin in Image J [48]. From an average of 200 sperm tracks analysed per slide, average sperm speed and percentage of motile sperm was determined for each replicate (slide).

## 2.4. Bioassays for fertilization and embryonic development

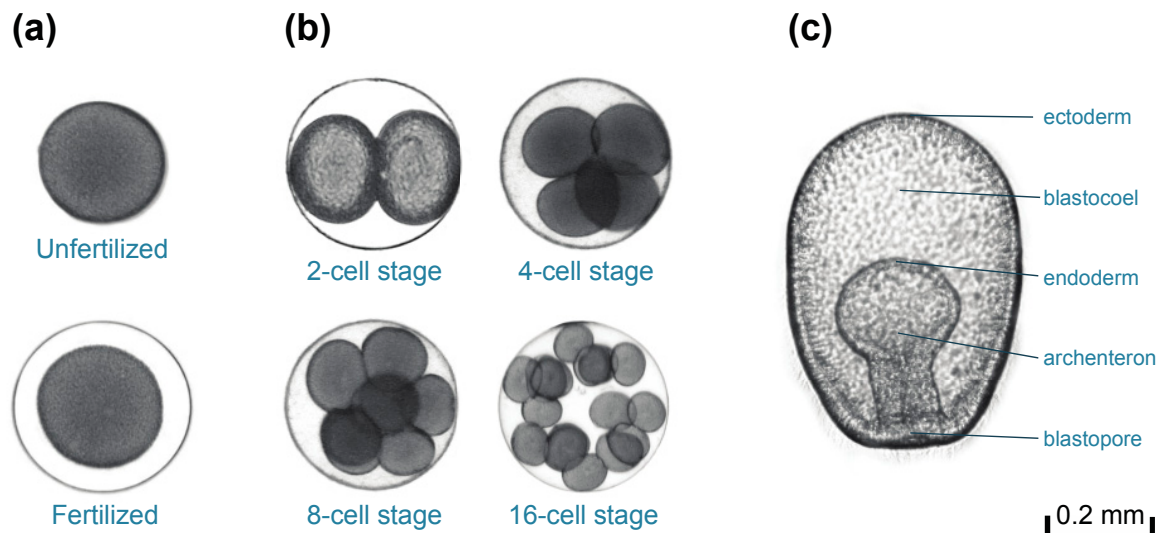
Three sets of experiments were conducted to quantify fertilization, cleavage, and gastrulation rates in response to different levels of (1) temperature, (2) salinity, and (3) pH. Ripe ovary lobes were dissected from two female starfish and gently placed in glass dishes with 0.2-µm filtered seawater (FSW) at 28°C, to which, 1-methyladenine (1-MA) as added at a final concentration of  $1 \times 10^{-4}$  M. Eggs were spawned after 60 min and pooled by transferring to a large glass beaker with FSW. For each experiment, eggs were split into triplicate containers (with 150-mL experimental seawater) for

each treatment level. Approximately 300 eggs were rinsed with experimental seawater and transferred to beakers so that final density was  $\sim 2$  eggs  $\text{ml}^{-1}$ . Testes lobes were dissected from three male starfish and sperm that were shed after  $\sim 3$  min were pooled together and placed in experimental seawater for  $\sim 10$  s at a concentration of  $1 \times 10^4$  sperm  $\text{ml}^{-1}$  to ensure appropriate treatment conditions when added to containers with eggs. There was no water movement in the beaker at this point to minimize immotile sperm from artificially coming in contact with eggs. After 30 min, eggs were rinsed three times in experimental FSW to remove excess sperm and resuspended in experimental FSW. Gametes were pooled to reflect a population of spawners, as might occur in nature, and to record the mean response of the system under investigation. Gamete concentrations used in this study resulted in high fertilization rates ( $> 95\%$ ) during procedural control experiments and none of the eggs showed fertilization envelopes without the addition of sperm, demonstrating that there was no contamination during the preparation and handling of gametes. After two hours,  $\sim 100$  eggs from each replicate was placed in a scintillation vial and 7% formalin was added to prevent further development. Fertilization (presence of fertilization envelope, **Figure 1a**) and/or holoblastic radial cleavage (cell division, **Figure 1b**) were assessed in the first 50 eggs seen across a gridded slide viewed under a compound microscope at low power. Beakers containing the remaining embryos were then resealed and maintained in experimental temperature, salinity, or pH conditions. After 24 hours, 50 embryos were scored as either "gastrula" if they had developed archenteron, or "non-gastrula", where invagination had not occurred (**Figure 1c**). Five independent runs using different sets of gamete sources were undertaken with full replication for each treatment. Mean values from three containers within runs were used as replicates in each experiment ( $n=5$ ). Temperature, salinity, and temperature-compensated pH measurements of seawater in experimental beakers were monitored using a HI 9828 multiparameter probe (Hanna Instruments, RI, USA), with only minimal fluctuation from set values ( $<0.1$ ).

### 2.5. Statistical analyses

Statistical comparisons of sperm speed between combinations of environmental treatment (temperature, salinity, or pH) and water-soluble egg extracts was performed using a two-factor analysis of variance (ANOVA) followed by *post hoc* pairwise comparisons using the 'lsmeans' function in R with Tukey's adjustment [49]. No significant departures from normality and homogeneity of variance were detected for all data. A generalized linear model (GLM) with binomial errors and logit link function was used to analyze the effect of each environmental treatment and water-soluble egg extracts (fixed categorical predictors) on the proportion of motile sperm. Significant overall tests were followed by *post hoc* pairwise comparisons between different levels of temperature, salinity, or pH with corrected p-values [50] using the 'glht' function from the 'multcomp' package in R [51]. Average sperm speed and motility for each male ( $n = 5$ ) across 3 replicate dilutions (slides) were used in these analyses.

A generalized linear model (GLM) with binomial errors and logit link function was used to analyze the effect of temperature, salinity, or pH (categorical predictors) on fertilization, cleavage, or gastrulation rates (binomial response variables). Quasibinomial error distributions were used in place of binomial errors to correct for overdispersion when detected [52]. This was followed by *post hoc* multiple comparisons with corrected p-values [50] using the 'glht' function from the 'multcomp' package in R (Hothorn et al. 2008). Data from within treatments that had zero variance were excluded in the analyses.



