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Hold the Lube? A Preliminary Investigation of the Implications of Water-Accommodated Petro-diesel Fractions on Egg Fertilization Success in *Dendraster excentricus*

ALYSSA BALL[1]*
PERSEPHONE SPURGEON[1]

[1]Simon Fraser University, *Department of Biological Sciences*
[2]University of Alberta, *Department of Biological Sciences*

Abstract

Echinoderms are highly sensitive to hydrocarbons because they cannot metabolize such substances, especially during early stages of development. With expanding pipelines, crude oil import and export via boat vessels in the Pacific Northwest will likely increase, endangering native marine species and their respective communities. The sand dollar *Dendraster excentricus* is among species likely to be affected and provides a good indicator species for modeling the potentially negative effects of hydrocarbons (in this case specifically petro-diesel) on marine communities, given its array of ecological interactions with other marine species. Few studies have examined how petro-diesel exposure impacts egg fertilization in *D. excentricus*. We exposed *D. excentricus* gametes to water accommodated fractions (WAFs) of petro-diesel, and measured the resulting density of successfully fertilized eggs. Stock solutions of gametes were collected from twenty different sand dollars per trial, mimicking natural broadcast spawning, which results in a diverse gene pool at spawning time. Different combinations of diesel exposed gametes and non-exposed gametes were mixed into the following treatments: unexposed sperm and egg, diesel exposed sperm and unexposed egg, unexposed sperm and diesel exposed egg, and diesel exposed sperm and egg. Statistical modelling does not support an effect of diesel treatment on the number of successful fertilization events in *D. excentricus*.

Keywords — Echinoderms, Fertilization, Sand dollar, Teratogenic

1. INTRODUCTION

PETROLEUM hydrocarbons (PHCs) enter the marine environment through oil spills, crude oil production, drilling, and transport [1, 127-128]. Local examples include: the tugboat fuel spill off the coast of Haida Gwaii [2], the diesel spill off Bella Coola [3], and the risk of increases in tanker traffic in the Northwestern Pacific from the proposed expansion of the Kinder Morgan pipeline [4, 1]. In marine environments, PHCs are generally unstable, and introducing such substances causes them to undergo "weathering" [5, 6]; whereby, PHCs adhere to sediments, mix with saltwater, and

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*Corresponding Author: alyssa_ball@sfu.ca photo-degrade, losing their evaporative constituents as they breakdown over time [1]. Weathering of hydrocarbons such as petro-diesel also releases low boiling point aromatic and saturated hydrocarbons [7, 202-203], which mix with seawater incorporating such substances weakly into the water column. In the laboratory- media created using low-energy (no vortex) mixing of poorly soluble solutes (e.g., oil or petroleum products) that are essentially free of bulk particles that effectively model the naturally occurring processes by which hydrocarbons are incorporated loosely into the water column are called water accommodated fractions (WAF) [8, 1270]. WAFs of hydrocarbons (including petro-diesel) are toxic to invertebrate (particularly echinoderm) embryos, and gametes [9, 1, 10], therefore they are of concern to biodiversity in marine environments. Weathering occurs more slowly at low temperatures [11], and therefore petroleum will remain longer in the water column in cold water regions. Therefore, cold water marine species will experience a greater exposure time to hydrocarbon WAFs, which may have a negative effect on their population densities.

There is a general acknowledgement in invertebrate embryology that gamete, embryo, and larval tolerances to environmental stressors differ significantly from adults' [10, 302]. The ability to successfully survive, colonize, fertilize and overall be viable when facing abiotic stress is more paramount for echinoderms in their gametes and juvenile stages than during adulthood [12]. Therefore, focusing on early life history stages of echinoderms may be more effective when developing strategies for their conservation.

Echinoderms are highly sensitive to increases of hydrocarbons in water, as they are unable to metabolize these substances [13]. The effects of hydrocarbons including petro-diesel on fertilization have been explored in the tropical sand dollar *Melitta quinquiesperforata* [10], and in several sea urchins [14, 15, 16, 17, 18]. One study has also briefly touched the effects of hydrocarbon exposure on *Dendraster excentricus* larvae [19]. However, we are unaware of any previous research that explores the effects of petro-diesel on egg fertilization success in *D. excentricus*. Given that a negative impact of petro-diesel on egg fertilization is found in *M. quinquiesperforata*, it seems appropriate to study if similar effects of WAFs of petro-diesel on fertilization success are also found in *D. excentricus*.

