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Jonathan D. Allen

College of William & Mary, jdallen@wm.edu

Kharis R. Schrage

College of William and Mary, krschrage@email.wm.edu

Shawna A. Foo

University of Sydney

Sue-Ann Watson

3 ARC Centre of Excellence for Coral Reef Studies, James Cook University

Maria Byrne

3 ARC Centre of Excellence for Coral Reef Studies, James Cook University

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The effects of salinity and pH on fertilisation, early development and hatching in the
Crown-of-Thorns Seastar

Jonathan D. Allen^{1*}

Kharis R. Schrage¹

Shawna A. Foo²

Sue-Ann Watson³

Maria Byrne^{2*}

¹College of William and Mary, Williamsburg, Virginia, 23187, USA

²School of Medical and Biological Sciences, University of Sydney, Sydney 2006,
Australia

³ ARC Centre of Excellence for Coral Reef Studies, James Cook University,
Townsville, Queensland 4811, Australia

*Correspondence: jdallen@wm.edu; mbyrne@anatomy.usyd.edu.au

Abstract

Understanding the influence of environmental factors on the development and dispersal of crown-of-thorns seastars is critical to predicting when and where outbreaks of these coral-eating seastars will occur. Outbreaks of the crown-of-thorns seastars are hypothesized to be driven by terrestrial runoff events that increase nutrients and the phytoplankton food for the larvae. In addition to increasing larval food supply, terrestrial runoff may also reduce salinity in the waters where seastars develop. We investigated the effects of reduced salinity on the fertilisation and early development of seastars, up to and including their hatching from the fertilisation envelope. We also tested the interactive effects of reduced salinity and reduced pH on the hatching of crown-of-thorns seastars. Overall, we found that reduced salinity has strong negative effects on fertilisation and early development, as has been shown in other echinoderm species. We also found that reduced salinity delays hatching but that reduced pH, in isolation or in combination with lower salinity, had no detectable effects on this developmental milestone. Models that assess the positive effects of terrestrial runoff on the development of crown-of-thorns seastars should also consider the strong negative effects of lower salinity on early development including lower levels of fertilisation, increased frequency of abnormal development and delayed time to hatching.

Keywords: fertilisation, embryonic development, salinity, pH, hatching, crown-of-thorns seastar, *Acanthaster*

INTRODUCTION

Coral reefs, among the world's most diverse and valuable ecosystems, are under threat from global stressors associated with climate change and local stressors such as overfishing, pollution and outbreaks of the coral-eating, crown-of-thorns seastars *Acanthaster cf. solaris* (COTS) [1,2]. The vulnerability of coral reef ecosystems to global change is seen in the mass bleaching and coral mortality across the tropics caused by the 2016 El Nino-driven ocean warming [3]. Australia's Great Barrier Reef (GBR), and Indo-Pacific reefs in general, are in the midst of a multi-decadal decline in coral cover [4,5]. Outbreaks of COTS cause major damage to coral reefs [5-7], prompting large-scale removal programs of this sea star, albeit with equivocal effectiveness [8]. Analysis of data from the GBR monitoring program estimates that 30-40 percent of the decline in coral cover can be attributed to COTS predation [5,9]. In addition, coral recovery after bleaching and cyclones is greatly reduced if these events are followed by *A. cf. solaris* predation [9,10].

It is unclear whether outbreaks of COTS are becoming more common or are simply reported with increasing frequency [8]. However, there is evidence that COTS outbreaks on the GBR occurred historically every 50-80 years, while today they occur approximately every 15 years [12]. Regardless of their frequency, given the far-reaching consequences of COTS outbreaks [e.g. 13], understanding the factors underlying increases in abundance of this species is critical to predicting when and where loss of coral cover due to COTS will occur. Despite decades of research, the factors behind the boom and bust population cycles of COTS are not understood. The very high fecundity (up to 200 million eggs/female) and resilient larval stage indicate

that success in the plankton is a key consideration in determining the causes of recruitment pulses [14-17].

There are two primary hypotheses for the increasing frequency of COTS outbreaks: the predator removal hypothesis (top-down) and the terrestrial runoff hypothesis (bottom-up). While there is some evidence for both hypotheses [reviewed by 8], a consensus seems to be emerging that the terrestrial runoff hypothesis best explains outbreak dynamics on the GBR, possibly combined with local hydrodynamic conditions that govern secondary outbreaks [18]. In brief, the terrestrial runoff hypothesis attributes outbreak events to increasing agricultural runoff, which in turn increases the abundance of nutrients in the waters surrounding the GBR. The addition of limiting nutrients enhances the growth of phytoplankton, providing more food for *A. cf. solaris* larvae, and thereby increasing larval survival and ultimately recruitment into the reproductive population [19].

In addition to increasing nutrients, freshwater runoff from terrestrial sources simultaneously reduces seawater salinity [19, 20]. In large flood events that often occur around the time that COTS larvae are in the plankton, flood plumes can extend up to 100 km offshore in GBR waters causing pulses of low salinity extending to mid-shelf reefs [21-23]. These low salinity events are predicted to increase by recent projections of global climate change [24]. As most echinoderms have limited tolerance for low salinity as adults and larvae [25, 26], even short-term exposure to low salinity may be detrimental to COTS development. For example, even small reductions in salinity cause abnormal developmental phenotypes in sand dollars and sea urchins, beginning at fertilization and continuing at least through hatching [24,

25]. However, in experiments where larval COTS were transferred to a range of salinity treatments, reduced salinities of 30 psu actually enhanced survival relative to 32 and 35 psu treatments [29]. Lucas (1973) also found that development was completed in larval COTS transferred to 26 psu, but not 22 psu. Developmental resilience to reduced salinities might be another trait of the life history of COTS contributing to its success during flooding events. However the tolerance of gametes and early developmental stages (e.g. zygotes, cleavage stage embryos, blastulae) of COTS to lower salinity has not been determined.