**Figure 1.** Early life history processes or stages assessed in this study: (a) fertilization, (b) early cleavage, and (c) gastrulation.

### 3. Results

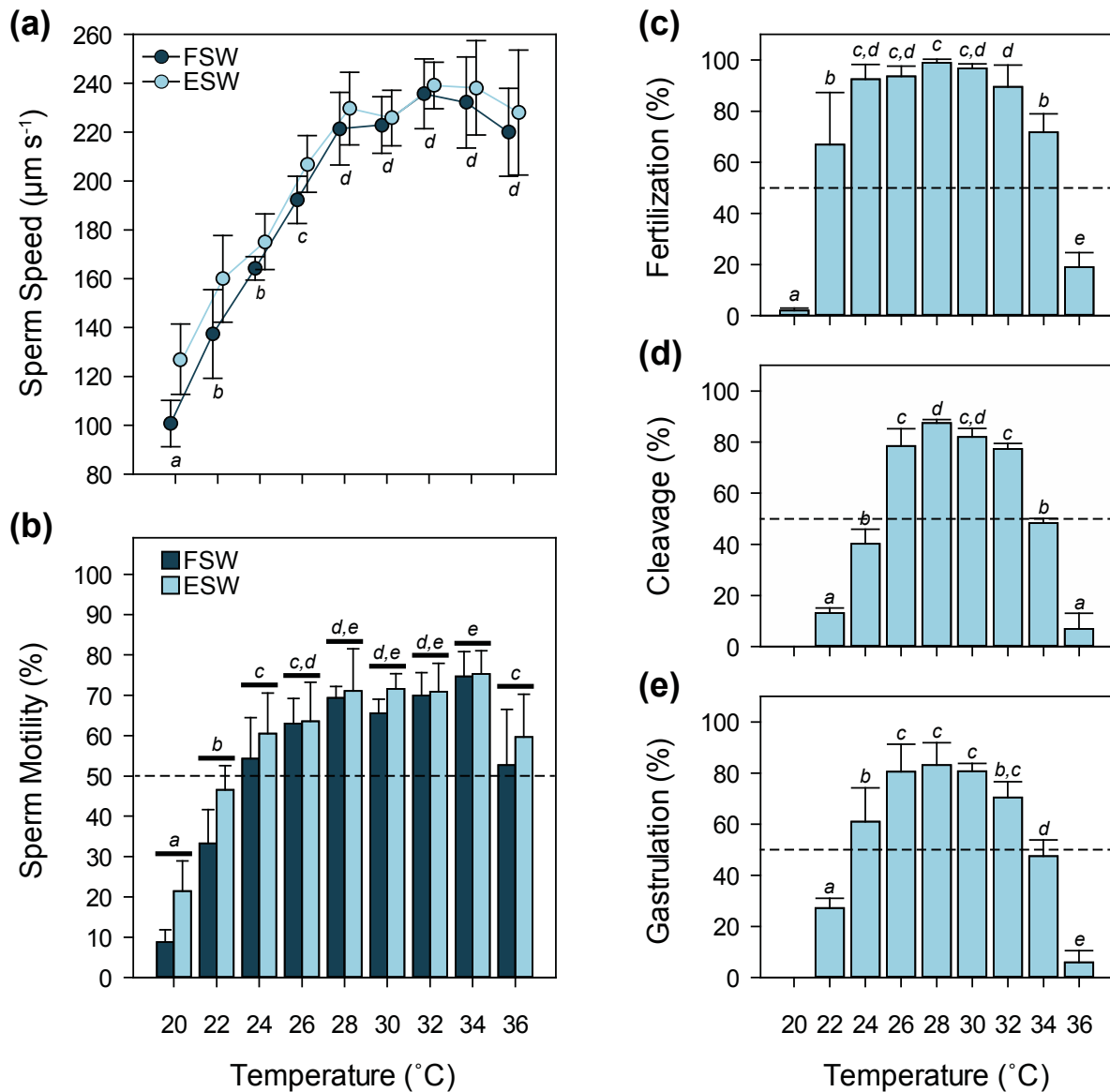
#### 3.1. Temperature

##### 3.1.1. Sperm swimming speed and motility

Seawater temperature ( $F_{8,72} = 85.96, p < 0.0001$ ) and exposure to water-soluble egg extracts ( $F_{1,72} = 13.16, p = 0.0005$ ) had a significant effect on sperm swimming speeds (**Table A1**). Sperm velocity was lowest at the minimum temperature tested, 20°C (FSW:  $100.75 \mu\text{m s}^{-1} \pm 9.48 \text{ SD}$ ; ESW:  $127.05 \mu\text{m s}^{-1} \pm 14.40 \text{ SD}$ ), and peaked at a temperature range of 28°C to 34°C (FSW:  $>221 \mu\text{m s}^{-1}$ ; ESW:  $>225 \mu\text{m s}^{-1}$ ) before slightly dropping back to  $219 \mu\text{m s}^{-1} \pm 96 \text{ SD}$  (FSW) and  $228.01 \mu\text{m s}^{-1} \pm 25.59 \text{ SD}$  (ESW) at 36°C (**Figure 2a**). Sperm exposed to water-soluble egg extracts had consistently faster swimming speeds compared to controls, but this difference is most prominent between 20°C and 26°C where sperms swimming speeds are relatively slow in controls (**Figure 2a**). We found a significant variation in sperm motility between temperature treatment levels ( $\chi^2 = 1233.07, df = 8, p < 0.0001$ ) and between control and water-soluble egg extract treatments ( $\chi^2 = 31.34, df = 1, p = 0.0008$ ). The proportion of motile sperm was steadily increasing from a minimum of  $8.80\% \pm 3.02 \text{ SD}$  (FSW) and  $21.47\% \pm 7.49 \text{ SD}$  (ESW) at 20°C then peaking at  $>65\%$  (FSW) and  $>70\%$  (ESW) for temperatures between 28°C and 34°C (**Figure 2b**).