Echinoderms, like *D. excentricus*, can serve as indicators of health in benthic communities due to their susceptibility to toxins [20]. Other echinoderms such as sea urchins are proven to be an integral part of marine kelp communities, and therefore, are a good indicator species [21]. *D. excentricus* is an echinoderm that interacts with many marine species in subtidal areas where it congregates in dense beds [22]. It is found from southern Alaska to Baja, California [23], within range of the Haida Gwaii and Bella Coola oil spills and therefore likely to come into contact with hydrocarbons such a petro-diesel. Given that *D. excentricus* beds provide habitat and concealment from predators for various marine organisms [24] and *D. excentricus* juveniles are involved in complex food web interactions (i.e., they are an important food source for zooplankton [25]. Further, given that the successful survival of *D. excentricus* gametes to the juvenile and adult stages is conducive to food availability for their zooplankton predators and intraspecific prey hiding habitat, respectively we expect trickle down effects of diesel exposure of gametes to the wider ecological relationships held by *D. excentricus*.

Therefore, knowledge of what effects (if any) petro-diesel has on *D. excentricus* gametes is paramount to predicting the consequences that spills of this hydrocarbon might have in Northwestern Pacific marine communities.

We explore the effects WAFs of petroleum have on fertilization success in *D. excentricus*. Our study builds upon that of Steffansson *et al.* [19], whom use field collected samples from a pre-existing spill (i.e., the Deep-Water Horizon incident), but differs in that we use a hydrocarbon concentration (0.06ml/L) based on reported values following experimental oil spill in the Raged Channel in the Arctic [26, 127]. Our study aims to determine at the ability of *D. excentricus* eggs to successfully fertilize in the presence of diesel WAFs, using twenty sand dollars per treatment as a proxy for genetically variable "populations".

We generate hypotheses based on known mechanisms that may reduce fertilization success in sand dollars, such as the inhibition of protein synthesis in gonads [27], decreased sperm motility [16], and gamete mortality. Our hypotheses are as follows:

H_0) Exposure to diesel has no effect on successful fertilization.

1) Exposure to diesel in both egg and sperm reduces the likelihood of successful fertilization, as the WAF of hydrocarbons alters the properties of the eggs and sperm, causing both gametes to become inviable, preventing fertilization.

2) Exposure of only eggs to diesel reduces the likelihood of successful fertilization, because the hydrophobic properties of boat fuel may detach the jelly layer (lipid layer that surrounds the egg [28] from the egg, essential in fertilization, based on our observations.

3) Exposure to diesel in only sperm affects fertilization, because sperm motility is decreased at concentrations of crude oil of 0.05 ml/L [10, 314].

2. MATERIALS AND METHODS

Sixty *D. excentricus* were collected subtidally by SCUBA from Brady's Beach, Bamfield, BC (48.8271°N, 125.1531°W) (Figure 1). We conducted three separate experimental trials, with 20 individuals per trial to mimic natural broadcast spawning [29], which leads to a naturally diverse gene pool [30]. Each trial consisted of four treatments (see Figure 2 & Figure 3), interspersed through time using a random number generator [31] to control for the effect of exposure time among trials for a total of thirty replicates per treatment in each experimental trial. All fertilization trials and husbandry of the sand dollars was conducted in the laboratory at room temperature (24-25°C), and husbandry of *D. excentricus* was carried out according the Bamfield Marine Science Centre's Animal Care protocols. As per Spiegler and Oppenheimer's [32] study in sea urchins, viability time of eggs and sperm was maximized by storing gametes in an ice bath (0 °C) during experimentation.

We induced gamete production in *D. excentricus* using Tyler's [33] method by injecting 0.2 ml of 0.5 M KCl into five gonads through the mouth with a sterile needle and 3cc syringe. Gamete release occurred within five minutes of the injection. Males and females were differentiated according to the colour of gametes they produced (males produced white and females produced orange-red gametes), which were collected from the aboral side. Gametes were then placed in a 200-ml pre-made stock solution of either filtered seawater or a WAF of diesel obtained from the Bamfield Marine Science Centre's boat fuel pump (Figure 3) to model their natural positions in the water column at spawning [34] in the event of an diesel spill. Diesel stock solutions (Figure 3) were created using a modified version of Nicol et. al.'s [10] method. A concentration of 0.024 mg/L of diesel was dissolved in 400 ml of filtered seawater, and shaken for 5-10 minutes.