To understand the effects of freshwater runoff on COTS reproductive success, we examined the effects of reduced salinity conditions on fertilization, normal development, and hatching in this seastar across a salinity gradient (19 to 34 psu). Based on previous studies demonstrating the negative effects of low salinity on echinoderm development [25, 26] we predicted that decreased salinity would lower fertilization success and reduce the percentage of embryos exhibiting normal development. We also predicted that decreased salinity would cause a delay in hatching as shown in other echinoderms [28, 30]. The potential for polyembryony, the phenomenon where low salinity induces fission of early embryos to generate multiple embryos per egg, as described for echinoid embryos [31] was also investigated. Finally, we examined the effect of salinity reductions in combination with shifts in pH to explore how changes in water chemistry more generally could affect development to hatching. We use our data to address the possibility that the resilience of the planktonic phase of *A. cf. solaris* to decreased salinity may contribute to its success in runoff conditions.

METHODS

Adult collection and maintenance

Crown-of-thorns seastars were collected on snorkel in December 2015 as encountered on reefs around Lizard Island (14°40'44.0"S 145°26'53.7"E), Northern Great Barrier Reef, Australia. Upon collection, the animals were transported by boat to the Lizard Island Research Station and the gender of each specimen was determined by gonad biopsy with the tissue removed using forceps through a small incision at the base of the arms. The males and females were placed in separate large tanks of ambient flow-through seawater at 28°C and ~34 psu salinity. Animals were kept in flow-through tanks and used within a week of collection for experiments.

To obtain gametes for fertilization, a small portion of gonad was removed through an incision. The ovaries were rinsed with 1 μ m filtered seawater (FSW) and placed in 10⁻⁵M 1-methyl adenine in FSW to induce ovulation. After 30-40 min the eggs were collected and placed in ~ 100ml of FSW. Eggs were checked microscopically for quality and to confirm germinal vesicle breakdown. Sperm was collected directly from dissected testes and placed in a small dish at room temperature (~28°C). Each sperm source was checked microscopically for motility and used promptly. For each fertilisation sperm a single males was combined to fertilise the eggs of a single female (salinity only experiments) or sperm from 2-3 males was combined with eggs from 2-3 females (salinity plus pH experiments) at a sperm to egg ratio of 100:1. Fertilisation was checked microscopically and confirmed to be >90% before the eggs were rinsed

in FSW to remove excess sperm. For pH experiments, levels of salinity, pH and DO were measured using a Hach Hqd Portable temperature-compensated multiprobe.

Effects of salinity on fertilization, development and hatching

For all experiments where salinity was manipulated, FSW at ambient temperature (~28°C) and salinity (34 psu) was mixed with deionized water to create treatment salinities of 19, 23, 25, 27, 29, 31, and 34 psu. New seawater was mixed each day for a complete water change in experiments, thereby minimizing changes in salinity due to evaporation. To determine the effects of salinity on fertilization success, development and hatching, embryos from single females were reared in water at a range of salinities. Use of one female and 2-3 males for each fertilization generated populations of embryos for the salinity experiment. The eggs were pipetted into 250 ml plastic beakers with ~100ml FSW at salinities of 19, 23, 25, 27, 29, 31, and 34 psu. After adding eggs to each beaker, a few drops of dilute sperm solution were added to each beaker. After a brief stir, gametes were left for a few minutes before checking for fertilisation. A subsample of eggs were photographed using a dissecting microscope to score the number of fertilised eggs as indicated by the presence of a fertilisation envelope. All eggs in focus were scored (n=39-97 per picture). Initial trials (2-3 per salinity) revealed that development was inhibited at 19, 23, or 25 psu and so those salinities were not used in subsequent trials. We then conducted 11 fertilisations and for each of these had one well of embryos for each salinity level. Variable numbers of crosses were examined for measures of fertilization (n = 3-11 crosses), normal development (n = 2-9 crosses) and hatching (n = 5 crosses) for each salinity treatment.

Six-well plates were prepared with four wells in each plate filled with 10 ml of experimental seawater (27, 29, 31, or 34 psu) and fertilised eggs from respective salinity treatments pipetted into individual wells. Plates were covered and left at ambient temperature (~28°C). At 14, 16, and 18 hours post fertilisation (hpf), a subsample of 30 embryos from each well were examined microscopically to score developmental stage. Scoring categories were as follows: unfertilised (no fertilisation envelope), fertilised (1 cell), dead, early cleavage (2 cell to blastula), abnormal cleavage (blastomeres varying in size and shape), abnormal blastula (irregular shapes with blebbing cells), blastula, gastrula, and hatched. The frequency of normal development (the sum of blastula, gastrula and hatched categories) was determined in counts of 30 embryos per well. The percentage of normal development was calculated by dividing the number of normally developing embryos by the total number of embryos that were fertilised, in order to avoid confounding the failed development of fertilised eggs with the previously observed failure of eggs to fertilise at low salinities. All cultures were examined closely for the incidence of polyembryony.

