

**Questioning success: Fertilisation outcomes in non-standard experiments in the sea-cake
*Dendraster excentricus***

Luisa Kumpitsch¹, Sophie Hanson², Lara Maleen Beckmann¹

¹Tjärnö Marine Laboratories, University of Gothenburg, Sweden

²Duke University, Durham, North Carolina, USA

ABSTRACT

Fertilisation in a laboratory environment is often not representative of ecological field conditions in which fertilisation naturally occurs, especially for broadcast-spawning species that release eggs and sperm into the water. Some challenges of broadcast-spawning include dispersal of gametes in the surrounding water, ability of eggs and sperm to interact, and competition from neighbouring male individuals. Here, we study the sea-cake or sand dollar *Dendraster excentricus*, a model study system, to understand how fertilisation success is affected by volume of water, varying sperm concentration, and mixed sperm from multiple males. Furthermore, we report sperm motility for *D. excentricus*. We found that with equal sperm concentration, fertilisation success was significantly lower in a larger volume of water compared to a standard 6-well laboratory plate. Finally, fertilisation was reduced by sperm from multiple males compared to sperm from one male at similar total concentrations. Our results elucidate how fertilisation success is related to conditions under which eggs and sperm meet and interact, and further highlight the challenges and remaining issues of gamete dispersal and gamete competition in external fertilisers, as is the case for many other echinoderms. Standard methods could potentially overestimate fertilisation success or increase the likelihood of polyspermy, and new techniques that more realistically reflect natural

conditions could open new avenues for studying reproductive strategies and life histories beyond model organisms such as *D. excentricus*.

INTRODUCTION

Early life history of marine external fertilisers

The successful fertilisation of an egg by sperm is essential for the maintenance of the reproductive cycle of most metazoans (Wassarman, 1987). Early life history stages are among the most sensitive and crucial processes in nature. Fertilisation is a complex process, especially in marine external fertilisers that release gametes into the water column and sperm cells must find eggs via chemotaxis (Alvarez et al., 2014). External fertilisation is the ancestral mode of metazoan reproduction and the most common mode of fertilisation found in the sea (Levitan, 1996; McHugh et al., 1998). Fertilisation success, or the fraction of eggs that are fertilised, depends on gamete abundance, gamete compatibility, and spawning synchrony (Bode & Marshall, 2007; Birkhead et al., 1998). However, our established understanding of these processes is largely based on internal fertilisers, key aspects of which may be inapplicable to external fertilisers (Levitan & Petersen, 1995; Bode & Marshall, 2005).

Studies on early life history

To understand the basic principles and mechanisms of fertilisation processes, experiments with subsequent investigation of embryogenesis are carried out in standardised laboratory environments. A common approach is a set-up with 6-well plates or 15–20 ml conical tubes, where eggs and sperm are mixed and evaluated for the ratio between fertilised and unfertilised eggs (Ericson et al., 2010; Graham et al., 2016; Kupriyanova & Havenhand, 2010; Levitan et al., 2007).

Although it is an easy method to investigate fertilisation success, there has been criticism on how applicable such studies are to natural conditions, where biological and physical conditions play a role (Thomas et al., 2013). Standard laboratory procedures involve predetermined concentrations of eggs and sperm from a pair of individuals in 5 ml of water (the volume of one well in a standard 6-well plate). While this setup is useful for conducting experiments and calculating comparative statistics, well-plates differ from natural conditions in two major aspects: 1) in small wells, the water volume is extremely reduced, unnaturally confining gametes and introducing wall effects; 2) moreover, only gametes of individual males and females are combined, while in nature multiple spawners occur simultaneously (Levitan, 2005). Investigating the fertilisation success of marine external fertilisers is crucial to both understanding their reproductive biology and estimating their effective population size as a metric of genetic diversity, as these highly depend on the dispersal of fertilised eggs and subsequent stages (Levitan, 2005).

Sperm competition in marine external fertilisers

Sperm competition is a post-copulatory sexual selection that drives the evolution of several sperm traits (Parker & Pizzari, 2010). The common theory of sperm competition developed by Parker (1970) postulates that 100% of a batch of eggs are fertilised when sperm compete for eggs, following Bateman's principle of sperm being excessive and eggs limited (Parker, 1982). However, this is not applicable to external fertilisers, where incomplete fertilisation is common (Bode & Marshall, 2007). Polyspermy, sperm limitation, and cryptic female choice are the mechanisms that can lead to incomplete fertilisation of the eggs released by the female (Bode & Marshall, 2007). Polyspermy occurs when more than one sperm penetrates the egg. Subsequently, a triploid nucleus forms in the egg which leads to the egg's death or abnormal development (Bode

& Marshall, 2007). Sperm limitation occurs when sperm are diluted in the water column after spawning (Vogel et al., 1982). Another sperm limitation scenario is when sperm cells are attracted to eggs but are too late to be the first to penetrate, and hence remaining eggs potentially compete for sperm (Marshall & Evans, 2005). Cryptic female choice is driven by the female's selection for favourable sperm cells and affects gamete compatibility, such as through certain egg-recognition proteins that select for sperm (Bode & Marshall, 2007; Levitan et al., 2007).

Sea urchins as model organisms for fertilisation studies

Among marine invertebrates, echinoderms are well-studied model organisms for fertilisation studies (e.g., Emler, 1986; Leuchtenberger et al., 2022; Levitan et al., 2007). These animals are easy to collect as they often live in the intertidal or benthos, and there are well-established techniques for husbandry to induce spawning and therefore obtain gametes in a controlled setting. Moreover, genomes are available for several echinoderm species (Cary et al., 2018), facilitating studies on genetic diversity, population dynamics, and climate change adaptations.

Dendraster excentricus as study organism

A well-studied echinoderm species is the sea-cake or sand dollar *Dendraster excentricus*, a common intertidal echinoderm on the northeast Pacific coast that was named after its sand burrowing lifestyle. Sand dollars sexually reproduce as broadcast spawners, with peak spawning occurring in early spring (Niesen, 1977). The eggs are covered with a gelatinous, pigmented coating whose purpose is still unknown. Because populations can occur in dense aggregations, many individuals can spawn at the same time. In addition, gamete fertilisation is subject to a variety

of environmental factors characteristic of tidal shores (Emlet, 1986). Therefore, fertilisation of gametes in the field presents far greater challenges that should be considered in laboratory-based, standardised fertilisation experiments.

Studies have already compared development of the planktonic larvae in the field to lab-reared larvae for *D. excentricus* (Cameron & Rumrill, 1982; Emlet, 1986). However, to our knowledge, fertilisation success has not been studied directly in the field, although there has been observation of spawning or the attempt to quantify it by measuring gonad size in adults (Niesen, 1977).

Fertilisation success of D. excentricus

In this study, we examine two aspects of *D. excentricus* reproduction that are "assumed away" in the standard assay. First, we compare assessment of fertilisation success using the standard methodology (6-well plates) to a set-up that represents a more natural approach by using a larger volume of water in a larger container (water column). We measure fertilisation success in (a) standard 6-well plates and (b) high-volume water columns but using proportionally identical concentrations. We hypothesise that fertilisation success will be higher in the 6-well plates, as the sperm-egg encounter rates should increase with smaller volume. Secondly, we examine the fertilisation success and sperm motility of individual males versus a mix of three males. We hypothesise that the fertilisation success and sperm motility of the three males will be the average of the individual males, as variation in fertilisation success will be averaged out in a mixed-male scenario. As fertilisation success varies between males due to traits like motility, cryptic female choice, and gamete compatibility, we assume that when sperm from different males is mixed, these

interactive effects average each other out and lead to our hypothesised average fertilisation success.

METHODS

Sampling

D. excentricus individuals ($n = \sim 60$) were collected at a field site in East Sound, Orcas Island, WA in April 2023. We kept organisms in unfiltered flow-through seawater tanks for several weeks at Friday Harbor Laboratories, San Juan Islands, WA, where we conducted the present study.