Due to time constraints only those eggs which had been fertilized successfully were counted. Counting of fertilized eggs was initiated after ten minutes of exposure, slides were then prepared according to treatment (as described below), allowed to sit for 30 seconds, and the number of fertilized cells were counted. We used a hemocytometer to count the number of eggs fertilized following the introduction of each of the four sperm and egg treatments at a standard interval of 30 seconds after the respective sperm was introduced. Use of the hemocytometer allowed us to later convert these counts into more realistic cell densities. From each treatment, we obtained 10 μL of one egg and one sperm stock solution per replicate (Figure 3), placed the two solutions on a slide to allow fertilization to occur, and pipetted this mixture into the hemocytometer. This entire process took approximately 6 hours per experimental trial and every three hours the person counting was alternated. Abnormalities such as the loss or partial destruction of the egg's jelly layer, were considered unfertilized eggs, as without this layer the egg lacks the cross-sectional diameter to fertilize successfully [34, 2479], and therefore not included in final fertilized egg counts (Figure 4).

2.1. Statistical Analysis

Statistical analysis was conducted in R- studio (version 1.0.44). In R, 18 linear models were used to compare the effects of and interactions between exposure time, experimental trial, and treatment on the resulting fertilized egg density. An Akaike's Information Criterion (AIC) table (Table 1) was used to compare the effects of each factor on egg fertilization success. Data trends were further compared between trials to account for potential differences due to the different stock solutions created for each experimental trial (i.e., differences in initial concentration of eggs and sperm (# of eggs/sperm per unit volume) (Table 2), and/or genetic based differences in tolerance to hydrocarbons between groups of sand dollars used for each trial). Mean and standard errors (Table 3), were calculated using Excel version 2016.

3. RESULTS

Qualitative analysis of pooled data, some trends among treatments (Figure 4). In order, of least to greatest fertilized egg densities: diesel exposed egg and normal sperm treatment (ED+S) was the lowest; the diesel exposed sperm and normal egg (SD+E); both diesel exposed gametes treatment (SD+ED); and finally, the regular egg and sperm treatment (S+E) (Figure 5). Quantitative analysis does not support this, as AIC modelling showed no effect of treatment on cell density of fertilized eggs (Table 1). Mean fertilized egg densities show little differences between our control, and the

Table 1: *Table representing the best 6 out of 18 Akaike's Information Criterion (AIC) models fitted against the response variable (cell density). The best fit model is given a ΔAIC score of 0, with all other models compared to it. Substituted variables in model description represent:* y = *cell density,* a = *effect of treatment,* b = *effect of experimental trial,* c = *effect of time,* $b : c$ = *interaction between experimental trial and time.*

Model Description	Model #	# of Param.	AIC	ΔAIC	AIC Weight	Cml. Weight
$y = b$	3	4	74.43	0.00	0.49	0.49
$y = b + c$	6	5	75.28	0.85	0.32	0.80
$y = b + c + b: c$	10	7	77.35	2.91	0.11	0.92
$y = a + b$	5	7	79.75	5.32	0.03	0.95
$y = a + b + c$	7	8	80.29	5.86	0.03	0.98
$y = a + b + c + b: c$	13	10	82.02	7.58	0.01	0.99

diesel exposed and regular egg treatment (means = 3003.90±, 303.10 & 3619.60 ± 403.88, respectively). Comparison of all means and standard errors, shows no significant difference among all treatments (Table 3).

Table 2: *Initial concentrations of eggs and sperm used to form stock solutions for each of the three experimental trials. Values are based on the initial concentration of egg and sperm in the stock solutions produced from twenty different sand dollars per each experimental trial.*

Trial	Concentration of eggs per 1mL	Concentration of Sperm per 1mL	Percentage of sperm compared to egg concentration (%)
1	0.080	0.033	40.625
2	0.078	0.046	59.677
3	0.054	0.055	1.035

Given there is lacking support for an effect of treatment on cell density, when the data was pooled (Table 1), we explored other factors that may have altered our results, by viewing experimental trials separately (Figure 6). When all experiments were compared, trials one and three showed a similar trend despite being started at different time intervals (18 hours after exposure, and immediately after exposure, respectively). Trial 2, however, had the largest cell density of the 3 trials (Figure 6). SD+E had the highest total density of fertilized eggs in experimental trial 2, followed by ED + S, SD + ED, and finally the control (S+E) (Figure 6). These trends differ from those in trials 1 and 3.