Effects of salinity and pH on development to hatching

The salinity-pH experiment used four salinity (27, 29, 31, 34 psu) and two pH_{NIST} levels (Mean ± SE, control 8.07 ± 0.02, and 7.61 ± 0.01, $n = 12$). The pH treatments were within model projections for near-future (2300) conditions [24]. Unmanipulated FSW served as the control. The water was first conditioned to achieve the salinity levels as above and then the pH was adjusted. To achieve experimental pH levels, FSW was bubbled with 100% CO₂ and pH adjustment was tracked using a using a

Hach Hqd Portable Multiprobe. Probes were calibrated using NIST high precision buffers pH 4.0, 7.0 and 10.0 (ProSciTech). To ground truth pH_{NIST} values, pH on the total scale was checked for adjusted FSW samples across all salinities. The spectrophotometric approach was used with *m*-cresol purple indicator dye (Acros Organics lot AO321770) and a USB4000 spectrophotometer following the procedures outlined in SOP 6b of [32] and the equations of [33]. All values fell in the expected range, confirming the accuracy of the pH_{NIST} values. Water samples (250 ml) collected for each pH level and fixed with 100 μ l of saturated HgCl were used to determine total alkalinity (TA) by potentiometric titration. Experimental $p\text{CO}_2$ was determined from pH_{NIST} , TA, temperature, and salinity data using CO2SYS [34] (Table 1) applying the dissociation constants of [35] as refitted by [36].

Use of gametes from multiple males and females (n= 2-3 each) males for each fertilisation generated populations of embryos for the salinity-pH experiment. Thus each experimental container was considered to be a replicate. Combined eggs of three females were placed in plastic beakers containing 50ml of experimental salinity-pH seawater at 460 eggs/ml as determined in egg counts and fertilised with dilute sperm to achieve ~ 90% fertilisation. Approximately 1 ml of these eggs was then pipetted into full 40-80 ml containers (~ 12 eggs/ml) of the same experimental water conditions and sealed. They were left in water baths maintained at ambient temperature (~ 28°C).

At 14 and 24 hpf, 30 embryos were pipetted from each container and scored as dead, abnormal blastula, blastula, gastrula, or hatched (as above). They were sampled in order of replicate (all replicate one pots, then two, then three), so that time was not a

confounding variable. This experiment was repeated three times with different gamete sources.

Statistical Analysis

Data describing the effects of salinity on fertilisation success were analyzed using a nonparametric Kruskal-Wallis test since the distribution of residuals resulting from ANOVA tests were non-normal, even following standard (arc-sine square root) transformations. The percentage of embryos that exhibited normal development at 14 hours post fertilisation under different salinity conditions were normally distributed and were analyzed using a mixed-model ANOVA where female was modeled as a random factor and salinity was modeled as a fixed factor. Data describing the effects of time and salinity on the percentage of embryos exhibiting normal development were non-normally distributed, preventing analysis in an ANOVA framework. Instead, we used a binomial logistic regression to analyze these data. A Hosmer-Lemeshow test was used to analyze the fit of our binomial regression model to the data, although the Hosmer-Lemeshow test is known to yield significant departures from a perfect fit when observations exceed a few hundred (our $N = 2140$) [37].

For the data on the percentage of hatching in salinity and pH treatments a mixed-model ANOVA was used with data at two different time points (14 and 24 hpf). In this case, we modeled pH, salinity, time and their interactions as fixed effects and block (different runs of the experiment) was modeled as a random effect. Each block was conducted on independent days using unique and non-overlapping combinations of male and female gametes. In all cases, normality of residuals was assessed using a

Kolmogorov-Smirnov test with an alpha level of 0.05 and in all cases the test yielded a $p \geq 0.200$ meeting this basic assumption of ANOVA. In cases where post-hoc tests were used to assess differences among levels of a significant main effect, we used the Bonferroni adjustment to correct p-values for multiple pairwise comparisons [38]. The one exception to this was analysis of pairwise comparisons following the Kruskal-Wallis nonparametric test where we used Dunn's adjustment [39] to correct for family-wise type I error. All analyses were conducted using IBM SPSS Statistics (version 23).

RESULTS

Effects of salinity on fertilization, early development and hatching time of crown-of-thorns seastars

Salinity had a significant effect on the percentage of eggs that were fertilized (Figure 1) as determined by a Kruskal-Wallis test ($H = 14.636$, d.f. = 5, $p = 0.021$). Multiple pairwise comparisons across all salinity levels revealed that fertilization at 23 psu was significantly lower than fertilization success at 34 psu. All other pairwise comparisons were not significantly different from one another after the p-values were corrected for multiple comparisons. While not statistically significant, the decrease in fertilization at 25 psu in some crosses indicates that this salinity level may approach the threshold for fertilization success.

Salinity had a significant effect on the proportion of embryos that exhibited normal development. Using a mixed-model ANOVA, we found that the proportion of

embryos exhibiting normal development was significantly reduced when salinity was reduced ($F_{1,4} = 9.989$; $p < 0.001$; Figure 2). Posthoc tests with Bonferroni corrections revealed that the frequency of normal development at 25 psu was significantly lower than at all other salinities ($p < 0.001$) except for 27 psu ($p = 0.070$). The frequency of normal development at 27 psu was also lower than the frequency at 29 psu ($p = 0.028$) and 31 psu ($p = 0.039$) but no different from the frequency at 34 psu ($p = 0.110$; Figure 2). The decrease in the frequency of normal development at 27 psu indicates that this salinity level approximates the embryo tolerance levels for reduced salinity.

We found that salinity, time and male/female cross all had significant effects on the percentage of embryos that hatched between 12 and 16 hpf (Figure 3). We initially tested for the main effects of salinity, time and male/female cross using a full model with all 2-way and 3-way interactions included, but found that none of the interactions were significant and all exceeded a threshold p-value of 0.250 for removal from the model to create a reduced model focused on testing for our main effects of interest [40]. The results of the reduced model are shown in Table 2 and show that all three main effects were highly significant predictors of hatching status in our experiments. We tested whether the fit of our model departed significantly from the data using a Hosmer-Lemeshow test and found that it did ($p < 0.002$), however our model correctly predicted hatching status in 82.3% of cases.