Fertilisation success curve

We spawned and paired ten male and eight female sand dollars randomly for each fertilisation experiment. We measured the size of each selected individual (cross-section at largest width and perpendicular length) with a standard ruler. Size was subsequently analysed as a potential confounding predictor of fertilisation success rate. We conducted all fertilisation experiments at room temperature (16.3–16.4°C).

We compared two different assays to assess how fertilisation success varies with water volumes (see Fig. 1) namely: (1) Standard assay, following typical protocols used in current fertilisation studies that use a 6-well plate with $V = 5$ ml/well; (2) Column assay, using a 200-ml water column where gametes are floating during the fertilisation experiment. For both assays we used proportionally identical concentrations, to be able to compare the two methods.

We induced spawning by injecting ~ 1 ml potassium chloride (KCl) 0.55 M into the gonadal region through the mouth and subsequent shaking of the sand dollar. Then, we collected eggs by

placing the aboral side of the individual facing down in a beaker filled with filtered sea water such that the aboral side was touching the top of the water and collected the eggs at the bottom of the beaker. We collected sperm dry directly from the aboral side of the male and then kept on ice until further processing. Individuals were only spawned once.

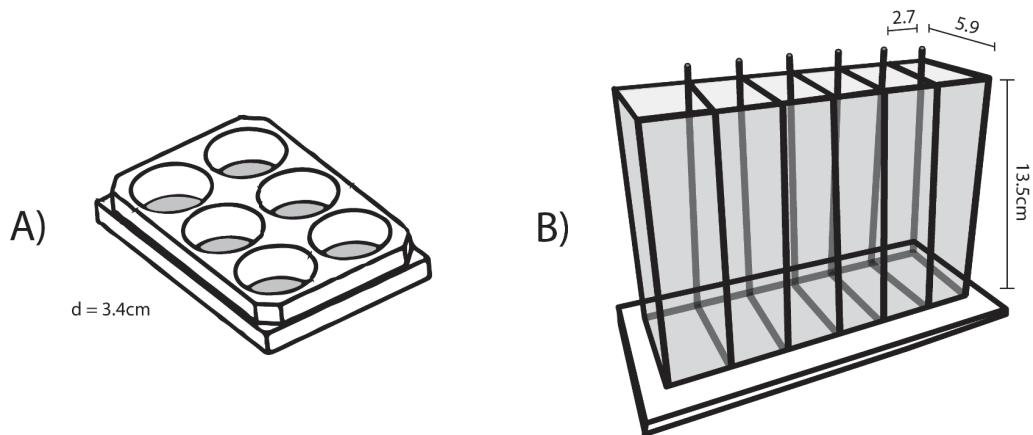


Figure 1. Illustrations of the two different setups. A) A standard 6-well plate with a diameter of 3.4 cm. B) Our water column is constructed out of transparent acrylic plastic and has 6 columns, each $13.5 \times 5.9 \times 2.7$ cm.

The number of eggs in $20 \mu\text{L}$ of the collected egg solution was counted and used to estimate the required volume for the experiments. For the 6-well plates, a total volume of 5 ml per well and 200 eggs per well were used (i.e., 0.04 eggs per $1 \mu\text{L}$). For the water columns, a total volume of 200 ml per column and 8000 eggs per column were used (i.e., 0.04 eggs per $1 \mu\text{L}$).

We diluted dry sperm with filtered seawater to a volume of 1.5 ml in an Eppendorf tube. Respective sperm concentrations were counted with a Hemacytometer counting chamber

(Improved Neubauer chamber; Bright-Line Hausser Scientific PA). We estimated the concentration by counting sperm within 20 small squares and calculating the average per square. The average was then extrapolated to the chamber's volume (0.00025 mm^3 / over each square; can be multiplied by 10^4 for cells per ml). The cytometer was loaded with a 1% dilution, using 5 μL sperm stock solution in 500 μL Lugol stain.

Standard assay (Figure 1, A): We filled all 6 wells with 5 mL of filtered seawater. To maintain a total volume of 5 mL, the volume of water equal to the added egg solution was removed. The volume of egg solution resulting in 200 eggs (dependent upon egg concentration for each female individual) was pipetted into the top of each water column. Sperm was then added in the following concentrations: (well 1) 1000 sperm/ μL , (2) 200 sperm/ μL , (3) 50 sperm/ μL , (4) 10 sperm/ μL , (5) 2 sperm/ μL , and (6; control) 0 sperm/ μL . The experiment was performed for 25 minutes and started simultaneously for both assays (standard and control). After the set fertilisation time, we immobilised the sperm by adding 1 ml KCl to each well (see Leuchtenberger et al., 2022). A prior test in this study showed that adding KCl at this time has no effect on the gametogenesis, larval development, and survival in *D. excentricus*.

Columns assay (Figure 1, B): We filled all 6 columns with 200 mL of filtered seawater. To maintain a total volume of 200 mL, the volume of water equal to the added egg solution was removed. The volume of egg solution resulting in 8000 eggs (dependent upon egg concentration for each female individual) was pipetted into the top of each water column simultaneously with respective sperm concentrations. Sperm was then added in the following concentrations: (column 1) 1000 sperm/ μL , (2) 200 sperm/ μL , (3) 50 sperm/ μL , (4) 10 sperm/ μL , (5) 2 sperm/ μL , and (6; control) 0 sperm/ μL . The experiment was performed for 25 min. Afterwards, the eggs were mixed in the water column and 5 ml of the mixed water was extracted and pipetted into a 6-well plate.

To prevent further fertilisation, we immobilised the sperm by adding 1 ml of KCl to each well in a similar manner as above.

We checked and counted all treatments after 2 h and 24 h after fertilisation. After 2 h, we counted a total of 200 eggs and noted the ratio of fertilised to unfertilized eggs. After 24 h, we counted a total of 200 embryos and noted the ratio of gastrulae to undeveloped embryos. A decline in the 24 h ratio compared to the 2 h ratio indicated polyspermic eggs, as they will develop fertilisation envelopes and divide after sperm penetration (Steve Paxton, Evolutionary Developmental Biology Lecture 11. (n.d.)) but stop development or continue abnormal development before they reach the gastrula stage, as the zygotic DNA then takes over.

To compare success rates between the 6-well plate and water column experiments, we created fertilisation success curves. We used the 50% fertilisation success averaged over all 9 replicates for further experiments. Fertilisation curves are commonly used to determine an optimal sperm concentration that neither exceeds nor limits fertilisation success of eggs (most commonly the 50% threshold; Leuchtenberger et al., 2022).

Sperm competition experiments

Gametes of four randomly selected females and three males per female were collected, counted, and measured as described above. The experimental design for one 6-well plate was (1) Male 1 \times Female, (2) Male 2 \times Female, (3) Male 3 \times Female, (4) Males 1+2+3 \times Female, and (5) Control (Female without sperm), summing up to four treatments per female and one control. We replicated the experiment with different males and females for four well-plates. For each male, we determined and diluted the sperm concentration to the concentration that results in approximately 50% of fertilised eggs (based on the results of the fertilisation curve described above). All

following steps were performed as described in the methods above. We then compared fertilisation curves compared between the five treatments. We then combined data from all individual males and mixed males per replicate to calculate the mean and standard deviation.

Sperm motility

We measured sperm motility using a high-speed camera (Photron MC1 HSV camera; 250 frames s⁻¹) mounted to a compound microscope (Olympus BH2 series microscope; 25X magnification). Dry sperm from 12 males was diluted into 1.5 ml of filtered seawater in an Eppendorf tube so that the final concentration was 10⁴ sperm ml⁻¹. To prevent water movement on the filmed microscope slide, 350–450 µL of suspended sperm stock was pipetted into the centre of a 11/16”-outer diameter O-ring, which was glued to the microscope slide using acrylic cement 24 h prior to filming. We used fresh saliva as a lubricant on the slide to prevent sperm from sticking to the slide itself. A cover slip was gently placed on top of the O-ring such that it touched the fluid’s surface. We maintained focal depth and lighting across all trials to avoid differences in subsequent digital analysis. For each male, we filmed 10 image sequences (1–2 seconds each), exported these as AVI files with Fastcam Viewer version 3 (Photron Inc., Tokyo, Japan), and then imported them into ImageJ (Version i.52, National Institutes of Health, USA) for all following tracking analysis. We used the Computer Assisted Sperm Analyzer (CASA) package (Wilson-Leedy & Ingermann, 2006) as a plugin in ImageJ to automatically track sperm head movement to extract kinetics. We used the following motility parameters for analysis: velocity curvilinear (VCL), velocity average path (VAL), velocity straight line (VSL), and motile sperm (%). VCL describes the point-to-point velocity (total distance travelled) per second, VAL the point to point velocity on a path constructed using a roaming average, and VSL the velocity measured using the

first point, the average path and the point reached that is farthest from the origin during the measured time period.