##### 3.1.2. Fertilization, cleavage, and gastrulation

Water temperature, ranging from 20 to 36°C, had a significant affect on fertilization, cleavage and gastrulation for *A. cf. solaris*, whereby reproductive performance would be maximised at intermediate temperatures (26–30°C). For fertilization, there was significant variation across the full range of temperatures tested (**Table A1**;  $\chi^2 = 1316.20, df = 8, p < 0.0001$ ), mainly due to low fertilization under low (20°C:  $2.00\% \pm 0.94 \text{ SD}$ ; 22°C:  $66.93\% \pm 20.33 \text{ SD}$ ) and high (34°C:  $71.73\% \pm 7.24 \text{ SD}$ ; 36°C:  $18.93\% \pm 5.75 \text{ SD}$ ) temperature extremes. Fertilization rates were  $>89\%$  between 24°C to 32°C (**Figure 2c**). For cleavage, there was significant variation with temperature (**Table A1**;  $\chi^2 = 521.09, df = 7, p < 0.0001$ ). Cleavage was  $>75\%$  for 26°C to 32°C (**Figure 2d**), but greatly reduced at lower (20°C: 0%; 22°C:  $13.12\% \pm 1.98 \text{ SD}$ ; 24°C:  $40.23\% \pm 5.69 \text{ SD}$ ) and higher (34°C:  $48.31\% \pm 1.84 \text{ SD}$ ; 36°C:  $6.98\% \pm 6.04 \text{ SD}$ ) temperatures. Temperature also had a significant effect on gastrulation rates (**Table A1**;  $\chi^2 = 822.66, df = 7, p < 0.0001$ ). The proportion of embryos undergoing gastrulation was maximised between 26°C and 32°C (**Figure 2e**).

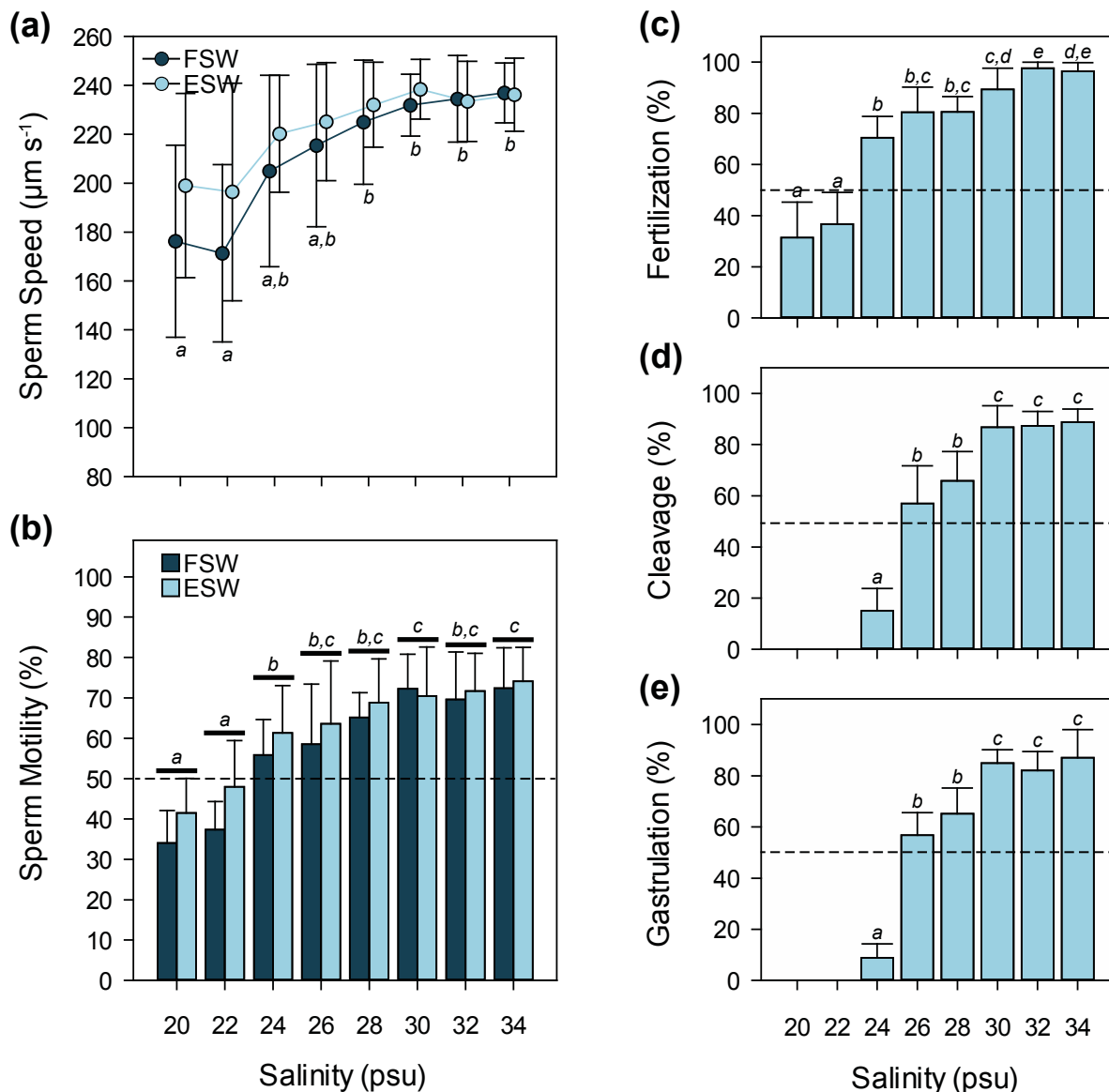


**Figure 2.** Thermal tolerance of sperm, fertilization, and embryonic development: (a) sperm speed (points slightly displaced for clarity), (b) sperm motility, (c) fertilization, (d) cleavage, and (e) gastrulation ( $n = 5$ ). Letters next to error bar caps ( $\pm$  SD) indicate significant differences based on *post hoc* pairwise comparisons with corrected  $p$ -values. FSW = 0.2- $\mu$ m filtered seawater (control); ESW = solution with water-soluble egg extract.