Models were fitted to observe the interactions and effects of the explanatory variables (treatment, time, and experimental trial) on the response variable (fertilized cell density) (Table 3). The top three best fit models, all eliminate treatment as influencing cell density (Table 1). According to Akaike weights there is a 49% probability that the data

Table 3: *Mean and standard error of fertilized cell density for each treatment: untreated sperm and untreated egg, untreated sperm and diesel exposed egg, diesel exposed sperm and egg, and egg and sperm both exposed to diesel.*

Treatment	Mean Fertilized Cell Density (# cells/mL)	Standard error of the mean (SE)
S+E	3003.90	303.10
S+ED	1151.76	160.32
SD+E	3619.60	403.88
SD+ED	1728.61	196.35

is accounted for by the effect of experimental trial (Table 1), giving further support that model three is the best candidate model. The second best fit model (model six) assesses the effect of both time and experimental trial on cell density (Table 1). The calculated evidence ratio, which determines how much better the best model is in comparison to other models is 1.53 between models three and six; this depicts that model three is 1.53 times better at estimating our data than model six, meaning that there is strong evidence that only experiment trial (model three) rather than experiment and time affected the resulting cell density of successfully fertilized eggs. The final three models presented in Table 1 include the effect of treatment. These models have high delta AIC's; therefore, treatment does not explain the resulting fertilized cell density. Pseudo r^2 (0.95) for model three, supports that experimental trial influences cell density.

4. DISCUSSION

Model analysis of our data does not support our alternative hypotheses (i.e., hypotheses 1, 2, and 3) (Table 1), as no models support treatment as a factor that affects fertilized cell density. Therefore, we fail to reject the null hypothesis. Our results suggest diesel exposure at a level of 0.06 mL/L does not decrease the likelihood of fertilization events in *D. excentricus* (Table 1). Therefore, food web interactions between *D. excentricus* prey juveniles and zooplankton, described by Pennington et al [25], based on our data may be unaffected by the exposure of *D. excentricus* gametes to diesel. Thus, the ecological impacts of oil spills on marine life in areas surrounding Brady's Beach may not be as great as we predict.

The effect of experimental trial on cell density based on our models may be an important factor for explaining observed densities of fertilized eggs (Table 1). General trends (Figure 6) across trials show trial 2 has a higher overall cell density in both the SD+E and S+ED treatments than experimental trials 1 and 3. Experimental trials 1 and 3 show trends that suggest treatment does affect fertilization succession, with ED+S having the most negative effect on resulting fertilized egg density. Given that trials start at different times after exposure, yet share similar trends in treatment, there may be need to investigate in future experiments if treatment influences fertilization success. Further, since experimental trial 2 does not show a similar trend among its treatment groups, and given the results of Table 1, further investigation of other factors not examined in our study, that may confound the effect of treatment on fertilization success in *D. excentricus* are required.

The models that fit the best according to Table 1 (models 3 and 6), suggest experimental trial may have the biggest effect on cell density, suggesting there may be differences between trials 1, 2, and 3. Trial 2 has the most outliers, suggesting some error was made during data collection (e.g., counting error). However, careful counts of cell density are checked prior to recording and all experimental procedures were kept constant between experimental trials, except sand dollar groups used to form stock solutions. Therefore, counting error seems an unlikely source of error. More likely, stock solution (initial concentration of eggs and sperm) may affect observed cell densities.

4.1. Effect of initial concentration of eggs vs. sperm

Presumably, as the proportion of sperm to eggs increases, so does the probability of polyspermy, (fertilization of an egg by multiple sperm resulting in unsuccessful fertilization [34, 2482]. Therefore, a higher concentration of sperm relative to the concentration of eggs would presumably decrease the probability of successful fertilization events. However, in our study, variation in the ratio of sperm: egg between experimental trials is not significant (Table 2). Further, a greater number of successful fertilization events in experimental trial 2, does not appear to be due to the initial concentration of sperm and eggs since experiment 2 was 59.6% sperm (Table 2), similar to that of trial 1 (40.6% sperm), even though we know from Figure 6 that their observed fertilized cell densities differ between these trials. Therefore, initial concentration of sperm and eggs is a poor explanation for differences among experimental trials.