Plotting the mean success of fertilisation and normal development by female shows different effects of salinity treatments across the male/female crosses (Figure 4). At fertilisation, performance across all 11 females was reduced at all experimental

salinity levels. At 14hpf, however, it can be seen that developmental success was dependent on female identity where females 7 and 8 showed enhanced development with respect to the control salinity (Figure 4B).

Effects of salinity and pH on early development of crown of thorns seastars

Salinity and time, but not pH had a significant effect on the percentage of hatched embryos (Figure 5). We tested for the main fixed effects of salinity, pH, time and for all possible interactions of these variables, along with the random effect of block. After running the full model described above, we removed all interactions where $p > 0.250$ and re-ran the reduced model presented in Table 3. The main effects of salinity and time both significantly affected the percent of embryos hatched ($p < 0.001$), but pH did not (Table 3). The random effect of block was also a significant factor affecting the percent of embryos hatched ($F_{2,136} = 5.550$; $p = 0.005$). For the main effect of salinity, 27 psu yielded significantly lower percentages of hatching ($p < 0.004$) from all other levels, while 29 psu was significantly lower ($p < 0.005$) than 31 psu and the highest salinities of 31 and 34 psu were not different from one another ($p > 0.9$).

DISCUSSION

We found that salinity has a strong negative effect on the early development of COTS, beginning with fertilisation and continuing through to hatching of embryos from the fertilization envelope. Fertilisation was relatively resilient to reductions in salinity, remaining above 90% until salinity dropped to 25 psu or below. However, by

14 hpf embryos began to exhibit sensitivity to salinity, as seen in the presence of high numbers of abnormal phenotypes at salinities < 27 psu. This pattern of increasing sensitivity to lower salinities continued into hatching. We detected negative effects of reduced salinity on the proportion of embryos hatching at < 29 psu, relative to embryos at 31 and 34 psu. Overall, this suggests that salinity is an important factor to include in models of reproductive success in COTS, especially as it is likely to covary with other environmental conditions such as nutrient runoff and subsequent phytoplankton blooms that are currently associated with COTS outbreaks on the GBR [19].

As seen here for COTS development, echinoderms generally exhibit a narrow tolerance for decreased salinity during both planktonic and benthic life stages although there is considerable variation among species [25,26]. Previous studies of the impact of lower salinity on fertilisation (where gamete union was conducted in treatment water) in asteroids indicate that 24-26 psu approximates the salinity when the percentage of fertilisation decreases and this is also influenced by temperature (eg. 23% at 5°C, 69% at 10°C in *Asterias amurensis*) [41, 42]. For COTS at 23-25 psu fertilisation was fairly high (~ 75%), although it does appear that this level of decreased salinity approximates a threshold tolerance level. Similar results were obtained for fertilization in *Acanthaster cf. solaris* from Guam, where fertilisation drops below 50% between 24 and 22 psu [43]. By comparison it appears that echinoid fertilisation is more sensitive to decreased salinity with significant deleterious effects at salinities below 28-29 psu for sea urchins [44-46] and at 25 psu for a sand dollar [27]. For *Lytechinus variegatus*, fertilisation was reduced to ~ 10% at 25 psu [41].

The percentage of normal development for COTS was significantly lower at 27 psu and it appears that this level of reduced salinity may be a tipping point for deleterious effects. Similarly increased embryo abnormality was observed in the asteroids *Pisaster ochraceus* and *Asterias amurensis* at ~ 25-26 psu [41,42,44] and in the echinoid *Evechinus chloroticus* at 29 psu [43]. While reduced salinity has been reported to induce polyembryony in echinoid species [31], the phenomenon of polyembryony was not observed in COTS under any experimental treatment. There was variation in the performance with the progeny of some females showing enhanced development with respect to the control salinity. As seen in studies of other stressors, the variable performance of progeny with respect to parental source indicates that there may be standing genetic variation in tolerance to salinity and potential for adaptation [48].

In general, decreased salinity is well known to delay or retard development in echinoderms [25, 27, 28, 44, 46, 47, 49-51]. For COTS, hatching was significantly delayed in embryos reared from fertilization at 27 psu. Similarly for *Lytechinus variegatus* time to hatching time was delayed by ~ 5 days later at 28 psu (6d vs 1 d) [44]. Although we did not rear our cultures to the larval stage, delayed hatching in COTS due to low salinity is likely to be associated with larval abnormality as shown for *Pisaster ochraceus* and *Echinaster* sp. [47, 52]. Despite our finding of delayed hatching of COTS, no signs of changes in stage at hatching were observed, as has been found in some echinoids [28]. Regardless, any delay of hatching would be expected to increase the time spent in the risky planktonic phase [53-55] as it delays the onset of larval feeding.

In contrast to salinity, we found no effects of lowered pH on hatching in COTS. This is in contrast with prior work showing that decreased pH has strong effects on COTS fertilization and early development [56]. In this latter study however fertilization was conducted in single male-female crosses where differences in gamete compatibility among pairs can influence the outcome. Some single male-female crosses are more resilient to decreased pH than others [56]. Our experiments used multiple parents to reduce the influence of individual gamete compatibility [see 57]. Decreased pH may affect sperm in isolation [53] but this may be ameliorated by the presence of egg chemicals [43].

With respect to hatching it appears that this stage is resilient to changes in pH while other stages of development are more vulnerable in both earlier (eg. gametes) and later (eg. larvae) life stages of COTS [43, 56, 58] with potential benefits of near future ocean acidification for the early juvenile [59]. Our results suggest that decreased salinity has a greater deleterious effect on hatching than decreased pH, as also found for *Acanthaster cf. solaris* in Guam [43].