Statistics

All statistical analyses were carried out in RStudio (v. 2023.06.0). Plots and graphs were created using the ggplot2 package (Wickham, 2016) and pictures edited in Adobe Illustrator 2023 (v. 27.6.1). We used the R packages tidyverse (Wickham et al., 2019) and dplyr (Wickham et al., 2023) to calculate standard statistical measures, including mean, standard deviation (hereafter SD), standard error from the mean (hereafter SE), and minimum and maximum values. We conducted a Wilcoxon test for a one-factor analysis using wilcox.test(), a Shapiro test for multiple factors to check for data normality (i.e., parametric vs. non-parametric) using shapiro.test(), and a Levene test to check for variance homogeneity using LeveneTest(). To test if our data were suitable for an ANOVA, we performed the Brown-Forsythe test using bf.test(). For analysis of more than two independent variables, we performed a two-way ANOVA using the R function aov().

RESULTS

Fertilisation success curve

Out of ten replicates, only eight were used for further analysis. Experiments 1 and 2 served as pilot studies and were performed with higher concentrations (10-fold, starting from 10^4 sperm/ μ l). To obtain better resolution from the fertilisation curve, the highest concentration was lowered to 10^3 / μ l and decreased 5-fold to 2 sperm/ μ l. For both experimental set-ups, the fertilisation rate increased with sperm concentration and then decreased at the highest concentrations for both the 6-well plate and water column experiments. All following numbers are

presented as mean \pm SD. Fertilisation success after 2 h (Fig. 2) and 24 h (Fig. 3) was higher in the 6-well plates ($97.2\% \pm 4.14$ and $79.3\% \pm 33.2$, respectively) than in the water columns ($40.5\% \pm 22.5$ and $37.2\% \pm 21.6$, respectively). The success rate decreased in all treatments with the highest sperm concentration (1000 sperm/ μ l) compared to the second highest (100 sperm/ μ l). All data is presented in Table 1 and 2. There was no significant effect of replicates or individual males on the success rates ($p > 0.05$).

Based on the determined fertilisation curve for both volumes, the intersection at 50% success to get viable gastrulae gives an average sperm concentration of 45 sperm/ μ l for wells and 38 sperm/ μ l for columns. These values for fertilised eggs after 2 h are similar with 40 sperm/ μ l for wells and 42.4 sperm/ μ l for columns (Fig. 2 and 3).

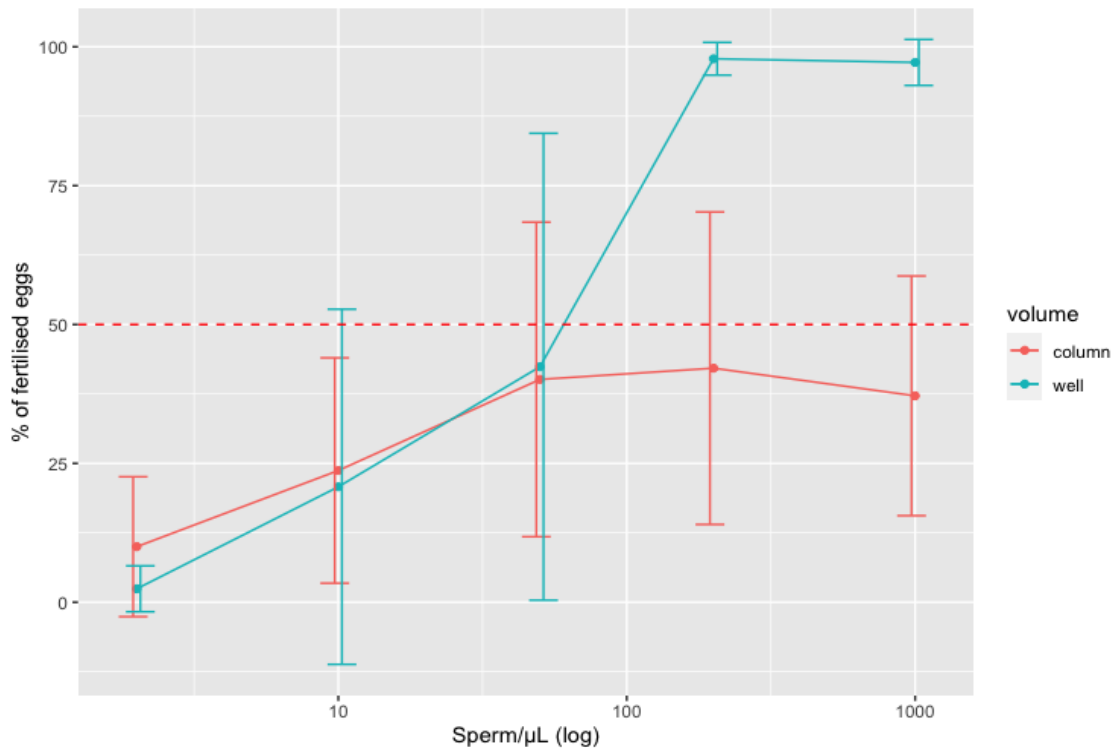


Figure 2. Plot based on the mean of all 8 replicates. Error bars show standard deviations from the mean. Red line shows fertilisation success (%) after 2 h for fertilisations performed in columns. Blue line shows fertilisation success (%) after 2 h performed in 6-well plates.

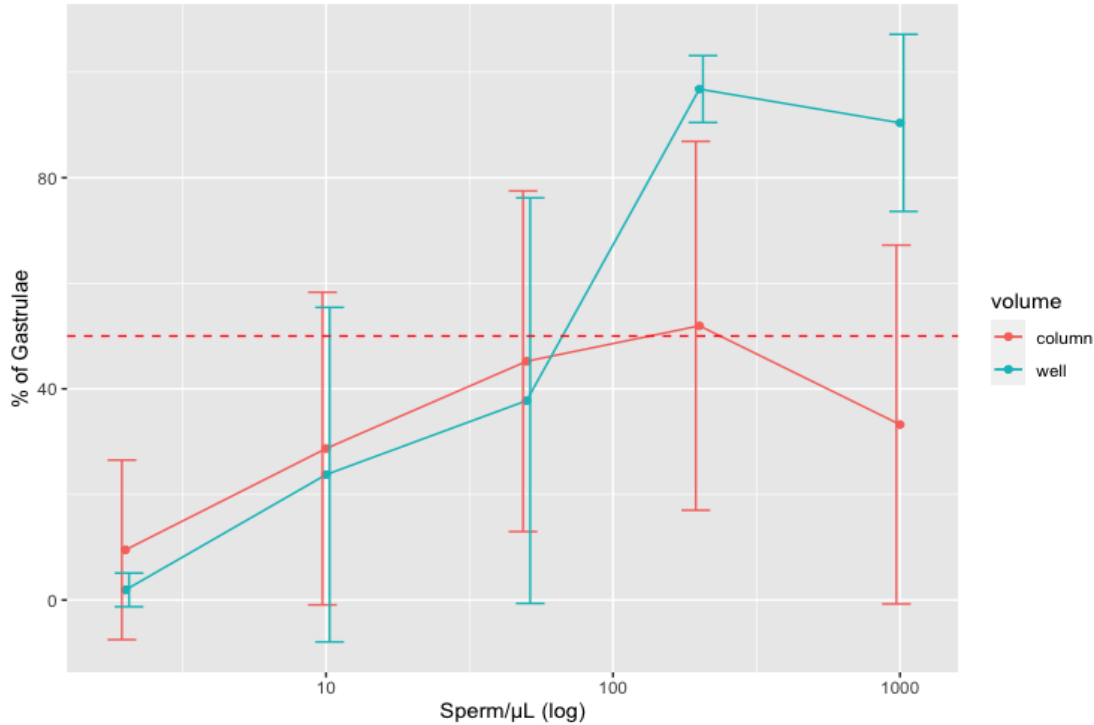


Figure 3. Plot based on the mean of all 8 replicates. Error bars show standard deviations from the mean. Red line shows fertilisation success (%) after 24 h for fertilisations performed in columns. Blue line shows fertilisation success (%) after 24 h performed in 6-well plates.