4.2. Genetic variability among individuals used in stock solutions

In animals, tolerance to toxins, such as hydrocarbons, are associated with, intraspecific, and interspecific differences in abilities to metabolize toxic compounds (e.g., in polychaetes: [35]; in mice: [36]). The ability to metabolize hydrocarbons, in echinoderms have been linked to the aryl hydrocarbon receptor (AHR), which mediates transcriptional response to hydrocarbons, regulates developmental processes, and determines gene expression [37, 10]. Therefore, differences between trials may be due to variability in the functioning of the AHR among individual sand dollar gametes in the three different stock solutions. Differences in AHR may additionally explain why certain gametes have greater tolerances to hydrocarbons than others, because the more sensitive AHR is to hydrocarbons, the more gene transcription and normal development are negatively affected [37, 10]. If this is the case, then the AHR in sand dollar gametes in experimental trial two maybe less sensitive to hydrocarbons than experimental trials 1 or 3. However, further investigation of the function of AHR in echinoderm species aside from sea urchins is needed to test this in *D. excentricus*.

4.3. Broader Ecological Relationships

Although the results of linear modelling found no effect of treatment, the implications of exposing *D. excentricus* gametes to 0.06 mL/L of petro-diesel on this species' ecological relationships nonetheless warrants discussion. Earlier we suggested *D. excentricus* may provide a good indicator species of ecosystem health in its respective marine communities. If our suspicion is correct and this is in fact the case then our study suggests at a level of 0.06 mL/L such communities would be relatively unaffected. This is of course assuming that *D. excentricus* is the most sensitive organism (with regards to petro-diesel toxicity) in said community.

However, such an interpretation does not take into account potential for hydrocarbons (such as petro-diesel) to bioaccumulate [38, A1-A2]. Bioaccumulation refers to an organism usually further up the food chain accumulating a toxic substance into its own living tissues through ingestion of contaminated lower trophic organisms. Underlying this process is that the rate of intake of a substance is greater than the rate of excretion or metabolic transformation. In a study done by Almeda *et al.* mesozooplankton were found to bioaccumulate polyaromatic hydrocarbons (PAHs) (such as naphthalene) found in crude oil which negatively impacted their survival [39]. PAHs including naphthalene are also found in most diesel fuels used to power boat engines [40], and therefore may be found in the diesel used in our study. Thus, although our exposure of *D. excentricus* gametes to 0.06 ml/L of petro-diesel did not show significant negative effects on fertilization success, and would allow said sand dollars to presumably survive to be prey juveniles there may still be negative effects (due to the potential for this concentration to bioaccumulate and hence be higher) in organisms such as predatory zooplankton that feed on exposed *D. excentricus* individuals. We caution though that in the present study the potential toxic effects of bioaccumulation of boat fuel in high trophic marine organisms who have consumed exposed *D. excentricus* remains unclear.

5. Conclusion

Results of Nicol *et al.* 's [10] study with suggest that our concentration of diesel (0.06 ml/L) should have inhibited fertilization in *D. excentricus*. However, despite our attempts to separate out the effects of a 0.06 mL/L diesel treatment on eggs and sperm, modelling suggests lack of support for an effect of petro-diesel on fertilization success in *D. excentricus*. Best fit models (models 3 and 6) instead predict that experimental trial had the greatest effect on fertilization success. We conclude that treatment does not influence either sperm nor eggs, but given the limited nature of our study and the toxic nature of diesel we suggest that further research is required. Therefore, if we assume successful fertilization increases the probability of maturating to the juvenile stage then the ecological predator-prey relationship between zooplankton and *D. excentricus* juveniles would remain unaffected in the BradyâĂŹs beach population in the event of petro-diesel exposure at a level of 0.06 mL/L.