While we show that the benthic embryonic stages of COTS are sensitive to low salinity, Lucas (1973) reported that larvae are less so. Larvae transferred to 26 psu, a salinity level deleterious to embryos, were capable of completing development. This indicates that COTS larvae that encounter plumes of lower salinity water in nature might be robust. Similarly the bipinnaria larvae of *Asterias amurensis* tolerated being transferred from ambient salinity seawater to 20-32 psu [41]. After initial osmotic shock these larvae were able to restore swimming activity, although they swam more slowly at decreased salinity. Larval tolerance to transfer to low salinity increased

with larval age [41]. This indicates asteroid larvae, perhaps especially those of resilient boom and bust species such as *Asterias amurensis* and *Acanthaster cf. solaris* [14] can tolerate low salinity perturbations. An extreme example of salinity tolerance among asteroids is found in populations of the seastar *Asteria rubens* in brackish waters of the Baltic Sea where it occurs at salinities from 15-35 psu [60]. In some populations of this species, survival of fertilized eggs to embryos is actually highest at 24 psu [60] and development to metamorphosis can be completed at salinities as low as 15 psu [61].

Across all asteroids, even the extreme cases described above, there is a limit to larval low salinity tolerance that is likely to differ not only taxonomically, but also with respect to the duration of the perturbation and across larval ages. *Pisaster ochraceus* gastrulae and larvae exposed to low salinity (20 psu) for 20 days, as occurs during precipitation events, developed into shorter and wider larvae, while those exposed to shorter pulses of lower salinity (3 days) developed into longer and more slender larvae [62]. This salinity-induced morphological change in seastar larvae likely has consequences for swimming and feeding as both of these functions are strongly influenced by larval size and shape [63]. The ability of asteroid larvae to change shape with respect to salinity treatments may indicate an ability to phenotypically adjust their body profile to maximize feeding and swimming efficiency with regard to salinity conditions, albeit with a lower limit [62]. For COTS and other asteroid and echinoid larvae, phenotypic plasticity with respect to their food environment is a key mechanism of resilience [15,64,65]. That larvae may also be able to adjust their phenotype in response to the salinity environment warrants further investigation. In addition asteroid and echinoid larvae exhibit avoidance behaviour swimming away

from, or not swimming into, low salinity water [66, 67] and this ability has recently been shown to be affected by prior exposure to low salinity during early development [68]. This behavioral plasticity may increase larval survival and given the plasticity of echinoderm larval growth may also be associated with differing body profiles. Empirical data on the influence of the timing of exposure (with respect to larval stage/age) on low salinity tolerance of COTS larvae, the life stage most likely to encounter pulses of low salinity conditions during flooding periods, and the potential for salinity-induced phenotypic plasticity is needed to more fully understand the resilience of this species with respect to the influence of freshwater incursions.

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AUTHORS CONTRIBUTIONS

JDA, MB, KS, SF and SAW conceived and designed the experiments; KS, SF, MB, and SAW performed the experiments; JDA and SF analyzed the data; MB and SAW contributed reagents/materials/analysis tools; JDA, MB, KS, SF and SAW wrote the paper.

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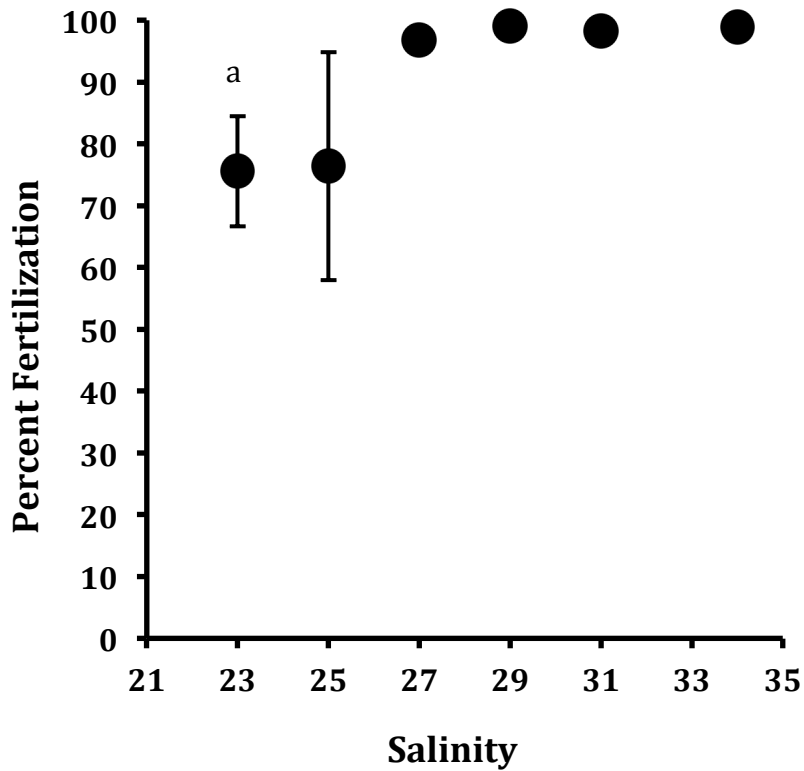


Figure 1: Fertilization percentage of *A. cf. solaris* eggs across a range of salinities. Circles represent the mean (\pm SE) percent fertilization of 3-11 independent, replicate crosses for each salinity treatment. Numbers beneath each data point indicate the number of replicate females for that point. Letters above each data point indicate significant differences in percent fertilization among salinities based on a Kruskal-Wallis H test and post-hoc tests (see text for details). For symbols where error bars are not visible, the error bars are contained within that symbol.