Table 1. Fertilisation success (%) after 2 h (ratio of embryos to non-fertilised eggs) and 24 h (ratio of gastrulae to non-developed embryos) after fertilisation. sd = standard deviation, se = standard error, n = number of replicates.

Volume	Sperm/μL	n	Average fertilisation success 2h (%)	SD/SE	Average fertilisation success 24 h (%)	SD/SE
Column	2	8	10	12.6/4.45	10	12.6/4.45

(V = 200 ml)	10	8	26.6	20.8/6.94	23.7	20.3/7.17
	50	8	40.1	28.3/10	40.1	28.3/10
	200	8	42.1	28.1/9.95	42.1	28.1/9.95
	1000	8	40.5	22.5/7.5	37.2	21.6/7.63
Well (V = 5 ml)	2	8	2.42	4.13/1.56	12.3	29.5/10.4
	10	8	20.8	32/12.1	31.6	36.2/12.11
	50	8	42.4	42/15.9	44.3	40.1/14.2
	200	8	97.8	2.95/1.11	95.6	6.8/2.41
	1000	8	97.2	4.14/1.56	79.3	33.2/11.1

Table 2. Two-way ANOVA results of sperm concentration and volume on fertilisation success for 2 h and 24 h post fertilisation.

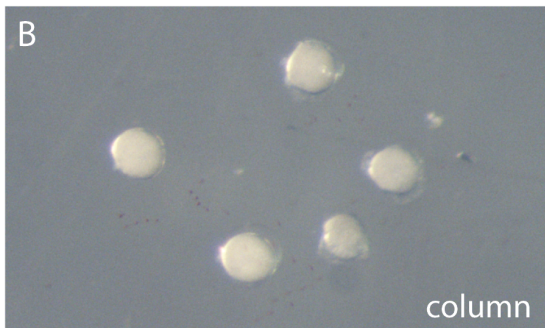
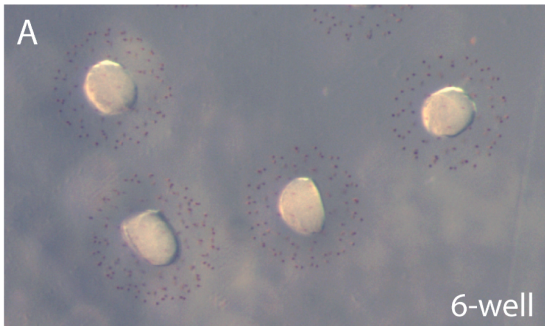
	Time post fertilisation (h)	Df	Sum sq	F value	Pr(>F)	Significance
Sperm concentration	2	1	18 376	17.272	8.81×10^{-5}	***
Volume	2	1	8 629	8.111	0.00573	**
Residuals	2	72	76 6601	1 064		
Sperm concentration	24	1	12 575	9.651	0.00271	**
Volume	24	1	5 023	3.855	0.05	*
Residuals	24	72	93 819	1 303		

We further related male sizes (height, width, and length) to the respective reproductive outcome to rule out any size effects on fertilisation success. Based on two-way ANOVA

(fertilisation percent \sim height+width+length), there was no significant effect on the fertilisation success ($p > 0.05$).

We observed differences in gamete distribution and appearance between well plate and column fertilisation. Gastrulae that resulted from fertilisation inside the water column gathered on the edges of the counting wells after 24 h and were heterogeneously distributed along the water column in the wells. Gastrulae that resulted from the 6-well plate fertilisation stayed in the centre and on the bottom of the well. In the water columns, we further observed broken eggs, embryos, and remains in the bottom of the wells after 2 and 24 h (Fig. 4B, C, and D). This was never observed for the well plate fertilisation replicates. Even 24 h after fertilisation, the eggs keep their gelatinous coat and are still arranged in a matrix-like orientation to each other (Fig. 4A). In the 6-well plates, the eggs seem to be distributed in chains or accumulations.

Eggs
Control, 2h



5 mm

Embryos
2h

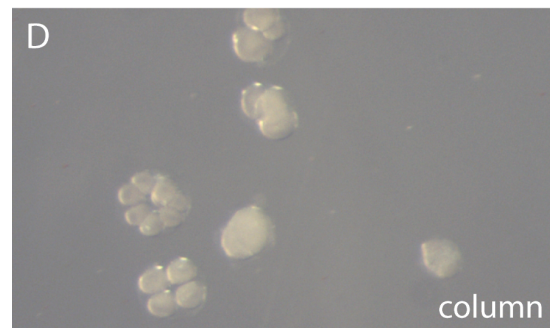
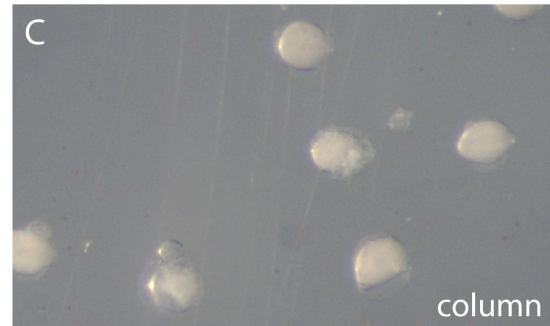


Figure 4. *D. excentricus* eggs used for the control of a fertilisation experiment in a 6-well plate (V= 5 ml). The eggs show an intact gelatinous coating with red pigments after 2 h in the experiment (A). The control conducted in a water column (V = 200 ml) resulted in eggs with a dissolved gelatinous coat and few red pigments (B). Deformed eggs and 2–6 cell stage embryos 2 h after fertilisation in a 200 ml water column (C, D). All images have the same scale (5 mm).

Sperm competition

Fertilisation success in the mixed males (n = 17) was significantly lower than in the individual males (n = 51) with $64.5\% \pm 26.4$ versus $81.3\% \pm 24.5$ ($p = 0.012$; Fig. 5). Note the high standard deviation for both treatments. There was no significant effect of replicate or well-plate and individual males' identification ($p > 0.05$). We also conducted one replicate in a water column (same specimens as in experiment 7, male ID 19–21) to compare the results with the well plates. Here, the average of all three individual males resulted in a success rate of 50.2% and the mixed male treatment showed a rate of 38% (data not shown in Fig. 5).