### 3.2. Salinity

#### 3.2.1. Sperm speed and motility

Salinity ( $F_{7,64} = 5.83$ ,  $p < 0.0001$ ) had a significant effect on sperm swimming speeds (Table A1). The disparity in sperm velocity between treatments exposed to water-soluble egg extracts and controls was progressively wider from high to low salinity, but differences were not statistically significant ( $F_{1,64} = 2.93$ ,  $p = 0.0918$ ) (Figure 3a). Variation between salinity treatments was mainly driven by differences between three groups – low sperm swimming speeds for treatments ranging from 20 to 22 psu, intermediate velocity at 24 and 26 psu, and significantly higher sperm velocity from 28 to 34 psu (Figure 3a). Sperm swimming speeds were relatively high across all treatments, with average sperm velocity all above  $170 \mu\text{m s}^{-1}$ . Salinity ( $\chi^2 = 523.43$ ,  $df = 7$ ,  $p < 0.0001$ ) also had a significant effect on sperm motility, but not water-soluble egg extracts ( $\chi^2 = 16.42$ ,  $df = 1$ ,  $p = 0.0682$ ) (Table A1). The proportion of motile sperm was above 40% for salinities ranging from 24 to 34 psu.



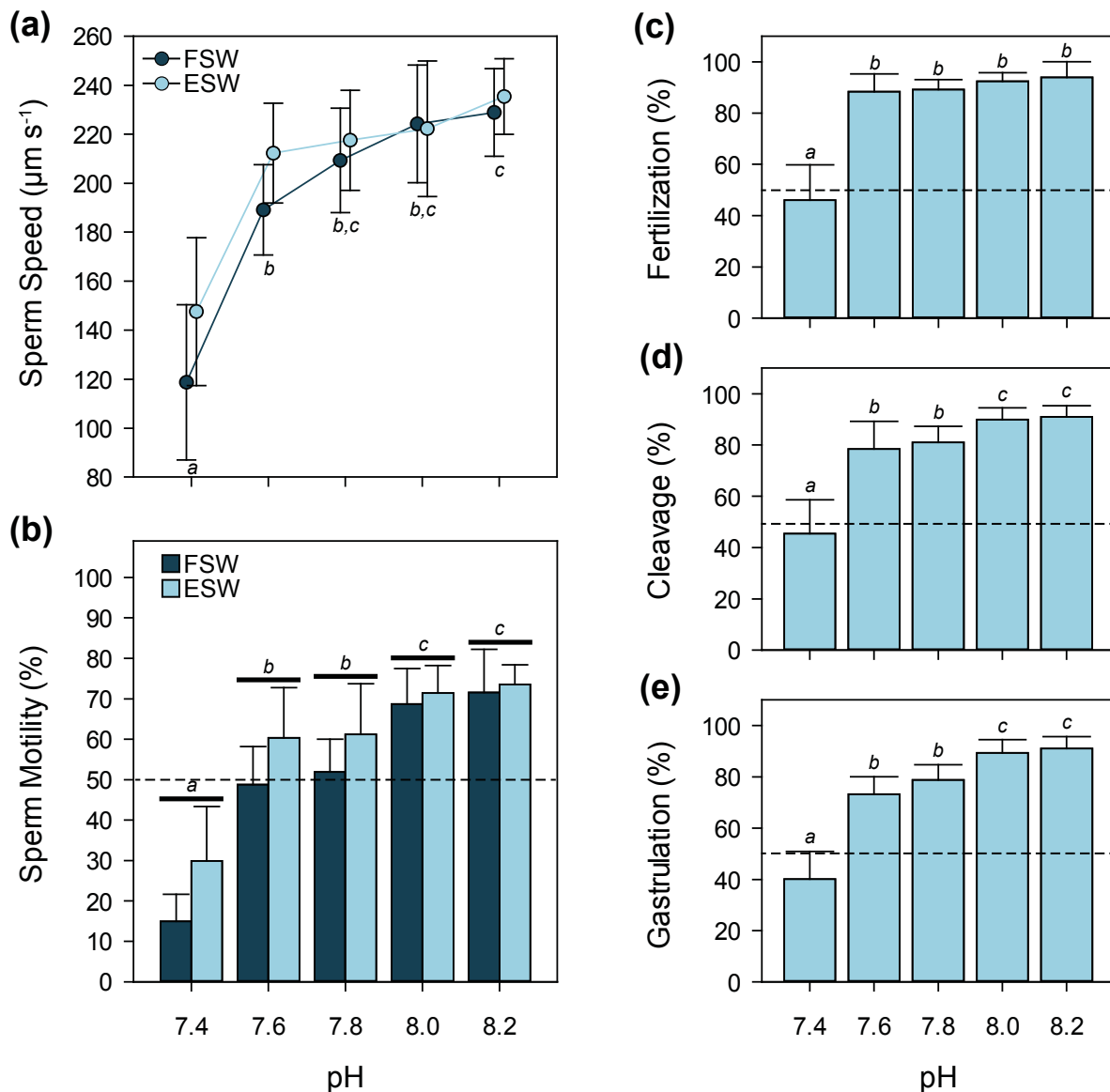
**Figure 3.** Effect of salinity on sperm behavior, fertilization, and early development: (a) sperm speed (points slightly displaced for clarity), (b) proportion of motile sperm, and proportion of eggs undergoing (c) fertilization, (d) cleavage, and (e) gastrulation ( $n = 5$ ). Letters above error bars ( $\pm$  SD) indicate significant differences based on *post hoc* pairwise comparisons with corrected p-values. FSW = 0.2- $\mu$ m filtered seawater (control); ESW = solution with water-soluble egg extract.