6. Future Research

Biodegradation of hydrocarbons (including petro-diesel) differs significantly between warm and cold water environments, as it can remain longer in the latter. Therefore, *D. excentricus* may be more tolerant to the effects of petro-diesel than other species of sand dollars accustomed to warmer climates (e.g. *M. quinquiesperforata*). For these reasons, and given that our study is the first of its kind in D. excentricus gametes, more research using higher concentrations and larger sample sizes are required to be certain of the effects petro-diesel will have on fertilization success in *D. excentricus*. Further, an in-depth analysis of what PAHs compose a particular boat petro-diesel being used would provide a clearer picture of the potential for bioaccumulation of petro-diesel and by consequence its toxic effects in marine food web systems than we can here. Moreover, to further assess how genetic variation may play a role in petro-diesel tolerance sampling of populations of *D. excentricus* from different study sites is required.

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8. APPENDIX

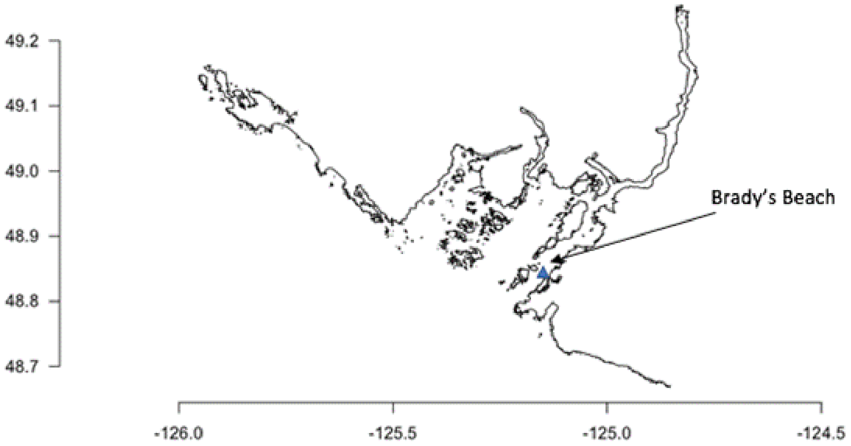

Figure 1: *Map marking the location of the collection site, Brady's Beach (48.8271°N, 125.1531°W) within the Barkley sound, BC area. The location is marked with a blue triangle.*