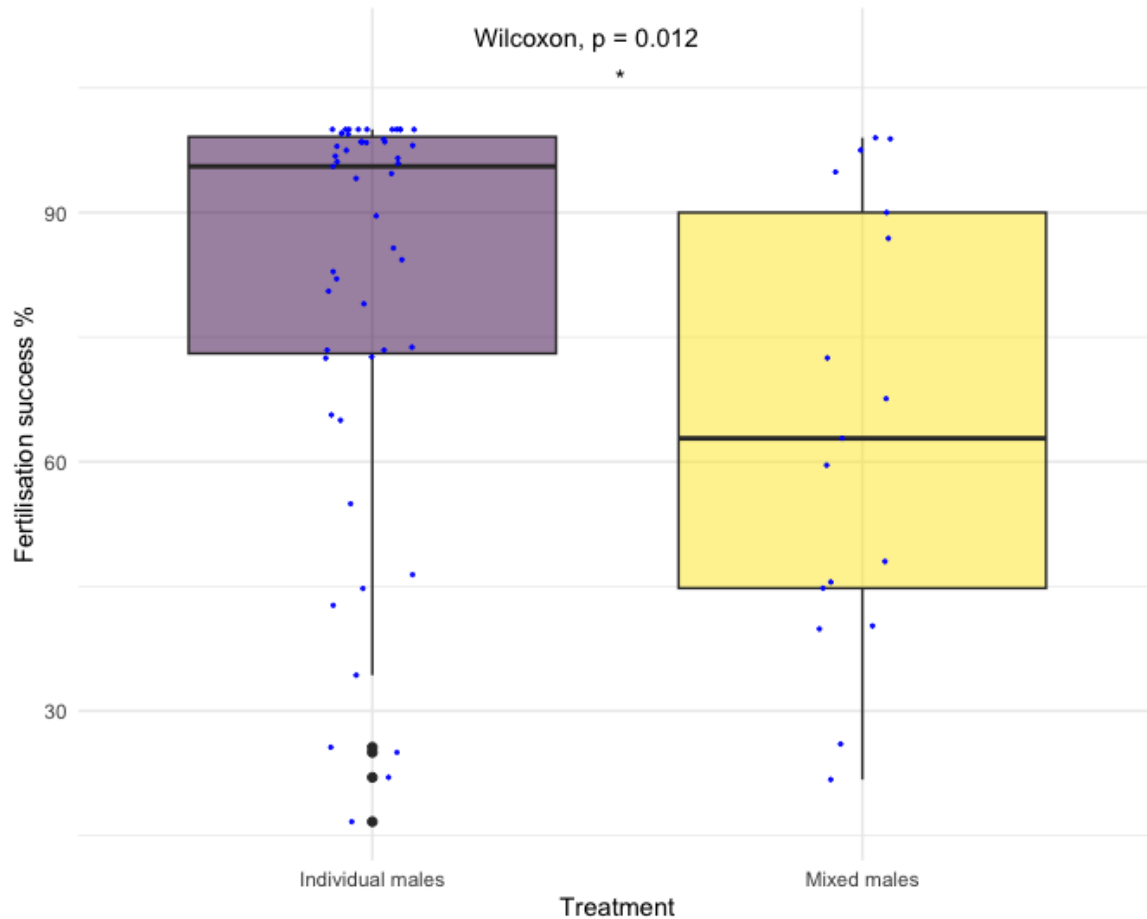


Figure 5. Fertilisation success (%) based on the treatment. The purple boxplot shows the individual male treatments ($n = 51$). The yellow box plot shows mixed-male treatments where three replicate males were mixed before fertilisation ($n = 17$). Blue dots represent success outcomes of the individual experiments.

Sperm motility

For individual males, the curvilinear velocity was $278 \mu\text{m/s} \pm 100$, average path velocity $91.6 \mu\text{m/s} \pm 21.8$, straight line velocity $69.4 \mu\text{m/s} \pm 17.1$, and motile sperm $27.8\% \pm 20.3$. For

mixed males, the velocity curvilinear was $246 \mu\text{m/s} \pm 62.6$, velocity average path $83 \mu\text{m/s} \pm 17.4$, velocity straight line $66 \mu\text{m/s} \pm 14$, and motile sperm $17.2\% \pm 14.4$.

The VAP and % of motile sperm were significantly lower in the mixed males versus the individual males (Table 3). There was no significant difference in the velocity curvilinear (VCL) or velocity straight line (VSL; Table 3). One male had to be excluded as the video showed irregularities and motility parameters could not be determined.

Table 3. Sperm motility parameters: velocity curvilinear ($\mu\text{m/s}$), velocity average path ($\mu\text{m/s}$), velocity straight line ($\mu\text{m/s}$), and motile sperm (%). t = t-value, df = degrees of freedom, W = Wilcoxon statistic, p = p-value.

	Test	t	df	W	p	Significance
VCL ($\mu\text{m/s}$)	Wilcoxon	-	-	179	0.17	
VAP ($\mu\text{m/s}$)	Wilcoxon	-	-	1900	0.05	*
VSL ($\mu\text{m/s}$)	T-test	1.057	120		0.26	
Motile sperm (%)	Wilcoxon	-	-	2354.5	0.003	*

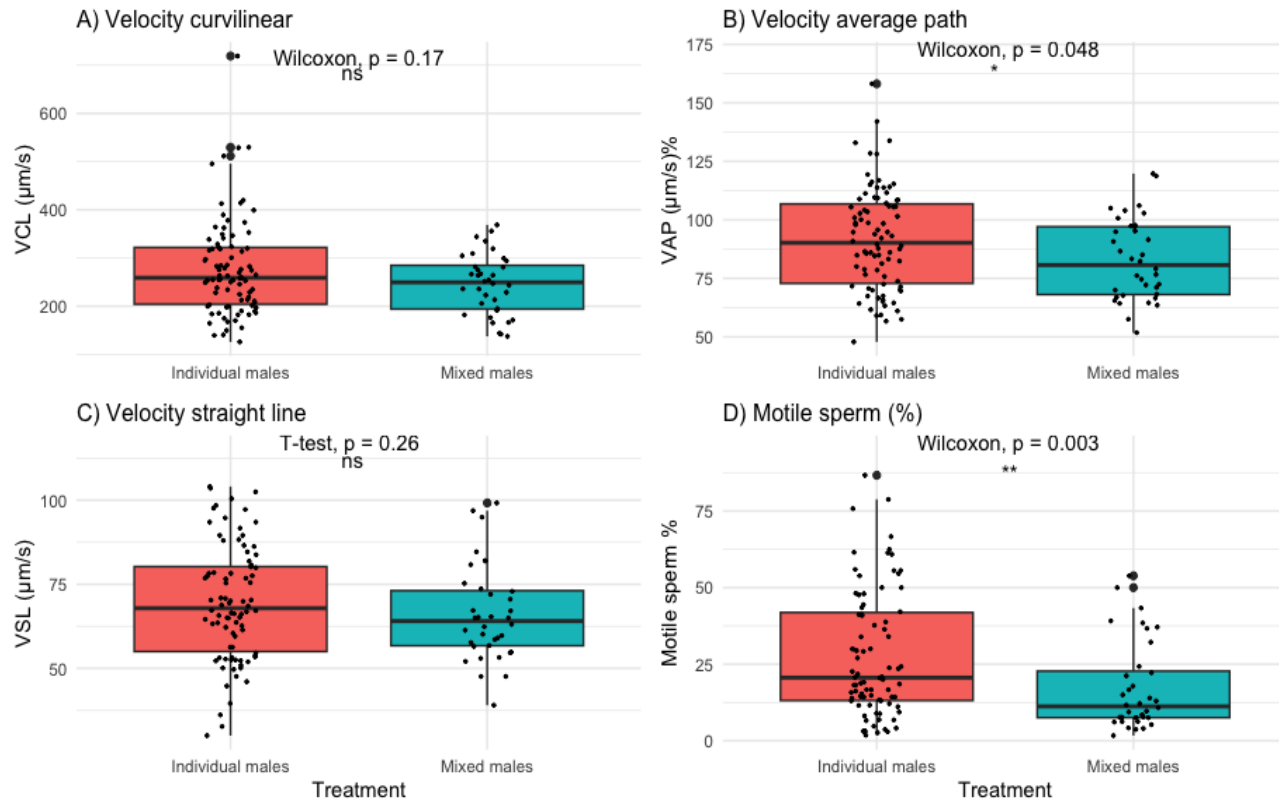


Figure 6. Boxplots show VCL (A), VAP (B), VSL (C) and motile sperm (%) (D) of individual males (red; $n = 86$) and mixed males (blue; $n = 36$). Black dots represent individual data points of all replicates. Signif. codes: 0.001 ‘***’ 0.01 ‘*’.

Fertilisation success and sperm motility

Fertilisation success increases with sperm motility when using VCL ($\mu\text{m/s}$) and motile sperm (%) as metrics of sperm motility (Fig. 7, Table 4).