### 3.2.2. Fertilization, cleavage, and gastrulation

Salinity had a significant effect on overall fertilization rates ( $\chi^2 = 597.86$ ,  $df = 7$ ,  $p < 0.0001$ ). Fertilization envelopes did not form at salinities  $< 20$  psu in preliminary experiments, while  $31.43\% \pm 13.89$  SD and  $36.67\% \pm 12.41$  SD of eggs were fertilized in 20 psu and 22 psu treatments, respectively. Highest fertilization rates were achieved at 30 psu ( $89.33\% \pm 8.29$  SD), 32 psu ( $97.60\% \pm 2.34$  SD), and 34 psu ( $96.40\% \pm 3.35$  SD). The proportion of embryos undergoing cleavage was significantly different between salinity treatments (**Table A1**;  $\chi^2 = 369.59$ ,  $df = 5$ ,  $p < 0.0001$ ). Fertilized eggs did not cleave at 20 and 22 psu, while only  $15.03\% \pm 8.76$  SD cleaved under 24 psu treatment. Percentage of normal cleavage in CoTS was optimal ( $> 85\%$ ) when exposed to salinities ranging from 30–34 psu. Cleavage rates at 26 psu ( $57.04\% \pm 14.64$  SD) and 28 psu ( $65.80\% \pm 11.50$  SD) treatments were significantly lower than those under 30–34 psu (**Figure 3d**). There was also a significant variation in the proportion of embryos undergoing gastrulation after 24 hours between salinity treatments (**Table A1**;  $\chi^2 = 504.40$ ,  $df = 5$ ,  $p < 0.0001$ ). As with cleavage rates, no gastrulation occurred at 20 and



22 psu, and the proportion of embryos at gastrula stage was significantly higher at salinities between 30 and 34 psu (30 psu:  $84.93\% \pm 5.26$  SD; 32 psu:  $82.13\% \pm 7.28$  SD; 34 psu:  $87.07\% \pm 10.97$  SD) compared to 26 psu ( $56.80\% \pm 8.81$  SD) and 28 psu ( $65.20\% \pm 10.07$  SD) treatments, which were also significantly higher than 24 psu treatment ( $8.80\% \pm 5.55$  SD) (Figure 3e)



**Figure 4.** Influence of pH on sperm behavior, fertilization, and early development: (a) sperm speed (points slightly displaced for clarity), (b) proportion of motile sperm, and proportion of eggs undergoing (c) fertilization, (d) cleavage, and (e) gastrulation. Letters above error bars ( $\pm$  SD) indicate significant differences based on *post hoc* pairwise comparisons with corrected p-values. FSW = 0.2- $\mu\text{m}$  filtered seawater (control); ESW = solution with water-soluble egg extract.