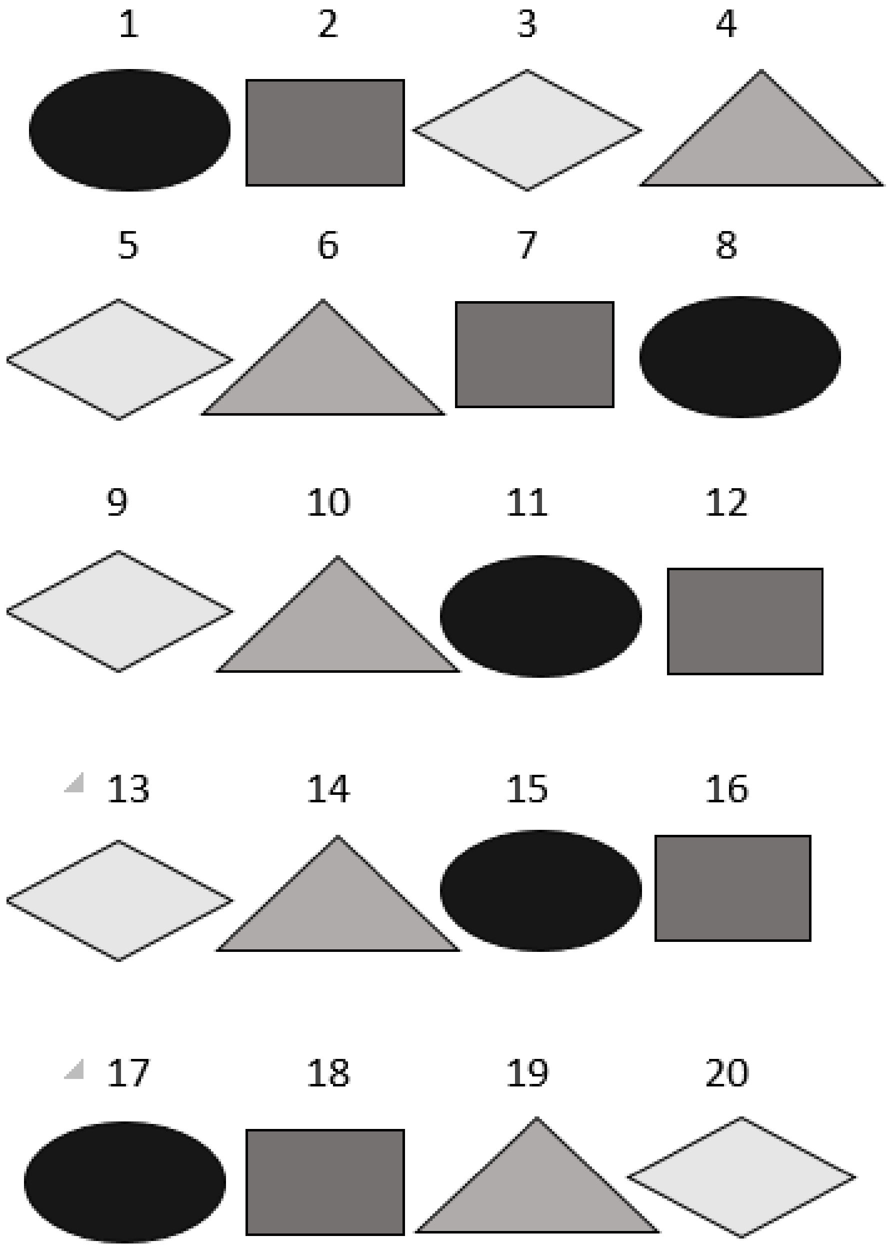

Figure 2: *Sample showing a subset (20 out of 120) trials used for each experimental trial. Each experimental setup consisted of four separate treatments, determined by a random number generator [39] regular sperm and egg (diamond), diesel sperm and diesel egg (circle), regular sperm and diesel egg (triangle), regular egg and diesel sperm (square).*

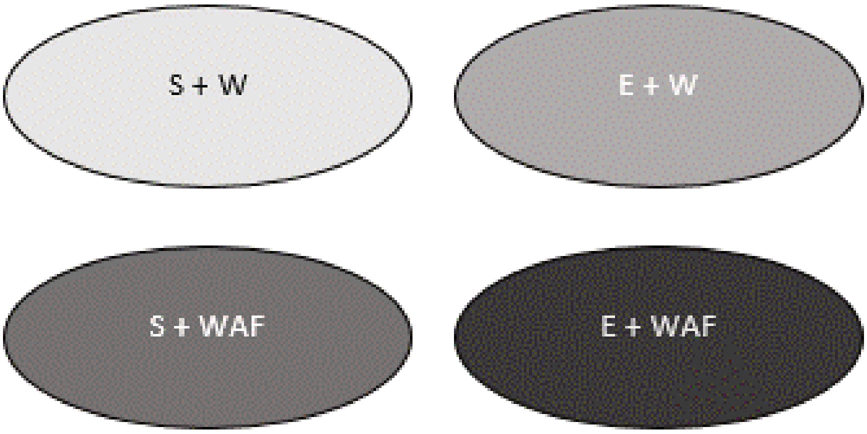

Figure 3: *Stock solutions used throughout each experimental trial: sperm and filtered sea water, egg and filtered sea water, sperm and water-accommodated fraction with diesel (WAF), egg and WAF. New stock solutions were created for each of the three trials. Egg and Sperm were evenly distributed between the two beakers.*