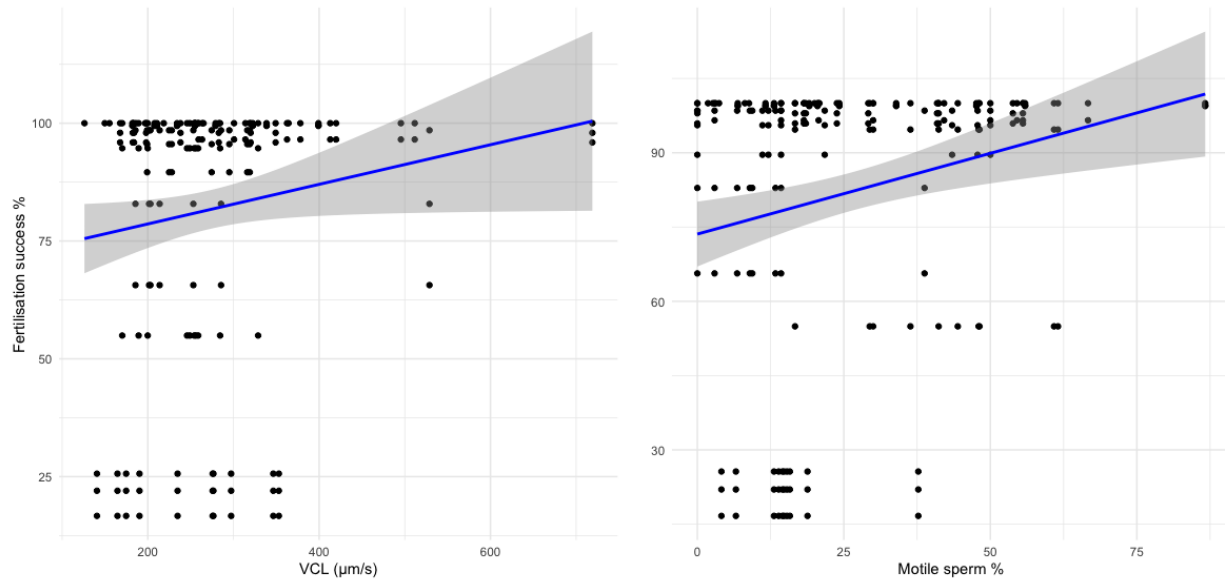


Figure 7. Correlation between fertilisation success (%) and sperm motility. The left plot shows velocity curvilinear (VCL) against fertilisation success and the right plot shows percentage of motile sperm against fertilisation success. The blue line is a significant linear model regression (lm). The grey area represents the 95% confidence level interval for predictions from the lm. Duplicate data points are a result from the individual replicate measurements. Signif. codes: 0.001 ‘***’ 0.01 ‘*’.

Table 4. Statistics for velocity curvilinear (VCL) and motile sperm (%) to fertilisation success in individual males. Df = degrees of freedom, Pr(>F) = p-value. Signif. codes: 0.001 ‘***’ 0.01 ‘*’.

	Df	Sum sq	Mean sq	F value	Pr(>F)	Significance
VCL (µm/s)	1	3235	3235	3.976	0.048	*
Residuals		186	151336	814		
motile sperm (%)	1	7889	7889	10.39	0.00149	**

Residuals		196	148844	759		
-----------	--	-----	--------	-----	--	--

DISCUSSION

A variety of factors control and mediate fertilisation success both in a laboratory setting and under natural field conditions. In our study, we demonstrated that fertilisation success varies at equivalent sperm and egg concentrations depending on the experimental set-up. Our results elucidated key mechanisms involved in natural fertilisation that are not reflected in standard fertilisation assays, indicating the importance of sperm-egg interactions dictated by gamete dispersal in broadcast-spawning echinoids.

Fertilisation success varies in different water volumes

We can accept our hypothesis that fertilisation success is higher in the 6-well plates compared to the columns. Eggs fertilised in 200 ml water columns generally showed lower fertilisation rates than the 6-well plates at the high sperm concentrations. At concentrations below 100 sperm/ μ l, the curves for both set-ups look similar. These results suggest that the volume in which fertilisation occurs has an impact on the fertilisation success, at least at higher sperm concentrations. In a small container, like a well, sperm encounters eggs quickly without having to swim far, which might lead to more sperm-egg encounters and hence high fertilisation success. On the other hand, sperm might need to swim faster in the water column to follow the sinking eggs and fertilise them, which might reduce fertilisation success. If there is chemotaxis in sperm towards eggs, the distribution of the attractant (released by the eggs) might be more intensive in the well compared to the column. In a study from Kiørboe et al. (2001), it was suggested that the wake of a small sinking particle has an attractive gradient laterally but almost no vertical gradient. This

implies that sperm would only be able to sense the chemical gradient of the egg in the wake of the egg. Even if the sperm could sense the egg, it may be further challenging to swim fast enough to match the eggs' sinking rate. To elucidate this theory further and be able to model this scenario, we measured egg sinking rates, however, did not incorporate it in this study due to time constraints.

It has been pointed out that the rate of sperm dispersal and the dynamics of gamete interactions become more important with larger amounts of water (Levitan and Petersen, 1995). However, the mechanism by which large amounts of water affect gamete interactions is somewhat unexplored. Given that the size of gametes are relatively small compared to the 5 ml 6-well plates, it is possible that the scale at which eggs and sperm interact with each other may not be strongly affected by hydrodynamic wall effects of the well, even in such a small container. However, we found that other hydrodynamic factors in the space of the 6-well plates, such as 3-dimensional diffusion and egg sinking rate, have the potential to affect the likelihood of gamete encounters. In the wells, we found that eggs lie flat on the well bottom and do not sink. Dispersal in the column is dominated by water depth, velocity, and potential turbulence, and gamete interactions are largely controlled by egg size and sperm swimming ability (Levitan and Petersen, 1995). Another factor that is often overlooked is the mobility of eggs in water, such as buoyancy and sinking rates. Sperm need to adjust their swimming behaviour to follow the eggs and fertilise them.

Given that sperm concentration was kept constant across the two different water volumes, it is likely that dispersal, sperm buoyancy, and gamete interactions affect fertilisation success differentially in the two set-ups. The columns are less artificial than the wells, which might provide a more realistic concept of how fertilisation success looks like in *D. excentricus*.

While sperm motility is certainly important in the context of sperm dispersal, the dispersal of eggs (i.e., the rate at which the eggs sink in the column) remains to be explored.

In the context of gamete interactions and egg–sperm motility, the rapid dilution of gametes in a water column is perhaps more relevant to pelagic spawners. We acknowledge that many echinoids, including *D. excentricus*, are benthic species that employ reproductive strategies to resist dispersal of gametes by retaining a mass of eggs on the aboral surface of the female instead of readily dispersing into the water column (Thomas et al., 2013; R. Emlet, personal communication; see Fig. 8). It has been shown in one species of sea urchins that fertilisation occurs at a higher rate in the egg mass adhered to the aboral surface in comparison within the water column (Thomas et al., 2013). This observation suggests that sperm must disperse into the water column to reach the eggs on the female. As eggs are limited and sperm excessive (Parker, 1970), sperm have the ability to actively move to the eggs laying on the female as well as disperse in the intertidal currents. Therefore, the ability to increase egg longevity and viability may be relevant life history characteristics of echinoids and in assessing their fertilisation rates. Further studies of natural spawning events and gamete dispersal in the field would help in elucidate this behaviour.

Considering a scenario where eggs remain on a female’s aboral surface, sperm might diffuse from the male to a mass of eggs on the female, which seems to be a more sperm-limited, egg-abundant scenario. Egg competition has been described as another fertilisation success driver, as eggs in a polychaete were shown to retain up to 140 sperm cells per egg (Marshall & Evans, 2005). Applying this extreme scenario to our experiments would add up to 28000 sperm cells that could be bound to eggs, which would exceed the numbers that we added even in the highest concentration. Egg competition in echinoderms remains understudied.