### 3.3. pH

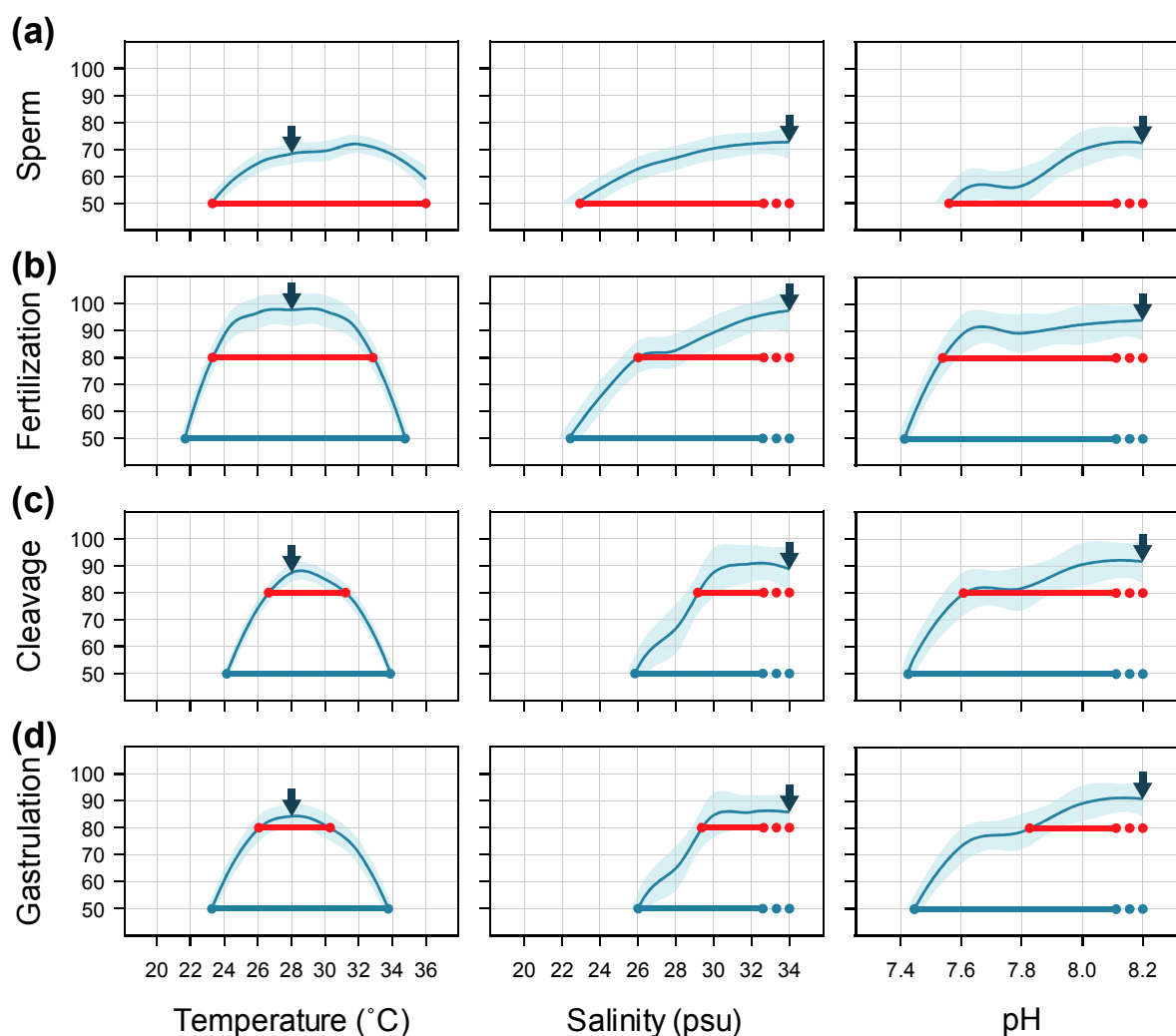
#### 3.3.1. Sperm speed and motility

Average sperm swimming speeds differed significantly (Table A1) between pH treatments ( $F_{4, 40} = 28.57$ ,  $p < 0.0001$ ), but not between egg-derived extracts and controls ( $F_{1, 40} = 3.85$ ,  $p = 0.0568$ ). For this experiment, sperm velocity was highest at pH 8.2 (FSW:  $228.89 \mu\text{m s}^{-1} \pm 17.89$  SD; ESW:  $235.40 \mu\text{m s}^{-1} \pm 15.44$  SD) and pH 8.0 treatments (FSW:  $224.23 \mu\text{m s}^{-1} \pm 24.05$  SD; ESW:  $222.23 \mu\text{m s}^{-1} \pm 27.65$  SD). Apart from pH 7.4 treatments, where sperm velocity was lowest (FSW:  $118.69 \mu\text{m s}^{-1} \pm 31.73$  SD; ESW:  $147.52 \mu\text{m s}^{-1} \pm 30.22 \mu\text{m s}^{-1}$ ), sperm swimming speeds were relatively high ( $> 180 \mu\text{m s}^{-1}$ ) for pH

levels ranging from 7.6 to 8.2 (**Figure 4a**). We also found significant variations in the proportion of motile sperm under different pH ( $\chi^2 = 669.24$ ,  $df = 4$ ,  $p < 0.0001$ ) and egg extract ( $\chi^2 = 38.11$ ,  $df = 1$ ,  $p = 0.0033$ ) treatments (**Table A1**). The proportion of motile sperm was consistently higher for treatments exposed to water-soluble egg extracts (**Figure 4b**). For sperm under pH levels ranging from 7.6 to 8.2, motility was over 50%, while the proportion of motile sperm was relatively low at pH 7.4 (FSW: 14.93%  $\pm$  6.74 SD; ESW: 29.87%  $\pm$  13.50 SD).

### 3.2.2. Fertilization, cleavage, and gastrulation

Percentage of fertilization was high across all pH levels tested (**Figure 4c**), except for eggs in pH 7.4 (46.09%  $\pm$  13.73 SD), which was significantly lower than fertilization success at pH 7.6 to pH 8.2 (> 88%). The effect of low pH levels was more evident when looking at the frequency of normal cleavage (**Figure 4d**) and gastrulation (**Figure 4e**). Cleavage (45.48%  $\pm$  13.17 SD) and gastrulation rate (40.13%  $\pm$  10.75 SD) at pH 7.4 was lowest among all the pH levels tested. The range of pH levels for optimum normal cleavage and gastrulation (> 89%) was between pH 8.0 and pH 8.2. Proportion of embryos undergoing cleavage and gastrulation was significantly higher at optimum pH levels (8.0-8.2) compared to pH 7.6 and pH 7.8 (**Table A1**).



**Figure 5.** Environmental tipping points for (a) sperm motility, (b) fertilization, (c) cleavage, and (d) gastrulation. Arrows signify average ambient levels during spawning. Curves are loess smoothers fitted to dataset with proportions >50%; bold lines cover the range where (a) proportion of motile sperm were >50% (red) and (b) fertilization, (c) cleavage, and (d) gastrulation rates were >50% (dark blue) or >80% (red). Ellipses indicate that upper limits (above ambient) were not examined.

## 4. Discussion

This study shows that CoTS gametes, fertilization, and embryonic development are robust to a wide range of environmental conditions. Notably, these early life-stages could tolerate temperature, salinity, and pH conditions well beyond those experienced by *Acanthaster* spp. across their normal geographic range, even accounting for extreme anomalies in contemporary environmental conditions that are likely to occur at the end of this century [46]. If general to all populations, these findings have important implications for the reproductive success and dispersal of CoTS. A common pattern observed in this study was that sperm motility, fertilization, cleavage, and gastrulation were maximized at local summer temperature, salinity, and pH conditions, which generally coincides with periods of peak reproduction for CoTS [53,54](Figure 5). This suggests that spawning in CoTS occurs at an optimal time when environmental conditions favor enhanced fertilization and early development. Our results also show that chemoattractants (water-soluble egg extracts) plays some role in sperm activity across all environmental parameters tested.

### 4.1. Temperature

Fertilization rates for CoTS were high (>80%) over a wide temperature range (24-32 °C), but does appear to be adversely affected by even higher temperatures (34-36 °C), as shown for many other tropical echinoderms [30]. Thermal enhancement of fertilization as a result of increased motility and respiratory rates of spermatozoa, with concomitant decrease in ATP concentration, has been previously demonstrated in other echinoderms [55]. Thermal robustness of fertilization may be also due to the loading of protective maternal factors (e.g. heat shock proteins) during oogenesis [56,57]. This protection may be enhanced in species with large eggs – CoTS for example, have larger eggs compared to other planktotrophic tropical asteroids and maternal provisioning to the egg influences early larval development [5]. Increased temperature and associated decrease in viscosity increases fertilization success due to increased sperm swimming speeds [36]. This was evident for high fertilization rates achieved at temperatures above ambient levels (28°C) and low fertilization at lower temperature extremes (zero fertilization at 18°C and below). However, reduced sperm activity at 22°C and 24°C still resulted in relatively high fertilization rates, while heightened sperm activity at 34°C and 36°C did not correspond with significant reductions in fertilization rates. The limiting factor appears to be the restriction placed on the viability of sperm subjected to temperature extremes [55]. At temperature extremes above normal, reduced fertilization is associated with increases in the incidence of polyspermy and granular fertilization membranes that adhere to the egg [58]. Increased sperm activity due to elevated temperature, as observed in this study, could also result in mechanical damage to the sperm and incur metabolic costs and exhaustion of energy reserves [29]. Physiological and viscosity-based aspects of high temperatures can influence sperm longevity, and hence fertilization success, by directly affecting sperm velocity [36].