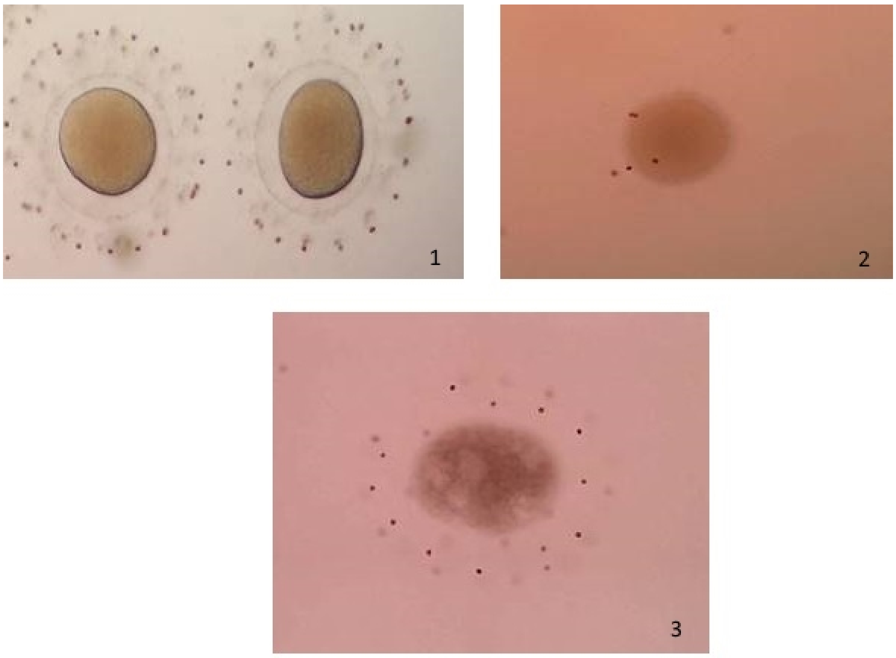

Figure 4: *Images comparing differences between a fertilized and unfertilized egg. 1) normal fertilized egg. 2) an egg that has lost its jelly layer. 3) an egg that contains missing portions within the egg itself. Image 2 and 3 are examples of unfertilized eggs, therefore, not included in final counts. Images courtesy of Persephone Spurgeon.*

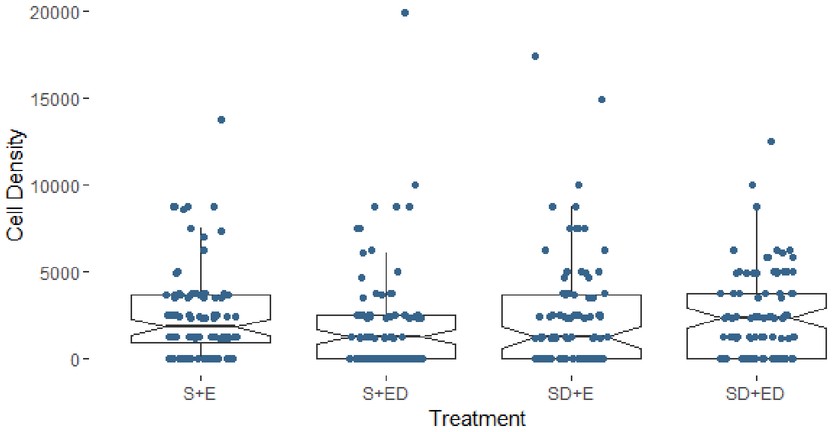

Figure 5: *Treatment vs. cell density among experimental trials pooled into one data frame. Data points represent raw data obtained with boxplots behind to better represent trends in data. Notches represent 95% confidence intervals of the median.*

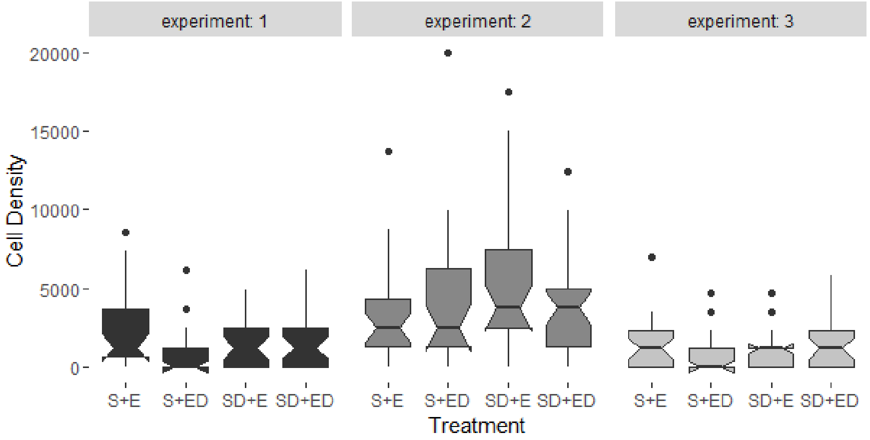

Figure 6: *Treatment vs. cell density box plots separating experimental trials (1,2,3) with 95% confidence intervals. Outliers are represented with dots above whiskers. Experiments 1,2,3 represent experimental trials.*