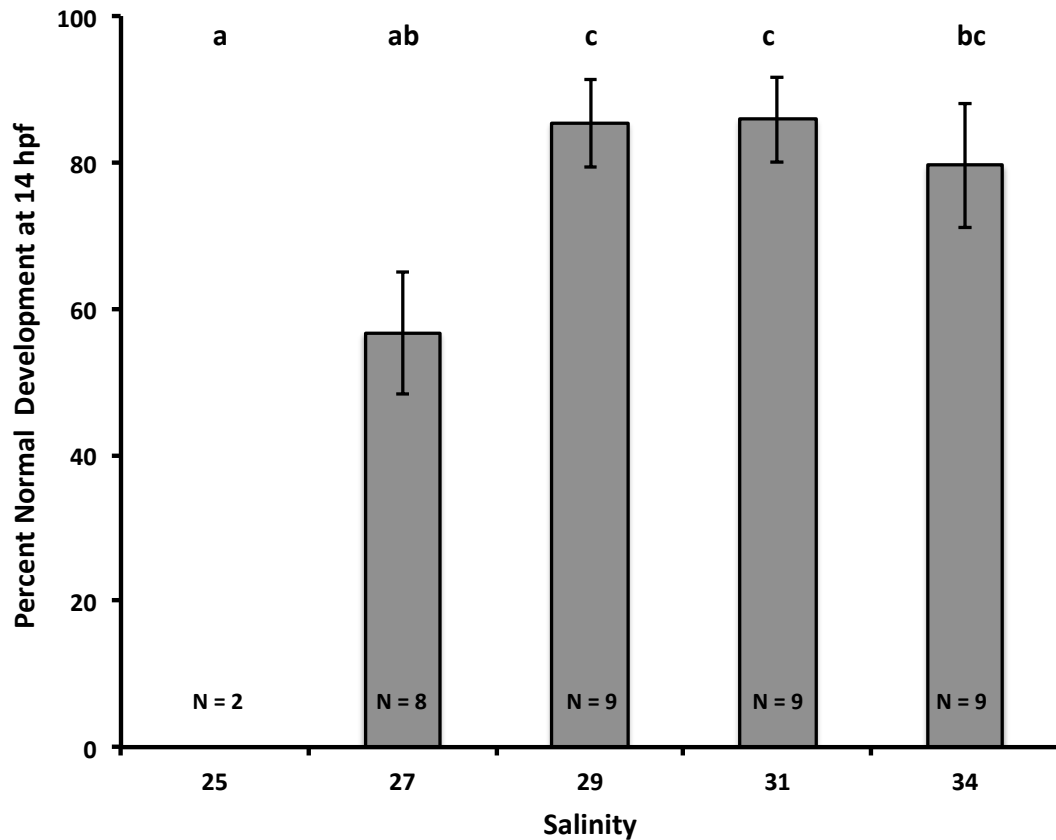


Figure 2: The percentage of embryos exhibiting normal development 14 hours post fertilization (hpf) across a range of salinities. Bars represent the means (\pm SE) for N = 2-9 independent, replicate crosses. The number of crosses tested for a given salinity is recorded within each bar. At low salinity (25) only two crosses were conducted as no normal development was seen in either of the first two replicates. Letters above each bar denote statistically significant differences in the percentage of embryos completing normal development based on a mixed model ANOVA and post-hoc tests (see text for details).

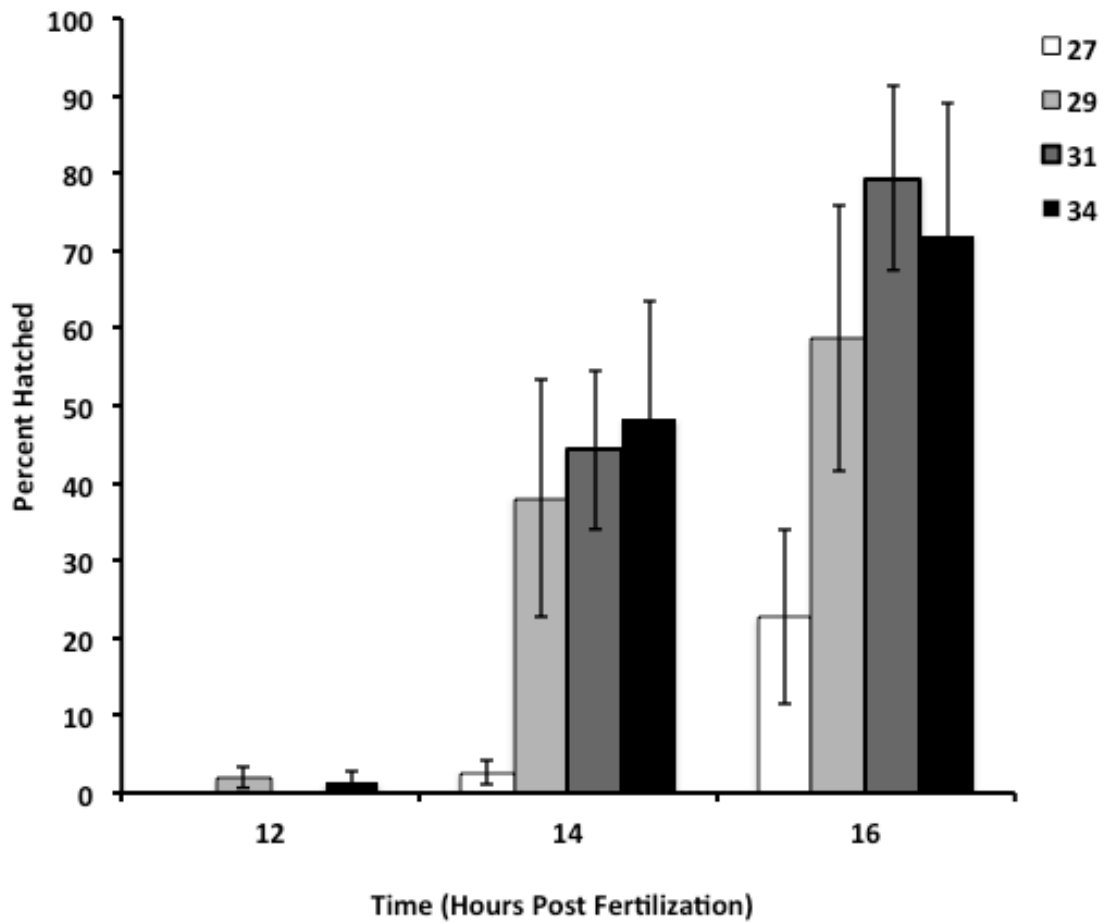


Figure 3: The relationship between percent hatching, salinity and time in *A. cf. solaris*. Percent hatching increases with both time and salinity and varied significantly with cross (see Table 1). Each bar represents the mean (\pm SE) for each of five replicate crosses, measured at three time points (12, 14 and 16 hours post fertilization).