Temperatures that do not restrict fertilization may nonetheless be detrimental for embryonic development [59]. Embryos of the temperate sea urchin, *Strongylocentrotus purpuratus*, subjected to seawater 8°C above ambient showed normal fertilization, but subsequently resulted in abnormal cleavage [60]. This is also consistent with earlier work by Rupp [30] where fertilization rates of CoTS decreased by 20% while cleavage fell by 60% at 34°C. Similarly, more recent work by Sparks et al. [61] showed that the proportion of cleaved embryos was significantly lower at 31°C compared to 27°C and 29°C treatments. Our study revealed that cleavage and gastrulation for CoTS, were maximised over a relatively narrow temperature range (26-32°C) and closely reflects the range of temperatures to which CoTS are likely to be exposed throughout their geographic range [3]. Conversely, Habe et al. [62] showed that gastrulation was possible at a wider temperature range (13 and 34°C) than cleavage. This suggests that if post-gastrula embryos are swept into cooler waters, normal development can proceed during transport and will have important implications for long-range dispersal. However, the proportion of embryos that successfully cleave limits the proportion of embryos undergoing gastrulation. In this study, embryonic development in CoTS ceased at 20°C and below, which is slightly higher than the lower thermal limit for embryonic development reported for CoTS from the GBR, which is between 18 and 19°C [33,63]. This might

reflect the less variable thermal environment of adult CoTS from Guam used in this study compared to CoTS from the GBR [3]. Thermal acclimatization of adults, particularly during gametogenesis, has been found to shift the thermotolerance of echinoderm embryos [33,64].

#### 4.2. Salinity

Out of the three pervasive environmental stressors investigated in this study, response to salinity is perhaps the least studied for CoTS. Here, we found that the lower salinity limit for successful fertilization (>50%) in *Acanthaster* spp. was about 24 psu (**Figure 5**). No fertilization occurred after 2 hours at salinities below 20 psu. At 20 and 22 psu, less than 10% of eggs produced fertilization envelopes. This range and lower salinity limit appears to be common in asteroids (24 to 32 psu in *Asterias amurensis* [31]; 22 to 34 psu in *Asterina pectinifera* [65]), echinoids (26 to 36 psu in *Echinocardium cordatum* [66]; 24 to 32 psu in *Echinarachnius parma* [45]), and holothuroids (24 to 32 psu in *Eupentacta fraudatrix* [67]). Fertilization was highest at average ambient salinity conditions experienced by adults in their natural habitat throughout most of the year. Dinnel et al. [37] found that fertilization of gametes of the sea urchin, *Strongylocentrotus purpuratus*, was best at the salinity at which the adults were held. Contrary to these observations, Roller and Stickle [68] found no evidence of acclimation of echinoid gametes when *Lytechinus variegatus* were exposed to different salinities prior to spawning.

Developmental failure at low salinity is often thought to reflect limited fertilization, possibly due to substantial reductions in sperm motility. There is a paucity of work on the response of echinoderm spermatozoa to salinity fluctuations and most examples come from research on sperm activity in commercially valuable teleost fishes [42,69,70]. Sperm swimming speeds and sperm motility were relatively high between 24 and 34 psu and decreased slightly at 20 and 22 psu, which partly mirrored the range observed for fertilization. Minor improvements in sperm activation when exposed to water-soluble egg extracts were observed, but were not significant. The influence of egg extracts on sperm velocity and motility was greater at lower salinities.

Although there was some fertilization at 20–22 psu, eggs failed to cleave at these salinities and less than 20% cleaved at 24 psu. The failure of eggs to develop at low salinity largely reflects an inability of fertilized eggs to complete meiosis and cleave, rather than simply an inability of eggs to become fertilized at those low salinities. Salinity changes appear to have most detrimental effects for ova, which are unable to control water flow in and out of the cell. Osmotic shock experiments on the spermatozoa and ova of the echinoid, *Parechinus angulosus*, prior to fertilization under optimal temperature and salinity conditions indicate that temperature gradients exert a greater effect on spermatozoa while low salinity is more deleterious to ova – at salinities below 15 psu, water was imbibed by the ova, which swelled and lysed. Salinity tolerance of gastrulation mirrored that of cleavage. Since embryos were not as tolerant to low salinities as previously expected [62], the timing of reduced salinity events will be critical in predicting the population response.

#### 4.3. pH

Our results show that fertilization in *Acanthaster* spp. is robust to reduced pH. Patterns of fertilization success in relation to pH were coincident with relatively high sperm swimming speeds and proportion of motile sperm down to 0.6 pH units below ambient (pH 8.2) and significant reductions at pH 7.4 (**Figure 5**). In looking at the potential effects of near-future ocean acidification on CoTS recruitment, Uthicke et al. [15] found that low pH reduced sperm motility and velocity, which resulted in reduction of fertilization rates by 0.7% at pH 7.9 and 25% at pH 7.7 across a wide range of sperm concentrations. It is not clear whether impaired sperm motility, resulting in reduced fertilization at low pH, may be due to acidosis or the narcotic effect of hypercapnia on sperm [71]. Seawater acidified by CO<sub>2</sub> had a more severe effect on fertilization in sea urchins (*Hemicentrotus pulcherrimus* and *Echinometra mathaei*) compared to HCl-acidified seawater, suggesting that hypercapnia may be more influential to fertilization. However, cross-factorial experiments showed no significant difference in fertilization rates between different combinations of temperature and pH (7.6 to 8.1) treatments [72]. This is consistent with our findings wherein no significant differences in

fertilizations rates were found for pH ranging from 7.6 to 8.2. The mechanism of hypercapnic stress on sperm involves the control intracellular pH; although these effects may be overcome through respiratory dilution effects when sperm is released into the water column [73]. Coelomic fluid surrounding gonads has a mean pH of 7.49 [15], which is relatively low, hence may be activated when seawater pH levels are above this. This could explain the robustness of sperm motility and fertilization in CoTS even at low pH. In addition, our results also demonstrated that water-soluble compounds derived from eggs also promoted sperm motility at low pH. Activation of nonmotile sperm by egg-derived compounds may provide a mechanism by which the energy reserves of sperm can be conserved in the absence of eggs, thereby maintaining sperm viability for extended periods [36,41]. This response has been reported for many species of corals, molluscs, echinoderms and ascidians [47].