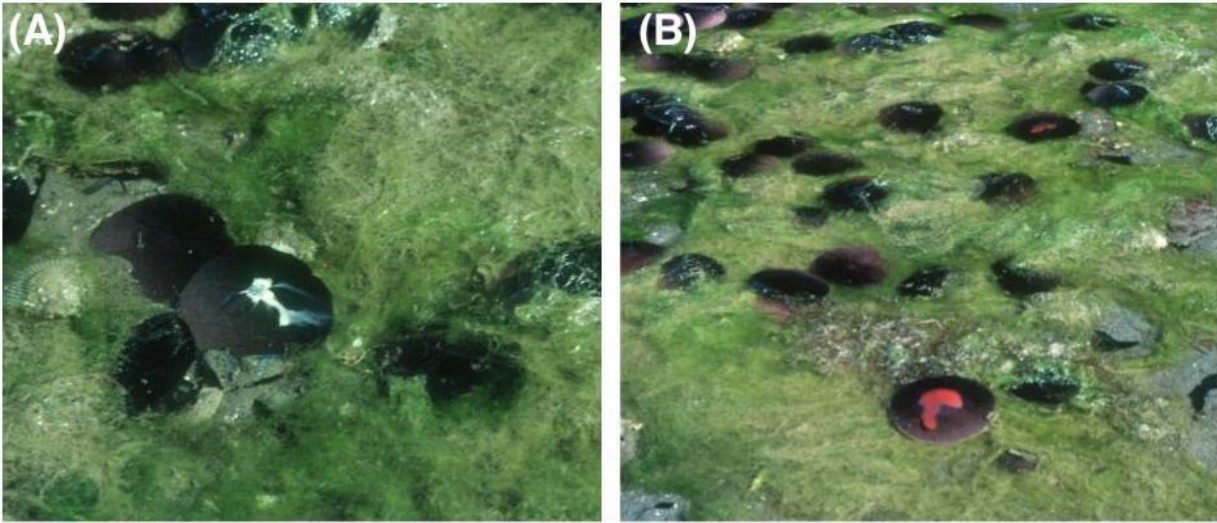


Figure 8. Natural spawning of (A) a male and (B) female *D. excentricus* at low tide. Photographs courtesy of Richard Emlet.

The importance of morphology and re-evaluating natural spawning behaviours

Despite observations of natural spawning in *D. excentricus*, it is not known how many eggs and sperm are actually distributed into the water column. In our experiments, we observed that eggs and embryos disintegrate in the water columns, leaving incomplete gelatinous coats and deformed embryos. There is not an apparent way by which damage to the eggs would be caused by our handling, since we used the same devices (e.g., pipettes, chemicals) and methods for both set-ups and performed the replicate experiments at the same time. The eggs in the 6-well control plates all displayed a matrix-like egg arrangement and were well-preserved for over 2 h after the start of the experiments. These observations may indicate that the gelatinous coating of *D. excentricus* eggs is fundamental to their integrity and longevity but is affected by diffusion in the water column. The eggs appeared to "stick" to each other, which also created some matrix alignment, which could indicate that the coating facilitates connectivity and prevents dehydration.

Our observations could indicate that spawning and subsequent suspension of eggs in the water in *D. excentricus* may be actually rare and that eggs rather accumulate on top of the female. A jelly layer increases the total surface area of the egg; thus, a removed gelatinous coat reduces the chances of sperm encountering the egg. Studies have shown that artificially removing the gelatinous layer in echinoderm eggs leads to a reduction in fertilisation success (Farley & Levitan, 2001; Thomas et al., 1999). The jelly coat was shown to be even more variable than the actual egg size, making it an essential structure to consider when measuring eggs, referring to the target size for sperm and considering it in fertilisation kinetic models (Deaker et al., 2019).

Another possible explanation for lower fertilisation success at higher concentrations in the water columns is that eggs lose their gelatinous layer in the columns while sinking and thus the velocity around the eggs changes the sperm's ability to reach the egg. Without the coat, the viscosity is probably lower around the egg and the sperm can reach it faster. Therefore, at high concentrations, this would increase the likelihood of polyspermy in this setup. It is also possible that eggs send out chemoattractants through the jelly coat, making it more difficult for sperm to find them without a layer and so reducing the fertilisation success. However, we could not find any information about chemoattractants in *D. excentricus* or related species, and it is unknown if any attractants are released by the egg or the surrounding coat. The function of that gelatinous layer should be further examined to fully understand the observed phenomenon in this study.

Polyspermy could have occurred in both experimental set ups, since we observed fertilisation rates generally being lower at the highest concentration compared to the second highest. We did not test this hypothesis statistically and cannot rule out other mechanisms that can lead to mortality of embryos after 24h. However, the lower success in the highest concentration supports the assumption that fertilisation success in external fertilisers is not always 100 %.

Sperm competition reduces fertilisation success

We reject our hypotheses that the mix of sperm from three males would result in the average fertilisation success and sperm motility achieved by the three males individually.

Our data suggest that a sperm mix from multiple males can significantly reduce fertilisation success compared to individual male's sperm. These results may provide evidence of male–male competition, even with a sperm concentration that was presumed to result in a 50% fertilisation success based on our previously constructed fertilisation curves. Assessing multiple male sperm competition at different sperm concentrations may reveal whether competition is a product of sperm proximity or perhaps an interaction between proximity and polyspermic effects. We also note that individual males result in a highly variable range of fertilisation success, supporting the presence of gamete compatibility mechanisms between individual male and female gametes (Bode & Marshall, 2007; Levitan et al., 2007).

Sperm motility might play a role, as the average percentage of motile sperm and the velocity average path were significantly lower in the mixed compared to the individual sperm. Males with faster sperm were more successful in fertilising eggs in the treatments with individual males. However, they could not succeed with the same speed in the mixed treatment. Sperm from different males might show characteristics that inhibit each other's swimming speed and/or ability to follow chemoattractants of the eggs. Seminal fluid that is released with the sperm could contain proteins that act as a defence mechanism against competitor sperm. Seminal fluids of external fertilisers contribute to the sperm's motility, or olfactory signalling (Hopkins et al., 2017), which mediates the sperm–egg encounter. It has been suggested that males might not spawn all at the same time when in proximity, but rather use a time frame when other males are not spawning to

increase their paternity chances. Our findings support previous observations in the Atlantic sea urchin *Paracentrotus lividus* where fertilisation success was lower on average in the mixed sperm versus an individual male's sperm (Kumpitsch et al., unpubl. data).

Assessing natural field conditions

Assessing fertilisation levels in field conditions has been advocated for in several model study systems, such as sea urchins (Yund and Meidel, 2003; Thomas et al., 2013). Such focus has been on the longevity of gametes and mechanisms to resist diffusion of gametes from the aboral surface. We show that a laboratory setting is capable of testing factors inherently related to the field in a controlled manner without the variability that comes with direct field experiments. By isolating one ecological challenge at a time in a laboratory setting, we can then draw connections to other factors—such as diffusion and sperm motility—that could influence fertilisation rates between the 6-well plate and water column experiments. In addition, computational and mathematical modelling of sperm kinetics and egg-sperm interaction dynamics have been fundamental tools in providing insight into fertilisation probability, trade-offs, differences under different flow regime, and sperm limitations (e.g., Vogel et al., 1982; Bode & Marshall, 2007; Parker & Pizzari, 2010; Leuchtenberger et al., 2022). These approaches will likely reveal new limits and trade-offs to ecological challenges and life history traits across this impressive strategy of broadcast-spawning in a highly variable environment.