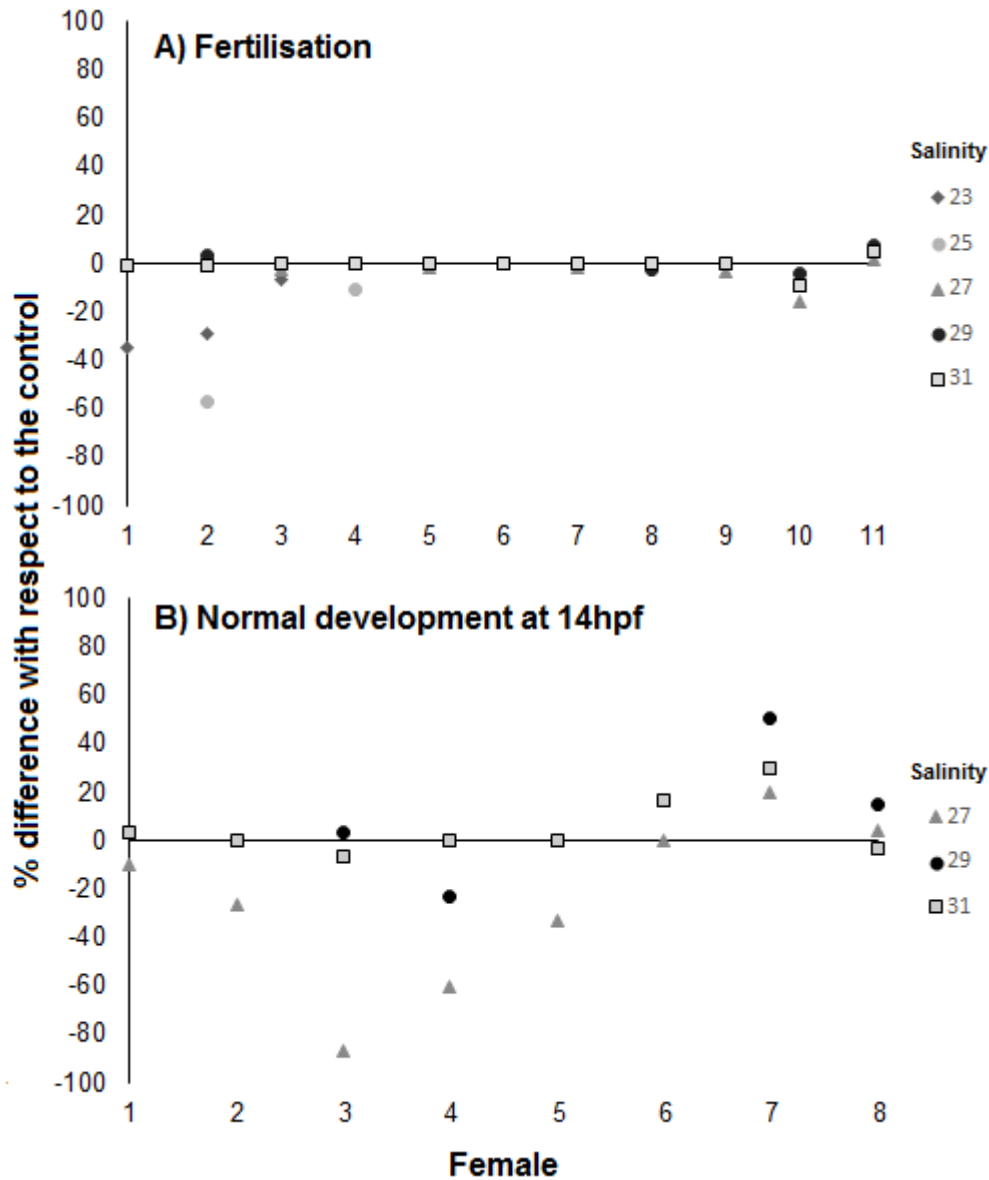


Figure 4: The difference in fertilisation and early developmental success with respect to the control treatment (salinity of 34) grouped by female across experimental salinity treatments. Mean success per female is displayed for the different salinity levels across fertilisation (A) and normal development 14 hours post fertilisation (B). Symbols above the line display higher success than the control, while success was lower than the control for those symbols below the line.