The pH tolerance range for cleavage and gastrula embryos coincided with that of fertilization, albeit with slight reductions in frequency. Similarly, Kanya et al. [72] reported that pH had no significant effect on gastrulation in CoTS. Marine invertebrates that do not calcify during early developmental stages are generally robust to reduced pH [23,32]. Later stages (bipinnaria and brachiolaria) in the life history of CoTS are more sensitive to reduced pH and have been shown to suffer high rates of larval abnormality and mortality at low pH [15,72].

## 5. Conclusions

Taken together, our results show that CoTS gametes, fertilization, and embryonic development are robust to a wide range of temperature, salinity, and pH levels, well beyond environmental conditions found within the current geographical distribution of *Acanthaster* spp. Majority of sperm are motile at temperatures between 24 and 36°C, salinities between 24 and 34 psu, and pH between 7.6 and 8.2. Over 50% of eggs are fertilized at wide range of temperature (22-34), salinity (24-34), and pH (7.6-8.2) levels. The robustness of fertilization to these pervasive environmental stressors may be attributed to the molecular predisposition of CoTS sperm [74], which possesses an enhanced capacity for high fertilization rates, compared to other echinoderms [22]. Compared to fertilization, tolerance range for was mostly narrower for temperature (26-32°C), salinity (26-34 psu), and pH (7.6-8.2). Gastrulation under salinity and pH levels tested coincided with cleavage rates, while thermotolerance range for gastrulation was slightly wider than cleavage (24-32°C). In general, the effects of temperature and pH on fertilization and early development mostly coincided with the sensitivity of sperm to these stressors, while response to salinity was largely due to detrimental effects on osmotic balance in eggs. Water-soluble compounds associated with eggs also enhanced sperm activity, particularly in environmental conditions where sperm motility is initially limited. Although the response to multiple environmental stressors was tested in this study, these pervasive environmental parameters impact marine organisms simultaneously. Future work should include cross-factorial studies to tease out additive, antagonistic, and synergistic interactions between these factors [24]. The tolerance of the earliest stages of development to a wide range of environmental stressors suggests that later ontogenic stages (larvae, juveniles, adults) may be more vulnerable to small fluctuations in environmental conditions.

**Acknowledgments:** The ARC Centre of Excellence for Coral Reef Studies provided funding for this study through a PhD research allocation to CFC and annual research appropriation to MSP. We are grateful to A.M. Kerr, L.J. Raymundo, R.G. Rowan, M. Kitamura, and M. Byrne for technical advice. We also thank J. Miller, J. Cummings, T. Reynolds, J. Gault and the staff at University of Guam Marine Laboratory for field and laboratory assistance.

**Author Contributions:** C.F.C., M.S.P., and J.R.P. conceived and designed the experiments; C.F.C and M.L.R. performed the experiments; C.F.C. analyzed the data; M.S.P. and J.R.P. contributed reagents, materials, and analysis tools; C.F.C., M.S.P., M.L.R., J.R.P. wrote the paper.

**Conflicts of Interest:** The authors declare no conflict of interest. The funder had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

## Appendix A

Table A1. Results of statistical analyses on the effects of temperature, salinity, and pH on sperm behavior, fertilization, and early development.

SOURCE	DF	Statistic (F, $\chi^2$ )	P
<b>Temperature</b>			
Sperm Speed <sup>1</sup>			
<i>temperature</i>	8	85.96	< 0.0001
<i>egg extract</i>	1	13.16	0.0005
<i>temperature</i> × <i>egg extract</i>	8	0.76	0.6353
Sperm Motility <sup>2</sup>			
<i>temperature</i>	8	1233.07	< 0.0001
<i>egg extract</i>	1	31.34	0.0008
<i>temperature</i> × <i>egg extract</i>	8	32.79	0.1612
Fertilization <sup>2</sup>	8	1316.20	< 0.0001
Cleavage <sup>2</sup>	7	521.09	< 0.0001
Gastrulation <sup>2</sup>	7	632.82	< 0.0001
<b>Salinity</b>			
Sperm Speed <sup>1</sup>			
<i>salinity</i>	7	5.83	< 0.0001
<i>egg extract</i>	1	2.93	0.0918
<i>salinity</i> × <i>egg extract</i>	7	0.31	0.9449
Sperm Motility <sup>2</sup>			
<i>salinity</i>	7	525.43	< 0.0001
<i>egg extract</i>	1	16.42	0.0682
<i>salinity</i> × <i>egg extract</i>	7	9.62	0.9626
Fertilization <sup>2</sup>	7	597.86	< 0.0001
Cleavage <sup>2</sup>	5	369.59	< 0.0001
Gastrulation <sup>2</sup>	5	504.40	< 0.0001
<b>pH</b>			
Sperm Speed <sup>1</sup>			
<i>pH</i>	4	28.57	< 0.0001
<i>egg extract</i>	1	3.85	0.0568
<i>pH</i> × <i>egg extract</i>	4	0.74	0.5706
Sperm Motility <sup>2</sup>			
<i>pH</i>	4	669.24	< 0.0001
<i>egg extract</i>	1	38.11	0.0033
<i>pH</i> × <i>egg extract</i>	4	18.05	0.3943
Fertilization <sup>2</sup>	4	234.28	< 0.0001
Cleavage <sup>2</sup>	4	95.37	< 0.0001
Gastrulation <sup>2</sup>	4	213.24	< 0.0001

<sup>1</sup> Two-way Analysis of Variance (ANOVA): *F* value<sup>2</sup> Analysis of Deviance for generalized linear models (GLM):  $\chi^2$  value

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