REFERENCES

- Alvarez, L., Friedrich, B. M., Gompper, G., & Kaupp, U. B. (2014). The computational sperm cell. *Trends in cell biology*, *24*(3), 198–207.
- Birkhead, T. R. (1998). Cryptic female choice: Criteria for establishing female sperm choice. *Evolution*, *52*(4), 1212–1218. <https://doi.org/10.1111/j.1558-5646.1998.tb01848.x>
- Bode, M., & Marshall, D. J. (2007). The quick and the dead? Sperm competition and sexual conflict in the sea. *Evolution*, *61*(11), 2693–2700.
- Cameron, R. A., & Rumrill, S. S. (1982). Larval abundance and recruitment of the sand dollar *Dendraster excentricus* in Monterey Bay, California, USA. *Marine Biology*, *71*, 197–202.
- Cary, G. A., Cameron, R. A., & Hinman, V. F. (2018). *EchinoBase: Tools for Echinoderm Genome Analysis*. Eukaryotic Genomic Databases (M. Kollmar, Ed.), 1757, 349–369, Springer New York. https://doi.org/10.1007/978-1-4939-7737-6_12
- Deaker, D. J., Foo, S. A., & Byrne, M. (2019). Variability in egg and jelly-coat size and their contribution to target size for spermatozoa: A review for the Echinodermata. *Marine and Freshwater Research*, *70*(7), 995. <https://doi.org/10.1071/MF18134>
- Emlet, R. B. (1986). Larval production, dispersal, and growth in a fjord: A case study on larvae of the sand dollar *Dendraster excentricus*. *Marine Ecology Progress Series*, *31*, 245–254.
- Ericson, J. A., Lamare, M. D., Morley, S. A., & Barker, M. F. (2010). The response of two ecologically important Antarctic invertebrates (*Sterechinus neumayeri* and *Parborlasia corrugatus*) to reduced seawater pH: Effects on fertilisation and embryonic development. *Marine Biology*, *157*(12), 2689–2702. <https://doi.org/10.1007/s00227-010-1529-y>

- Farley & Levitan (2001). The Role of Jelly Coats in Sperm-Egg Encounters, Fertilization Success, and Selection on Egg Size in Broadcast Spawners. *The American Naturalist*, 157(6), <https://doi.org/10.1086/320619>
- Graham, H., Rastrick, S. P., Findlay, H. S., Bentley, M. G., Widdicombe, S., Clare, A. S., & Caldwell, G. S. (2016). Sperm motility and fertilisation success in an acidified and hypoxic environment. *ICES Journal of Marine Science*, 73(3), 783–790.
- Hopkins, B.R., Sepil, I., Wigby, S. (2017). Seminal fluids. *Current Biology*, 27(11), R404–R405, <https://doi.org/10.1016/j.cub.2017.03.063>
- Kjørboe, T., Ploug, H., Thygesen, U. H. (2001). Fluid motion and solute distribution around sinking aggregates. I. Small-scale fluxes and heterogeneity of nutrients in the pelagic environment. *Marine Ecology Progress Series*, 211, 1-13. <https://www.jstor.org/stable/24864138>
- Kupriyanova, E. K., & Havenhand, J. N. (2005). Effects of temperature on sperm swimming behaviour, respiration and fertilization success in the serpulid polychaete, *Galeolaria caespitosa* (Annelida: Serpulidae). *Invertebrate Reproduction & Development*, 48(1–3), 7–17. <https://doi.org/10.1080/07924259.2005.9652166>
- Leuchtenberger, S. G., Daleo, M., Gullickson, P., Delgado, A., Lo, C., & Nishizaki, M. T. (2022). The effects of temperature and pH on the reproductive ecology of sand dollars and sea urchins: Impacts on sperm swimming and fertilization. *Plos one*, 17(12), e0276134.
- Levitan, D. R., & Petersen, C. (1995). Sperm limitation in the sea. *Trends in Ecology & Evolution*, 10(6), 228–231. [https://doi.org/10.1016/S0169-5347\(00\)89071-0](https://doi.org/10.1016/S0169-5347(00)89071-0)
- Levitan, D. R. (1996). Effects of gamete traits on fertilization in the sea and the evolution of sexual dimorphism. *Nature*, 382(6587), 153–155. <https://doi.org/10.1038/382153a0>

- Levitan, D. R. (2005). The Distribution of Male and Female Reproductive Success in a Broadcast Spawning Marine Invertebrate. *Integrative and Comparative Biology*, 45(5), 848–855. <https://doi.org/10.1093/icb/45.5.848>
- Levitan, D. R., TerHorst, C. P., & Fogarty, N. D. (2007). The risk of polyspermy in three congeneric sea urchins and its implications for gametic incompatibility and reproductive isolation. *Evolution*, 61(8), 2007–2014.
- Marshall, D. J., & Evans, J. P. (2005). Does egg competition occur in marine broadcast-spawners?: Egg competition in broadcast-spawners. *Journal of Evolutionary Biology*, 18(5), 1244–1252. <https://doi.org/10.1111/j.1420-9101.2005.00947.x>
- McHugh, D., & Rouse, G. W. (1998). Life history evolution of marine invertebrates: New views from phylogenetic systematics. *Trends in Ecology & Evolution*, 13(5), 182–186. [https://doi.org/10.1016/S0169-5347\(97\)01285-8](https://doi.org/10.1016/S0169-5347(97)01285-8)
- Niesen, T. M. (1977). Reproductive cycles in two populations of the Pacific sand dollar *Dendraster excentricus*. *Marine Biology*, 42, 365–373.
- Parker, G.A. (1970). Sperm competition and its evolutionary consequences in the insects. *Biological reviews*, 45, 525–567. <https://doi.org/10.1111/j.1469-185X.1970.tb01176.x>
- Parker, G. A. (1982). Why are there so many tiny sperm? Sperm competition and the maintenance of two sexes. *Journal of Theoretical Biology*, 96(2), 281–294. [https://doi.org/10.1016/0022-5193\(82\)90225-9](https://doi.org/10.1016/0022-5193(82)90225-9)
- Parker, G. A., & Pizzari, T. (2010). Sperm competition and ejaculate economics. *Biological Reviews*, 85(4), 897–934.
- RStudio Team (2020). RStudio: Integrated Development for R. RStudio, PBC, Boston, MA URL <http://www.rstudio.com/>

- Thomas, F. I., Edwards, K. A., Bolton, T. F., Sewell, M. A., & Zande, J. M. (1999). Mechanical resistance to shear stress: the role of echinoderm egg extracellular layers. *The Biological Bulletin*, 197(1), 7–10.
- Thomas, F. I., Kregting, L. T., Badgley, B. D., Donahue, M. J., & Yund, P. O. (2013). Fertilization in a sea urchin is not only a water column process: effects of water flow on fertilization near a spawning female. *Marine Ecology Progress Series*, 494, 231–240.
- Vogel, H., Czihak, G., Chang, P., & Wolf, W. (1982). Fertilization kinetics of sea urchin eggs. *Mathematical Biosciences*, 58(2), 189–216.
- Wassarman, P. M. (1987). The biology and chemistry of fertilization. *Science*, 235(4788), 553–560.
- Wickham H (2016). *ggplot2: Elegant Graphics for Data Analysis*. Springer-Verlag New York. ISBN 978-3-319-24277-4, <https://ggplot2.tidyverse.org>
- Wickham, H., Averick, M., Bryan, J., Chang, W., McGowan, L., François, R., Grolemund, G., Hayes, A., Henry, L., Hester, J., Kuhn, M., Pedersen, T., Miller, E., Bache, S., Müller, K., Ooms, J., Robinson, D., Seidel, D., Spinu, V., ... Yutani, H. (2019). Welcome to the tidyverse. *Journal of Open Source Software*, 4(43), 1686.
- Wickham, H., François, R., Henry, L., & Müller, K. (2023). Vaughan D. *dplyr: A Grammar of Data Manipulation*.
- Wilson-Leedy, J. G., & Ingermann, R. L. (2007). Development of a novel CASA system based on open source software for characterization of zebrafish sperm motility parameters. *Theriogenology*, 67(3), 661–672. doi:10.1016/j.theriogenology.2006.10.003

Yund, P. O., & Meidel, S. K. (2003). Sea urchin spawning in benthic boundary layers: are eggs fertilized before advecting away from females?. *Limnology and Oceanography*, 48(2), 795-801.

Steve Paxton (2023, June 27). Evolutionary Developmental Biology Lecture 11. <https://www.leeds.ac.uk/chb/lectures/edb11.html>