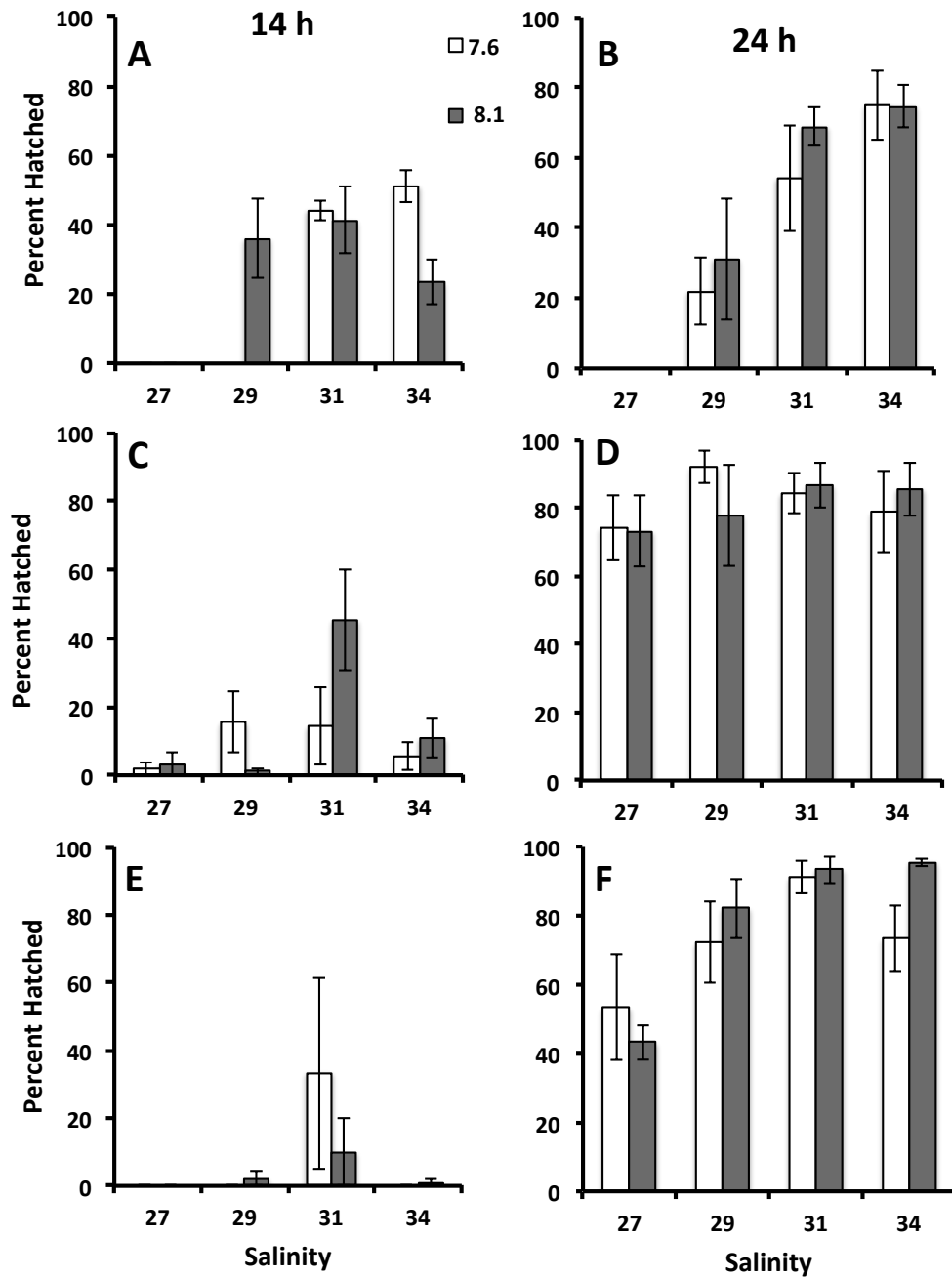


Figure 5: The effect of salinity and pH on the percent of hatched *A. cf. solaris* embryos at 14 and 24 hours post fertilization. Each bar represents the mean (\pm SE) of three replicate blocks. Panels A and B represent the first block. Panels C and D represent the second block and panels E and F represent the third block. The color of the bars represents the pH treatments. Dark grey = pH 8.1 and white = pH 7.6.

Table 1. Experimental pH conditions in experiments with *Acanthaster planci*. Mean values for pH_{NIST} ($\pm\text{SE}$, $n = 3$) for each salinity across experimental runs is presented, as well as the overall mean pH_{NIST} ($n = 12$). $p\text{CO}_2$ was calculated in CO2SYS using data on total alkalinity (TA, $n = 2-4$ per salinity), salinity and pH_{NIST} .

	pH 8.1				pH 7.6			
	27	29	31	34	27	29	31	34
	8.08 ± 0.02	8.07 ± 0.02	8.07 ± 0.02	8.07 ± 0.01	7.61 ± 0.01	7.61 ± 0.01	7.61 ± 0.01	7.61 ± 0.02
pH_{NIST}	8.09 ± 0.01				7.61 ± 0.00			
TA ($\mu\text{mol/kg}$)	1968.3 ± 12.0	2026.9 ± 7.1	2166.4 ± 12.9	2309.4 ± 2.0	1968.3 ± 12.0	2026.9 ± 7.1	2166.4 ± 12.9	2309.4 ± 2.0
$p\text{CO}_2$ (ppm)	492.4	493.1	514.3	529.9	1682.1	1696.2	1779.6	1851.0

Table 2. Binomial logistic regression of hatching probability in *Acahaster cf. solaris*. A full model found that all possible interactions yielded $p > 0.250$ and so the results of a reduced model using only main effects are presented. Significant effects are in **bold**.

Variable	β	p-value	Exp(β)
Salinity	0.295	>0.001	1.343
Male/Female Cross	-0.146	>0.001	0.864
Time	1.025	>0.001	2.788

Table 3. ANOVA for the effects of salinity, pH, time, block (male/female cross) and their interactions on the percent of embryos hatched. Data were arcsine-squareroot transformed prior to analysis. Significant effects ($p < 0.05$) are in **bold**.

<i>Dependent variable</i>	<i>Fixed effects</i>	<i>df</i>	<i>F</i>	<i>p</i>
Percent embryos hatched	Salinity	3,136	18.244	<0.001
	Time	1,136	196.418	<0.001
	pH	2,136	1.094	0.298